01-137-01p

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Dennis P. Ward, Ph.D.
Regulatory Affairs Manager
(636) 737-6631

MONSANTO COMPANY
700 CHESTERFIELD PARKWAY NORTH
ST. LOUIS, MISSOURI 63198
PHONE (314) 694-1000
http://www.monsanto.com

May 15, 2001

James L. White, Ph.D.
Senior Operations Officer
Animal and Plant Health Inspection Service
U.S. Department of Agriculture
4700 River Road, Unit 147
Riverdale, MD 20737

Subject: Petition for Determination of Nonregulated Status for the genetically modified corn product: Corn Rootworm Protected Corn Event MON 863

Dear Dr. White,

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are protected from damage due to feeding by corn rootworm larvae (Coleoptera, *Diabrotica* sp.). The tissues of these plants have been genetically engineered to produce a modified *Bacillus thuringiensis* Cry3Bb1 protein that is lethal to corn rootworm larvae.

Monsanto requests that the Administrator of APHIS make a determination of nonregulated status for Corn Rootworm Protected Corn Event MON 863, any progeny derived from crosses between MON 863 and other corn varieties, and any progeny derived from crosses of MON 863 with other transgenic corn varieties that have also been granted nonregulated status under 7 CFR Part 340.

The enclosed "Petition for Determination of Nonregulated Status for the Regulated Article: Corn Rootworm Protected Corn Event MON 863" contains relevant information upon which to make a determination. We are claiming the appendices to this petition as confidential business information. Therefore, we are also submitting a CBI Deleted version of this petition that can be released to the public. Data reports from completed field trials with MON 863 have been sent to the attention of Mrs. Dianne Hatmaker.

Contained within this package are:

- 5 copies of the petition containing confidential information
- 2 complete sets of the confidential appendices
- 2 copies of a CBI Deleted version of the petition

5/17/01
Received
MKP

Please feel free to contact me directly if you have any questions regarding this petition. Thank you.

Sincerely,

Dennis P. Ward

Encl: Petition

cc: Russ Schneider

01-137-01p

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Petition for Determination of Nonregulated Status for the Regulated Article:

Corn Rootworm Protected Corn Event MON 863

[CBI Deleted Version]

Submitted by:

Monsanto Company
600 13th Street, N.W.
Suite 660
Washington, DC 20005

May 15, 2001

Monsanto Petition #

01-CR-050U

5/17/01
MKP

Request for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

SUMMARY

Corn (*Zea mays* L.) is the largest crop grown in the United States in terms of acreage planted and net value. In 2000, corn production covered 79.5 million acres, yielding 10 billion bushels with a net value of $18.4 billion (NCGA, 2001). Corn yields are negatively impacted by a number of insect pests. One of the most pernicious in the U.S. Corn Belt is the corn rootworm (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. Corn Belt from the standpoint of chemical insecticide usage (NASS, 2000). Over 15 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 pest complex (Metcalf, 1986), based on costs associated with the purchase of soil insecticides and crop losses due to pest damage.

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are protected from damage due to CRW feeding. The tissues of corn plants have been genetically engineered to produce a modified *Bacillus thuringiensis (*subspecies *kumamotoensis)* Cry3Bb1 protein that is selectively toxic to CRW species. Transformation of these plants has resulted in the creation of event MON 863. This transformation event affords corn a level of protection from CRW damage that is comparable or superior to that offered by currently available synthetic insecticides.

Event MON 863 was produced by particle acceleration technology using a purified linear DNA fragment containing a *cry3Bb1* coding cassette, as well as a cassette coding for the selectable marker, neomycin phosphotransferase type II (NPTII). Southern blot analyses confirmed that event MON 863 contains one copy of the transformation cassette 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 two full-length proteins, Cry3Bb1 and NPTII, will be 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 fingerprint analysis of DNA extracted from plants spanning three generations further confirmed the stability of the inserted genes in MON 863. Plant production of Cry3Bb1 was determined in multiple tissues collected from replicated field trials. Levels of the Cry3Bb1 variant produced in MON 863 were found to be in the range of 3 to 93 µg/g of fresh tissue weight. Cry3Bb1 levels in root tissue were sufficient to confer protection against CRW feeding damage. Two years of replicated field efficacy trials have demonstrated that

MON 863 provides comparable or superior control of CRW larvae compared to currently available insecticide products.

Agronomic, morphological and pest susceptibility observations have been recorded in multiple field trials conducted across major corn growing regions of the United States. These trials confirmed that MON 863 is phenotypically equivalent to nontransgenic corn except for its tolerance to CRW. Neither the *cry3Bb1* nor *nptII* coding sequences, their regulatory sequences, or the proteins that they express are expected to confer any plant pest characteristics to MON 863. Seeds are the only known survival structures of corn. The dormancy and germination characteristics of MON 863 seed are comparable to that of nontransgenic corn seed. Corn would not be competitive as a weed due to past selection for traits designed to improve its performance in production agriculture. The insertion of *cry3Bb1* and *nptII* coding sequences into the plant genome is not expected to impart an increased weediness potential to corn event MON 863. Movement of these transgenes from MON 863 to related species, other than corn, is unlikely.

Bacillus thuringiensis (B.t.) Cry proteins have a long history of safe and widespread use in agriculture (McClintock *et al.*, 1995). The deduced amino acid sequence for the crystal protein expressed in MON 863 is 98.9% identical to that of the Cry3Bb1 protein contained in the topically applied commercial product, *Raven*® Oil Flowable Bioinsecticide. Based on toxicological evaluations, the Cry3Bb1 protein variant produced in MON 863 poses minimal risk to mammals, wildlife and nontarget insects. It is not expected to accumulate or persist in the environment. The introduction of this product will offer U.S. farmers a safe and effective alternative to the use of synthetic chemical insecticides for control of the CRW pest.

The use of corn hybrids containing Cry3Bb1 protein is expected to select for CRW that are tolerant to the toxin, a phenomenon that also occurs with conventional chemical insecticides. However, selection pressure will not be significant until the product is in widespread use many years after its introduction. Resistance management tactics based on the use of refuges planted to nontransgenic corn, monitoring for resistant insects, use of complementary integrated pest management (IPM) practices, and grower education will be put in place to minimize the risk of widespread resistance developing.

The information contained within this petition demonstrates that Corn Rootworm Protected Corn Event MON 863 does not represent a plant pest risk, nor does it pose any significant risk to the environment. Therefore, Monsanto requests that the Administrator of the Animal and Plant Health Inspection Service (APHIS) make a determination of nonregulated status for MON 863, any progeny derived from crosses between MON 863 and other corn varieties, and any progeny derived from crosses of MON 863 with other transgenic corn varieties that have also been granted nonregulated status under 7 CFR Part 340.

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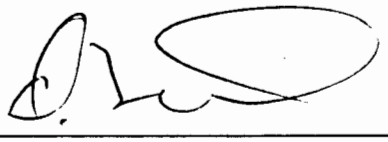
Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

CERTIFICATION

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner that are unfavorable to the petition.



Dennis P. Ward, Ph.D.
Regulatory Affairs Manager
Monsanto Company
700 Chesterfield Parkway
St. Louis, MO 63198
Tel. 636-737-6631
Fax. 636-737-5943
email: dennis.p.ward@monsanto.com

Author

Dennis P. Ward

Contributors

F. Behr, T. Cavato, Y. Dudin, G. Head, R. Hileman,
R. Knutson, M. McKee, C. Pilcher, R. Sidhu and C. Wiltse

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Request for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

CLAIM OF DATA CONFIDENTIALITY

In response to a USDA request dated August 17, 2000, we are hereby supplying copies of all unpublished study reports cited in the main body of this document. Monsanto is claiming the entire contents of each study report as Confidential Business Information (CBI). This claim is being made for 25 reports, all of which can be found in Appendix C of this petition. The following justification is made for this CBI claim.

i. Legal Background

The Freedom of Information Act (FOIA), 5 U.S.C. § 552, specifically exempts from release "trade secrets and commercial or financial information obtained from a person and privileged or confidential" ("Exemption 4") 5 U.S.C. § 552(b)(4). Exemption 4 applies where the disclosure of information would be likely to cause substantial harm to the competitive position of the owner, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to share data with the agency voluntarily. National Parks & Conservation Association v. Morton, 498 F.2d 765, 770 (D.C. Cir. 1974); Gulf & Western Industries, Inc. v. U.S., 615 F.2d 527, 530 (D.C. Cir. 1979).

A party seeking to demonstrate "substantial competitive harm" need not show actual competitive harm, but must only demonstrate the presence of competition and the likelihood of substantial competitive injury. Id. at 530; National Parks & Conservation Association v. Kleppe, 547 F.2d 673, 679 (D.C. Cir. 1976); Miami Herald Pub. Co. v. U.S. Small Business Administration, 670 F.2d 610, 614 (5th Cir. Unit B 1982).

For the purposes of FOIA, courts have defined the term "trade secret" to mean a "secret, commercially valuable plan, formula, process, or device that is used for the making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort." Public Citizen Health Research Group v. FDA, 704 F.2d 1280, 1288 (D.C. Cir. 1983); Anderson v. Dept. of Health & Human Services, 907 F.2d 936, 943-44 (l0th Cir. 1990).

Where, as in the case of the Monsanto products subject to an FOIA request, the development time and costs of the products have been substantial and the information can only be obtained by competitors at considerable cost, disclosure is prohibited. Greenberg v. Food and Drug Administration, 803 F.2d at 1213, 1216-1218 (D.C. Cir. 1986); Worthington Compressors, Inc. v. Costle, 622 F.2d 45, 51-52 (D.C. Cir. 1981).

The U.S. Department of Agriculture's APHIS has defined "Confidential Business Information" for the purposes of biotechnology submissions within the boundaries of these statutory and court interpretations of Exemption 4. "Policy Statement on the

Protection of Privileged or Confidential Business Information," (the CBI Policy Statement), 50 Fed. Reg. 38561 (Sept. 23, 1985). The CBI Policy Statement defines CBI to consist of "Trade Secrets" and "Commercial or Financial Information." "Trade Secrets" are, in turn, defined as: "information relating to the production process. This includes production data, formulas, and processes, and quality control tests and data, as well as research methodology and data generated in the development of the production process. Such information must be (1) commercially valuable, (2) used in one's business and (3) maintained in secrecy."

The CBI Policy Statement states that "Commercial or Financial Information" will also be deemed confidential if review establishes that substantial competitive harm would result from disclosure. Information such as safety data, efficacy or potency data, and environmental data may be such confidential information. Persons desiring protection for confidential information must submit a detailed statement containing facts to show that the person faces active competition in the area to which the information relates, and that substantial competitive harm would result from disclosure."

Disclosure of these types of materials is also prohibited under another exemption from FOIA's disclosure provisions. This exemption prohibits the disclosure of materials specifically exempted from disclosure by another federal statute ("Exemption 3"), 5 U.S.C. § 552(b)(3). Here, APHIS is seeking information required and protected by the U.S. Environmental Protection Agency (EPA) for purposes of pesticide registration under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). Exemption 3 provides additional authority for APHIS to protect the materials at issue here from disclosure.

***ii.* Justification**

The study reports contained in Appendix C to this petition (the "Study Reports") fall squarely within the well-established boundaries of CBI as recognized by the federal courts and by APHIS. All of the Study Reports at issue here either constitute Monsanto trade secrets or commercial or financial information, as APHIS and the courts have defined those terms. As discussed more fully below, the Study Reports comprise the results of extensive research and intellectual property required both for the commercial viability and regulatory authorization of this product. This information would be worth millions of dollars to one of Monsanto's competitors in this field, and should be accorded the protections due such confidential and valuable information.

Monsanto is at the leading edge in the development of biotechnology products in a rapidly growing and highly competitive industry. Monsanto faces a number of strong, multinational competitors in this field, including Aventis, Dow AgroSciences, Syngenta and DuPont. Monsanto's competitors, both domestic and international, have the expertise not only to replicate Monsanto's products, but also to use Monsanto's technology to develop other, competing products, thereby saving millions of dollars and years of development efforts.

Monsanto has been working on the development of transgenic crops since the early 1980s, and has become a leader in the field through the expenditure of several billion dollars in research and testing costs. Monsanto can document the development and testing costs by means of monthly summaries of the worker hours devoted to these projects, budgetary documents, field test agreements and project documents.

Presently, Monsanto's competitors cannot duplicate Monsanto's commercially valuable products from information in the public domain without going through the same painstaking trial and error development and testing that Monsanto has undertaken. Although certain information regarding Monsanto products has been made available, *e.g.*, in the context of patent applications, this information is voluminous and general in nature, and does not identify information Monsanto has found most effective for a particular product. A competitor cannot determine from the patent applications which particular combination of genes and transgenic products will prove to be commercially valuable.

Access to the information contained in the Study Reports could be used by competitors to create essentially "copy-cat" products (avoiding any technical patent infringement) that would result in a market share loss for Monsanto of millions of dollars. By performing simple copy work, these competitors would avoid the millions of dollars and many years of research and development effort expended by Monsanto to develop its commercial products. The risk of this type of intellectual property usurpation is even more heightened in the international arena, where patent protections are not as fully developed and strictly enforced as they are domestically.

The release of the Study Reports could provide competitors with commercially valuable knowledge regarding the characteristics of particular products Monsanto is planning to commercialize and the likely time frame for commercialization. This information would be extremely helpful to these companies in developing their own marketing strategies and development plans in a highly competitive market.

The commercial value of the information contained in the Study Reports has been recognized by Congress in its enactment of FIFRA and the FFDCA. Section 3 of FIFRA sets up an elaborate system of protections for these types of data, protecting them from any use by other manufacturers for a period of ten years, and requiring compensation for the use of these data by competitors after that initial ten year period. In 1996, Congress amended the FFDCA to provide both disclosure protections and compensation equivalent to that provided by FIFRA for health and safety data submitted to support pesticide residue tolerance and tolerance exemption applications. FFDCA § 408(i). APHIS should recognize the Congressional action to protect the commercial nature of these types of documents. APHIS's failure to do so could result in the loss of millions of dollars to Monsanto in data use and compensation rights.

In addition to the compensation provisions for these types of data set forth by FIFRA and FFDCA, each statute contains independent provisions for the protection from disclosure

of this information. FIFRA § 10(g); FFDCA § 408(i). FOIA prohibits the disclosure of information specifically protected by statutes such as these. 5 U.S.C. § 552(b)(3). This prohibition provides additional justification for the protection of these data.

In summary, the Study Reports contained in Appendix C of the petition are required in order for Monsanto to obtain nonregulated status of MON 863, and thereby, commercial approval, for this product. The information regarding study design, detailed research methodology, contract laboratory and report construction information available from these Study Reports could save such competitors millions of dollars in research. Monsanto has demonstrated, and Congress has recognized, the commercial value and confidential nature of these data. The Study Reports are an integral part of Monsanto's business and should be protected as such.

KEY TO ABBREVIATIONS

2219-4A1 (COL-026)	An alternative Monsanto code designation for event MON 863
2,4-D	The herbicide 2,4-dichlorophenoxyacetic acid
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
A. tumefaciens	*Agrobacterium tumefaciens*
APHIS	Animal and Plant Health Inspection Service
~	Approximately
AS1	Activating sequence-1
ATP	Adenosine triphosphate
BC_1F_1	The first filial generation derived from back crossing to a recurrent nontransgenic parent
BC_2F_1	The first filial generation derived from a second back crossing to a recurrent nontransgenic parent
BC_2F_2	The second filial generation derived from a second back crossing to a recurrent nontransgenic parent
BC_2F_3	The third filial generation derived from a second back crossing to a recurrent nontransgenic parent
BC_2F_4	The fourth filial generation derived from a second back crossing to a recurrent nontransgenic parent
ble	The gene that encodes for bleomycin binding protein
BLMT	Bleomycin binding protein from Tn5
bp	Base pairs
B.t.	*Bacillus thuringiensis*
CAB	Chlorophyll a/b binding protein
CaMV	Cauliflower mosaic virus
CBI	Confidential business information
COL-026	An alternative Monsanto code designation for event MON 863
CFR	Code of Federal Regulations (U.S.)
CRW	Corn rootworm, *Diabrotica* sp.
Cry	Crystal protein, a diverse group of insecticidal proteins produced by *B.t.*
Cry1	A class of Lepidopteran-specific *B.t.* Cry proteins that share >45% amino acid sequence homology
Cry1A	A class of Lepidopteran-specific *B.t.* Cry1 proteins that share >75% amino acid sequence homology

KEY TO ABBREVIATIONS (cont.)

Cry1Aa	A class of Lepidopteran-specific *B.t.* Cry1A proteins that share >95% amino acid sequence homology
Cry2	A class of Lepidopteran- and Dipteran-specific *B.t.* Cry proteins that share >45% amino acid sequence homology
Cry3	A class of Coleopteran-specific *B.t.* Cry proteins that share >45% amino acid sequence homology
Cry3A	A class of Coleopteran-specific *B.t.* Cry3 proteins that share >75% amino acid sequence homology
Cry3Aa4	A commercially available Cry3A protein that is active against the Colorado potato beetle
Cry3B	A class of Coleopteran-specific *B.t.* Cry3 proteins that share >75% amino acid sequence homology
Cry3Bb	A class of Coleopteran-specific *B.t.* Cry3B proteins that share >95% amino acid sequence homology
Cry3Bb1	A natural isolate, and holotype, of the Cry3Bb class of *B.t.* Cry proteins; a variant of this protein is produced in transgenic corn event MON 863
cry3Bb1	DNA sequence that encodes for the protein, Cry3Bb1
Cry4	A class of Dipteran-specific *B.t.* Cry proteins that share >45% amino acid sequence homology
df	Degrees of freedom
DNA	Deoxyribonucleic Acid
DT50	Degradation time to achieve 50% loss of bioactivity or concentration
E. coli	*Escherichia coli*
Eco RV	Restriction endonuclease that cuts DNA at specific locations
EG11098	A recombinant strain of *B.t.* that produces a variant of Cry3Bb1 with enhanced insecticidal activity
EG11231	A recombinant strain of *B.t.* that produces a variant of Cry3Bb1 with enhanced insecticidal activity
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (U.S.)
F_1	First filial generation
F_2	Second filial generation
FDA	Food and Drug Administration (U.S.)
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide Fungicide and Rodenticide Act
FOIA	Freedom of Information Act
FR	Federal Register (U.S.)
fw	Fresh weight
GCC	Guanine – Cytosine – Cytosine
GDU	Growing degree units
Hind III	Restriction endonuclease that cuts DNA at specific locations

KEY TO ABBREVIATIONS (cont.)

IPM	Integrated Pest Management
IRM	Insect Resistance Management
kb	Kilobase pairs
kDa	Kilodaltons
LC_{50}	Median lethal concentration
MCRW	Mexican corn root worm
Mlu I	Restriction endonuclease that cuts DNA at specific locations
MON 846	Nontransgenic parental hybrid corn line (A634 x A1)
MON 863	Transgenic corn event that produces a genetically enhanced variant of the insecticidal protein, Cry3Bb1
mRNA	Messenger RNA
MW	Molecular weight
NASS	National Agricultural Statistics Service
NCGA	National Corn Growers Association
Nco I	Restriction endonuclease that cuts DNA at specific locations
Nde I	Restriction endonuclease that cuts DNA at specific locations
NCR-46	North Central Regional Working Group-46; comprised of cooperators from 13 states and the USDA that work on corn rootworm
NCRW	Northern corn rootworm
NDF	Neutral detergent fiber
NOEC	No observable effect concentration
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	The protein neomycin phosphotransferase type II
OECD	Organization for Economic Cooperation and Development
ORF	Open reading frame
ori-pUC	Origin of replication
PCR	Polymerase chain reaction
pdegen	Percent degenerated seed
pfms	Percent viable firm-swollen
pgerm	Percent germinated
ppm	Parts per million
pvhs	Percent viable hard
PV-ZMIR13	Plasmid vector containing the genes of interest
PV-ZMIR13L	Linear fragment of the vector PV-ZMIR13 used for transformation of event MON 863
ract1 intron	Intron from the rice actin gene
RDR	Root damage rating
RNA	Ribonucleic acid
R_0	Designation for originally transformed plant

KEY TO ABBREVIATIONS (cont.)

SCRW	Southern corn rootworm
S.D.	Standard deviation
Sh*ble*	Bleomycin binding protein from *Streptoalloteichus hindustanus*
sp	Species
Subsp.	Subspecies
tahsp17 3'	Coding sequence for wheat heat shock protein 17.3
T-DNA	Transferred DNA
Tn5	Transposon 5
tRNA	Transfer RNA
U.S.	United States
U.S.C.	United States Code
USDA	United States Department of Agriculture
wt CAB	Leader of wheat chlorophyll a/b-binding protein
WCRW	Western corn rootworm

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

I. Rationale for Development of MON 863

A. Basis For Determination of Nonregulated Status Under 7 CFR Part 340.6

APHIS of the United States Department of Agriculture (USDA) has been delegated responsibility under the Federal Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. 7701-7772) for preventing the introduction and dissemination of plant pests in the United States. This statute vests APHIS with the authority to regulate the introduction of organisms and products altered through genetic engineering which are plant pests or which have the potential to be reasonably considered plant pests. APHIS has promulgated regulations entitled "Petition for Determination of Nonregulated Status" (7 CFR Part 340.6), which allow for any person to submit a petition to seek determination that an article should not be regulated under this statute. These regulations also define the nature of data and information that APHIS will consider in evaluation of a petition for nonregulated status. If APHIS determines that the regulated article does not present a plant pest risk, the petition will be granted, thereby allowing for unrestricted release and interstate movement of the article.

B. Corn Rootworm Protected Corn Event MON 863

Corn is the largest U.S. crop in terms of acreage planted and net crop value. According to statistics compiled by the National Corn Growers Association, the 2000 U.S. corn crop covered 79.5 million acres, had an overall yield of 10 billion bushels from an average yield of 137.1 bushels per harvested acre, and had a net value of $18.4 billion (NCGA, 2001).

Corn yields are negatively impacted by a number of insect pests. One of the most pernicious insect pests in the U.S. Corn Belt is the corn rootworm complex (CRW: Coleoptera, *Diabrotica* sp.), comprised primarily of *D. virgifera*, the western corn rootworm (WCRW), and *D. barberi*, the northern corn rootworm (NCRW). CRW larvae damage corn by feeding on the roots, which reduces the ability of the plant to absorb water and nutrients from the soil (Reidell, 1990), and causes harvesting difficulties due to plant lodging (Spike and Tollefson, 1991). CRW is the most significant insect pest problem for corn production in the U.S. Corn Belt from the standpoint of chemical insecticide use. Over 15 million acres of corn were treated with organophosphate, carbamate and pyrethroid insecticides to control CRW in 2000 (Thiemann, 2001). CRW has been described as the billion dollar pest complex, based on costs associated with the purchase of soil insecticides and crop losses due to CRW damage (Metcalf, 1986).

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are protected from damage due to CRW feeding. These plants have been genetically engineered to produce a modified *Bacillus thuringiensis* (subspecies *kumamotoensis*) Cry3Bb1 protein that is lethal to CRW species. Corn Rootworm Protected Corn Event MON 863, hereafter referred to as MON 863, is the subject of this petition for nonregulated status. The introduction of this product will offer U.S. farmers a

safe and effective alternative to the use of synthetic chemical insecticides for control of CRW pests.

B.t. proteins have a long history of safe and widespread use (EPA, 1998; McClintock *et al.*, 1995). The deduced amino acid sequence of the *B.t.* protein produced in MON 863 is 98.9% identical to that of the wild type *B.t.* Cry3Bb1 protein contained in the topically-applied commercial product, *Raven* Oil Flowable Bioinsecticide (EPA Reg. No. 55638-27). The protein poses minimal risk to mammals, wildlife and nontarget insects, and is not expected to accumulate or persist in the environment. In addition, corn containing event MON 863 has not been altered in any way other than by engineering of its resistance to CRW feeding damage. As such, corn event MON 863 is not altered in its plant pest potential relative to nontransgenic corn.

C. Coordination with Other U.S. Federal Agencies

Corn event MON 863 falls within the scope of the Food and Drug Administration's (FDA) policy statement concerning regulation of products derived from new plant varieties, including those produced through genetic engineering (FDA, 1992). Monsanto has voluntarily initiated and will complete a consultation process with FDA prior to commercial distribution of this product. A safety and nutritional assessment of food and feed derived from corn event MON 863 was submitted to FDA on September 25, 2000.

Substances that are pesticides as defined under the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. §136(u)) are subject to regulation by the Environmental Protection Agency (EPA). An application for registration of "*Bacillus thuringiensis* Cry3Bb Protein and the Genetic Material (Vector ZMIR13L) Necessary for its Production in Corn" was submitted to EPA on June 20, 2000. EPA has recently published a rule to establish an exemption from the requirement of a tolerance for *B.t.* Cry3Bb1 proteins, pursuant to §408(d) of the Federal Food Drug and Cosmetic Act (21 U.S.C. §346a(d)). EPA has previously established an exemption from the requirement of a tolerance for the NPTII protein and the genetic material necessary for its production in or on all agricultural commodities (40 CFR Part 180.1134). Field testing of MON 863 has been conducted under an experimental use permit issued by EPA (Permit No. 524-EUP-93).

II. The Biology of Corn

Corn (*Zea mays* L.), or maize, is one of the few major crop species indigenous to the Western Hemisphere. Corn is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) in total production. Corn has been studied extensively, and it seems the probable domestication of corn was in southern Mexico more than 7,000 - 10,000 years ago. The putative parents of corn have not been recovered, but it is likely teosinte played an important role in the genetic background of corn. The transformation from a wild, weedy species to one dependent on humans for its survival probably evolved over a long period of time by the indigenous inhabitants of the Western Hemisphere. Corn, as we known it today, cannot survive in the wild, because

the female inflorescence (the ear) restricts seed dispersal. Although grown extensively throughout the world, corn is not considered a persistent weed nor one difficult to control. A summary of the history, taxonomy, genetics, and life cycle of corn is presented, followed by a discussion of how the characteristics of cultivated corn affect gene flow between cultivated corn and its wild relatives.

This summary of corn biology was prepared with the assistance of Dr. Arnel R. Hallauer (Department of Agronomy, Iowa State University). Other summaries of corn biology are available in the public domain (Plant Biotechnology Office, 1994; Lackey, http://www.aphis.gov/biotech/corn). To date, a consensus document on the biology of corn has not been developed as part of the Organization for Economic Co-operation and Development (OECD) Series on Harmonization of Regulatory Oversight in Biotechnology.

A. History of Corn

Corn originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus discovered the Western Hemisphere, corn was being grown by the indigenous civilizations from Chile to southern Canada. Columbus noted the presence of corn on the north coast of Cuba on November 5, 1492 and introduced corn to Europe upon his return to Spain (Goodman, 1988). Within two generations after the introduction of corn to Europe, corn became distributed throughout those regions of the world where it could be cultivated. Today, corn ranks third after wheat and rice as one of the world's three leading food crops. However, unlike wheat and rice, the majority of corn produced in the U.S. is consumed by livestock. In the tropics and in the Southern Hemisphere corn is a significant component of the human diet.

The original corn growing areas did not include the north-central area (U.S. Corn Belt) of the United States. The highly productive U.S. Corn Belt dent corns were derived after the colonization of North America. The European settlers accepted the local native American varieties and incorporated them with other crops to provide food, feed, and fuel for their survival. The current U.S. Corn Belt dent corns evolved from the gradual mingling of those settlements that spread north and west from southeastern North America and those settlements that spread south and west from northeastern North America.

The corn types grown in the northeast are called northern flints; their origin is not clear, but races from the highlands of Guatemala have similar ear morphology (Goodman and Brown, 1988). Northern flints are largely eight-rowed with cylindrical ears, are early maturing, and are short statured plants with tillers. The southern dent corns grown in the southeast United States seemed to have originated from the southeast coast of Mexico. Southern dent corns are characterized as having tall, late maturing, non-tillered, poorly rooted plants with soft-textured white kernels on many-rowed, tapering ears. It seems the Tuxpeno race contributed to the development of southern dents. The intentional and/or unintentional crossing between the early northern flints and late southern dents led

eventually to the highly productive U.S. Corn Belt dent corns that are used extensively throughout the world today.

The origin of corn has been studied extensively, and hypotheses for the origin and for the parentage of corn have been advanced (Mangelsdorf, 1974). Hypotheses suggested for the origin of corn include the following: 1) cultivated corn is a descendent of pod corn; 2) corn originated by direct selection from teosinte; 3) corn, teosinte, and *Tripsacum* descended independently from a common, unknown ancestor; and 4) the tripartite theory: a) corn originated from pod corn, b) teosinte derived from a cross of corn and *Tripsacum*, and c) modern corn varieties evolved by corn intercrossing with teosinte or *Tripsacum* or both (Mangelsdorf, 1974).

It has been suggested that modern corn originated from corn grass by a single-gene mutation causing ear development. Other suggestions have included *Coix* and species of the genus *Manisuris* in the tribe *Andropogoneae* for contributing to the genome of corn. The hypotheses have been tested by the study of crosses for genome commonality, fertility, variation, and segregation of morphological plant traits, by archeological evidence, and by use of molecular genetic markers.

Evidence has been reported to support the different hypotheses, but it seems that the preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). The teosinte genome is similar to corn, teosinte easily crosses with corn, and teosinte has several plant morphological traits similar to corn. Teosinte has a more weedy appearance and more tillers than modern corn varieties. The one major distinguishing difference between corn and teosinte is the female inflorescence, or ear. Modern corn varieties have 1 to 3 lateral branches that terminate in an ear with 8 to 24 kernel rows of 50 seeds, and the ear is enclosed in modified leaves or husks. Teosinte also has lateral branches, but they terminate in two-rowed spikes of perhaps 12 fruit cases, with each fruit case having one seed enclosed by an indurated glume (Goodman, 1988).

B. Taxonomy of the Genus *Zea*

Corn is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae (Table 1). The genera included in the tribe Maydae include *Zea* and *Tripsacum* in the Western Hemisphere and *Coix*, *Polytoca*, *Chionachne*, *Schlerachne*, and *Trilobachne* in Asia. Although the Asian genera have been implicated by some in the origin of corn, the evidence for them is not as extensive and convincing as for the genera located in the Western Hemisphere.

There has been some fluctuation in Latin binomial designations of the species included in *Zea* in recent years and the classification will be used herein (Doebley and Iltis, 1980). The genus *Zea* includes two subgenera: *Luxuriantes* and *Zea*. Corn (*Zea mays* L.) is a separate species within the subgenus *Zea*, along with three subspecies. All of the species

Table 1. Taxonomic classification of corn and its closely related relatives.

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

T. andersomii (2n = 64)	*T. latifolium* (2n = 36)
T. australe (2n = 36)	*T. percuvianum* (2n = 72, 90, 108)
T. bravum (2n = 36, 72)	*T. zopilotense* (2n = 36, 72)
T. cundinamarce (2n = 36)	*T. jalapense* (2n = 72)
T. dactyloides (2n = 72)	*T. lanceolatum* (2n = 72)
T. floridanum (2n = 36)	*T. fasciculatum* (2n = 36)
T. intermedium (2n = 72)	*T. maizar* (2n = 36, 72)
T. manisuroides (2n = 72)	*T. pilosum* (2n = 72)

    - Asia:
    - I. Genera—

Chionachne (2n = 20)	*Schlerachne* (2n = 20)
Coix (2n = 10, 20)	*Trilobachne* (2n = 20)
Polytoca (2n = 20)	

  - Tribe—Andropogoneae
    - I. Genus - *Manisuris*

within the genus *Zea*, except corn, are different species of teosinte. Until recently, the teosinte species were included in the genus *Euchlaena* rather than the genus *Zea*.

The other genus included in the Maydae tribe is *Tripsacum*. *Tripsacum* includes 16 species with a basic set of 18 chromosomes (n = 18), and the different species of *Tripsacum* include multiples of 18 chromosomes ranging from 2n = 36 to 2n = 108.

Five genera are included in the tribe Maydeae that originated in Asia. Except for *Coix*, the basic chromosome number is n = 10. Within *Coix*, n = 5 and n = 10 have been reported.

C. Genetics of Corn

Corn is genetically one of the best developed and best characterized of the higher plants. Because of the separation of male and female inflorescence, number of seeds produced on female inflorescence, ease in handling (growing and hand pollinating), nature of the chromosomes, and low basic chromosome number (n = 10), corn has been accessible for study at all levels of genetics.

Corn was one of the first crop species included in genetic laboratories to obtain a basic understanding of mitosis, meiosis, chromosome segregation, linkage and effects of crossing-over, and transposable elements. Because of the importance of corn in the U.S. and world economies, and the genetic information obtained since 1900, corn has continued to receive extensive study in modern genetic laboratories.

Molecular geneticists have developed extensive genetic maps of corn to complement those developed by the early corn geneticists. Corn has been used in tissue culture research, in extensive studies to relate molecular markers to qualitative and quantitative traits, in sequencing of genes, in study of transposable elements for gene tagging and generating genetic variability, and in gene transformation (Coe *et al.*, 1988; Carlson, 1988; Phillips *et al.*, 1988; Walbot and Messing, 1988).

D. Life Cycle of Corn

Corn is an annual plant and the duration of its life cycle depends on the cultivars and on the environments in which the cultivars are grown (Hanway, 1966). Corn cannot survive temperatures below 0°C for more than 6 to 8 hours after the growing point is above ground (5 to 7 leaf stage). Damage from freezing temperatures, however, depends on the extent of temperatures below 0°C, soil condition, residue, duration of freezing temperatures, wind movement, relative humidity, and stage of plant development. Light frosts in the late spring of temperate areas can cause leaf burning, but the extent of the injury usually is not great enough to cause permanent damage, although the corn crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. The completion of the life cycle of corn, therefore, is dictated by the duration of the average number of frost-free days.

The number of frost-free days dictates that corns with differences in length of their life cycles be grown in north-to-south directions of temperate areas. In the United States, corns with relative maturities of 80 days or less are grown in the extreme northern areas, and corns with relative maturities of more than 125 days are grown in the southern areas. Corns having relative maturities of 100 to 115 days are typically grown in the U.S. Corn Belt. Relative maturities, however, are not parallel lines east-to-west because they are dependent on prevailing weather patterns, topography, large bodies of water, and soil types (Troyer, 1994).

Another measure used to judge the relative maturities of corns is the number of growing degree units (GDU) required from emergence to maturity. Based on GDU required to mature, corns are assigned to areas that have, on the average, less than 1850 GDU in the extreme northern areas of the United States to corns that require more than 2750 GDU in more southern areas. Assume a 115-day maturity hybrid is grown in central Iowa. Average last frost date is May 1 and average first frost date is October 5, resulting in an expected 158 frost-free days. If average emergence is May 15 and average flowering is July 15, 60 days are required from emergence to flowering. Corn requires 50 to 60 days to attain physiological maturity. If physiological maturity occurs 55 days after flowering, physiological maturity will occur on or about September 10, or 115 days from emergence to physiological maturity.

If one considers the central U.S. Corn Belt as an example, the following time-frame for each stage of corn development could be as follows:

Planting date: May 1 ± 10 days
Date of emergence: May 10 ± 4 days
Date of flower: July 20 ± 10 days
Physiological maturity: September 10 ± 5 days
Harvest maturity: October 10 ± 10 days

These estimated time frames could vary within the same year among locations and among years at the same location, depending on the environmental conditions experienced from planting to harvesting.

E. Hybridization

Hybridization is a fundamental concept used in the breeding, production, and growing of corn in the United States. Corn evolved as an open-pollinated (cross-fertilizing) crop species and until the 20th century the corn cultivars were what we designate today as open-pollinated corn varieties. Because corn is essentially 100% cross pollinated, the corn varieties were a collection of heterozygous and heterogeneous individuals (genotypes). Varieties were developed by simple mass selection by the indigenous natives prior to the arrival of Columbus. Their methods of selection were simple by present-day standards, but they were obviously effective in developing races, varieties, and strains to satisfy their food, fuel, feed, and cultural needs. Hybridization occurred between varieties as cultures moved within the Western Hemisphere, releasing genetic variability to develop other unique varieties.

The fundamental concepts for development of hybrid corn were defined by 1920 (Sprague, 1946). Basic studies on the genetic composition of a corn variety were conducted to determine the effects of self pollinating (or inbreeding which is the opposite of outcrossing) within a corn variety (Shull, 1908). Because corn naturally cross-fertilizes, the genetic composition of each plant is not known. Continuous selfing of individuals for 7 to 10 generations resulted in pure lines (or inbred lines) within which

every plant had similar traits. The correct interpretation of what occurred during inbreeding was based on Mendelian genetics: the heterozygous loci were eliminated by inbreeding to homozygous loci of either one of the two alleles at each locus. The fixation of alleles in pure lines caused a general reduction in vigor and productivity.

It was found upon crossing two pure lines that vigor was restored. If no selection occurred during inbreeding, the average performance (*e.g.*, grain yield) of all possible crosses was similar to performance of the original variety in which inbreeding was initiated. Some crosses, however, were better than the original open-pollinated variety and could be reproduced from the cross of the pure-line parents of the cross. Hence, the concept of hybrid corn was determined: self to develop pure lines, cross the pure lines to produce hybrids, evaluate hybrids to determine the best hybrid, and use of pure-line parents to reproduce the superior hybrid and distribute it for use by the growers (Shull, 1909).

Hybridization is used in many phases of corn breeding because of the expression of heterosis. Hybridization is used to produce breeding populations (*e.g.*, F_2) to develop inbred lines for use in hybrids, and hybridization is used to produce the crosses of superior lines for distribution to growers. Hybridization is easily accomplished either by hand pollination or by wind pollination in large crossing fields (male and female inbred lines) to produce large quantities of high quality hybrid seed.

F. Pollination

1. Outcrossing with Wild *Zea* Species

Annual teosinte (*Zea mays* subsp. *mexicana*) and corn are wind pollinated, self-compatible, and are highly variable, interfertile species (Wilkes, 1972 and 1989). Corn and teosinte are genetically compatible, and in areas of Mexico and Guatemala they freely hybridize when they are in proximity to each other and other conditions are favorable. Teosinte exists primarily as a weed around the margins of corn fields, and the frequency of hybrids between teosinte and corn has been studied. A frequency of one F_1 hybrid (corn x teosinte) for every 500 corn plants or 2 to 5% of the teosinte population for the Chalco region of the Valley of Mexico has been reported (Wilkes, 1972). This frequency of hybrids represents a significant gene exchange between a wild weedy plant (*i.e.*, teosinte) and a cultivated relative (*i.e.*, corn). The F_1 hybrid of teosinte by corn is robust and fertile and is capable of backcrossing to corn. Intercrossing and gene exchange between teosinte and corn occurs freely, and, accompanied by selection, teosinte had a significant role in the evolution of corn.

Although corn easily crosses with teosinte, teosinte is not present in the U.S. Corn Belt. The natural distribution of teosinte is limited to the seasonally dry, subtropical zone with summer rain along the western escarpment of Mexico and Guatemala and the Central Plateau of Mexico (Wilkes, 1972; Gonzalez and Corral, 1997). Dependent upon the human characterization of teosinte with its local environment, it may be considered a

weed. However, it has been noted that populations of teosinte have been in decline for several decades due to increased grazing and urbanization in Mexico (Wilkes, 1995). Except for special plantings, there are no reports of teosinte occurring in the United States.

Tripsacum-corn hybrids have not been observed in the field and *Tripsacum*-teosinte hybrids have not been produced (Wilkes, 1972). *Tripsacum* evolved by polyploidy, whereas corn and teosinte have undergone introgressive hybridization at the diploid level (2n = 20). The diploid forms of *Tripsacum* (2n = 36) are morphologically distinct and allopathic in their distribution (Wilkes, 1989). *Tripsacum* species are perennials and seem to be more closely related to the genus *Manisuris* than to either corn or teosinte (Goodman, 1976). *Tripsacum* received greater interest in the evolution of corn after Mangelsdorf and Reeves (1931) successfully crossed corn and *Tripsacum dactyloides* (2n = 36). The cross by Mangelsdorf and Reeves (1931) was made with the diploid *Tripsacum dactyloides* (2n = 36) as the male parent. Silks of the female corn parent were cut to permit successful pollination. The cross had 28 chromosomes and was male sterile. Five other *Tripsacum* species have been crossed with corn, and Galinat (1988) has mapped more than 50 homologous loci on the chromosomes of corn and *Tripsacum*. In contrast with corn and teosinte, which can be easily hybridized, both in the wild and by controlled pollinations, special techniques are required to hybridize corn and *Tripsacum*. Except for *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with corn, and the offspring of the cross show varying levels of sterility. Small portions of *Tripsacum* genome can be incorporated by backcrossing.

Sixteen species of *Tripsacum* have been described (Table 1). Five species are found in the U.S. (USDA, 2001). *Tripsacum floridanum* is native to the southern tip of Florida. *Tripsacum lanceolatum* is native to Arizona and New Mexico. *Tripsacum dactyloides* is native to the mid-west, eastern and southern U.S.. *Tripsacum fasciculatum* is native to Puerto Rico. *Tripsacum hermaphrodita* (*Anthephora hermaphrodita*) is native to Florida, Puerto Rico, the Virgin Islands and Hawaii. Twelve of 16 *Tripsacum* species are native to Mexico and Guatemala. *Tripsacum australe* and two other species are native to South America. The center of variation for *Tripsacum* is the western slopes of Mexico, the same area where teosinte is frequently found. The habitat preferences of *Tripsacum* are similar to those for teosinte: seasonally dry, summer rains, elevation of 1500 m, and limestone soils (Wilkes, 1972).

2. Outcrossing with Cultivated *Zea* Varieties

Corn is wind pollinated, and the distances that viable pollen can travel depend on prevailing wind patterns, humidity, and temperature. Occasionally it has been found that corn pollen can travel up to 3.2 km (2 miles) by wind under favorable conditions. All corns will interpollinate, except for certain popcorn varieties and hybrids that have one of the gametophyte factors (Ga^S, Ga, and ga allelic series on chromosome 4). Pollen of a specific hybrid can be carried by wind to pollinate other dent corn hybrids, sweet corn, and popcorn if the popcorn does not carry the dent-sterile gametophyte factor. Corn

pollen, therefore, moves freely within an area, lands on silks of the same cultivar or different cultivars, germinates almost immediately after pollination, and within 24 hours completes fertilization. Although there may be some minor differences in rate of pollen germination and pollen tube elongation on some genotypes, corn pollen is very promiscuous. It is estimated each corn plant can shed more than 10 million pollen grains.

Certification standards for distances between different corn genotypes have been established to assist in the production of hybrid corn having desired levels of purity. A specific isolation field to produce commercial hybrid seed shall be located so that the seed parent is no less than 200 m (640 feet or 40 rods) from other corn of a similar type (*i.e.*, if seed parent is a yellow, dent corn it should be isolated at least 200 m from other yellow, dent corns). The distance of 200 m can be modified because of size of field, number of border rows, and different maturity dates of flower, provided no receptive silks are available at the time pollen is being shed from the contaminating field. If the hybrid seed being produced is of a different color or texture from neighboring contaminating fields, the distances and the number of border rows should be increased.

G. Weediness of Corn

Modern day corn cannot survive outside of cultivation (Gould, 1968). One does not find volunteer corn growing in fence rows, ditches, and roadsides as a weed. Although corn from the previous crop year can overwinter and germinate the following year, it cannot persist as a weed. The appearance of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with a hoe or use of herbicides to kill the corn plants in soybean fields, but the plants that remain and produce seed usually do not persist inthe following years.

It is difficult for corn to survive as a weed because of past selection in the evolution of corn. In contrast with weedy plants, corn has a polystichous female inflorescence (or ear) on a stiff central spike (or cob) enclosed with husks (modified leaves). Consequently, seed dispersal of individual kernels naturally does not occur because of the structure of the ears of corn. Individual kernels of corn, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities. In neither instance (natural or mechanical harvesting) does corn become a troublesome weed. Corn cannot survive without human assistance and is not capable of surviving as a weed.

H. Characteristics of the Recipient Corn Material

The 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 GDU to flower. In the most popular hybrid, Mo17 x A634, it is used as the male parent. In the early 1980's, A634 was among the five most popular public inbreds used in U.S. hybrid production. It has also been widely used to develop new inbred lines.

III. Description of the Transformation System

A purified DNA fragment of the Monsanto plasmid vector, PV-ZMIR13, was used for transformation of corn to create event MON 863. Figure 1 displays a plasmid map of PV-ZMIR13. This plasmid was amplified in *E. coli* and purified from bacterial lysates.

DNA was introduced into tissue from inbred corn line A634 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 one or more plant chromosomes. The plant cells were incubated on a tissue culture medium containing 2,4-D that supported callus growth. The introduced DNA contained genes encoding resistance to the antibiotic, paromomycin (*i.e.*, *nptII*). When grown in the presence of paromomycin, only successfully transformed cells continued to grow. Plants were regenerated from the tolerant callus tissue and assayed for the presence of modified Cry3Bb1 by enzyme-linked immunosorbant assay (ELISA) methods.

IV. Donor Genes and Regulatory Sequences

A purified DNA fragment of the Monsanto plasmid vector, PV-ZMIR13, was used for transformation of corn event MON 863.

A. Vector PV-ZMIR13L

Corn event MON 863 was produced by particle gun acceleration technology using a purified DNA fragment obtained from a restriction enzyme digestion of plasmid PV-ZMIR13. This linear DNA fragment is designated PV-ZMIR13L and is diagrammatically displayed in Figure 2. PV-ZMIR13L was prepared by digestion of the plasmid with the restriction endonuclease *Mlu* I. 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, rice actin intron, and tahsp17 3' transcriptional termination sequence. The *nptII* expression cassette consists of the *nptII* coding region

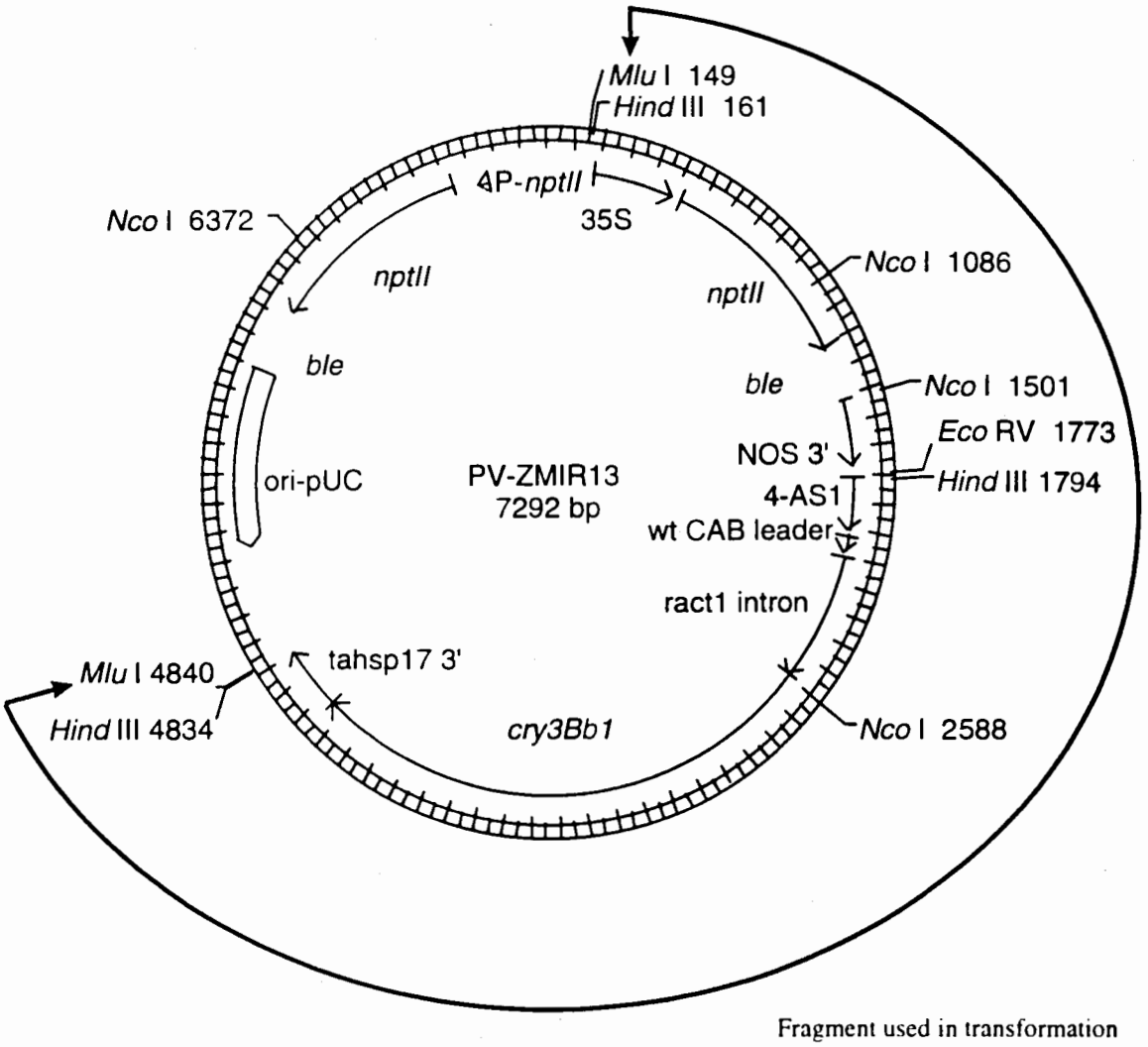
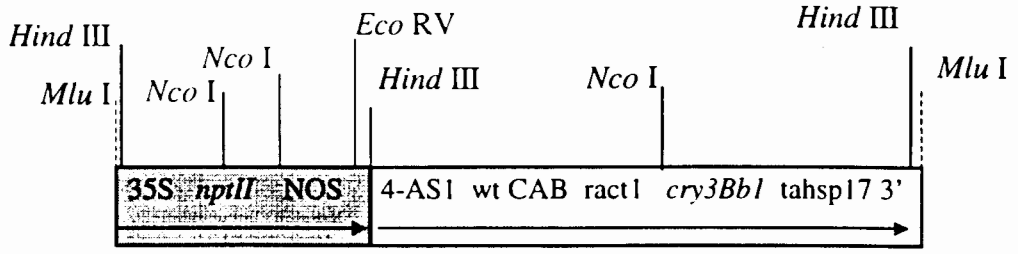


Figure 1. Plasmid map of PV-ZMIR13. Plasmid PV-ZMIR13 was the source of the *Mlu* I fragment used in the transformation of corn event MON 863.

regulated by the 35S promoter, and the NOS 3' transcriptional termination sequence. A description of each of these elements is contained in Table 2.

B. The *cry3Bb1* Coding Sequence

In 1991, Rupar *et al.* reported discovery of a novel *B.t.* strain (EG4691) that produced a crystal protein which displayed 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 (GenBank Accession No. M89794). Following the adoption of standardized nomenclature for identifying *B.t.*



PV-ZMIR13L
4691 bp

Figure 2. Linear map of DNA fragment PV-ZMIR13L. The purified linear fragment, PV-ZMIR13L, was introduced by particle acceleration technology into corn tissue to produce transgenic event MON 863. The dashed lines represent the remaining *Mlu* I half sites following digestion of the plasmid.

crystal proteins, the protein isolated from strain EG4691 was renamed Cry3Bb1.

Wild type Cry3Bb1 is present in the commercial product, *Raven* Oil Flowable Bioinsecticide, which has been sold in the U.S. since 1995 for control of Coleopteran pests. Cry3Bb1 shares approximately 67% amino acid sequence identity with another Cry3 protein, Cry3Aa4 (GenBank Accession No. M30503), which has been commercially used in the U.S. and other countries for control of the Colorado potato beetle, a major pest of potatoes (Perlak *et al.*, 1993).

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. Linking the genes to a strong constitutive plant expressible promoter and optimizing their codons for expression in monocotyledonous plants were necessary to achieve meaningful levels of foreign protein production in applicable tissues (Fischhoff and Perlak, 1996; Brown *et al.*, 1997). To facilitate linkage of the redesigned *cry3Bb1* coding sequence 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 2 of the encoded protein. When sequence aligning *Bacillus* and plant-produced proteins, an adjustment needs to be made to account for this frame shift alteration.

Table 2. Genetic elements present in the *Mlu* I restriction fragment PV-ZMIR13L.

Genetic Element	Size (kb)	Function
***cry3Bb1* gene cassette:**		
4-AS1	0.22	Promoter containing four tandem copies of AS1 and a single portion of the 35S promoter of CaMV (Lam and Chua, 1990; Odell *et al.*, 1985)
wt CAB	0.06	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa *et al.*, 1985)
ract1 intron	0.49	Intron from the rice actin gene (McElroy *et al.*, 1990)
cry3Bb1	1.96	Coding sequence for a synthetic variant of Cry3Bb1 protein from *Bacillus thuringiensis* subsp. *kumamotoensis* (English *et al.*, 2000)
tahsp 17 3'	0.23	3' nontranslated region of the coding sequence for wheat heat shock protein 17.3 which ends transcription and directs polyadenylation (McElwain and Spiker, 1989)
Selectable marker elements:		
35S	0.35	CaMV promoter (Odell *et al.*, 1985)
nptII	0.97	Coding sequence for the enzyme NPTII from an *Escherichia coli* transposon (Tn5). The DNA derived from *E. coli* also includes a 0.153 kb segment of the *ble* gene (Beck *et al.*, 1982)
NOS 3'	0.26	3' nontranslated region of the nopaline synthase gene of *Agrobacterium tumefaciens* T-DNA that ends transcription and directs polyadenylation (Bevan *et al.*, 1983)

Nucleotides comprising the transgenic insert in event MON 863 have been sequenced by Monsanto (Hileman and Astwood, 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 Time of Flight mass spectrometric techniques (Thoma *et al.*, 2001). The Cry3Bb1 variant produced in MON 863 differs from the wild type Cry3Bb1 protein by seven amino acids. These differences are: 2A, D166G, H232R, S312L, N314T, E318K and Q349R. The amino acid sequence for the MON 863 protein is displayed in Table 3. This variant shares 98.9% sequence identity with the wild type Cry3Bb1 protein.

Table 3. Deduced amino acid sequence of the Cry3Bb1 protein produced in event MON 863. Amino acid differences from the wild type protein are underscored (_). The position designation for each amino acid substitution differs from that in the *B.t.* produced-protein due to the insertion of an alanine residue at the #2 position in the plant produced- protein.

1	MANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT
51	EDSSTEVLDN STVKDAVGTG ISVVGQILGV VGVPFAGALT SFYQSFLNTI
101	WPSDADPWKA FMAQVEVLID KKIEEYAKSK ALAELQGLQN NFEDYVNALN
151	SWKKTPLSLR SKRSQGRIRE LFSQAESHFR NSMPSFAVSK FEVLFLPTYA
201	QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ YTDHCVNWYN
251	VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE
301	LTRDIFTDPI FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP
351	GYFGKDSFNY WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK
401	VYRTIANTDV AAWPNGKVYL GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH
451	VSAQDSIDQL PPETTDEPLE KAYSHQLNYA ECFLMQDRRG TIPFFTWTHR
501	SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN LLFLKESSNS
551	IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN
601	KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP
651	VQL

C. 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 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 that 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 the *nptII* coding sequence under the control of a 35S CaMV promoter (Odell, *et al.*, 1985). The *nptII* gene originated from the *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 segment 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). 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 sequence. In prokaryotic organisms, mRNAs that contain tandem ORFs are termed polycistronic and each ORF in a polycistron is translated independently, yielding unique proteins (Kozak, 1999). Polycistrons of nonviral origin are extremely rare in eukaryotic organisms. Due to differences in the mechanism for initiation of translation between prokaryotic and eukaryotic organisms, it is highly unlikely that the partial *ble* ORF will be translated into protein in MON 863. Specifically, in eukaryotic organisms such as corn, the small ribosomal subunit identifies a unique nucleotide, the mRNA 5' 7-methylguanylate cap, binds and migrates toward the start codon. As the small subunit migrates it is joined by large ribosome subunits resulting in ribosome assembly and initiation of protein translation at the start codon (Kozak, 1999). In contrast, the ribosome binding sites contained on prokaryotic mRNA function as the site of ribosome assembly. Since protein translation in eukaryotic organisms is dependent upon recognition of a unique 5' mRNA cap and scanning for a start codon to complete ribosome assembly, only the proximal ORF of a polycistron will be translated. For MON 863, this means that *nptII* will be translated, but the *ble* fragment will not.

Bleomycin is a glycopeptide antibiotic that forms a complex with iron (Fe^{+2}). In the presence of molecular oxygen, the bleomycin-iron complex causes nucleotide sequence specific DNA and RNA cleavage. It is this ability to cleave DNA and RNA that forms the basis of bleomycin's antibiotic activity and the reason that it has found only limited therapeutic use as an antineoplastic drug. Bleomycin binding protein from Tn5 (BLMT) is a 14.1 kDa protein that forms homodimers that are capable of binding two molecules of bleomycin (Kumagai *et al.*, 1999). BLMT does not have any intrinsic enzymatic activity, but rather confers bleomycin resistance by binding the bleomycin-iron complex, thereby inhibiting the production of hydroxyl radicals that cause DNA and RNA cleavage.

Like BLMT, Sh*ble*, the bleomycin binding protein from *Streptoalloteichus hindustanus* is a homodimer that binds two bleomycin molecules. X-ray crystallographic analysis has identified 21 amino acids in monomeric Sh*ble* that interact with bleomycin (Dumas, 1994). Furthermore, the Sh*ble* monomer folds into a structure that is composed of symmetrical halves that share an identical α/β fold structure. Amino acids one through

eight of one Sh*ble* monomer interact with a β-strand composed of residues 62 to 68 in the second monomer of a dimer resulting in a tight association between the two monomers due to chain exchange. Using site-directed mutagenesis, Kumagai *et al.*, (1999) have shown that chain exchange is important for stabilizing the BLMT dimer and that BLMT dimerization is necessary for bleomycin binding.

Alignment of the predicted BLMT protein fragment sequence with Sh*ble* shows the following: the breakpoint in the BLMT coding sequence is located at amino acid 51. When aligned with Sh*ble*, amino acid 51 of BLMT is predicted to lie in a loop that joins the two symmetrical halves of the BLMT monomer. Therefore, in the absence of the two symmetrical halves, it is unlikely that the BLMT fragment will dimerize because it can not undergo chain exchange. Futhermore, based upon alignment, the BLMT fragment is predicted to contain only 11 of the 21 residues that are involved in bleomycin binding. If translated, the BLMT fragment will not bind bleomycin because it is unable to dimerize and approximately 50% of the residues that are involved in bleomycin binding are absent.

An origin of replication sequence (*ori*-pUC) is present in PV-ZMIR13 to allow for replication of the plasmid in *E. coli*. Preceding the *ori* region is the sequence that codes for the enzyme NPTII. This enzyme confers resistance to aminoglycoside antibiotics, a feature that is used in bacterial selection during construction of the plasmid.

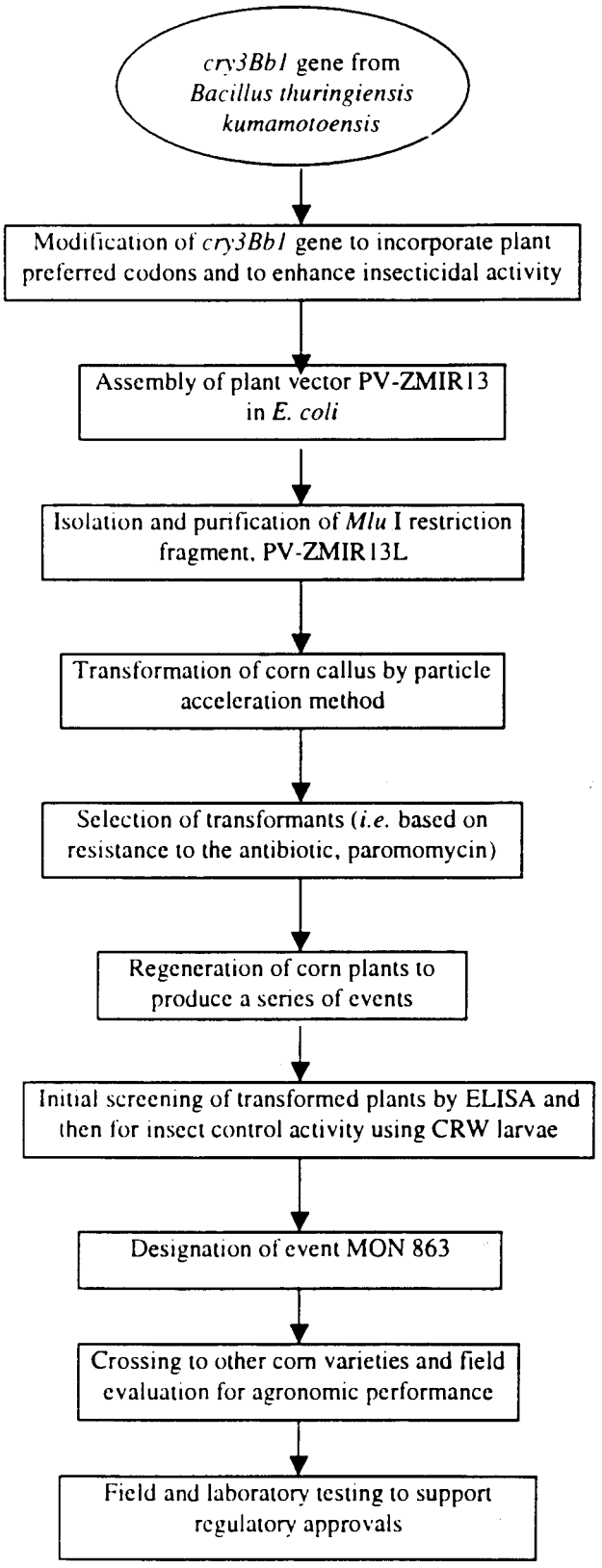
D. NPTII Selectable Marker

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). 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 *nptII* coding sequence is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982). 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 insecticidal coding sequence and that can be used in bacterial selection during construction of the plasmid. In general, the frequency of cells that are transformed is often as low as $1x10^{-4}$ 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.

E. MON 863 Development Process

In the development of a transgenic product such as MON 863, thousands of plant cells are transformed and screened for incorporation of the genes of interest. Figure 3 displays a flow chart of the major steps involved in the transformation, selection, and development of corn event MON 863.

Figure 3. Steps in the Development of MON 863.



V. Genetic Analysis

Molecular analysis was performed to characterize the DNA inserted into corn to produce event MON 863. This analysis demonstrated that MON 863 contains: 1) the *cry3Bb1* coding sequence whose transcription is directed by 4 copies of CaMV activating sequence-1 and a single 35S promoter, a wheat chlorophyll binding protein leader sequence and intron from the rice actin gene, and whose termination and polyadenylation sequences are derived from wheat heat shock protein gene 17.3; 2) the *nptII* coding sequence whose transcription is directed by the 35S CaMV promoter and whose termination sequences are derived from the nopaline synthase gene of *A. tumefaciens*; This transformation cassette was randomly inserted into the corn genome and results in the synthesis of both Cry3Bb1 and NPTII proteins.

A. Molecular Characterization of MON 863

Laboratory analyses were conducted to characterize the inserted DNA in MON 863 (Cavato *et al.*, 2001). 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 to characterize the DNA that was integrated into the plant genome (Southern, 1975). 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 retain all restriction fragments on the gel. The locations of the restriction sites utilized for Southern analyses are shown in the linear map of the DNA fragment PV-ZMIR13L displayed in Figure 2. 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.

MON 863 corn grain from the $A634F_{2a}$ generation was used for the Southern and PCR analyses. Tissue samples were collected under APHIS Notification #99-106-16n. Nontransgenic corn line MON 846 served as a control for the molecular characterization and protein expression studies. MON 846 was produced by crossing inbred line A634 to A1. Table 4 has been prepared as a guide for interpretation of MON 863 Southern blots.

1. Insert Number

The number of integration sites for transgenic DNA in the corn genome was evaluated by digesting MON 863 and control DNA with the restriction endonuclease *Nde* I. This enzyme does not cleave within the DNA fragment used for transformation and would therefore, release a fragment containing both the inserted DNA and adjacent plant genomic DNA. Plasmid PV-ZMIR13 plus control DNA were digested with both *Nde* I and *Eco* RV. Since *Nde* I does not cleave within the plasmid, a second enzyme, *Eco*

Table 4. Guide for interpretation of MON 863 Southern blots. The probe used for hybridization, the restriction enzyme used to cut the DNA, and the expected and observed sizes of hybridization bands are shown for each parameter evaluated in the molecular analysis of corn event MON 863.

Parameter	Probes	Restriction Enzyme	Expected Size of Hybridization Band(s)	Observed Band Size(s)
Insert number	PV-ZMIR13L	*Nde* I	>4.691 kb	5.0 kb
Copy number	PV-ZMIR13L	*Eco* RV	>3.067 kb; >1.624 kb	9.6 kb; 3.7 kb
cry3Bb1 cassette intactness	4-AS1+wtCAB+ract1 *cry3Bb1* tahsp17 3'	*Hind* III	>3.040 kb	3.2 kb
nptII cassette intactness	35S *nptII* NOS 3'	*Hind* III	1.633 kb	1.6 kb
Plasmid backbone	Backbone sequence minus *nptII*	*Hind* III	2.619 kb†	No band

† This band is expected in the plasmid positive hybridization control lanes only, not in the lanes for MON 863.

RV, was added to linearize the plasmid. This was necessary to facilitate migration of the plasmid through the gel so that it could serve as an accurate estimator of fragment size. The blot was probed with radiolabeled PV-ZMIR13L. The number of fragments detected indicates the number of inserts present. The results of this Southern blot are shown in Figure 4. As expected, control DNA produced no detectable bands (lane 1). Plasmid PV-ZMIR13 DNA mixed with control DNA produced a band of approximately 7.3 kb corresponding to the size of the entire plasmid (lanes 3 & 4). MON 863 DNA produced one band of approximately 5.0 kb size (lanes 2 & 5). Since the linear DNA fragment used for transformation was approximately 4.7 kb in size, this result indicates that event MON 863 contains a single insertion of transgenic DNA.

2. Copy Number

The number of copies of the whole transformation cassette inserted into one locus was determined by Southern blot analysis following digestion of genomic DNA with the restriction endonuclease, *Eco* RV. This enzyme cuts only once in the linear DNA fragment used for transformation. MON 863 DNA, control DNA, and control DNA mixed with plasmid PV-ZMIR13 DNA were digested with *Eco* RV. The blot was probed

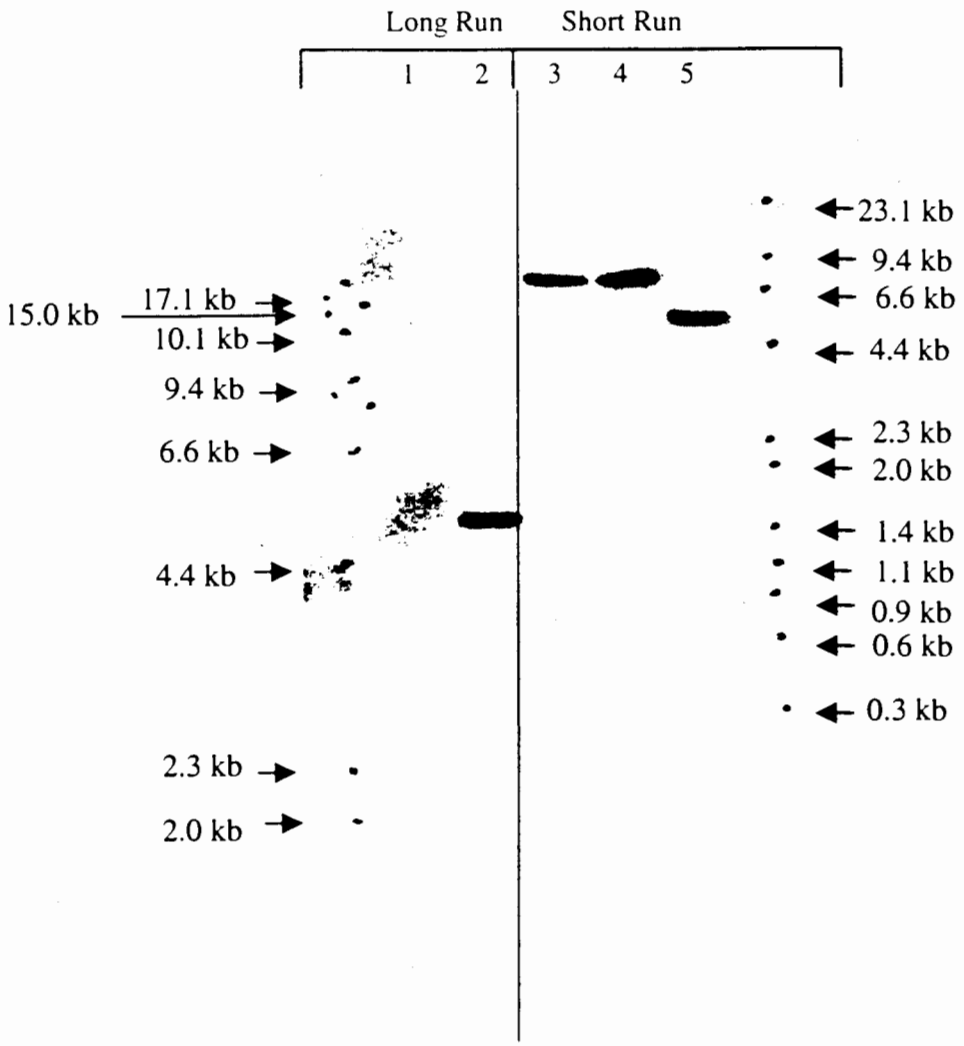


Figure 4. Southern blot analysis of event MON 863: Insert number analysis. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Nde* I. Plasmid PV-ZMIR13 DNA mixed with MON 846 DNA was digested with *Nde* I and *Eco* RV. The blot was probed with ^{32}P-labeled PV-ZMIR13L. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 (1.0 copy)
5: MON 863 [10 µg]

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

with the entire PV-ZMIR13 plasmid and is displayed in Figure 5. One copy of the transformation cassette would be expected to produce two bands, each containing a portion of the inserted DNA and flanking corn genomic DNA. The appearance of additional bands would indicate the presence of multiple copies of the transformation cassette.

Control DNA produced a very faint band at 9.3 kb which is likely due to nonspecific hybridization to the genomic DNA (lane 1). This conclusion is supported by the observation of a similar faint band in the MON 863 lanes (2 & 5). Control DNA mixed with PV-ZMIR13 DNA produced a band of the expected size of 7.3 kb, representing the linearized plasmid (lanes 3 & 4). MON 863 DNA produced two unique bands of size 3.7 kb and 9.6 kb (lanes 2 & 5). This finding supports a conclusion that only a single copy of the transformation cassette is present at the insertion locus.

3. *cry3Bb1* Cassette Intactness

Integrity of the *cry3Bb1* cassette was evaluated by Southern blot following digestion with the restriction endonuclease, *Hind* III, which cleaves at the 5' and 3' ends of the cassette. Digested control DNA was spiked with a 3.0 kb *Hind* III restriction fragment from plasmid PV-ZMIR13 containing the *cry3Bb1* cassette. Individual Southern blots were probed with the 4-AS1 promoter / wt CAB leader / ract1 intron, the *cry3Bb1* coding region, or the tahsp17 3' transcriptional terminator. The results are displayed in Figures 6, 7 and 8, respectively. Bands corresponding to the expected size for an intact *cry3Bb1* cassette were identified. These data support the conclusion that event MON 863 contains only the intact *cry3Bb1* cassette.

a. 4-AS1 Promoter

The lane for control DNA showed no hybridization band, as expected (Figure 6, lane 1). PV-ZMIR13 DNA mixed with control DNA produced a single band of approximately 3.0 kb corresponding to the size of the intact *cry3Bb1* cassette (lanes 3 & 4). MON 863 DNA produced a single band of approximately 3.2 kb which is slightly larger than expected (lanes 2 & 5). Genomic flanking sequence data (discussed in Section V.6) indicates that a small portion (10 bp) of the 3' end of the PV-ZMIR13L linear fragment was not incorporated into the plant genome. This missing portion included the *Hind* III site. There is however, a genomic *Hind* III site found approximately 175 bp downstream of the tahsp17 3' transcription termination sequence. The presence of this additional genomic DNA explains the larger band observed for blots analyzing *cry3Bb1* cassette intactness in MON 863 DNA. The absence of additional bands indicates a single insertion of an intact *cry3Bb1* gene cassette and that MON 863 does not contain any detectable 4-AS1 promoter / wt CAB leader / ract1 intron elements other than those associated with an intact cassette.

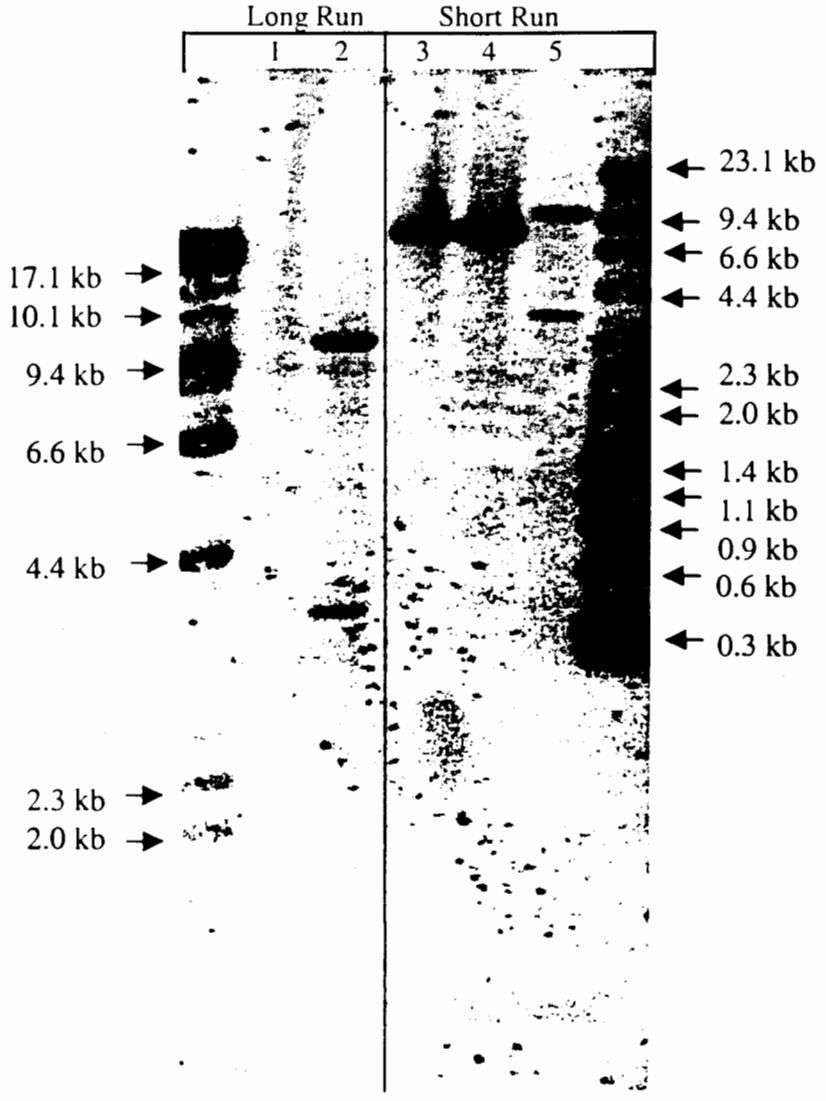


Figure 5. Southern blot analysis of event MON 863: Copy number analysis. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Eco* RV. The blot was probed with ^{32}P-labeled PV-ZMIR13. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg] (Long Run)
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 (1.0 copy)
5: MON 863 [10 µg]

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

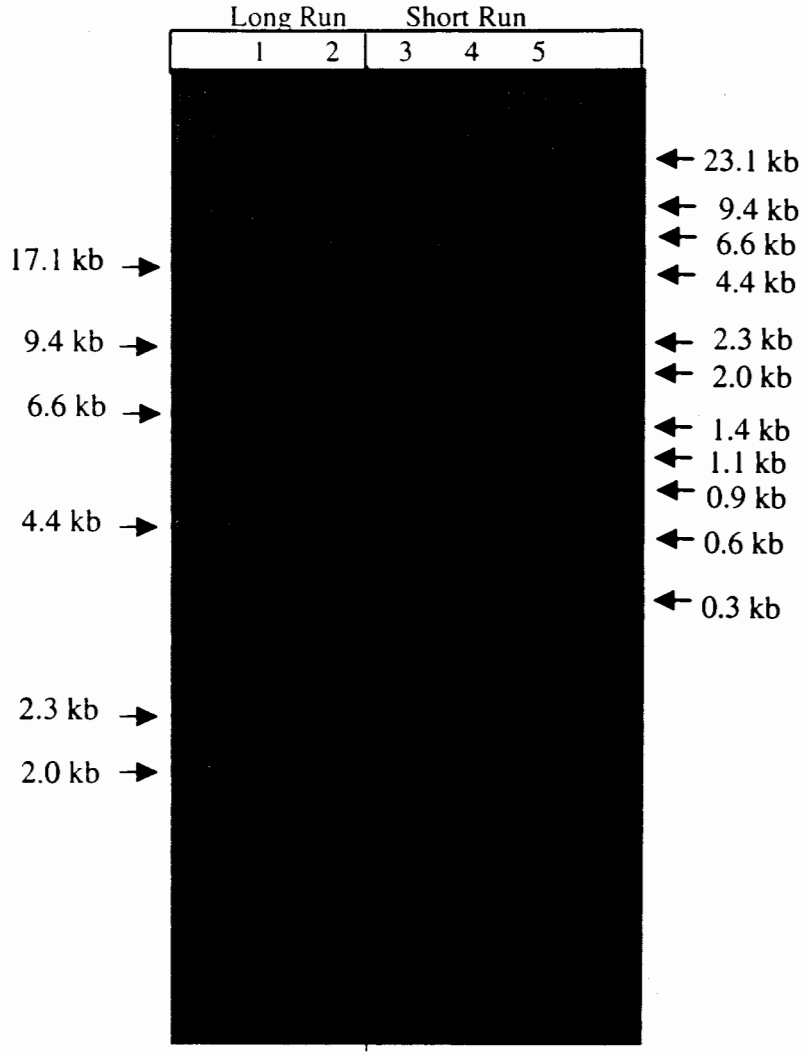


Figure 6. Southern blot analysis of event MON 863: *cry3Bb1* cassette intactness probed with the 4-AS1 promoter / wt CAB leader / ract1 intron. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with ^{32}P-labeled full-length 4-AS1 promoter / wt CAB leader / ract1 intron. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *cry3Bb1* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *cry3Bb1* cassette (1.0 copy)
5: MON 863 [10 µg]

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

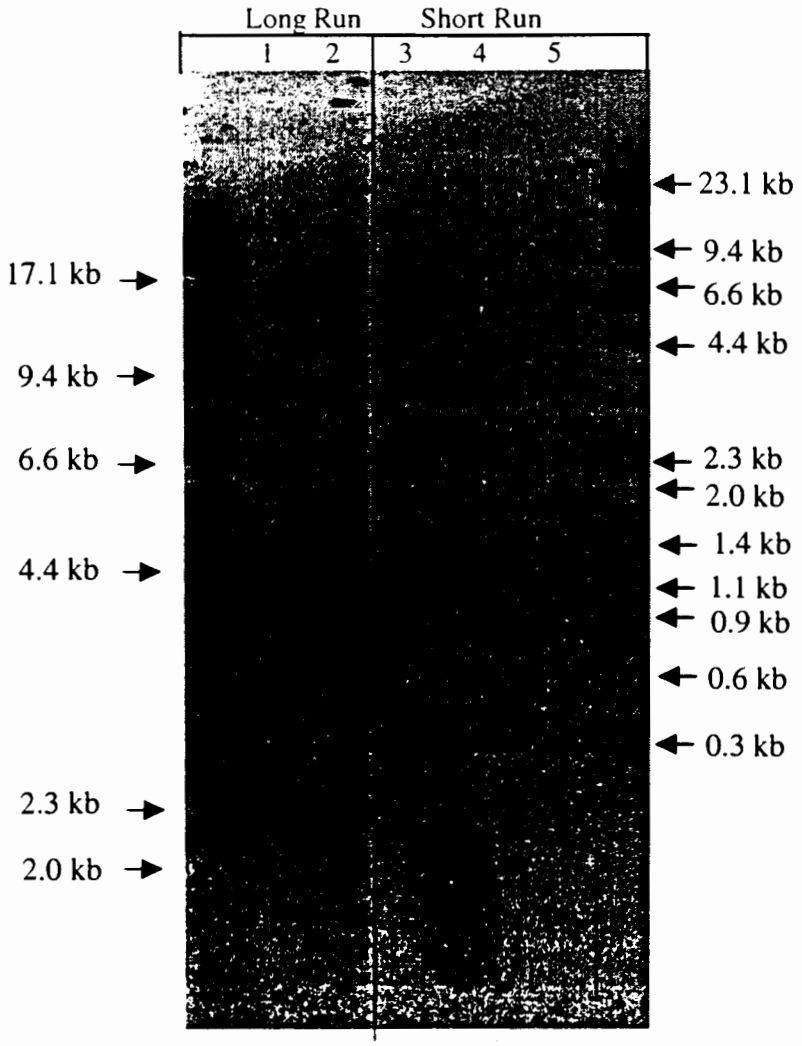


Figure 7. Southern blot analysis of event MON 863: *cry3Bb1* cassette intactness probed with the *cry3Bb1* coding region. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with the ^{32}P-labeled full-length *cry3Bb1* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *cry3Bb1* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *cry3Bb1* cassette (1.0 copy)
5: MON 863 [10 µg]

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

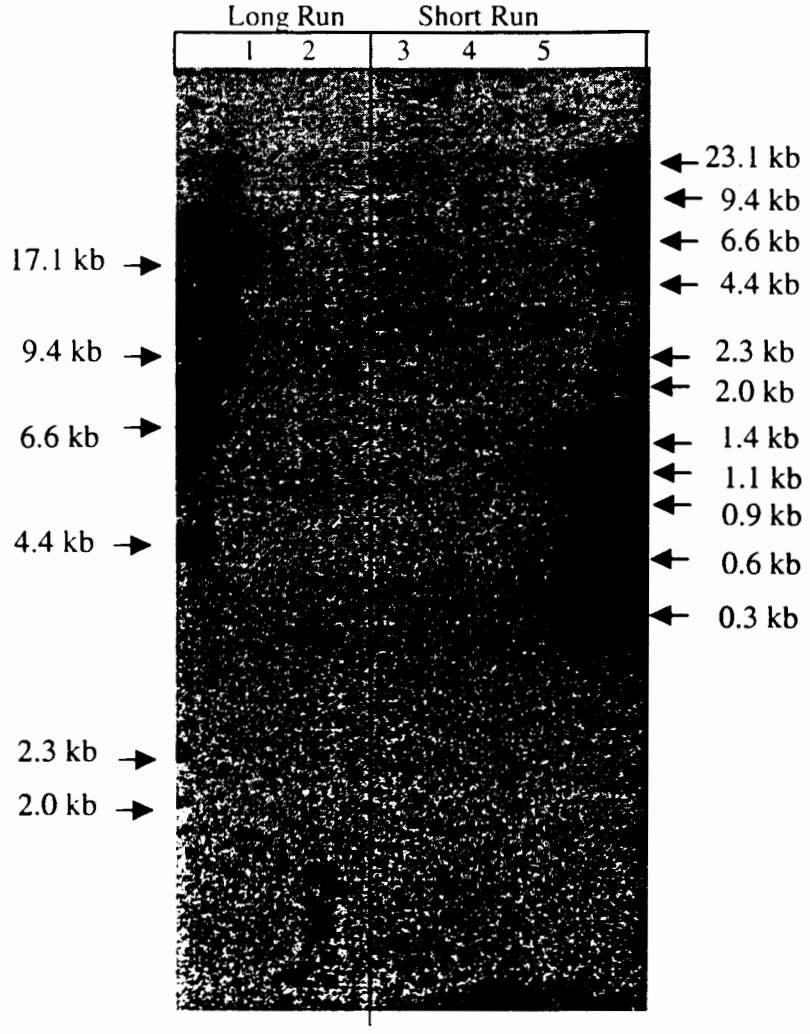


Figure 8. Southern blot analysis of event MON 863: *cry3Bb1* cassette intactness probed with the tahsp17 3' transcriptional termination sequence. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with the ^{32}P-labeled full-length tahsp17 3' termination sequence. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *cry3Bb1* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *cry3Bb1* cassette (1.0 copy)
5: MON 863 [10 µg]

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

b. *cry3Bb1* Coding Region

The lane for control DNA showed no detectable hybridization bands, as expected (Figure 7, lane 1). PV-ZMIR13 DNA mixed with control DNA produced a single band of approximately 3.0 kb corresponding to the size of the intact *cry3Bb1* cassette (lanes 3 & 4). MON 863 DNA produced a single band of approximately 3.2 kb (lanes 2 & 5). As discussed above, this band corresponds to the intact *cry3Bb1* cassette plus a portion of plant genomic DNA. The absence of additional bands indicates a single insertion of an intact *cry3Bb1* cassette and that MON 863 does not contain any detectable *cry3Bb1* elements other than those associated with an intact cassette.

c. tahsp17 3' Transcriptional Terminator

The lane for control DNA showed no hybridization bands, as expected (Figure 8, lane 1). PV-ZMIR13 mixed with control DNA produced a single band of approximately 3.0 kb corresponding to the size of the intact *cry3Bb1* cassette (lanes 3 & 4). MON 863 DNA produced a single band of approximately 3.2 kb (lanes 2 & 5). As discussed above, this band corresponds to the intact *cry3Bb1* cassette plus a portion of plant genomic DNA. Genomic flanking sequence indicates that while the *Hind* III site at the 3' end of PV-ZMIR13L is missing, the entire tahsp17 3' transcriptional termination sequence is present in MON 863. The absence of additional bands indicates a single insertion of an intact *cry3Bb1* cassette and that MON 863 does not contain any detectable tahsp17 3' transcriptional termination elements other than those associated with an intact cassette.

4. *nptII* Cassette Intactness

The integrity of the *nptII* cassette was evaluated by digestion with the restriction endonuclease, *Hind* III, which cleaves the DNA at both the 5' and 3' ends of the cassette. Digested control DNA was spiked with a 1.6 kb *Hind* III restriction fragment from PV-ZMIR13 containing the *nptII* cassette. Individual Southern blots were probed with 35S promoter, *nptII* coding region, or NOS 3' transcriptional termination sequence. The results are displayed in Figures 9, 10 and 11, respectively. The presence of a single band corresponding to the expected size of the cassette (*i.e.*, 1.6 kb) indicates that the *nptII* cassette and each of its elements are intact.

a. 35S Promoter

The lane for control DNA showed no detectable hybridization bands, as expected (Figure 9, lane 1). PV-ZMIR13 DNA mixed with control DNA produced a single band of approximately 1.6 kb corresponding to the size of the intact *nptII* cassette (lanes 3 & 4). MON 863 DNA produced a single band of approximately 1.6 kb (lanes 2 & 5). The absence of additional bands indicates a single insertion of an intact *nptII* cassette and that MON 863 does not contain any detectable 35S promoter elements other than those associated with an intact cassette.

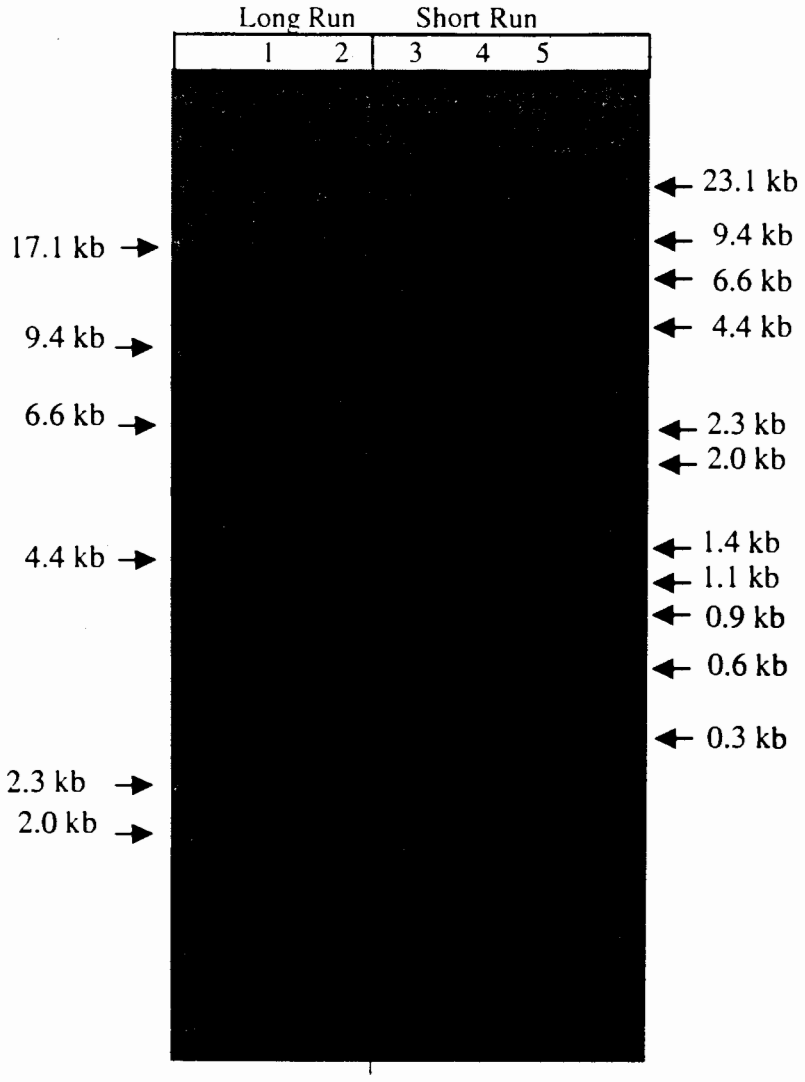


Figure 9. Southern blot analysis of event MON 863: *nptII* cassette intactness probed with the 35S promoter. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with the ^{32}P-labeled-full length 35S promoter. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *nptII* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *nptII* cassette (1.0 copy)
5: MON 863 [10 µg]

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

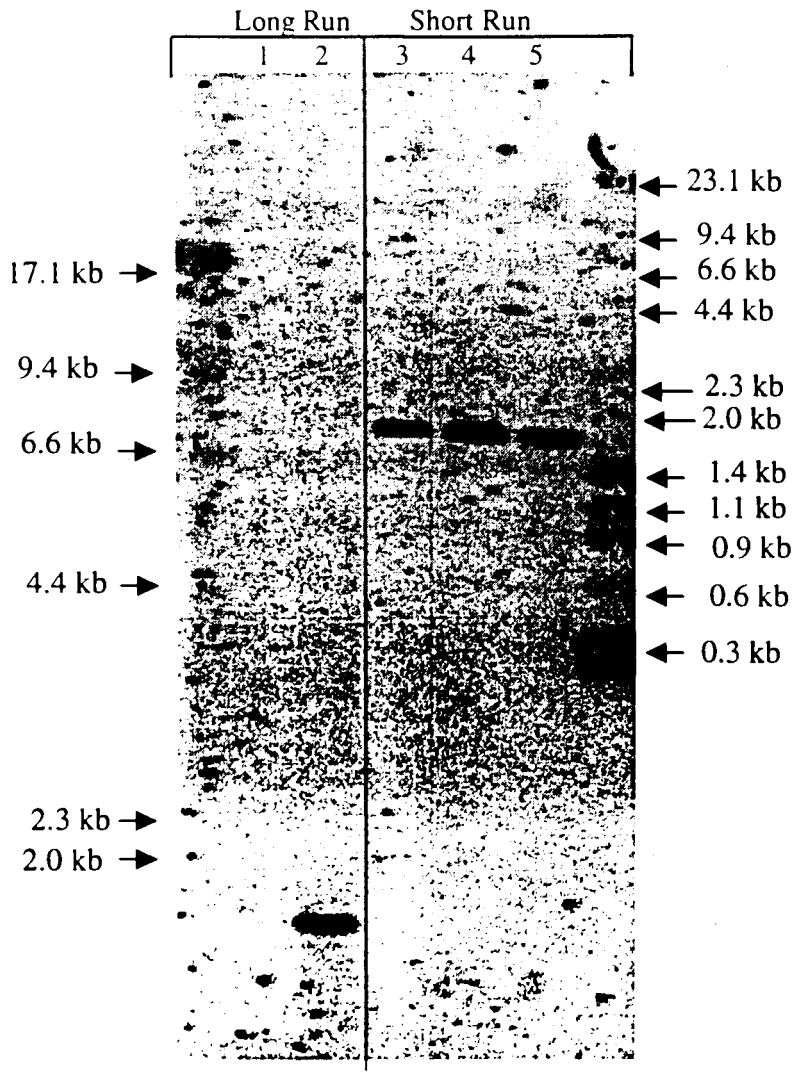


Figure 10. Southern blot analysis of event MON 863: *nptII* cassette intactness probed with the *nptII* coding region. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with a ^{32}P-labeled full-length *nptII* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *nptII* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *nptII* cassette (1.0 copy)
5: MON 863 [10 µg]

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

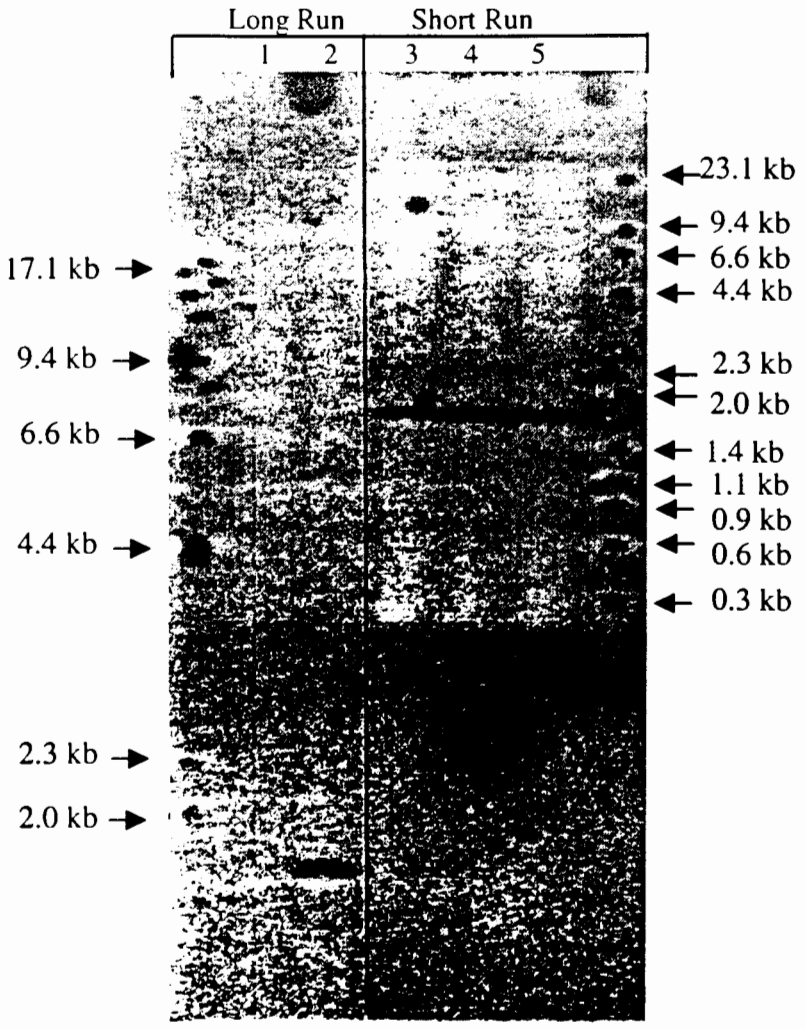


Figure 11. Southern blot analysis of event MON 863: *nptII* cassette intactness probed with the NOS 3' transcriptional termination sequence. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with a ^{32}P-labeled full-length NOS 3' transcriptional termination sequence. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 *nptII* cassette (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 *nptII* cassette (1.0 copy)
5: MON 863 [10 µg]

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

b. *nptII* Coding Region

The lane for control DNA showed no detectable hybridization bands, as expected (Figure 10, lane 1). PV-ZMIR13 DNA mixed with control DNA produced a single band of approximately 1.6 kb corresponding to the size of the intact *nptII* cassette (lanes 3 & 4). MON 863 DNA produced a single band of approximately 1.6 kb (lanes 2 & 5). The absence of additional bands indicates a single insertion of an intact *nptII* cassette and that MON 863 does not contain any detectable *nptII* elements other than those associated with an intact cassette.

c. NOS 3' Transcriptional Terminator

The lane for control DNA showed no hybridization bands, as expected (Figure 11, lane 1). PV-ZMIR13 mixed with control DNA produced a single band of approximately 1.6 kb corresponding to the size of the intact *nptII* cassette (lanes 3 & 4). MON 863 DNA produced a single band of approximately 1.6 kb (lanes 2 & 5). The absence of additional bands indicates a single insertion of an intact *nptII* cassette and that MON 863 does not contain any detectable NOS 3' transcriptional terminator elements other than those associated with an intact cassette.

5. Analysis for Backbone Fragments

The plasmid backbone region is defined as that portion of the DNA which is not necessary for transformation of the target plant cell and consists of the ***Mlu* I - *Mlu* I** restriction fragment. The backbone of plasmid PV-ZMIR13 consists of *ori*-pUC and an *nptII* coding region regulated by a bacterial promoter (refer to Figure 1). Genomic DNA was digested with the restriction endonuclease, *Hind* III, and probed with two PCR-generated probe templates to confirm the absence of plasmid backbone in event MON 863. One probe covered the unique backbone sequence located upstream of the 5' end of the *nptII* coding region, while the second probe covered the remaining unique backbone sequence located downstream of the 3' *nptII* coding region. Together, these two probes encompassed the entire backbone sequence except for the *nptII* coding region. The linear DNA fragment used to transform the plants also contained the *nptII* coding region. Therefore, this sequence could not be used as part of the backbone probes. However, the *nptII* probe used to evaluate *nptII* coding region intactness would have shown backbone sequence containing that element had it been present in the event.

The lane for control DNA showed no hybridization bands, as expected (Figure 12, lane 1). A 2.6 kb *Hind* III restriction fragment from PV-ZMIR13 containing the plasmid backbone sequence mixed with control DNA produced a single band of approximately 2.6 kb corresponding to the size of the intact plasmid backbone (lanes 3 & 4). MON 863 DNA showed no detectable hybridization bands (lanes 2 & 5). This result, in conjunction with the Southern blot analysis for the *nptII* coding region (Figure 10), establishes that MON 863 does not contain any detectable plasmid backbone sequences.

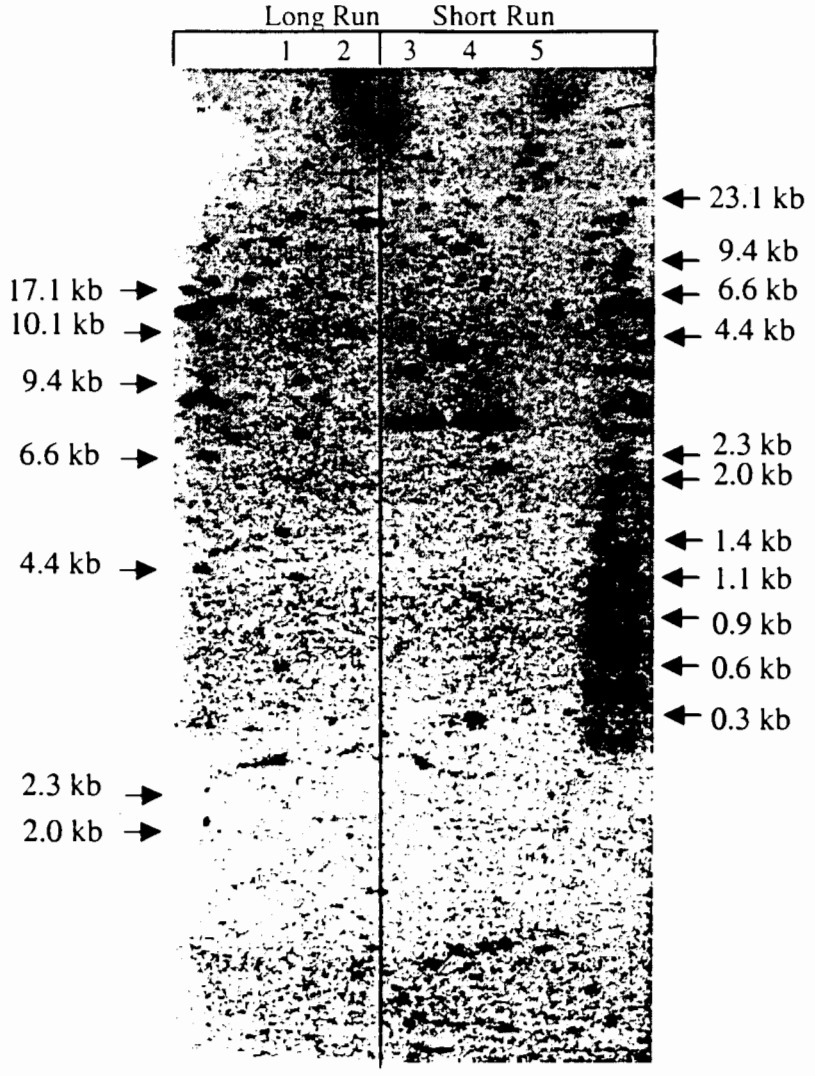


Figure 12. Southern blot analysis of event MON 863: Backbone analysis. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Hind* III. The blot was probed with two ^{32}P-labeled backbone probes encompassing the entire backbone except for the *nptII* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg]
2: MON 863 [10 µg]
3: MON 846 [10 µg] spiked with ~9.5 pg PV-ZMIR13 backbone region (0.5 copy)
4: MON 846 [10 µg] spiked with ~19 pg PV-ZMIR13 backbone region (1.0 copy)
5: MON 863 [10 µg]

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

6. Genomic Flanking Sequence

PCR and DNA sequencing were performed on genomic DNA to confirm the unique junction sequences at the 5' and 3' ends of the MON 863 insert (Cavato *et al.*, 2001). The results of these PCRs are shown in Figure 13. The negative controls MON 846, distilled water and DNA from an unrelated transgenic corn event did not yield a PCR product with either the 5' or 3' primer sets (lanes 1, 3, 4, 6, 8 & 9). MON 863 genomic DNA yielded the correct size products of 312 bp for the 5' PCR (lane 2) and 363 bp for the 3' PCR (lane 7) as predicted by sequence analysis. These results demonstrate the specificity of the primer pairs to MON 863. The sequences of these PCR products were compared to previously obtained sequence data for MON 863 and found to match. Therefore, this PCR analysis verified the DNA sequence of the 5' and 3' borders in event MON 863.

7. Conclusions

MON 863 was produced by particle acceleration technology using an *Mlu* I DNA restriction fragment from plasmid PV-ZMIR13 that contained both *nptII* and *cry3Bb1* cassettes. 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 5.

Table 5. 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 PV-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

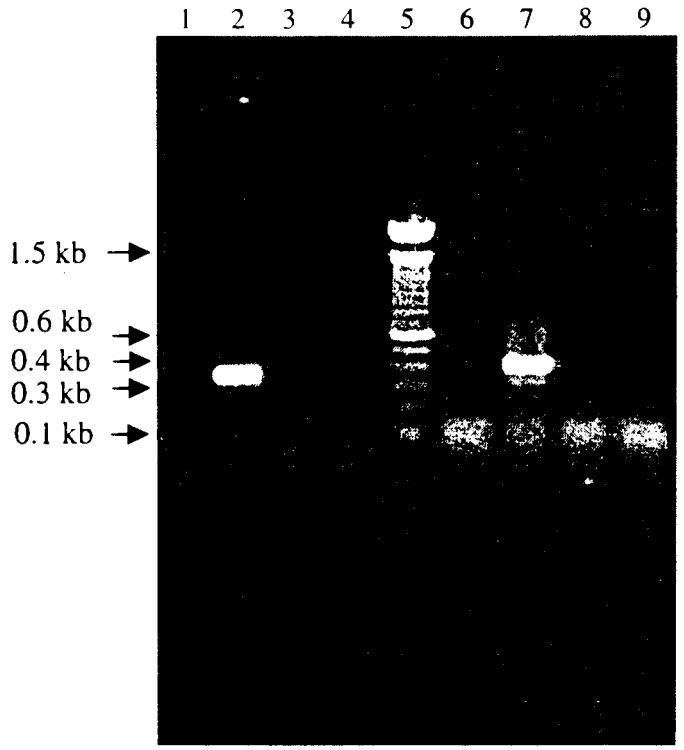


Figure 13. PCR verification of the 5' and 3' border sequences of the MON 863 insert. PCR was performed using primers specific to the 5' and 3' border sequences for MON 863 on genomic DNA extracted from grain from MON 846 (control), an unrelated transgenic corn line, and MON 863. DNAs were amplified with primers A and B from the 5' end of MON 863, amplifying a 312 bp product, and primers C and D from the 3' end of MON 863, amplifying a 363 bp product (Figure 14). Lane designations are as follows:

Lane 1: 10 µl of 5' PCR, MON 846 (control)
2: 10 µl of 5' PCR, MON 863
3: 10 µl of 5' PCR, an unrelated transgenic corn line
4: 10 µl of 5' PCR, no template control
5: Gibco BRL 100 bp DNA Ladder
6: 10 µl of 3' PCR, MON 846 (control)
7: 10 µl of 3' PCR, MON 863
8: 10 µl of 3' PCR, an unrelated transgenic corn line
9: 10 µl of 3' PCR, no template control

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

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 14.

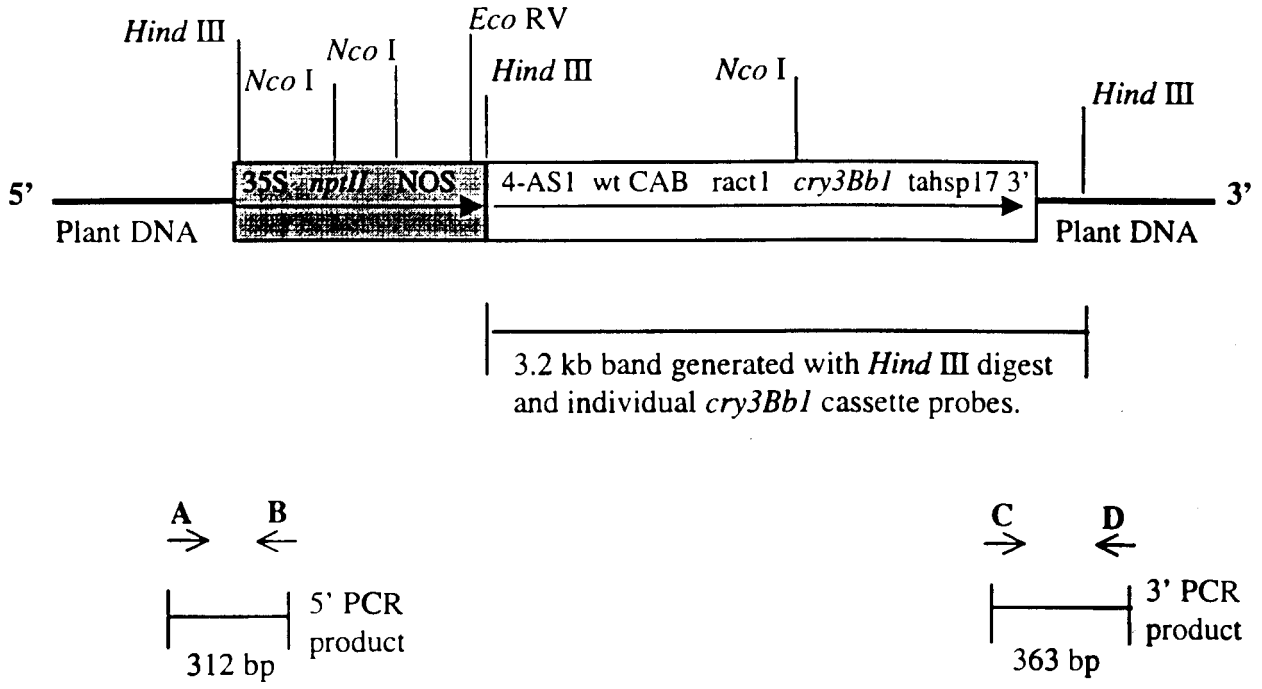


Figure 14. Schematic representation of the MON 863 insert. This figure depicts the predicted insert in corn event MON 863 based on data from Southern blot analyses and PCR confirmation of the sequences at the 5' and 3' ends of the insert. There is one copy of the PV-ZMIR13L fragment that was used to 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.

B. Heritability and Stability of Gene Transfer for MON 863

Chi square analysis of Mendelian inheritance data over five generations was performed to determine the heritability and stability of the *cry3Bb1* gene in MON 863. Expected and observed segregation frequencies of MON 863 progeny that are positive for the CRW-protected phenotype are presented in Table 6. Analysis of leaf tissue for Cry3Bb1 protein was conducted by ELISA. The $A1F_1$ generation was derived from cross-pollinating the original transformed plant with an 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 nontransgenic inbred line A1. The $A1BC_2F_1$ generation was derived from back-crossing $A1BC_1F_1$ plants to nontransgenic inbred line A1. The $A1BC_2F_2$ generation was derived from self-pollinating individual $A1BC_2F_1$ plants. Figure 15 diagrammatically presents the breeding history for MON 863 and indicates the generations used for segregation, Southern blot and expression analyses.

Table 6. Comparison of expected and observed segregation frequencies for MON 863 progeny. (+) denotes plants that are positive for the trait; (-) denotes plants that are negative for the trait.

	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); not significant at $p \leq 0.01$ (Chi square = 6.63)

Genotype frequencies were compared by means of a Chi square test (Little and Hills, 1978a). 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 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. 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.

Southern blot fingerprint analysis of DNA extracted from plants spanning three

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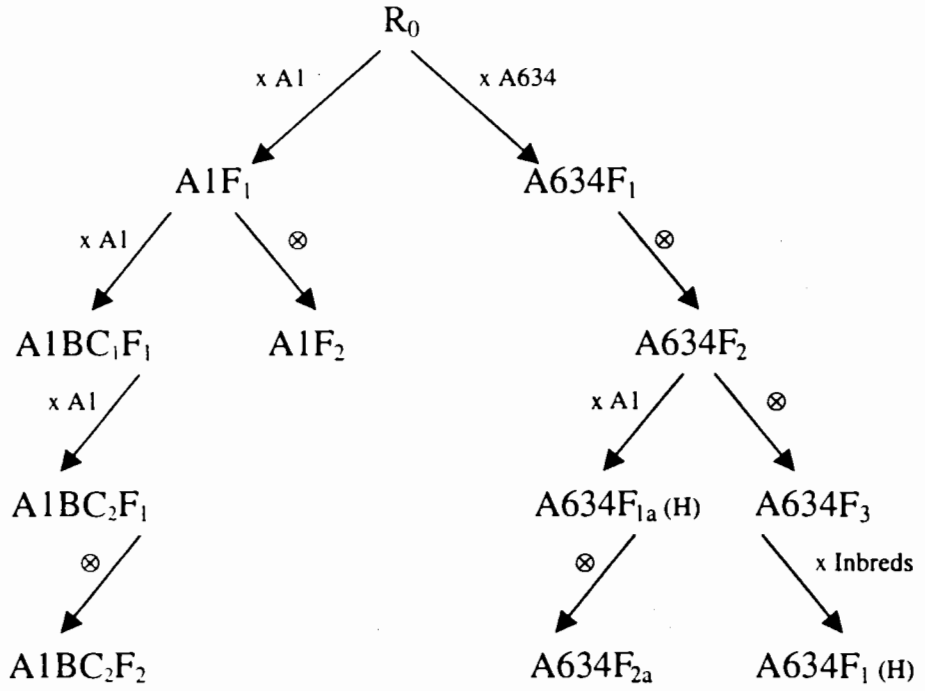
MON 863 Breeding History



R_0 - originally transformed plant
⊗ - self-pollinated
(H) - hybrid

Figure 15. 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 $A634F_2$ and $A634F_{2a}$. Molecular characterization was performed on generation $A634F_{2a}$. Gene expression and compositional analyses were performed on generations $A634F_{1a}$ and $A634F_{2a}$.

generations was also conducted to evaluate the stability of the inserted DNA in corn event MON 863. Genomic DNA was extracted from MON 863 grain tissue, digested with *Nco* I, blotted, and probed with the full-length ^{32}P-labelled *nptII* coding region. Grain from nontransgenic corn line MON 846 served as a negative control. Figure 16 displays the Southern blot result for generations A634F$_2$ and A634F$_{2a}$. The breeding history of these generations can be found in Figure 15. 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 are most likely attributable to partial digestion of the plasmid. Blots of MON 863 DNA from generations A634F$_2$ and A634F$_{2a}$ showed bands at approximately 0.4 and 8.0 kb, 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.

C. Expression of the Inserted *cry3Bb1* and *nptII* Genes

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 investigation. 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 12 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.

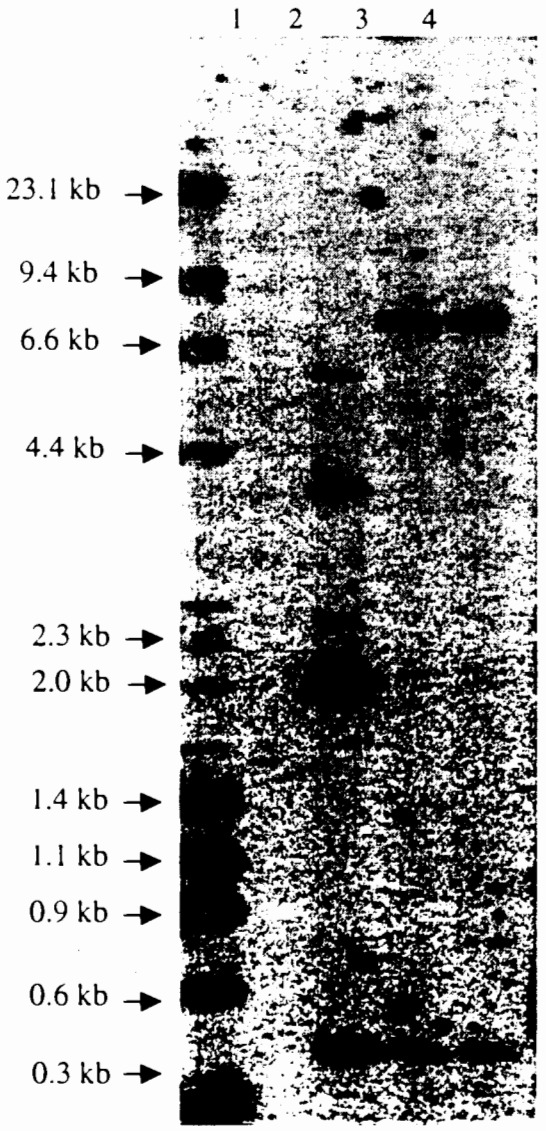


Figure 16. Southern blot analysis of event MON 863: Stability of inserted DNA. MON 846 (control) and MON 863 genomic DNA extracted from grain were digested with *Nco* I. The blot was probed with a ^{32}P-labeled full-length *nptII* coding region. Lane designations are as follows:

Lane 1: MON 846 [10 µg] grain DNA
2: MON 846 [10 µg] grain DNA 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.

Direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) methods were developed and validated to quantify the levels of Cry3Bb1 and NPTII proteins in tissue extracts of MON 863 and control plants. Protein 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 7 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 7. 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	81 ± 11	0.98 ± 0.27
	Range	65 – 93	0.74 – 1.4
	n	4	4
Forage (90 days)	Mean ± SD	39 ± 10	0.19 ± 0.03
	Range	24 – 45	0.17 – 0.23
	n	4	4
Mature Root (90 days)	Mean ± SD	41 ± 13	Not
	Range	25 – 56	Analyzed
	n	4	
Grain (125 days)	Mean ± SD	70 ± 17	<0.076†
	Range	49 – 86	
	n	4	4
Silk (58 days)	Mean ± SD	10	Not
	n	1	Analyzed
Pollen (60 days)	Mean ± SD	62 ± 18	Not
	Range	30 – 93	Analyzed
	n	13	

* SD = standard deviation of the mean; n = number of replicates analyzed

† Limit of detection for corn grain = 0.076 µg/g fw

‡ Collected under APHIS Notification #99-106-16n

Table 8 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 8. Summary of Cry3Bb1 protein levels measured in MON 863 tissue samples collected over the 1999 growing season.

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

* SD = standard deviation of the mean; n = number of replicates analyzed
† NC = not collected
‡ Collected under APHIS Notification #99-106-16n

CRW species that colonize mid-western corn 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 (Steffy *et al.*, 1999). The pupal stage typically lasts 10-14 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 8, Cry3Bb1 protein levels are seen to decline after 49 days post-planting.

In one of the 2000 season field efficacy trials, corn plants were infested with western CRW larvae at the V2, V4 and V6 growth stages. No differences in root feeding damage were observed. Stage V6 would typically be reached by 42 days post-planting. Mean root tissue levels of Cry3Bb1 at this stage (*i.e.*, 57 µg/g) are sufficient to provide protection. As the plants mature, a decline in protein levels becomes irrelevant because the CRW larvae are no longer capable of inflicting any meaningful damage to the larger root mass. It is during the early stages of root development that the high levels of Cry3Bb1 are needed to guard against larval feeding damage.

VI. Agronomic Performance

A. Disease and Pest Susceptibility

MON 863 has been field tested in the United States since 1998 under USDA notifications. As part of these tests, researchers are requested to monitor field sites for disease and insect susceptibility. Visual observations were made while walking the fields and, in almost all circumstances, the observations were qualitative as opposed to quantitative. Depending on the nature, length and number of plantings, observations ranged from one to twenty per site. Nets or beat sheets were not employed. The diseases and insects observed in MON 863 field trials reported through October 2000 are summarized in Tables 9 and 10, respectively.

There were no apparent differences in disease occurrence noted between MON 863 and nontransgenic control plants at 30 of 31 U.S. sites (Table 9). In one of three plantings at the Bo Finca Alomar site in Puerto Rico (Notification #99-063-04n), corn stunt disease was observed on 2% of MON 863 plants but not on parental controls. This difference was based on an evaluation of 200 to 300 plants across plots containing 20 different inbred conversions of MON 863 versus a single plot of the parental control. Seventy-five percent of the field was planted to MON 863 with the remainder planted to the nontransgenic control. This observation does not support a conclusion of increased corn stunt disease susceptibility for MON 863 plants because overall disease incidence was low, there was a greater probability of observing the disease in the larger population of MON 863 plants compared to the smaller population of control plants, and because the disease was not observed at any of the other 30 sites where MON 863 was planted.

There were no apparent differences in insect occurrence between MON 863 and nontransgenic control plants at 29 of 31 U.S. sites (Table 10). In two of three plantings at the Bo Finca Alomar site in Puerto Rico (Notification #99-063-04n), minor differences (≤6%) in the level of armyworm infestation were observed. At the Juana Diaz site in Puerto Rico (Notification #99-056-04n), fall armyworm was observed on 3% of control plants but not on MON 863 plants. This difference was based on evaluation of 100

Table 9. Evaluation of disease susceptibility for corn event MON 863 in multiple U.S. field trials.

					Diseases Observed							
USDA#	*Year*	*State*	*County*	*# obs*	*SLB*	*GLS*	*CR*	*SR*	*MMV*	*CS*	*PM*	*SW*	Δ
98-033-01n	1998/99	PR	Bo Guerro	4									no
98-040-04n	1998	IL	Jersey	5	√								no
98-098-01n	1998/99	HI	Maui	2				√	√				no
98-229-02n	1999	HI	Maui	2				√	√				no
98-229-05n	1998/99	HI	Maui	4							√		no
98-229-09n	1999	IN	Tippecanoe	1									no
98-288-11n	1999	IA	Story	5			√						no
98-288-11n	1998/99	PR	Isabella	20									no
99-056-09n	1999/00	HI	Oahu	10	√								no
99-055-07n	1999	NE	Clay	6		√		√					no
99-055-07n	1999/00	HI	Maui	5									no
99-071-41n	1999	IA	Hamilton	3									no
99-071-41n	1999	IA	Story	3									no
99-071-41n	1999	IA	Cass	1									no
99-071-41n	1999	IL	Champaign	5		√	√	√				√	no
99-071-41n	1999	IL	Douglas	4									no
99-071-41n	1999	IL	Warren	3		√							no
99-071-41n	1999	IL	Jersey	12									no
99-071-41n	1999	MO	Boone	1									no
99-071-41n	1999	SD	Brookings	3									no
99-071-41n	1999	NE	York	1									no
99-106-16n	1999	IA	Jefferson	4									no
99-106-16n	1999	IA	Benton	4									no
99-106-16n	1999	IL	Warren	4		√							no
99-106-16n	1999	NE	York	4									no
99-056-07n	1999/00	HI	Maui	5	√								no
99-056-07n	1999/00	IN	Tippecanoe	1				√					no
99-056-04n	2000	PR	Juana Diaz	2									no
99-063-04n	2000	PR	Bo F. Alomar	9						√			yes
99-095-13n	2000	HI	Honolulu	4									no
99-116-05n	1999	HI	Maui	3				√	√				no

Legend

obs - number of observations
SLB - southern corn leaf blight (*Cochliobolus heterostrophus)* including blight
GLS - gray leaf spot (*Cercospora zeae-maydis*)
CR - common rust (caused by *Puccinia sorghi*)
SR - southern rust (caused by *Puccinia polysora*)
MMV - maize mozaic virus
PM - Psuedomonas
SW - Stewart's wilt
CS - corn stunt disease
Δ - difference observed between MON 863 and control plants

Table 10. Evaluation of insect susceptibility for corn event MON 863 in multiple U.S. field trials.

USDA#	Year	State	County	Insects Observed													Δ
				#obs	ECB	CEW	FAW	AP	CRW	LB	JB	TP	CRB	BA	LH	GH	SM	
98-033-01n	1998/99	PR	Bo Guerro	4														no
98-040-04n	1998	IL	Jersey	5	√	√												no
98-098-01n	1998/99	HI	Maui	2		√		√				√	√	√				no
98-229-02n	1999	HI	Maui	2		√		√				√	√	√				no
98-229-05n	1998/99	HI	Maui	4				√				√			√			no
98-229-09n	1999	IN	Tippecanoe	1														no
98-288-11n	1999	IA	Story	5														no
98-288-11n	1998/99	PR	Isabella	20														no
99-056-09n	1999/00	HI	Oahu	10					√			√						no
99-055-07n	1999	NE	Clay	6	√	√			√									no
99-055-07n	1999/00	HI	Maui	5				√							√			no
99-071-41n	1999	IA	Hamilton	3					√									no
99-071-41n	1999	IA	Story	3			√											no
99-071-41n	1999	IA	Cass	1														no
99-071-41n	1999	IL	Champaign	5	√				√									no
99-071-41n	1999	IL	Douglas	4														no
99-071-41n	1999	IL	Warren	3														no
99-071-41n	1999	IL	Jersey	12														no
99-071-41n	1999	MO	Boone	1	√		√											no
99-071-41n	1999	SD	Brookings	3	√													no
99-071-41n	1999	NE	York	1														no
99-106-16n	1999	IA	Jefferson	4														no
99-106-16n	1999	IA	Benton	4	√				√	√						√		no
99-106-16n	1999	IL	Warren	4														no
99-106-16n	1999	NE	York	4					√									no
99-056-07n	1999/00	HI	Maui	5		√		√							√			no
99-056-07n	1999/00	IN	Tippecanoe	1							√							no
99-056-04n	2000	PR	Juana Diaz	2			√											yes
99-063-04n	2000	PR	Bo. F. Alomar	8		√	√											yes
99-095-13n	2000	HI	Honolulu	4				√				√			√		√	no
99-116-05n	1999	HI	Maui	3		√		√				√	√	√				no

Legend

# obs	- number of observations	ECB	- European corn borer (*Ostrinia nubilalis*)
CEW	- corn earworm (*Heliothis zea*)	FAW	- fall armyworm (*Spodoptera frugiperda*)
AP	- aphids (*Rhopalosiphum maidis*)	CRW	- corn rootworm beetles (*Diabrotica* sp.)
LB	- ladybird beetle (*Hippodamia convergens*)	JB	- Japanese beetle
TP	- thrips (*Anaphothrips* and *Frankliniella* sp.)	CRB	- Chinese Rose beetle (*Adoretus sinicus*)
BA	- beet armyworm (*Spodoptera* sp)	LH	- leaf hooper
GH	- grass hopper	SM	- spider mite
Δ	- difference observed between MON 863 and control plants		

randomly selected plants from each of the MON 863 and control plots. These differences are minor and probably fall within the range of normal biological variation; they were not observed at the other 29 sites.

Disease and insect susceptibility of MON 863 were also evaluated in four trials conducted at three sites in Argentina during the 1999-2000 growing season. Each trial included four replicate plots planted with MON 863 and its parental control. MON 863 and control plots were treated in a comparable manner. Evaluation of disease occurrence involved two observations taken at each site, one at 60 days post-planting (V10 growth stage) and the other at 83 days post-planting (R3 growth stage). Evaluation of insect occurrence involved one observation taken at each site at 83 days post-planting. Common corn rust, fall armyworm and European corn borer (ECB) were observed at all sites. The frequency of these pests in each trial is summarized in Table 11. Analysis of these data in a Duncan Multiple Range test revealed no statistically significant difference ($p \leq 0.05$) between pest incidences on MON 863 and control plants.

Table 11. Evaluation of disease and insect susceptibility for corn event MON 863 in 1999-2000 field trials conducted in Argentina. Reported values represent the percentage of MON 863 and control plants observed with insects or disease in four replicated field trials conducted at three sites.

Site	Armyworm & ECB (%) at R3 stage	Common Corn Rust (%) at V10 Stage	Common Corn Rust (%) at R3 Stage
Fontezuela 1			
Control	7.0	10	31.3
MON 863	1.5	10	26.3
Fontezuela 2			
Control	4.0	0	28.7
MON 863	2.5	0	27.5
Salto			
Control	2.5	13	15
MON 863	1.5	13	16.2
Rojas			
Control	3.2	25	22.5
MON 863	4.0	20	20.0

Collectively these data support a conclusion of no difference in disease and pest susceptibility for MON 863 when compared to conventional corn, except for its resistance to CRW larval feeding damage.

A complete list of USDA Notifications approved for MON 863 and a status report on the trials conducted under these Notifications are contained in Appendix A.

B. Lack of Toxicants in Corn

Corn has a long history of safety in terms of production and as a food and feed source. Toxicants are not considered a significant component of healthy corn (White and Pollack, 1995; Watson, 1987). Compositional analyses were performed on tissues collected from event MON 863, its nontransgenic parental control line, and 18 commercial varieties grown under typical field conditions. These field trials were conducted in the U.S. in 1999 at four replicated sites in the Midwest. A detailed report describing the materials and methods employed in this study and the analytical results has been submitted to the U.S. FDA as part of the premarket notification procedure. A compositional analysis summary is provided herein as Appendix B. No meaningful differences between MON 863 and control values were observed. All MON 863 values fell within reference ranges. The results of these analyses demonstrate that MON 863 is compositionally equivalent to nontransgenic corn.

Cry3Bb1 protein variants produced by bacteria and plants have been well characterized (Hileman *et al.*, 2001a). Small differences in amino acid sequence occur between these variants. The Cry3Bb1 variant produced in MON 863 corn differs in sequence from the wild type Cry3Bb1 protein by only seven amino acids. It differs by only two amino acids from another Cry3Bb1 variant isolated from recombinant *B.t.* strain, EG11098. The bacterial-produced protein from strain EG11098 has been shown to be physiochemically and functionally equivalent to the Cry3Bb1 variant produced in MON 863 (Holleschak *et al.*, 2001a & 2001b).

When administered orally at the highest dose feasible, the EG11098 variant of Cry3Bb1 produced no adverse effects in a rodent acute toxicity bioassay (Bechtel, 1999). The no observable effect level (NOEL) in this study was found to be ≥2980 mg/kg, the highest dose level tested. This NOEL is five orders of magnitude greater than an upper bound estimate of dietary exposure for Cry3Bb1 in humans[†]. The protein is very degraded in digestive tract matrices (Leach *et al.*, 2001). The Cry3Bb1 variant produced in MON 863 lacks sequence similarity to known toxins, allergens and pharamacologically active proteins relevant to human and animal health (Hileman *et al.*, 2001b). *B.t.* Cry proteins have a 40-year history of safe use in agriculture (EPA, 1998; McClintock *et al.*, 1995). Collectively, these findings indicate that food and feed derived from *cry3Bb1*-expressing corn will be as safe for consumption as traditional corn varieties.

C. Efficacy of MON 863

Numerous variants of the *B.t.* protein, Cry3Bb1, have shown high levels of pesticidal activity against the CRW complex. The two most economically important *Diabrotica* species in corn crops are the WCRW and the NCRW. These species feed primarily on corn and evolve through a diapausing egg stage. Both species are widely distributed throughout the U.S. and account for significant crop losses and increased production costs. The southern corn rootworm (SCRW) is a minor pest of corn, except in coastal

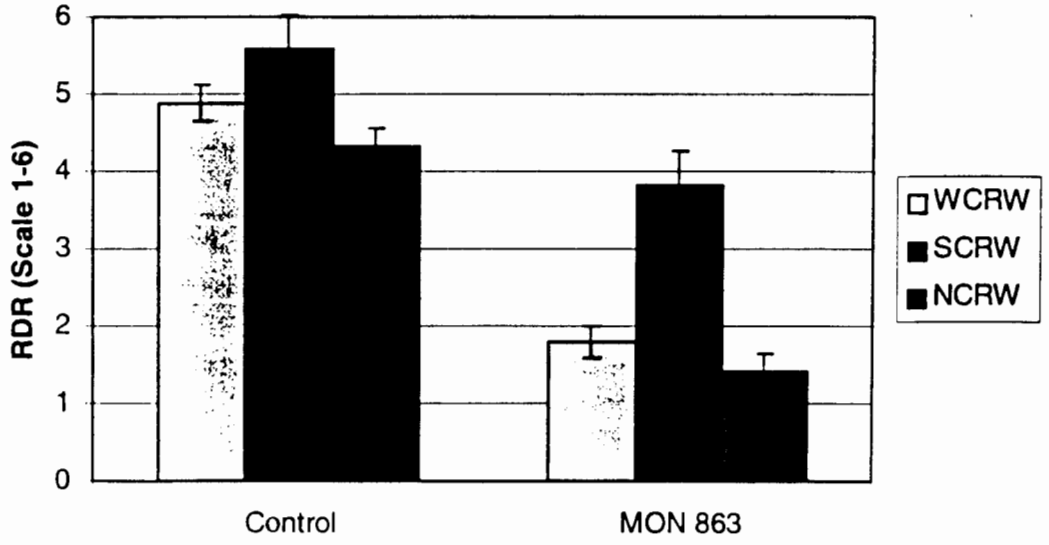
[†] Margin of exposure is $\geq 2.13 \times 10^{5}$ based on estimated dietary intake of 1.40×10^{-2} mg/kg Cry3Bb1

areas of Texas and Louisiana. The SCRW life cycle differs from that of the WCRW and NCRW. SCRW eggs do not require a diapause, they have a faster growth rate, and they feed on many types of plants, rather than strictly on corn.

The efficacy of event MON 863 against these three CRW species has been evaluated under growth chamber conditions. WCRW, NCRW and SCRW eggs were obtained from a commercial source and were infested onto corn two weeks after planting when the plants were at approximately the V2 growth stage. The eggs hatched and larvae began feeding approximately one week after infestation. Feeding was allowed to occur for three to four weeks at which time roots were dug, rinsed and rated. Ratings were based on the Iowa root damage rating criteria (Hills and Peters, 1971). A root damage rating (RDR) was assigned on a scale of 1-6 as follows: 1 = no feeding scars; 2 = visible scars, but no roots pruned to within 4 cm of the stalk; 3 = one or more nodal roots pruned to within 4 cm of the stalk, but less than one nodes' worth of roots; 4 = one node worth of pruned roots; 5 = two nodes' worth of pruned roots; 6 = three or more nodes' worth of pruned roots. MON 863 and control root ratings were averaged for each insect species.

The mean RDR for each insect pest is graphically presented in Figure 17. The results of these growth chamber trials reveal that event MON 863 has activity against all three species of corn rootworm. Root damage inflicted by the WCRW and NCRW was notably lower in MON 863 plants compared to controls, as evidenced by the significant reduction in mean RDR values. The level of protection afforded by MON 863 against these two CRW species is well below the generally recognized 3.0-RDR threshold for economic damage. Some level of protection was provided against SCRW larval feeding damage, but to a lesser degree than for the other two CRW species. The faster growth rate of SCRW larvae may provide an explanation for the greater level of damage it inflicts on corn roots.

Figure 17. Quantitative comparison of root damage inflicted by three species of rootworm in control and MON 863 corn.



In 1999, MON 863 was evaluated at seven field locations in the U.S. for control of WCRW. The limited availability of NCRW and SCRW eggs precluded large scale field trials with these two species. All locations were planted in a randomized complete block design with four replicates per treatment. Each replicate was comprised of approximately 30 plants. In order to compare the efficacy of event MON 863 against a commercial standard, tefluthrin formulated as *Force® 3G* insecticide was applied at labeled rates, in-furrow, over plots planted to nontransgenic seed. Untreated plots planted to nontransgenic seed served as controls.

All plots were mechanically infested at the V2 growth stage with 1,200-1,600 WCRW eggs per square foot. Plants were analyzed for the presence of Cry3Bb1 protein using field ELISA kits; negative plants were removed. Plants were dug for evaluation at maximum damage (*i.e.*, when half of the larvae in adjoining test rows were pupating). Roots were evaluated for damage using the Iowa classification system.

MON 863 and *Force 3G* treated plants sustained less damage than unprotected control plants and maintained low damage ratings in locations where feeding on controls was significant. MON 863 tended toward a reduced RDR by more than 0.5 points versus *Force 3G* insecticide treated plants. The results of these efficacy trials are summarized in Table 12.

Table 12. Summary of efficacy data collected in 1999 field trials with MON 863. Mean root damage ratings (RDR) are presented for MON 863, *Force 3G* insecticide treated plants, and unprotected controls at each trial location.

Location †	Mean RDR ± S.D. (n) *		
	Control	**Force 3G**	**MON 863**
Monmouth, IL	3.78 ± 0.10 (40)	2.38 ± 0.10 (40)	1.70 ± 0.11 (33)
Williams, IA	3.95 ± 0.09 (39)	2.45 ± 0.09 (40)	1.82 ± 0.09 (38)
Atlantic, IA	4.08 ± 0.12 (40)	2.48 ± 0.12 (40)	1.93 ± 0.13 (30)
Brookings, SD	4.35 ± 0.12 (40)	2.10 ± 0.12 (40)	1.50 ± 0.12 (35)
Columbia, MO	4.44 ± 0.17 (39)	2.55 ± 0.17 (39)	1.86 ± 0.17 (24)
Tuscola, IL	4.98 ± 0.26 (40)	2.15 ± 0.26 (40)	1.00 ± 0.26 (35)
Thomasboro, IL	5.03 ± 0.13 (35)	2.33 ± 0.12 (39)	2.33 ± 0.12 (39)
Overall Mean (N)	4.37 (273)	2.35 (278)	1.73 (234)

* S.D. - standard deviation; n - number of plant roots evaluated

† - Collected under APHIS Notification #99-071-41n

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Year 2000 field efficacy trials were conducted utilizing three different protocols, at multiple locations in the Midwest, and included performance comparisons against three commercial insecticide standards (*i.e.*, tefluthrin, terbufos and chlorpyrifos). Many of these trials were conducted in collaboration with university scientists. The results of these trials demonstrate that corn event MON 863 provides superior protection against CRW feeding damage when compared to standard insecticide treatments and unprotected control corn. MON 863 displayed a greater consistency in its performance by providing a 5-15% greater probability of maintaining CRW damage below the economic threshold (RDR $\leq$ 3.0) than standard insecticide treatments (Pilcher, 2001).

D. Agronomic Characteristics of MON 863

Event MON 863 has been incorporated into commercial corn breeding lines. Demonstration of agronomic equivalency between MON 863 and nontransgenic corn of comparable germplasm is a critical part of the product development process. It is one of the final phases to be completed prior to commercialization because of the extended time required for integration of the 'genes of interest' into elite corn germplasm. The purpose of these trials is to determine if the transformation of corn to produce event MON 863 results in any unintended effects on plant agronomic performance. Measurement of appropriate agronomic characteristics is also an accepted component of assessing the weediness potential of a genetically modified crop. Evaluation of agronomic parameters that indicate alterations in crop dormancy, growth rate, reproductive potential, and ability to disperse and persist are the basis of determining weediness potential.

Agronomic equivalency trials are designed to evaluate corn development through its entire life cycle. Phenotypic assessments that include measurements of plant structure and timing of growth activities provide indications of any functional discrepancies in plant growth. Yield, which is the culmination of many physiological processes, is assessed because it is a major trait of interest in the commercial product, particularly for hybrids. Trials are conducted at multiple locations to evaluate equivalency under a variety of environmental conditions. An assessment of agronomic equivalency under field conditions has been conducted for both inbred and hybrid corn varieties containing event MON 863.

1. Inbred Agronomic Equivalency

Experimental studies were designed to evaluate agronomic characteristics in side-by-side comparisons of a MON 863 inbred and its near isogenic control. These trials were conducted in 1999 at two locations in Illinois under APHIS Notification 99-112-07n. The data from one location was not used due to poor germination caused by dry soil conditions at the time of planting. Field trials were conducted using a randomized split-block design. Each plot was planted to either the positive inbred isoversion of MON 863 or its negative near isogenic control. Each plot was comprised of two rows, of approximately 200-250 ft^2 (10' x 20-25' ft), and was replicated four times. Pre-emergent herbicide and fertilizer applications were made based on typical farming practices at the

location. A soil insecticide was used to control any background population of corn rootworm larvae. However, no natural population was expected due to a nonhost crop being planted the previous season. In addition, potential secondary pest injury to the plots was prevented by use of foliar insecticide and fungicide sprays. The parameters evaluated, their timing, and a description of the measurements taken in each trial are described in Table 13.

Table 13. Agronomic parameters evaluated in MON 863 inbred equivalency trials.

Parameter	Timing	Description
Seedling Vigor	Stage V2-V3	Scale 1-9 1=poor, 9=vigorous
Early Stand Count	Stage V4-V6	# Plants/row
GDU[1] to 50% Pollen Shed	Pollen shed	50% Pollen shed/tassel
GDU[1] to 50% Silk	Silking	50% Plants showing silks
Ear Height	Maturity	Soil surface to ear attachment node (inches)
Plant Height	Maturity	Soil surface to top flag leaf attachment (inches)
Stay Green	Maturity	Scale 1-9 1=all leaves dead 9=all leaves green
Grain Moisture	Harvest	% Moisture
Yield	Harvest	Bushels/acre

1 - Growing degree units (GDU) = [(Tmax + Tmin)/2] – 50; where Tmax ≤ 88° F and Tmin ≥ 55° F

A summary of inbred agronomic data collected is presented in Table 14. The parameter mean for each MON 863 inbred plot was computed and compared to its side-by-side near isogenic control by a standard t-test. An analysis of variance was conducted on means for each replicate (Little and Hills, 1978b). Statistical significance was preset at the 5% level ($p<0.05$). No statistically significant differences between MON 863 and control parameters were observed. However, there did appear to be a 31% drag on yield for the MON 863 inbred when compared to the average control yield. This difference was an artifact of an abnormally high value for yield in one of the control replicates. Yield in control replicate #2 was 70.9 bu/A, a value more than 3-fold higher than the average yield of 19.9 bu/A recorded for the other control replicates. Collectively, these limited data indicate that under field test conditions, the MON 863 inbred line is phenotypically equivalent to the near isogenic control and provides no indication of increased weediness potential.

2. Hybrid Agronomic Equivalency

Hybrid agronomic equivalency can be demonstrated in different ways. One approach involves the use of converted inbreds. These inbreds are created by crossing MON 863

Table 14. Summary of agronomic data collected in inbred evaluations of MON 863. Mean values are presented for MON 863 and its near isogenic control.

Parameter [1]	Reps	MON 863	Control	% Δ^2	*p*-value
Seedling Vigor	4	6.8	6.3	7.9	0.410
Early Stand Count	4	46	46.8	1.7	0.850
GDU to Pollen Shed	4	1334	1358	1.8	0.183
GDU to Silk	4	1365	1352	1.0	0.415
Ear Height	4	27.8	27.5	1.1	0.936
Plant Height	4	56.5	60	5.8	0.208
Stay Green	4	6	6	0	1.000
% Grain Moisture	4	15.9	16.2	1.9	0.933
Yield	4	25.3	36.9	31.4	0.348

1 - Refer to Table 13 for units of measure
2 - [(MON 863 – Control) / Control] x 100

donor material to a commercial inbred line of interest, repeatedly backcrossing the progeny to the same line of interest (*i.e.*, the recurrent parent), and then allowing the progeny to self-pollinate through successive generations to bring the gene of interest into a homozygous state. The resulting homozygous MON 863 inbred is then crossed to a different nontransgenic inbred to produce a hybrid. The MON 863 hybrid is placed into agronomic equivalency trials alongside its nontransgenic parental control hybrid. The parental control is produced by crossing the recurrent parent line to the second inbred.

In another approach, the progeny of a single heterozygous MON 863 plant is self-pollinated through successive generations to allow the gene of interest to segregate into homozygous positive and negative plants. These positive and negative 'isolines' are crossed to another inbred line to create a pair of hybrids that are compared in agronomic equivalency trials. Under this approach, both positive and negative plants can trace their ancestry back to a common parent (*i.e.*, the original heterozygous MON 863 plant). Both approaches for evaluating equivalency have been used in the development of event MON 863.

Six hybrids developed by Monsanto were placed into agronomic equivalency trials during the year 2000 growing season. These hybrids are designated: H-1, H-2, H-3, H-4, H-5 and H-6.

H-1, H-2 and H-3 are hybrids derived from positive and negative isolines. These were compared under typical field production conditions at multiple locations in Illinois, Iowa and Nebraska using a randomized split-plot design. Each plot was comprised of two rows ranging in size from 88-115 ft^2. Plots were replicated four times at each location.

H-4, H-5 and H-6 are hybrids derived from converted inbreds. These were evaluated for

equivalency to their respective parental controls at multiple locations in Illinois and Iowa using a balanced lattice square design with two replications per site. Each plot was comprised of two rows measuring 115 ft^2. The positive and negative isolines of hybrid H-1 were also evaluated under this balanced lattice square design protocol.

Herbicide and fertilizer applications were made in accordance with typical farming practices at each location. A soil insecticide was applied at planting to control corn rootworm larvae, although no significant infestations were expected since a nonhost crop was planted the previous season. In addition, plots were sprayed with foliar insecticides and fungicides. The parameters evaluated, the timing of evaluations, and a description of the measurements taken are described in Table 15. Every parameter was not necessarily evaluated in all trials. These hybrid equivalency trials were conducted under the following APHIS Notifications: #00-088-35n, #00-088-16n and #00-088-13n.

Table 15. Agronomic parameters evaluated in MON 863 hybrid equivalency trials.

Parameter	Timing	Description
Seedling Vigor	Stage V2-V3	Scale 1-9 1=excellent; 9=poor growth
Early Stand Count	Stage V4-V6	#Plants/row
GDU[1] to 50% Pollen Shed	Pollen Shed	50% Pollen shed/tassel
GDU[1] to 50% Silk Emergence	Silking	50% Plants showing silks
Ear Height	Maturity	Soil surface to ear attachment node (in.)
Plant Height	Maturity	Soil surface to flag leaf attachment (in.)
Final Stand Count	Harvest	# Plants/row
Test Weight	Harvest	Weight/unit volume (lb/bushel)
Grain Moisture	Harvest	% Moisture
Yield	Harvest	Bushels/acre

1 – Growing degree units (GDU) = [(Tmax + Tmin)/2] – 50; where Tmax ≤ 88° F and Tmin ≥ 55° F

Statistical analyses were based on location means for each hybrid pair (*i.e.*, MON 863 hybrid versus control hybrid). A standard Student's t-test was used to assess the significance of any differences observed. Statistical significance was predetermined to be at the 5% level ($p \leq 0.05$).

Summary results from the year 2000 agronomic equivalency trials for these six hybrids are displayed in Tables 16 through 22. Least square means are presented for each MON 863 and control parameter evaluated. Nine statistically significant differences were observed in 64 comparisons made between MON 863 hybrids and their respective hybrid controls; of these, 5% (*i.e.*, 3) are expected to show a significant difference based on chance alone.

Table 16. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-1. In a randomized split-plot design MON 863 was compared to its negative isogenic control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-1				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	7	2.7	3.1	12.9	0.14
Early Stand Count	5	62.2	61.6	1.0	0.69
GDU to Pollen Shed	5	1343	1350	0.5	0.34
GDU to Silk	5	1325	1332	0.5	0.54
Ear Height	8	46	44.1	4.3	0.05
Plant Height	8	94	92.4	1.7	0.02
Final Stand	5	66.1	64.8	2.0	0.22
Test Weight	5	53	53.7	1.3	0.17
% Grain Moisture	8	19.4	19.7	1.5	0.63
Yield	8	146.5	133	10.2	0.12

1 – Refer to Table 15 for units of measure
2 – [(MON 863 – Control) / Control] x 100

Table 17. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-2. In a randomized split-plot design MON 863 was compared to its negative isogenic control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-2				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	10	2.3	2.1	9.5	0.37
Early Stand Count	11	70.6	71.6	1.4	0.41
GDU to Pollen Shed	9	1272	1272	0	0.76
GDU to Silk	9	1259	1256	0.2	0.51
Ear Height	15	44.3	43.3	2.3	0.34
Plant Height	15	86.2	85.3	1.1	0.18
Final Stand	12	69.3	69.2	0.1	0.69
Test Weight	9	55.4	56.4	1.8	0.01
% Grain Moisture	14	19.6	19.2	2.1	0.31
Yield	14	136.2	125.9	8.2	0.03

1 – Refer to Table 15 for units of measure
2 – [(MON 863 – Control) / Control] x 100

Table 18. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-3. In a randomized split-plot design MON 863 was compared to its negative isogenic control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-3				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	9	2.4	2.8	14.3	0.21
Early Stand Count	11	69.9	69.3	0.9	0.55
GDU to Pollen Shed	8	1316	1321	0.4	0.32
GDU to Silk	8	1304	1315	0.8	0.17
Ear Height	14	48.2	46.3	4.1	0.01
Plant Height	14	94	95.6	1.7	0.18
Final Stand	11	67.3	68.2	1.3	0.14
Test Weight	9	54.7	55	0.5	0.52
% Grain Moisture	14	22.8	22.6	0.9	0.60
Yield	14	154.3	151	2.2	0.37

1 – Refer to Table 15 for units of measure
2 – [(MON 863 - Control) / Control] x 100

Table 19. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-4. Utilizing a balanced lattice square design, MON 863 was compared to its parental control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-4				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	7	2.2	2.9	24.1	0.05
GDU to Pollen Shed	8	1254	1239	1.2	0.06
GDU to Silk	6	1231	1231	0	0.96
Ear Height	10	48.1	47	2.3	0.46
Plant Height	10	95.8	96.6	0.8	0.33
Final Stand	13	67.9	68.5	0.9	0.08
Test Weight	11	55.7	56	0.5	0.21
% Grain Moisture	13	18.5	18.6	0.5	0.60
Yield	13	147	145.6	1.0	0.61

1 – Refer to Table 15 for units of measure
2 – [(MON 863 - Control) / Control] x 100

Table 20. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-5. Utilizing a balanced lattice square design, MON 863 was compared to its parental control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-5				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	7	2.8	3.1	9.7	0.10
GDU to Pollen Shed	8	1265	1274	0.7	0.06
GDU to Silk	6	1246	1247	0.08	0.84
Ear Height	10	48.4	44.2	9.5	0.04
Plant Height	10	98.1	99	0.9	0.47
Final Stand	13	67.9	68.7	1.2	0.38
Test Weight	11	54.7	55.5	1.4	0.23
% Grain Moisture	13	17.5	18.4	4.9	0.01
Yield	13	154.5	155.6	0.7	0.78

1 – Refer to Table 15 for units of measure
2 – [(MON 863 – Control) / Control] x 100

Table 21. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-6. Utilizing a balanced lattice square design, MON 863 was compared to its parental control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-6				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	7	3.0	3.0	0	1.0
GDU to Pollen Shed	8	1277	1281	0.3	0.28
GDU to Silk	6	1246	1248	0.2	0.50
Ear Height	10	47.4	46	3.0	0.19
Plant Height	10	97.8	97.4	0.4	0.85
Final Stand	13	69.5	69.1	0.6	0.45
Test Weight	11	55.4	55.2	0.4	0.65
% Grain Moisture	13	19.4	20.1	3.5	0.03
Yield	13	153.5	149.2	2.9	0.20

1 – Refer to Table 15 for units of measure
2 – [(MON 863 – Control) / Control] x 100

Table 22. Summary of agronomic equivalency trials conducted at multiple locations with the MON 863 hybrid, H-1. Utilizing a balanced lattice square design, MON 863 was compared to its negative isogenic control. The number of sites from which data were collected, the parameter least square mean, the percent difference from control (%Δ), and the calculated *p*-values are presented.

Parameter [1]	Hybrid H-1				
	# Sites	MON 863	Control	% Δ [2]	*p*-value
Seedling Vigor	8	3.4	3.4	0	0.86
Ear Height	9	49.2	47.8	2.9	0.32
Plant Height	9	95.8	95.1	0.7	0.53
Final Stand	11	68.6	69.3	1.0	0.32
Test Weight	11	53.7	54.2	0.9	0.45
% Grain Moisture	11	17.2	16.5	4.2	0.07
Yield	11	133.3	126.9	5.0	0.18

1 – Refer to Table 15 for units of measure
2 – [(MON 863 – Control) / Control] x 100

For the MON 863 H-1 hybrid a 4.3% increase in ear height and a 1.7% increase in plant height were observed in the randomized split-plot trials (Table 16). These differences are small and of no agronomic significance. Additionally, they were not observed in the H-1 hybrid trials planted in a balanced lattice square design (refer to Table 22).

When the MON 863 H-2 hybrid was compared to its negative isoline, a 1.8% reduction in test weight was observed. Such a small difference is of no agronomic significance; furthermore, no effect on test weight was observed with the other five hybrids. When the MON 863 H-2 hybrid was compared to its negative isoline, an 8.2% increase in yield was observed. Significant yield differences were not observed at all locations. This difference may be attributable to inadvertent plant selection bias during the breeding process. In all other hybrid comparisons there were no differences in yield performance observed.

A 4.1% increase in ear height was observed in the MON 863 H-3 hybrid when compared to its isogenic control. As test weight and yield were unaffected, this difference is of no agronomic significance.

Equivalence between the MON 863 H-4 hybrid and its parental control was observed for all parameters except seedling vigor. The MON 863 seedlings were found to be more vigorous (*i.e.*, lower rating) than control seedlings. This may be attributable to differences in seed source (*i.e.*, nursery location and timing of production).

When MON 863 hybrid H-5 was compared to its parental control, a 9.5% increase in ear height and 4.9% reduction in grain moisture were observed. A 3.5% reduction in grain

moisture was also observed with the MON 863 H-6 hybrid (the only difference observed for this hybrid). At the ten sites where H-5 ear height was recorded, the MON 863 hybrid had an elevated ear height compared to parental controls at nine sites. This is most likely an interaction effect of the converted inbred with the tester line used to make the H-5 hybrid. Such an effect has not been seen in other test crosses made with the same MON 863 inbred. The lower grain moisture in the H-5 and H-6 hybrids could be a spurious finding as it was not observed in the other four hybrids. Furthermore, it is of no agronomic concern because there was no corresponding effect on test weight or yield in these two hybrids.

It is quite common to see variation in traits with converted inbreds and the hybrids produced from them, such as H-4, H-5 and H-6. The converted line will differ from a recurrent parent not only for the trait of interest, but also in having segments of DNA surrounding the transgene which traces back to the genome of the MON 863 donor line. This phenomenon is known as "linkage drag". It may result in a converted line differing from the original line for a segment of DNA that is large enough to contain many genes. The segment of DNA that remains linked to a trait of interest through the backcrossing process may introduce natural variation in corn for genes affecting any number of agronomic traits of complex inheritance. This phenomenon is a natural consequence of the breeding process and is not preventable. Thorough testing and selection of converted lines eliminates any linkage drag that may be present in a given transgenic event.

Collectively, the results of these trials support a conclusion of agronomic equivalence for MON 863 hybrids and their respective controls. In multiple field trials with six MON 863 hybrids, statistically significant differences were observed for very few of the 64 parameters evaluated. These differences were uniformly small, not consistently observed across locations or other MON 863 hybrids, and none would be considered to be of adverse agronomic consequence. Furthermore, there is no correlation between these small differences that would be indicative of increased weediness potential.

VII. Environmental Consequences of Introduction of MON 863

A. *B. t.* Insect Control Proteins

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 a wide variety of insect species. The appearance of parasporal inclusions distinguishes *B.t.* from the other common soil bacterium, *Bacillus cereus*. The proteins contained within the parasporal crystals can account for as much as one-third the weight of the bacterial cell.

Corporate, institutional, and government strain 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* 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, cotton bollworm). Biopesticidal products based on recombinant *B.t.* strains have also been commercialized. 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).

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, 1993; Feitelson *et al.*, 1992). 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 (Figure 18). 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 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 different 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. The coleopteran-toxic Cry3 proteins are 73-74 kDa in molecular mass and share significant (~36%) amino acid sequence identity with the amino terminal toxin domain of the Cry1-type proteins (Schnepf *et al.*, 1998).

A review of the research characterizing the mechanism of action for *B.t.* crystal proteins has been recently 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: 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).

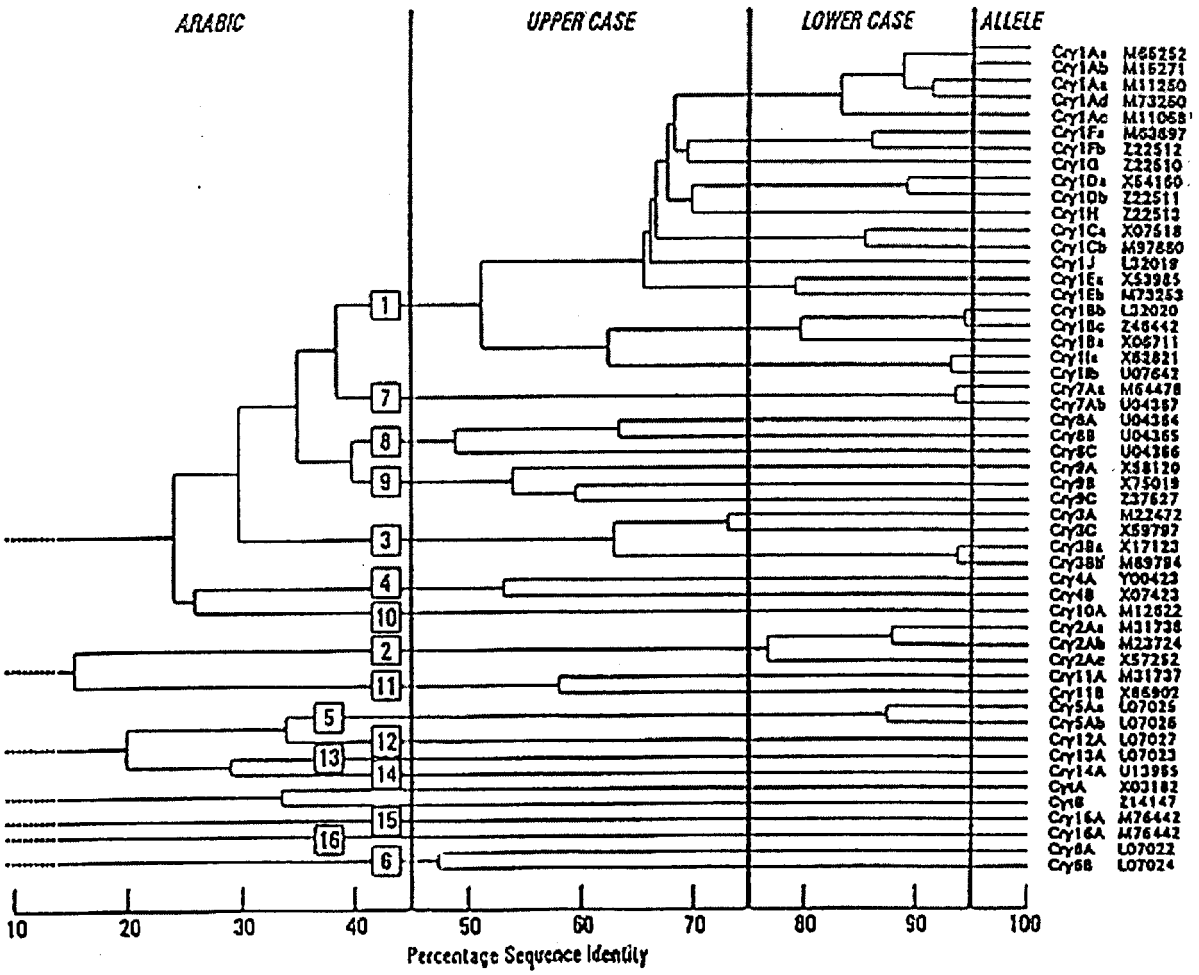

Figure 18. Dendrogram showing sequence relatedness among Cry Proteins as determined by ClustalV multiple alignment analysis. Vertical bars demarcate levels of sequence identity chosen in the standardized nomenclature to define the four levels of rank - Arabic numerals, upper and lower case letters, and allele numbers. Boxed numerals indicate the first level (Arabic numeral) groupings of proteins (*e.g.*, Cry1, Cry2, Cry3, *etc.*). Individual proteins are designated by their revised nomenclature assignment and database accession numbers. (Crickmore *et al.*, 1998).

B. Specificity of Cry3Bb1 Protein

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 that membrane (English and Slatin, 1992). 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 (Schnepf *et al.*, 1998). 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 (Noteborn, 1994; Sacchi *et al.*, 1986; 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.

B.t. Cry proteins have been generally classified based on their insecticidal activity. For example, Cryl proteins are toxic to lepidopteran pests, Cry2 proteins are toxic to lepidopteran and dipteran pests, Cry3 proteins are toxic to coleopteran pests, and Cry4 proteins are toxic to dipteran pests (Bravo, 1997; Höfte and Whiteley, 1989). The Cry3 class protein, Cry3Bb1, has natural insecticidal activity against the coleopteran pest, corn rootworm (Von Tersch *et al.*, 1994).

A series of dietary bioassays has been conducted to characterize the insecticidal specificity of Cry3Bb1. These bioassays demonstrate that Cry3Bb1 insecticidal activity is limited to select beetle species within the family Chrysomelidae, namely the CRW and the Colorado potato beetle. Bioassays on pest insects have been performed by Monsanto with the Cry3Bb1 protein variant extracted from recombinant *B.t.* strain, EG11231. This variant of Cry3Bb1 differs from the wild type protein by only four amino acids and from the variant produced in MON 863 by three amino acids (2A, D166G and Q349R). Cry3Bb1 protein was isolated and purified from sporulated lysed cultures of recombinant *B.t.* strain EG11231 utilizing methods reported by Donovan *et al.* (1992) and Malvar *et al.* (1994). The following species were tested: Colorado potato beetle, western corn rootworm, cowpea weevil, red flour beetle, cotton boll weevil, pepper weevil, rice weevil, corn earworm and European corn borer. Multiple concentrations of Cry3Bb1 protein were used in these assays, typically ranging from 1 to 200 ppm (except in the case of boll weevil where 50 ppm was the highest dose tested). The upper limit of 200 ppm was chosen based on an expectation that this would represent the maximum achievable level of Cry3Bb1 production in plants.

Insects were obtained from commercial suppliers as eggs that were then held in incubators until eclosion. Neonates were infested into the test diets. Mortality and weight measurements were recorded seven days after infestation. All assays were replicated. When adverse effects were observed, concentration-mortality regressions were estimated assuming a probit model and body mass reduction was used to calculate effective concentrations (using a nonlinear regression model). When no treatment-related adverse effects were detected, the highest dose tested was designated the no observable effect concentration (NOEC). The results of these bioassays are summarized in Table 23.

C. Current Uses of Insecticides for Corn Rootworm Control in Corn

Corn yields are adversely impacted by a number of insect pests. One of the most pernicious pests in the U.S. Corn Belt is the corn rootworm complex, comprised primarily of the WCRW and NCRW. Damage is caused by the larvae feeding on the corn roots,

Table 23. Susceptibility of various insect pests to Cry3Bb1 extracted from recombinant *B.t.* strain EG11231 as determined in laboratory dietary bioassays. With all species, except the cotton boll weevil, the highest concentration tested was 200 ppm.

Species Tested	LC_{50} (ppm)	NOEC (ppm)
Colorado potato beetle (*Leptinotarsa decimlineata*)	2.7	
Western corn rootworm (*Diabrotica virgifera*)	75	
Cowpea weevil (*Callosobruchus maculatus*)		200
Red flour beetle (*Tribolium castaneum*)		200
Cotton boll weevil (*Anthonomus grandis*)		50
Pepper weevil (*Anthonomus eugenii*)		200
Rice weevil (*Sitophilus oryzae*)		200
Corn earworm (*Helicoverpa zea*)		200
European corn borer (*Ostrinia nubilalis*)		200

which reduces the ability of the plant to absorb water and nutrients (Reidell, 1990) and causes harvesting difficulties due to plant lodging (Spike and Tollefson, 1991).

Growers mitigate CRW damage primarily through crop rotation and the use of soil-applied 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 to have the option of continuous (nonrotated) corn production, even if this practice requires increased chemical inputs for soil fertility and insect control. Second, researchers have confirmed that populations of both NCRW and WCRW can exhibit extended diapause, and in these instances some eggs 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, crop rotation is no longer effective as a cultural rootworm management option in east central Illinois and northern Indiana due to the rapid spread of a new strain of WCRW that, unlike previous populations, survives well on soybean (Onstad and Joselyn, 1999; O'Neal *et al.*, 1999). Based on the rapid expansion of this variant population since its initial discovery in 1993, it is expected to continue to spread throughout the Corn Belt.

Each of these factors has increased growers' reliance on synthetic chemical insecticides for CRW control. CRW is the most significant insect pest problem of corn in the U.S. Midwest from the standpoint of chemical insecticide use. 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 (NASS) of the USDA has compiled statistics on 1999 corn insecticide use across 15 states comprising 68.3 million acres of corn (NASS, 2000). Those insecticides that are registered for CRW control are shown in Table 24. NASS statistics indicate that chemical insecticides registered for CRW control were applied on over 30% of this corn acreage in 1999. These totals would also include acres treated with commercial insecticide combination products, as well as acres treated for other soil pests such as black cutworms. In Iowa, the leading state for corn production with 17.5% of U.S. production in 1999, a comprehensive crop pesticide use survey was conducted in 1995 (Hartzler, 1997). This survey found that growers applied insecticides for CRW control on 22% of corn acres. The most widely used products are from the organophosphate or synthetic pyrethroid classes of chemistry.

Table 24. Percent of corn acres in the U.S. Corn Belt treated in 1999 with insecticides registered for corn rootworm larval (L) and adult (A) control.

Active Ingredient Applied	Type[1]	% Acres Treated
Chlorpyrifos (L,A)	OP	5
Terbufos (L)	OP	5
Tebupirimfos (L)	OP	2
Chloroethoxyfos (L)	OP	1
Dimethoate (A)	OP	< 1
Fonofos (L)	OP	< 1
Methyl parathion (A)	OP	1
Phorate (L)	OP	< 1
Ethoprop (L)	OP	< 1
Tefluthrin (L)	SP	7
Cyfluthrin (L)	SP	2
Permethrin (A)	SP	3
λ-Cyhalothrin (A)	SP	3
Carbofuran (L)	C	1

1 - OP = Organophosphate; SP = Synthetic pyrethroid; C = Carbamate

Widespread use of synthetic chemical insecticides for CRW control is associated with worker safety, food residue and environmental risks that have been a concern to many,

including the U.S. EPA. There is a need for safe and effective alternatives to these chemical insecticides. MON 863 offers such an alternative.

D. Development of Resistance Management Strategies

The use of corn hybrids containing the Cry3Bb1 protein will eventually result in the selection of CRW that are tolerant to the toxin. In this sense, the protein is no different than any conventional insecticide. Selection pressure will not become significant until the product is in widespread use many years after its introduction. Monsanto is engaged in ongoing research to identify appropriate insect resistance management (IRM) strategies for MON 863. IRM strategies will be put in place at the time of product commercialization to minimize the risk of resistance developing. These strategies are being developed in concert with academia and USDA experts on CRW (*i.e.*, NCR-46) and will include: the use of a refuge planted to nontransgenic corn to ensure the survival of insects susceptible to the Cry3Bb proteins, monitoring for resistant insects, use of complementary IPM practices, and a comprehensive education program for growers and other stakeholders (*e.g.*, NCGA). A detailed IRM plan will be submitted to EPA for approval prior to commercialization.

E. Impact of Cry3Bb1 Proteins on Nontarget Organisms

Ecological risk associated with exposure to Cry3Bb1 protein variants has been evaluated in a series of studies with representative beneficial terrestrial invertebrates and aquatic species. These 'nontarget' organisms were exposed to high doses of leaf tissue, grain or pollen containing the same Cry3Bb1 variant as produced in MON 863 or to an artificial diet containing the purified variant from recombinant *B.t.* strain, EG11231. The results of these studies are summarized in Table 25. They demonstrate that Cry3Bb1 proteins pose no significant risk for harm to nontarget organism populations. In all studies conducted, a NOEC was established and found to equal or exceed predicted maximum environmental concentrations.

Feeding of Cry3Bb1-containing grain to channel catfish at 35% of their diets resulted in no adverse effects on growth rate or survival (Li and Robinson, 1999). The potential toxicity of Cry3Bb1 variants was also evaluated in seven species of beneficial invertebrates: cladoceran (Drottar and Krueger, 1999), collembola (Teixeira, 1999), adult and larval ladybird beetles of the specie, *Coleomegilla maculata* (Duan *et al.*, 2001a and 2001b), adult ladybird beetles of the specie *Hippodamia convergens* (Bryan *et al.*, 2001), adult and larval honey bees (Maggi, 1999a and 1999b), green lacewing larvae (Palmer and Krueger, 1999a), parasitic wasps (Palmer and Krueger, 1999b) and earthworms (Hoxter *et al.*, 1999).

No adverse effects were observed at the maximum predicted environmental concentration to which these beneficial organisms would be exposed. The maximum environmental concentration for organisms feeding on corn plants is predicted to be 93 µg/g based on the highest Cry3Bb1 expression level measured in pollen and leaf tissue of MON 863 plants

Table 25. Summary of results from ecological toxicity tests with Cry3Bb1 proteins. Plant tissue expressing the MON 863 variant of the *cry3Bb1* gene served as the test substance for some assays. All other assays employed an artificial diet containing the EG11231 variant of Cry3Bb1. Risk conclusions are based on protein concentrations in plant tissues from event MON 863.

Test Organism	Test Substance	Results	Conclusions
Cladoceran (*Daphnia magna*)	Pollen containing Cry3Bb1	NOEC ≥ 2.26 µg/l	NOEC > 141X predicted maximum concentration in surface water
Collembola (*Folsomia candida*)	Leaf containing Cry3Bb1	NOEC ≥ 872.5 µg protein/g diet	NOEC > 66X predicted maximum concentration in soil
Channel Catfish	Grain containing Cry3Bb1	No effect on growth or survival at 35% of diet	No significant risk
Larval Ladybird Beetle (*C. maculata*)	Pollen containing Cry3Bb1	No effect on development or survival at 50% of diet	No significant risk
Adult Ladybird Beetle (*C. maculata*)	Pollen containing Cry3Bb1	No effect on survival at 50% of diet	No significant risk
Adult Ladybird Beetle (*H. convergens*)	Pollen containing Cry3Bb1	No effect on survival at 50% of diet	No significant risk
Adult Honey Bee (*Apis mellifera*)	EG11231 in an artificial diet	NOEC ≥ 360 µg/ml in diet	NOEC > 3.9X predicted maximum concentration in pollen
Larval Honey Bee (*Apis mellifera*)	EG11231 in water	NOEC ≥ 1790 µg/ml as a single dose	NOEC > 19X predicted maximum concentration in pollen
Green Lacewing Larvae (*Chrysoperla carnea*)	EG11231 in an artificial diet	NOEC ≥ 8000 µg/g in diet	NOEC > 86X maximum environmental concentration predicted in diet
Parasitic Hymenoptera (*N. vitripennis*)	EG11231 in an artificial diet	NOEC = 400 µg/ml in diet	NOEC > 4.3X maximum environmental concentration predicted in pollen
Earthworm (*Eisenia fetida*)	EG11231 in soil	NOEC = 57 mg/kg in soil	NOEC ≥ 4.3X maximum estimated environmental exposure in soil

(refer to Tables 7 and 8). The maximum environmental concentration for soil-dwelling organisms is predicted to be 13.3 mg/kg based on an assumption that corn plants are tilled into the top 6" of soil at the time of maximum leaf expression for Cry3Bb1 (*i.e.*, 93 µg/g).

The maximum environmental concentration for aquatic organisms is predicted to be 0.016 µg Cry3Bb1 protein per liter based on the following assumptions: Cry3Bb1 concentration is 93 µg/g, an edge of field deposition rate of 0.02-0.03 mg pollen/cm^2, and draining into a body of water 2 meters in depth. The measured NOECs from these tests exceed the maximum predicted environmental concentration by 3.9 to 141-fold, thus demonstrating an adequate margin of safety for these organisms.

Cry3Bb1 proteins are selectively toxic to coleopteran insects such as CRW. Studies conducted to determine the insecticidal spectrum of activity for Cry3Bb1 have established that this *B.t.* protein exhibits excellent activity against beetles (order Coleoptera) of the family Chrysomelidae (Donovan *et al.*, 1992; Rupar *et al.*, 1991). However, it has no activity against other families of beetles or other insect orders, including Lepidoptera. Specifically, no effects on survival or growth have been seen in studies with corn earworm and European corn borer at dietary concentrations of 200 ppm (refer to Table 23). There is also no reason to believe the Cry3Bb1 proteins would represent a threat to butterflies, such as the Monarch butterfly. Lack of activity for Cry3Bb1 protein in Lepidoptera was confirmed in laboratory bioassays with *H. virescens* and *O. nubilalis* (refer to Table 23).

A review of the endangered or threatened species listed in 50 CFR 17.11 and 17.12 reveals that none of the members of the Chrysomelidae family are endangered or threatened, and only one member of the superfamily is so identified. That species is the valley elderberry longhorn beetle, *Desmocerus californicus dimorphus*, which is located in California and does not feed on corn. The valley elderberry longhorn beetle feeds solely on elderberry and is not likely to come in contact with Cry3Bb1-containing corn tissue. Thus, the risk is minimal to any endangered or threatened coleopteran species.

As further evidence of the protein's low risk, the results of an aerobic soil degradation study demonstrate that the Cry3Bb1 protein degrades rapidly in the environment (Martin *et al.*, 2000)[‡]. This study measured the decline in soil concentrations of Cry3Bb1, as well as its loss of insecticidal activity over time. Analysis of insect bioassay and ELISA data resulted in DT50 estimates of 2.37 and 2.76 days, respectively. The dissipation rate estimates resulting from both of these methods indicate that Cry3Bb1 dissipates rapidly in soil.

In summary, the results of toxicity studies with numerous nontarget organisms exposed to Cry3Bb1 proteins support a conclusion that MON 863 poses no significant risk to the environment. In fact, its introduction will likely reduce environmental risk in the U.S. corn belt through the displacement of synthetic chemical insecticide applications.

[‡] This study was conducted with plant tissue of corn event MON 859. This event was transformed with vector ZMIR13L and thus, produces the identical Cry3Bb1 variant as found in event MON 863.

F. Weediness Potential of MON 863

Modern corn cannot survive as a weed because of past selection in the evolution of corn. In contrast with weedy plants, the corn ear is enclosed with husks. Consequently, seed dispersal of individual kernels does not occur naturally because of the structure of ears of corn. Even if individual kernels of corn were distributed in the fields and main avenues of travel from the fields to storage facilities, volunteer corn is not found growing in fence rows, ditches, and road sides as a weed. Although corn seed can overwinter into a crop rotation with soybeans, mechanical and chemical measures can be utilized to control volunteers. Corn is poorly suited to survive without human assistance and is not capable of surviving as a weed (Galinat, 1988). Insertion of the *cry3Bb1* gene into the plant genome will not impart an increased weediness potential to corn line MON 863.

A growth chamber study has been conducted to evaluate dormancy and germination characteristics of MON 863 seed (Wiltse *et al.*, 2001). Corn hybrids containing event MON 863 were compared to seeds of a nontransgenic hybrid control (RX670) and four conventional reference corn hybrids under eight different temperature regimes ranging from 5 to 40°C. MON 863, control and reference seeds were arranged in a complete random block design with four replications. The number of germinated and degenerated seeds was counted periodically throughout a 12-day experimental period. Ungerminated seeds remaining on the twelfth day were tested for viability using a tetrazolium test and characterized as hard or firm-swollen seed. The following parameters were evaluated: mean percent viable hard (pvhs), percent germinated (pgerm), percent viable firm-swollen (pfms), and percent degenerated seed (pdegen). Pdegen was defined as the sum of percentage of degenerated seed removed during the course of the study plus the percentage of nonviable hard and firm-swollen seed identified by the tetrazolium test at the end of the study. This study was conducted under standards established by the Association of Official Seed Analysts, a seed trade association.

The results of this study are summarized in Table 26. No statistically significant differences between MON 863 and control pvhs values were observed. One statistically significant difference was noted between MON 863 and control values for each of the other parameters evaluated. These differences were small (<0.5%) and the MON 863 values fell within the range of values obtained for the reference hybrids. These findings provide no evidence of an increase in weediness potential for MON 863 based on altered seed dormancy and germination. The extensive database of agronomic, phenotypic, compositional, and disease and insect susceptibility data developed for MON 863 corn collectively provides no evidence of increased plant pest risk.

G. Cross Pollination to Wild and Cultivated Related Species

The potential for pollen transfer from corn to other species is addressed in Section II of this document. Based on an extensive review of available literature on corn outcrossing potential and a history of nonregulated status for genetically modified corn phenotypes, movement of transgenes from corn to related plant species, other than corn, is unlikely.

Table 26. Comparison of seed dormancy and germination characteristics for MON 863 and nontransgenic corn seed. Mean values for pvhs, pgerm, pfms and pdegen parameters measured in MON 863 hybrids were compared to a nontransgenic control line of comparable germplasm (RX670), and to a range of values for reference commercial corn hybrids under multiple temperature regimes.

Temp.	Hybrid	pvhs [1] (Dormant) (%)	pgerm [1] (%)	pfms [1] (%)	pdegen [1] (%)
5° C	MON 863	0.0	0.0	97.4*	2.8*
5° C	RX670	0.0	0.0	97.9	2.3
5° C	Ref. Range	(0-0)	(0-0)	(97-100)	(0-3)
10° C	MON 863	0.0	99.6	0.0	0.8
10° C	RX670	0.0	98.8	0.3	1.5
10° C	Ref. Range	(0-0)	(94-100)	(0-3)	(0-4)
20° C	MON 863	0.0	99.6	0.0	0.8
20° C	RX670	0.0	99.7	0.0	0.5
20° C	Ref. Range	(0-0)	(97-100)	(0-0)	(0-3)
30° C	MON 863	0.0	99.1*	0.0	1.3
30° C	RX670	0.0	99.4	0.0	0.8
30° C	Ref. Range	(0-0)	(97-100)	(0-0)	(0-3)
40° C	MON 863	0.0	93.6	0.0	6.5
40° C	RX670	0.0	97.9	0.0	2.3
40° C	Ref. Range	(0-0)	(77-100)	(0-0)	(0-23)
5/20° C	MON 863	0.0	99.4	0.0	0.8
5/20° C	RX670	0.0	99.8	0.3	0.3
5/20° C	Ref. Range	(0-0)	(97-100)	(0-1)	(0-3)
10/20° C	MON 863	0.0	99.9	0.0	0.3
10/20° C	RX670	0.0	99.4	0.0	0.8
10/20° C	Ref. Range	(0-0)	(94-100)	(0-1)	(0-6)
20/30° C	MON 863	0.0	99.6	0.0	0.7
20/30° C	RX670	0.0	99.7	0.0	0.5
20/30° C	Ref. Range	(0-0)	(96-100)	(0-0)	(0-4)

* Indicates significant difference from RX670 at $p < 0.05$.

1 - pvhs = percent viable hard seed, pgerm = percent germinated seed, pfms = percent viable firm-swollen seed, pdegen = percent degenerated seed

1. MON 863 Outcrossing Potential to Wild *Zea* Species

For gene flow, in the form of successful introgression, to occur via normal sexual transmission, certain conditions must exist: 1) the two parents must be sexually compatible; 2) there must be overlapping phenology; and 3) a suitable pollen vector must be present and capable of transferring pollen between the two parents.

Corn and annual teosinte (*Zea mays* subsp. *mexicana* Schrad.) are genetically compatible, wind pollinated and, in areas of Mexico and Guatemala freely hybridize when in close proximity to each other. Corn easily crosses with teosinte; however, teosinte is not present in the U.S. other than as occasional botanical garden specimens. These specimens would only flower at the same time as corn (due to photoperiod reaction) if they were subject to artificial day length shortening for several weeks at a time (Wilkes, 1967). Differences in factors such as flowering time, geographical separation and development factors make natural crosses in the United States speculative.

The habitat preferences of *Tripsacum*, another related genus, are similar to those of teosinte, with twelve of the sixteen species native to Mexico and Guatemala. *T. dactyloides* is widespread in the U.S. but crosses in nature are unknown. *T. floridanum* (Florida Gamagrass) is native to the southern tip of Florida. Outcrossing with *Tripsacum* species is not known to occur in the wild. Only with extreme difficulty can corn be crossed with *Tripsacum* species. Furthermore, the offspring of this cross show varying levels of sterility (Galinat, 1988; Mangelsdorf, 1974; Russell and Hallauer, 1980). No cases of gene flow between corn and its wild relatives are known to occur in the U.S.

2. MON 863 Outcrossing Potential to Cultivated *Zea* Varieties

Gene exchange between cultivated corn and genetically modified corn would be similar to what occurs between conventional corn varieties. Wind blown pollen would move among plants within the same field and among plants in nearby fields. Free flow of genes would occur in a manner similar to what occurs in cultivated corn. The production of Cry3Bb1 in seed would not be of concern due to the lack of potential to cause harm given its demonstrated safety to humans and nontarget organisms.

3. Assessment of MON 863 Pollen

Reproductive potential is one of several factors affecting plant pest potential. Reproductive potential can be defined as the likelihood of successful hybridization with a sexually compatible species that yields viable seed and maintains the ability to reproduce in later generations. For U.S. corn, reproductive potential is limited to intraspecific hybridization. Changes in viability caused by the genetic transformation of corn could influence plant pest potential. Pollen viability and morphology for event MON 863 have been evaluated.

Differences in MON 863 pollen viability should impact heritability of the corn rootworm-protected trait. Segregation analysis of this trait over five generations has shown that the *cry3Bb1* gene follows a simple Mendelian inheritance pattern of a single dominant gene (see Table 6). These data suggest there is no selection for gametes (*i.e.*, pollen) carrying the transgene. Gamete selection would have skewed the segregation data towards more trait-positive or trait-negative progeny. Confirmatory data for the lack of altered reproductive potential for MON 863 corn comes from an assessment of gross pollen morphology. Light microscopic examination at 200x multiplication revealed no gross

differences between MON 863 and parental control pollen (Wiltse, 2001). The genetic modification needed to create event MON 863 has not resulted in an alteration to pollen morphology.

H. Transfer of Genetic Information to Species with Which Corn Cannot Interbreed

As stated in the USDA's Interpretative Ruling on Calgene, Inc., Petition for Determination of Regulatory Status (U.S. Federal Register **57,** No.202, pp. 47608-47616, October 19, 1992), "There is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from plants to other organisms. Evidence presented in the Calgene petition and supplementary information summarized in the FR Notice suggests that, based on limited DNA homologies, transfer from plants to microorganisms may have occurred in evolutionary time over many millennia. Even if such transfer were to take place, transfer of a *cry3Bb1* gene to a microbe would not pose a plant pest risk. Based on these considerations, transfer to microbes or other living species in nature is extremely unlikely and of no significant consequence from a plant pest perspective.

I. Environmental Benefits from the Introduction of MON 863

The delivery of an efficacious *B.t.* protein for CRW control in genetically modified plants integrates well with current agronomic practices and offers growers numerous advantages over the use of synthetic chemical insecticides. MON 863 provides an ideal fit with current IPM practices, which generally recommend scouting for adult CRW in the late summer and early fall and the use of economic thresholds to optimize insecticide application decisions for the following spring. More often, however, growers base their CRW management decisions on cropping history and always apply soil insecticides for corn following corn. The introduction of MON 863 provides growers with an alternative to the application of organophosphate and synthetic pyrethroid products. Moreover, growers will be able to use the same pest management and economic criteria for decisions on planting of CRW-protected corn seed.

Crops such as MON 863 that are improved through biotechnology provide totally new pest control options that are of equal benefit to large and small growers. No new equipment or specialized skills are needed. Use of MON 863 will reduce the need for manufacture, storage, transportation, and disposal of hazardous pesticides and pesticide containers, as well as the reliance on properly calibrated and maintained pesticide application equipment. Furthermore, the *B.t.* protein in MON 863 is expressed in the target tissue (*i.e.*, roots), thus eliminating the possibility of soil insecticide "misses" or the limitations of the chemical zone of protection around insecticide granules.

Based on the safety and efficacy of CRW-protected corn, and its fit into current agronomic practices, many growers are expected to adopt this technology as a replacement for traditional chemical control techniques. This product offers the potential to significantly reduce the amount of chemical pesticides applied in the U.S. Corn Belt,

benefiting the environment and leading to a reduction in real and potential adverse effects throughout the manufacturing, distribution and use chain.

VIII. Adverse Consequences of Introduction

Monsanto Company is unaware of any information indicating that MON 863 may pose a greater plant pest risk than nontransgenic corn. In addition, there are no adverse environmental consequences anticipated with its introduction. Therefore, on the basis of the substantial benefits that this product offers for U.S. agriculture and the environment, Monsanto requests that MON 863 be granted nonregulated status under 7 CFR Part 340.

IX. References

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APPENDIX A

USDA Notifications Approved for MON 863* and Status of Trials Conducted Under These Notifications

USDA Reference No.	Effective Date	Release Site (State)	Trial Status
98-033-01n	03/05/1998	PR, IA	Report submitted to USDA
98-040-02n	03/11/1998	HI, IA	Report submitted to USDA
98-040-04n	03/23/1998	IL, SD	Report submitted to USDA
98-085-28n	05/04/1998	KS, IA, IL, IN, NE, OH, MO	Report submitted to USDA
98-098-01n	05/11/1998	IL, HI	Report submitted to USDA
98-098-02n	05/08/1998	KS	Report submitted to USDA
98-128-13n	06/07/1998	MO	Report submitted to USDA
98-216-01n	09/03/1998	PR	Report submitted to USDA
98-229-02n	09/16/1998	HI	Report submitted to USDA
98-229-03n	09/16/1998	HI	Report submitted to USDA
98-229-04n	09/16/1998	HI	Report submitted to USDA
98-229-05n	09/16/1998	HI	Report submitted to USDA
98-229-07n	09/16/1998	HI	Report submitted to USDA
98-229-08n	09/16/1998	WI	Report submitted to USDA
98-229-09n	09/17/1998	HI, IA, IL, IN, PR	Report submitted to USDA
98-245-05n	10/02/1998	PR	Report submitted to USDA
98-247-01n	10/04/1998	HI	Report submitted to USDA
98-247-02n	10/09/1998	PR	Report submitted to USDA
98-266-03n	10/23/1998	CT, HI, IL	Report submitted to USDA
98-288-11n	11/14/1998	PR, IA	Report submitted to USDA
98-302-01n	11/29/1998	HI	Report submitted to USDA
99-040-06n	03/22/1999	IL, IN, WI, CA, FL	Report submitted to USDA
99-040-07n	03/11/1999	HI, IL	Report submitted to USDA
99-056-09n	03/27/1999	HI, IL	Report submitted to USDA
99-055-04n	04/06/1999	HI, MN, PR	Report submitted to USDA
99-055-06n	04/13/1999	TN, IA, IL, HI, AL, CT, PR	Report submitted to USDA
99-055-07n	04/05/1999	HI, NE	Report submitted to USDA
99-056-04n	03/27/1999	PR, HI	Report submitted to USDA
99-056-07n	03/27/1999	IN, HI, PR, IL, IA	Report submitted to USDA
99-060-05n	04/05/1999	MN, TX, IN, NY, OH, IA, PA	Report submitted to USDA
99-060-06n	04/01/1999	NE, SD, MI, IL, WI, VA	Report submitted to USDA
99-063-03n	04/03/1999	KS, VA	Report submitted to USDA
99-063-04n	04/23/1999	IL, MN, IA, PR	Report submitted to USDA
99-071-41n	04/11/1999	SD, IL, MO, IA, NE	Report submitted to USDA
99-091-07n	05/04/1999	NE, IN	Report submitted to USDA

USDA Reference No.	Effective Date	Release Site (State)	Trial Status
99-095-13n	05/07/1999	IL, IA, NE, MN, IN, HI	Report submitted to USDA
99-095-14n	05/05/1999	NY	Report submitted to USDA
99-095-15n	05/07/1999	IN	Report submitted to USDA
99-106-16n	05/16/1999	IA, IL, NE	Report submitted to USDA
99-111-04n	05/21/1999	IN	Report submitted to USDA
99-112-07n	05/22/1999	IA, IL, IN, NE	Report submitted to USDA
99-116-05n	05/26/1999	TN, HI, IL, IN, IA	Report submitted to USDA
99-116-08n	05/26/1999	IN, KS	Report submitted to USDA
99-116-09n	05/26/1999	MD	Report submitted to USDA
99-116-11n	06/03/1999	IL, WI, CA, FL, ID, PR, MN	Report submitted to USDA
99-124-01n	06/03/1999	NY	Report submitted to USDA
99-125-01n	06/04/1999	MO	Report submitted to USDA
99-126-08n	06/08/1999	TN, PR, HI, IL, IN, IA, CT	Report submitted to USDA
99-126-13n	06/05/1999	IA, IL	Report submitted to USDA
99-132-01n	05/26/1999	HI, IL, OH	Report submitted to USDA
99-133-05n	05/26/1999	HI, NE	Report submitted to USDA
99-244-08n	10/01/1999	HI, IN	Report submitted to USDA
99-244-10n	10/07/1999	PR	Report submitted to USDA
99-244-12n	10/07/1999	PR, IA	Report submitted to USDA
99-270-03n	10/27/1999	IN, NE	Report submitted to USDA
99-309-01n	12/06/1999	WI, PR, HI	Report submitted to USDA
00-031-04n	03/02/2000	HI, NE	In progress
00-031-06n	03/01/2000	HI, IL, IN, PR	In progress
00-031-08n	03/01/2000	HI, IA, IN	In progress
00-031-10n	03/01/2000	IL, PR	In progress
00-031-12n	03/01/2000	HI, IA, IL	In progress
00-031-14n	03/01/2000	IL, PR	In progress
00-038-04n	03/08/2000	LA	In progress
00-038-09n	03/09/2000	NY, OH, PA	In progress
00-038-11n	03/08/2000	MI, MN, SD, WI	In progress
00-038-14n	03/09/2000	CO, KS, OK	In progress
00-039-04n	03/09/2000	IA, IL, IN, MO, NE	In progress
00-042-09n	03/15/2000	HI, PR	In progress
00-053-19n	03/23/2000	HI	In progress
00-066-08n	04/05/2000	IL, IN, MS, OH, WI	In progress
00-066-10n	04/12/2000	CO, IA, KS, MO, NM	In progress
00-066-12n	04/18/2000	MI, MN, ND, NE, SD	In progress
00-082-08n	04/21/2000	IA, IL, KS, NE, OH	In progress
00-087-03n	04/27/2000	IL, NE	In progress
00-088-07n	05/03/2000	IA, IL, IN, MI, WI	In progress
00-088-09n	05/16/2000	NY, OH, PA, VA	In progress
00-088-13n	05/12/2000	IL, IN, PA, TN	In progress
00-088-16n	05/04/2000	MI, MN, SD, WI	In progress
00-088-20n	04/27/2000	IL, IN, MI, OH, PA	In progress
00-088-22n	05/12/2000	IA, KS, MO, NE, TX	In progress
00-088-24n	05/04/2000	MN, SD, WI	In progress

USDA Reference No.	Effective Date	Release Site (State)	Trial Status
00-088-26n	05/03/2000	CA	In progress
00-088-29n	05/05/2000	HI	In progress
00-088-32n	05/05/2000	PR	In progress
00-088-35n	05/12/2000	IA, IN, MN, MO, NE	In progress
00-088-39n	05/31/2000	CO, KS, MN, NE, SD	In progress
00-089-11n	05/05/2000	HI	In progress
00-095-07n	05/08/2000	HI, IN	In progress
00-095-09n	05/12/2000	IN	In progress
00-095-11n	05/08/2000	HI, IL	In progress
00-095-13n	05/19/2000	HI, IL, IN, MD, NE, PA, PR, WI	In progress
00-101-06n	05/15/2000	IN, MO, NE	In progress
00-103-06n	05/17/2000	IL	In progress
00-112-02n	05/12/2000	IA, IL	In progress
00-145-01n	08/03/2000	IN, NE	In progress
00-180-02n	07/28/2000	PR	In progress
00-214-04n	09/21/2000	IA, IL, MN, PR	In progress
00-279-03n	11/08/2000	HI, IA	In progress
00-336-01n	12/31/2000	AL	In progress
00-336-02n	12/31/2000	LA	In progress
00-336-03n	12/31/2000	TX	In progress
00-336-04n	12/31/2000	TN	In progress
00-336-05n	12/31/2000	GA	In progress
00-336-06n	12/31/2000	FL	In progress
00-339-01n	01/03/2001	TX	In progress
00-356-02n	01/31/2001	FL	In progress
00-356-03n	01/31/2001	HI	In progress
00-356-04n	01/31/2001	PR	In progress
00-356-07n	01/29/2001	TN	In progress
00-356-08n	01/26/2001	IL	In progress
00-356-19n	01/29/2001	IA	In progress
00-356-16n	01/22/2001	IN	In progress
00-356-17n	01/29/2001	OH	In progress
00-356-18n	01/29/2001	NE	In progress
01-010-01n	02/09/2001	VA	In progress
01-010-02n	02/09/2001	NC	In progress
01-010-03n	02/09/2001	NC	In progress
01-010-04n	02/09/2001	VA	In progress
01-010-05n	02/14/2001	AR	In progress
01-010-06n	02/09/2001	KY	In progress
01-010-07n	02/09/2001	TX	In progress
01-012-08n	02/21/2001	HI	In progress
01-012-09n	02/21/2001	NE	In progress
01-012-10n	02/21/2001	HI	In progress
01-012-11n	02/21/2001	IN	In progress
01-012-12n	02/21/2001	IL	In progress
01-012-13n	02/21/2001	HI	In progress
01-012-14n	02/11/2001	OH	In progress

USDA Reference No.	Effective Date	Release Site (State)	Trial Status
01-012-15n	02/11/2001	SD	In progress
01-016-07n	02/21/2001	PR	In progress
01-016-08n	02/21/2001	IL	In progress
01-016-09n	02/16/2001	HI	In progress
01-016-10n	02/15/2001	IL	In progress
01-016-11n	02/15/2001	MO	In progress
01-016-12n	02/15/2001	IN	In progress
01-016-13n	03/08/2001	NE	In progress
01-016-14n	03/12/2001	MN	In progress
01-016-15n	02/15/2001	WI	In progress
01-016-16n	02/15/2001	KS	In progress
01-016-17n	02/15/2001	IL	In progress
01-016-28n	02/15/2001	OK	In progress
01-016-29n	02/15/2001	OK	In progress
01-017-05n	02/16/2001	TX	In progress
01-017-06n	02/21/2001	HI	In progress
01-017-07n	02/16/2001	IN	In progress
01-017-10n	02/16/2001	IL	In progress
01-017-11n	02/28/2001	NE	In progress
01-018-04n	02/17/2001	CO	In progress
01-018-05n	02/17/2001	NY	In progress
01-018-06n	02/17/2001	NY	In progress
01-018-07n	02/17/2001	IA	In progress
01-018-09n	02/17/2001	IA	In progress
01-019-08n	02/18/2001	IL	In progress
01-019-09n	02/18/2001	IL	In progress
01-022-01n	02/21/2001	IA	In progress
01-022-02n	02/21/2001	IA	In progress
01-022-03n	02/21/2001	MO	In progress
01-022-04n	02/21/2001	SD	In progress
01-023-01n	02/22/2001	CA	In progress
01-023-02n	02/22/2001	HI	In progress
01-023-05n	03/08/2001	NE	In progress
01-023-06n	02/22/2001	IN	In progress
01-023-18n	02/22/2001	IL	In progress
01-023-19n	02/22/2001	IL	In progress
01-023-20n	02/22/2001	IN	In progress
01-023-21n	02/22/2001	WI	In progress
01-023-22n	02/22/2001	CO	In progress
01-023-23n	02/22/2001	SD	In progress
01-023-24n	02/23/2001	KS	In progress
01-023-25n	02/23/2001	KS	In progress
01-024-01n	02/23/2001	IL	In progress
01-024-02n	02/23/2001	PR	In progress
01-024-03n	02/23/2001	IL	In progress
01-024-04n	02/23/2001	CA	In progress
01-024-08n	02/28/2001	MN	In progress

USDA Reference No.	Effective Date	Release Site (State)	Trial Status
01-024-09n	02/28/2001	IA	In progress
01-024-10n	03/08/2001	NE	In progress
01-024-11n	02/28/2001	MO	In progress
01-024-12n	02/28/2001	MN	In progress
01-024-13n`	02/23/2001	IN	In progress
01-024-14n	02/23/2001	CO	In progress
01-024-15n	02/23/2001	MI	In progress
01-024-16n	02/23/2001	CO	In progress
01-024-17n	02/23/2001	MD	In progress
01-024-18n	02/23/2001	MD	In progress
01-024-19n	02/23/2001	SD	In progress
01-025-01n	02/24/2001	NY	In progress
01-025-02n	02/24/2001	IA	In progress
01-025-03n	02/24/2001	IL	In progress
01-025-04n	02/24/2001	MO	In progress
01-025-05n	03/08/2001	NE	In progress
01-025-06n	02/24/2001	NY	In progress
01-025-07n	02/24/2001	PA	In progress
01-025-08n	02/24/2001	SD	In progress
01-025-09n	02/28/2001	MN	In progress
01-029-05n	02/28/2001	IL	In progress
01-029-06n	02/28/2001	SD	In progress
01-029-07n	02/28/2001	PA	In progress
01-029-08n	03/09/2001	NE	In progress
01-029-09n	02/28/2001	NY	In progress
01-029-10n	02/28/2001	IA	In progress
01-029-11n	03/12/2001	MN	In progress
01-029-13n	03/12/2001	MN	In progress
01-032-03n	03/05/2001	PR	In progress
01-032-04n	03/05/2001	HI	In progress
01-032-05n	03/03/2001	HI	In progress
01-032-06n	03/03/2001	IA	In progress
01-032-07n	03/03/2001	IN	In progress
01-032-08n	03/05/2001	WI	In progress
01-032-09n	03/05/2001	PA	In progress
01-032-10n	03/09/2001	NE	In progress
01-032-11n	03/05/2001	IN	In progress
01-032-12n	03/05/2001	IL	In progress
01-037-01n	03/09/2001	MD	In progress
01-038-03n	03/09/2001	IN	In progress
01-038-04n	03/09/2001	IN	In progress
01-038-05n	03/09/2001	OH	In progress
01-038-06n	03/09/2001	OH	In progress
01-038-07n	03/09/2001	MI	In progress
01-038-08n	03/09/2001	IL	In progress

USDA Reference No.	Effective Date	Release Site (State)	Trial Status
01-038-09n	03/09/2001	IL	In progress
01-038-10n	03/09/2001	KS	In progress
01-038-11n	03/09/2001	SD	In progress
01-038-12n	03/09/2001	IA	In progress
01-038-13n	03/09/2001	MO	In progress
01-038-14n	03/27/2001	MO	In progress
01-038-15n	03/16/2001	NE	In progress
01-038-16n	03/17/2001	NE	In progress
01-043-02n	03/14/2001	IN	In progress
01-044-05n	03/15/2001	NE	In progress
01-044-06n	03/15/2001	PA	In progress
01-044-07n	03/15/2001	IN	In progress
01-044-08n	03/15/2001	IN	In progress
01-044-09n	03/15/2001	OH	In progress
01-044-10n	03/15/2001	MO	In progress
01-046-01n	03/17/2001	IL	In progress
01-046-02n	03/17/2001	IL	In progress
01-046-03n	03/17/2001	IL	In progress
01-046-04n	03/22/2001	OH	In progress
01-046-05n	03/17/2001	IA	In progress
01-046-06n	03/17/2001	IA	In progress
01-046-07n	03/17/2001	IA	In progress
01-046-08n	03/17/2001	IA	In progress
01-046-09n	03/17/2001	NE	In progress
01-046-10n	03/17/2001	NE	In progress
01-046-11n	03/17/2001	NE	In progress
01-046-12n	03/17/2001	NE	In progress
01-047-01n	03/26/2001	NE	In progress
01-047-02n	04/04/2001	ND	In progress
01-047-03n	03/26/2001	MO	In progress
01-047-04n	03/26/2001	MN	In progress

* Depending on its stage of development, Notifications for MON 863 could have been filed using one of the following Monsanto code designations: COL-026 or 2219-4A1(COL-026)

APPENDIX B

Compositional Analysis Summary of the Grain Collected from Corn Event MON 863, Nontransgenic Control Corn, and Commercial Corn Varieties [e]

Component	Unit[a]	MON 863 Mean[b]	Control Mean[b]	Commercial Range[c]
Ash	% dry wt.	1.35	1.41	0.62-1.53
Carbohydrates	% dry wt.	83.3	82.8	82.5 - 87.8
Acid detergent fiber	% dry wt.	4.45	4.50	3.65 - 6.09
Neutral detergent fiber	% dry wt.	11.6	12.0	9.50 - 15.0
Moisture	% fresh wt.	10.0	10.2	8.75 - 15.7
Total fat	% dry wt.	3.77	3.64	2.18 - 3.86
Protein	% dry wt.	11.6	12.2	7.95 - 13.8
Calcium	% dry wt.	0.005	0.005	0.004 - 0.006
Copper	mg/kg dry wt.	2.26[d]	2.19	1.03 - 2.15
Iron	mg/kg dry wt.	23.6	24.2	16.7 - 28.7
Magnesium	% dry wt.	0.13	0.14	0.091 - 0.14
Manganese	mg/kg dry wt.	5.81	6.15	3.51 - 9.80
Phosphorus	% dry wt.	0.40	0.42	0.27 - 0.41
Potassium	% dry wt.	0.43	0.44	0.33 - 0.43
Zinc	mg/kg dry wt.	22.2	23.7	12.8 - 31.2
Phytic acid	% dry wt.	1.11	1.23	0.73 - 1.17
Trypsin inhibitor	TIU/mg dry wt.	2.30	2.48	0.58 - 3.05
Vitamin E	mg/g dry wt.	0.011	0.013	0.004 - 0.014
16:0 Palmitic acid	% of total FA	12.0	11.9	9.07 - 12.1
18:0 Stearic acid	% of total FA	1.66	1.66	1.44 - 2.40
18:1 Oleic acid	% of total FA	22.0	21.9	21.3 - 32.1
18:2 Linoleic acid	% of total FA	62.2	62.5	54.2 - 63.6
18:3 Linolenic acid	% of total FA	1.20	1.24	0.97 - 1.36

a - wt. = weight; TIU = trypsin inhibitor units; FA = fatty acid.
b - The mean of 16 samples, four replicates collected from each of four sites.
c - The range of 18 commercial variety samples collected across the four sites.
d - This value was within the literature range of 0.9-10 mg/kg dry wt (see Watson, S.A. 1987. Structure and composition. *In* Corn: Chemistry and Technology. Watson, S.A. and Ramstad, R.E., Eds. American Association of Cereal Chemists, Inc. St. Paul, Minnesota, p. 73)
e – Plant tissue collected under APHIS Notification #99-106-16n

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C

Reports for Unpublished Studies Supporting Regulatory Approval of Corn Rootworm Protected Corn Event MON 863

SECTIONS 1 - 25

[CBI Deleted]

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C - SECTION 1

[CBI Deleted]

Amended Report for MSL-16505:
Molecular Analysis of Corn Event MON 863
(Cavato *et al.*, 2001)

Report No. MSL-17152
(60 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 2

[CBI Deleted]

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**
(Dudin *et al.*, 2001)

Report No. MSL-17181
(30 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 3

[CBI Deleted]

Amended Report for MSL-15531:
Characterization of *B.t.* Protein 11098 and *B.t.* Protein 11231
Produced by Fermentation
(Hileman *et al.*, 2001a)

Report No. MSL-17219
(36 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 4

[CBI Deleted]

Amended Report for MSL-16596:
Assessment of the Physiochemical Equivalence of Cry3Bb1.11098 and NPTII Proteins in Corn Event MON 863 to Microbial Sources
(Holleschak *et al.*, 2001a)

Report No. MSL-17220
(40 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 5

[CBI Deleted]

Amended Report for MSL-15835:
Assessment of the Equivalence of *B.t.* Protein 11098, *B.t.* Protein 11231 and NPTII Protein Expressed in Corn Events MON 853 and MON 860 to Microbial Sources (Holleschak *et al.*, 2001b)

Report No. MSL-17222
(53 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 6

[CBI Deleted]

Primary Structural Protein Characterization of Corn Event MON 863 Cry3Bb1.11098 Protein Using N-terminal Sequencing and MALDI Time of Flight Spectrometric Techniques (Thoma *et al.*, 2001)

Report No. MSL-17154
(21 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 7

[CBI Deleted]

Additional Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863 (Hileman and Astwood, 2001)

Report No. MSL-17137
(22 pages)

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APPENDIX C – SECTION 8

[CBI Deleted]

Acute Oral Toxicity Study of *B.t.* Protein 11098 in Mice (Bechtel, 1999)

Report No. MSL-16215
(212 pages)

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APPENDIX C – SECTION 9

[CBI Deleted]

Amended Report for MSL-15704:
Assessment of the *in vitro* Digestibility of *B.t.* Protein 11098 and *B.t.* Protein 11231 Utilizing Mammalian Digestive Fate Models
(Leach *et al.*, 2001)

Report No. MSL-17166
(41 pages)

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APPENDIX C – SECTION 10

[CBI Deleted]

Bioinformatics Evaluation of the Cry3Bb1 Protein Produced in Corn Event MON 863 Utilizing Allergen, Toxin and Public Domain Protein Databases (Hileman *et al.*, 2001b)

Report No. MSL-17140
(102 pages)

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APPENDIX C – SECTION 11

[CBI Deleted]

Efficacy of MON 863 Against Corn Rootworm and Comparison to Insecticide Treatments – Results of Year 2000 Field Trials (Pilcher, 2001)

Report No. MSL-17070
(10 pages)

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APPENDIX C – SECTION 12

[CBI Deleted]

Aerobic Soil Degradation of the *B.t.* Protein 11098 as a Component of Insect Protected Corn (Martin *et al.*, 2000)

Report No. MSL-16440
(70 pages)

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APPENDIX C – SECTION 13

[CBI Deleted]

Evaluation of Dietary Effect(s) of Purified *Bacillus thuringiensis* Protein 11231 on Adult Honey Bees (*Apis mellifera* L.) (Maggi, 1999a)

Report No. MSL-16169
(58 pages)

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APPENDIX C – SECTION 14

[CBI Deleted]

Evaluation of the Dietary Effects of Purified *Bacillus thuringiensis* Protein 11231 on Honey Bee Larvae (Maggi, 1999b)

Report No. MSL-16168
(46 pages)

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APPENDIX C – SECTION 15

[CBI Deleted]

Bacillus thuringiensis Protein 11231: A Dietary Toxicity Study with Green Lacewing Larvae (*Chrysoperla carnea*) (Palmer and Krueger, 1999a)

Report No. MSL-16165
(40 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 16

[CBI Deleted]

Bacillus thuringiensis Protein 11231: A Dietary Toxicity Study with the Parasitic Hymenoptera (*Nasonia vitripennis*) (Palmer and Krueger, 1999b)

Report No. MSL-16167
(50 pages)

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APPENDIX C – SECTION 17

[CBI Deleted]

Bacillus thuringiensis Protein 11231: An Acute Toxicity Study with the Earthworm in an Artificial Soil Substrate (Hoxter *et al.*, 1999)

Report No. MSL-16162
(45 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 18

[CBI Deleted]

Assessment of Chronic Toxicity of Corn Tissue Containing the *Bacillus thuringiensis* Protein 11098 to Collembola (*Folsomia candida*) (Teixeira, 1999)

Report No. MSL-15988
(55 pages)

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APPENDIX C – SECTION 19

[CBI Deleted]

Evaluation of Insect Protected Corn Lines MON 853 and MON 859 as a Feed Ingredient for Catfish (Li and Robinson, 1999)

Report No. MSL-16164
(32 pages)

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APPENDIX C – SECTION 20

[CBI Deleted]

Bacillus thuringiensis Protein 11098 in Corn Pollen: A 48-Hour Static-Renewal Acute Toxicity Test With the Cladoceran (*Daphnia magna*) (Drottar and Krueger, 1999)

Report No. MSL-16163
(31 pages)

APPENDIX C – SECTION 21

[CBI Deleted]

Dietary Effects of Transgenic *Bacillus thuringiensis* (Bt) Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Adults of the Ladybird Beetle, *Coleomegilla maculata*
(Duan *et al.*, 2001a)

Report No. MSL-16936
(35 pages)

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APPENDIX C – SECTION 22

[CBI Deleted]

Dietary Effects of Transgenic *Bacillus thuringiensis* (Bt) Corn Pollen Expressing a Variant of Cry3Bb1 Protein on Larvae of the Ladybird Beetle, *Coleomegilla maculata* (Duan *et al.*, 2001b)

Report No. MSL-16907
(39 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 23

[CBI Deleted]

Dietary Effects of Transgenic BT Corn Pollen Expressing a Variant of Cry3Bb1 Protein on the Ladybird Beetle, *Hippodamia convergens* (Bryan *et al.*, 2001)

Report No. MSL-17171
(36 pages)

Petition for Determination of Nonregulated Status for Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 24

[CBI Deleted]

Dormancy and Germination Evaluation of Corn Rootworm-Protected Corn for an Ecological Assessment of Plant Weediness (Wiltse *et al.*, 2001)

Report No. MSL-17054
(46 pages)

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Corn Rootworm Protected Corn Event MON 863

APPENDIX C – SECTION 25

[CBI Deleted]

Morphological Assessment of Event MON 863 Corn Pollen (Wiltse, 2001)

Report No. MSL-17231
(21 pages)