Food and Feed Safety and Nutritional Assessment of the Lepidopteran-protected Corn MON 89034 (OECD Unique Identifier MON-89Ø34-3)

Conclusion Based on Data and Information Evaluated According to FDA's Policy on Foods from New Plant Varieties

October 13, 2006

FDA BNF No. 00105

Monsanto 06-CR-161F

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Certification

Monsanto Company is submitting this food and feed safety and nutritional assessment in compliance with the FDA's 1992 policy statement regarding foods derived from new plant varieties (57 FR 22984). At the agency's request, and where appropriate, this submission also complies with the recommendations contained in the proposed rule for Premarket Biotechnology Notice (PBN) Concerning Bioengineered Foods (66 FR 4706).

Specifically, as recommended in the proposed 21 CFR §192.25(a), the undersigned attests to the following:

1. It is the view of Monsanto Company (hereafter referred to as Monsanto) that: (i) MON 89034 is as safe and nutritious as other commercially available corn; and (ii) the intended uses of the food and feed derived from MON 89034 are in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.
2. Monsanto will make available to FDA, upon request, relevant data or other information not included in this submission, either during the course of FDA's evaluation of the submission, or for cause.
3. Monsanto will make relevant data or other information not included in this submission available to FDA either: (i) by allowing FDA to review and copy these data or information at Monsanto's offices in St. Louis, MO, during customary business hours; or (ii) by sending a copy of these data or information to FDA.
4. Monsanto makes no claim of confidentiality regarding either the existence of this submission, or any of the data or other information contained herein. However, Monsanto reserves the right to make a claim of confidentiality regarding any relevant data or other information not included in this submission, but requested by FDA, either in the course of its review of this submission, or for cause. Any such claim of confidentiality will be made at the time such data or information is provided, along with an explanation for the basis of the claim.
5. To the best of Monsanto's knowledge, this submission is representative and balanced, including information, unfavorable as well as favorable, pertinent to the evaluation of the safety, nutritional, or other regulatory issues that may be associated with MON 89034.

[redacted]

Date:

October 13, 2006

Director, North American and Latin American North Biotechnology Regulatory Affairs
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Release of Information

Monsanto is submitting the information in this assessment for review by the FDA as part of the regulatory process. By submitting this information, Monsanto does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., § 552; FDA complies with the provisions of FOIA and FDA's implementation regulations (21 CFR Part 20); and this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

Abbreviations[1], Acronyms and Definitions

1× LB	Laemmli Buffer [62.5mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8]
5× LB	Five times concentrated 1× LB
2T-DNA	Plasmid vector containing two separate T-DNA regions each surrounded by left and right borders of the Ti plasmid
35S	Promoter and leader from the Cauliflower mosaic virus (CaMV) 35S RNA
AA or aa	Amino Acid
AACC	American Association of Cereal Chemists
aadA	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
ACB	Asian corn borer, *Ostrinia funicalis*
AEX	Anion Exchange Chromatography
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists Society
AD6	Allergen, gliadin, and glutenin protein sequence database
ADF	Acid Detergent Fiber
ALLPEPTIDES	Protein sequence database comprised of NRAA and SwissProt databases
ALLERGEN-SEARCH	Computer program for the search against known allergens
bp	base pair
BCW	Black cutworm, *Agrotis ipsilon*
BRS	Biotechnology Regulatory Sciences
BSA	Bovine Serum Albumin
Bt	*Bacillus thuringiensis*
bw	body weight
Cab	5' untranslated leader of the wheat chlorophyll a/b-binding protein
CaMV	Cauliflower Mosaic Virus
CAPS	3-[cyclohexyl amino]-1-propanesulfonic acid

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

CEW	Corn earworm, *Helicoverpa zea*
CFR	Code of Federal Regulations
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
CI	Confidence Interval
Cry	Crystal proteins from *Bacillus thuringiensis*
CS	Coding Sequence
Cry1A.105	A chimeric protein comprised of domains from the naturally occurring Cry1Ab, Cry1F, and Cry1Ac proteins of *Bacillus thuringiensis*
cry1A.105	Coding sequence for the Cry1A.105 protein
Cry2Ab2	A Cry2 class crystal protein from *Bacillus thuringiensis* subsp. kurstaki.
cry2Ab2	Coding sequence for the Cry2Ab2 protein
Cry1Ab	A naturally-occurring protein produced by Bt spp. *kurstaki* HD-1 with activity against certain Lepidopteran insects
Cry1Ac	A naturally-occurring protein produced by Bt spp. *kurstaki* HD-73 with activity against certain Lepidopteran insects
Cry3	A class of Bt crystal proteins with insecticidal activity against coleopteran insects
Cry3Bb1	A naturally-occurring protein produced by *B.t.* spp. *kumamotoensis* with activity against certain Coleopteran insects
CTP	Chloroplast Transit Peptide
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of Variation
DAP	Days After Planting
dCTP	Deoxycytidine triphosphate
DF	Dilution Factor
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
dw or DW	dry weight
dwt	dry weight of tissue
e35S	The promoter and leader from cauliflower mosaic virus (CaMV) 35S RNA containing the duplicated enhancer region
EC_{50}	Effective protein concentration to inhibit the growth of the target insect by 50%
ECB	European corn borer, *Ostrinia nubilalis*

E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
EPPS	4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid
FA	Fatty Acid
FAW	Fall armyworm, *Spodoptera frugiperda*
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FAARP	Food Allergy Research and Resource Program
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act (U.S.)
FMV	Figwort mosaic virus 35S promoter
FR	Federal Register
FW or fw	Fresh Weight
fwt	fresh weight of tissue
gDNA	genomic DNA
GLP	Good Laboratory Practice
HEPES	N-[2-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
Hsp17	3' nontranslated region of the gene for wheat heat shock protein 17.3 which ends transcription and directs polyadenylation
Hsp70	Maize heat shock protein 70 gene
I	Intron
I-*Ract1*	Intron from the rice actin gene
IgG	Immunoglobulin G
ILSI-CCD	International Life Sciences Institute-Crop Composition Database
kb	kilobase
kDa	kilodalton
L	Leader
Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
LOQ	Limit of Quantitation
LOD	Limit of Detection
mA	milliampere

MAFF	Ministry of Agriculture, Forestry and Fisheries (Japan)
MALDI-TOF	Matix Assisted Laser Desorption Ionization - Time of Flight
MH+	Protonated mass ion
MHLW	Ministry of Health, Labor and Welfare (Japan)
MMT	Million Metric Tonnes
MOE	Margin of Exposure
MOA	Mode of Action
MON 810	A Monsanto corn product, producing the insecticidal Bt Cry1Ab protein
MON 89034	A Monsanto corn product, and the subject of this application, which produces the Bt Cry1A.105 and Cry2Ab2 proteins
MS	Mass Spectrometry
MWCO	Molecular weight cut-off
MW	Molecular Weight
MWM	Molecular Weight Marker
n	Number of Observations
n.a. or na	not available
NCGA	National Corn Grower's Association
n.d. or nd	not detectable
NDF	Neutral Detergent Fiber
NFDM	Non-fat Dry Milk
NIST	National Institute of Standards and Technology
NMWC	Nominal molecular weight cut-off
NOEL	No Observable Effect Level
NRAA	A public protein database maintained by the NCBI at the National Institutes of Health, Bethesda, MD
nos	3' transcript termination sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*
nptII	Coding sequence of neomycin phosphotransferase II gene that confers resistance to neomycin and kanamycin
NPTII	Neomycin phosphotransferase II
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
OR	origin of replication
ori-PBR322	origin of replication from pBR322 for maintenance of plasmid in *E. coli*

ori V	origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2
OSL	Overseason Leaf
OSR	Overseason Root
OSWP	Overseason Whole Plant
P	Promoter
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline containing Tween-20
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
ppm	parts per million
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene Difluoride
PVPP	Polyvinylpolypyrrolidone
PV-ZMIR245	Plasmid vector used to develop MON 89034
Ract1	the rice actin gene
Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
rop	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
SAS	Statistical Analysis System, an integrated system of software products provided by the SAS Institute, Inc. headquartered in Cary, North Carolina, USA
SCB	Sugarcane borer, *Diatraea saccharalis*
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SE	Standard Error
SGF	Simulated Gastric Fluid
SOP	Standard Operating Procedure
sp.	species
subsp.	subspecies
SSU-CTP	DNA region containing the targeting sequence for the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit and the first intron

SWCB	Southwestern corn borer, *Diatraea grandiosella*
SwissProt	A public protein database maintained by the Swiss Institute of Bioinformatics, Geneva, Switzerland, and EMBL
Taq	*Thermus aquaticus*, a thermophilic bacterium
T	Terminator
TS	Targeting Sequence
TCA	Trichloroacetic Acid
T-DNA	Transfer DNA
T-DNA I	Transferred DNA containing the *cry1A.105* and *cry2Ab2* expression cassettes in plasmid vector PV-ZMIR245
T-DNA II	Transferred DNA containing the *nptII* gene cassette in plasmid vector PV-ZMIR245
TDF	Total Dietary Fiber
TI	Tolerance Interval
TFA	Trifluoroacetic Acid
TMB	3,3’,5,5’-Tetramethylbenzidene
TOXIN5	Toxin protein sequence database
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate
USDA-APHIS	United States Department of Agriculture – Animal and Plant Health Inspection Service
USDA-NASS	United States Department of Agriculture – National Agricultural Statistics Service
U.S. EPA	United States Environmental Protection Agency
U.S. FAS	United States Foreign Agriculture Service
U.S. FDA	United States Food and Drug Administration
UV	Ultraviolet
VOI	Verification of Identity
v/v	volume to volume ratio
w/v	weight to volume ratio
w/w	weight to weight ratio
wt	weight
wt CAB	A 5’ untranslated leader of the wheat chlorophyll a/b/-binding protein
WBCW	Western bean cutworm, *Striacosta albicosta*

Narrative Summary

Food and Feed Safety and Nutritional Assessment of the Lepidopteran-protected Corn MON 89034

Monsanto Company has developed, through the use of recombinant DNA techniques, MON 89034, a corn product that is protected from damage caused by larval feeding of lepidopteran insect pests. MON 89034 produces the *Bacillus thuringiensis* (Bt) Cry1A.105 and Cry2Ab2 proteins that are active against lepidopteran insects. MON 89034 is a second-generation product that will effectively address a corn grower's need to control a wide spectrum of lepidopteran pests. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides excellent control of lepidopteran insect pests and offers an effective insect resistance management tool.

Corn (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2005, 81.8 million acres of corn was planted in the U.S. The average yield of corn grain was 148 bushels per acre, with a total production of 11 billion bushels valued at $21 billion. The corn crop is susceptible to feeding damage from insect pests resulting in significant economic losses. In 1997, Monsanto commercialized the first-generation YieldGard® Corn Borer corn (hereafter referred to as MON 810), which produces a Cry1Ab protein that provides effective protection against damage caused by lepidopteran insect pests, especially the European corn borer (ECB, *Ostrinia nubilalis*) and the corn earworm (CEW, *Helicoverpa zea*). The benefits of MON 810 and other Bt corn products have included more effective control of lepidopteran larval pests, lower levels of harmful mycotoxins in grain, and reduction in the use of chemical insecticides. Bt corn products have been widely adopted by growers, and by 2006, insect-protected corn was planted in over 40% of the U.S corn acres.

The introduction of the second-generation product MON 89034 is expected to provide enhanced benefits for the control of lepidopteran pests of corn compared to existing products. MON 89034 will have a wider spectrum of activity against lepidopteran pests and strengthen insect resistance management, continue to reduce the potential for mycotoxins in grain, enable more efficient plant breeding of this multi-genic trait into superior hybrids compared to MON 810, and potentially reduce the refuge acreage required for resistance management purposes. Taken together, adoption of MON 89034 is likely to enhance economic and other benefits (occupational health, flexibility of use, etc) to farmers and improve the quality of grain and the safety of the derived food and feed products.

MON 89034 was produced by *Agrobacterium*-mediated transformation of corn with the PV-ZMIR245 vector, which is a binary vector containing two separate transfer DNA's (2T-DNA). The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the

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nptII (neomycin phosphotransferase II) expression cassette. During transformation, both T-DNAs were inserted into the genome. The *nptII* selectable marker gene was used for the selection of transformed cells in the presence of neomycin. Once the transgenic cells were identified, the selectable marker gene was no longer needed. Traditional breeding was used to produce plants that only contained the *cry1A.105* and *cry2Ab2* expression cassettes (T-DNA I) and did not contain the *nptII* expression cassette (T-DNA II), thereby, producing marker-free corn MON 89034.

The data and information presented in this summary demonstrate that the foods and feeds derived from MON 89034 are as safe and nutritious as the comparable foods and feeds derived from conventional corn. This conclusion is based on several lines of evidence. The first is the detailed molecular characterization of the inserted DNA. Results confirm the insertion of a single functional copy of the *cry1A.105* and *cry2Ab2* expression cassettes at a single locus within the genome. The second is a detailed biochemical characterization of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034. Data demonstrate that the two Cry proteins produced in MON 89034 are equivalent to their counterparts produced by recombinant strains of *Escherichia coli* for use in various safety assessment studies. The third is a thorough assessment of the allergenicity and toxicity potential of the Cry1A.105 and Cry2Ab2 proteins based on extensive studies. The results demonstrate the safety of the Cry1A.105 and Cry2Ab2 proteins due to the lack of allergenic potential and the lack of acute toxicity when ingested, and their similarity to Cry proteins that have a history of safe use. The fourth is the estimation of protein levels and a dietary safety assessment based on anticipated exposure and the results of acute toxicology tests. Results show that there are no meaningful risks to human or animal health from dietary exposure to the Cry1A.105 and Cry2Ab2 proteins based on the large margins of exposure (MOEs) obtained. The fifth is the compositional and nutritional assessment of MON 89034 grain and forage, which confirms that MON 89034 is compositionally equivalent to and as safe as conventional corn.

Molecular characterization of MON 89034 by Southern blot analyses was conducted to determine: 1) the number of inserts and copies in the genome, 2) intactness of the genetic elements within the insert, 3) absence of the T-DNA II encoding the selectable marker, 4) absence of backbone sequences, and 5) stability of the inserted DNA across multiple generations. Results demonstrated that the DNA inserted into the corn genome is present at a single locus and contains one functional copy of the *cry1A.105* and the *cry2Ab2* expression cassettes. All genetic elements were shown to be present in the inserted DNA as expected. However, the *e35S* promoter, which regulates expression of the *cry1A.105* gene, was modified and the Right Border sequence present in PV-ZMIR245 was replaced by a Left Border sequence in MON 89034. There were no other elements, either full length or partial, present other than those associated with the intended insert, and no backbone plasmid DNA or *nptII* sequences were detected. PCR and DNA sequence analyses provided the complete DNA sequence of the insert and confirmed the organization of the elements within the insert. The stability of the integrated DNA and absence of the T-DNA II and backbone sequences in multiple generations of MON 89034 was also confirmed. The heritability of the *cry1A.105* and *cry2Ab2* genes was confirmed by segregation analysis of several generations of MON 89034. These results are

consistent with the conclusion of a single active site of insertion that segregates according to the Mendelian laws of genetics.

Detailed biochemical characterization of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 confirmed their identity and equivalence to the corresponding *E. coli*-produced proteins. The characterization was based on: a) the source organism from which the two Cry proteins are derived; 2) identity and function; and, 3) physicochemical and functional equivalence to the *E. coli*-produced protein standards. The donor organism for the Cry1A.105 and Cry2Ab2 proteins, *Bacillus thuringiensis*, has been used commercially in the U.S. for over four decades to produce microbial pesticides. Cry proteins have a history of safe use since 1958 as active ingredients either in Bt microbial pesticides or in biotechnology-derived food and feed crops. Cry1A.105 is a chimeric protein comprised of domains I and II from Cry1Ab and Cry1Ac, domain III from Cry1F (Bt subsp. *aizawai*), and the C-terminal portion from Cry1Ac (Bt subsp. *kurstaki*). Domains I and II of Cry1A.105 are 100% identical in amino acid sequence to domains I and II of both Cry1Ab and Cry1Ac, domain III is 99% identifical to domain III of Cry1F, and the C-terminal portion is 100% identical to the C-terminal portion of Cry1Ac. The overall amino acid sequence identity to the Cry1Ab, Cry1Ac, and Cry1F proteins is 90.0%, 93.6%, and 76.7 % respectively. Bt microbial strains producing Cry1Ac and Cry1Ab, and Cry1F proteins have been used for decades as biopesticides.[1] The Cry1A.105 protein produced in MON 89034 is structurally and functionally similar to Cry1A proteins produced in a number of biotechnology-derived crops (e.g., YieldGard Corn Borer corn, Bollgard® cotton and Bollgard II® cotton) that have demonstrated history of safe use.

The Cry2Ab2 protein produced in MON 89034 is derived from the Bt subspecies *kurstaki* and its amino acid sequence differs from that of the wild-type protein by a single amino acid. The Cry2Ab2 protein has 88% amino acid sequence identity to the Cry2Aa protein which is present in commercial microbial pest control products such as Dipel® and Crymax®. The Cry2Ab2 proteins produced in MON 89034 and Bollgard II cotton have an identical amino acid sequence. Bollgard II cotton has been on the market since 2003 and there have been no adverse reports regarding its safety.

The characterization of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 confirmed that these proteins are equivalent to the respective *E. coli*-produced protein standards used in safety studies. Since the in planta expression of the Cry1A.105 and Cry2Ab2 proteins is low, it was necessary to produce these proteins in the high-expressing recombinant host organism, *E. coli,* so that they could be used in safety studies. The *E. coli*-produced proteins were engineered to have the identical amino acid sequences as their counterparts expressed in MON 89034. The MON 89034- and *E. coli*-produced proteins were then evaluated to ensure that they were physicochemically and

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[1] Cry1Ab and Cry1Ac are components of the microbial product Dipel®, and a Cry1Ac/Cry1F chimeric protein is a component of the microbial product Lepinox® (Ecogen Inc.).

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functionally equivalent based on the following analytical tests: a) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate approximate molecular weight; b) western blot analysis to demonstrate identity and immunoreactivity; c) N-terminal sequence analysis or western blot analysis to examine the intactness of the N-terminus; d) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to establish protein identity by peptide mapping; e) glycosylation analysis to determine the presence or absence of covalently-linked carbohydrates; and, f) insect bioassay to assess functional equivalence. These analyses provided a detailed characterization of the Cry1A.105 and Cry2Ab2 proteins isolated from MON 89034 and confirmed their equivalence to the *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins.

The assessment of potential allergenicity and toxicity showed there was a reasonable certainty of no harm to mammals from exposure to the Cry1A.105 and Cry2Ab2 proteins. These assessments were based on: a) an evaluation of potential allergenicity based on the source of the protein, structural similarities to known allergens, in vitro digestibility in simulated digestive fluids, and expected dietary exposure; and, b) an evaluation of potential toxicity based on history of use, similarity to known toxins or biologically active proteins, and evaluation of acute toxicity to mammals

As mentioned previously, *Bacillus thuringiensis*, the donor organism for these two Cry proteins, has been used commercially in the U.S. for over four decades to produce microbial pesticides, and there are no confirmed cases of allergic reactions to Cry proteins. Results of extensive bioinformatics assessments using FASTA sequence alignment and eight-amino acid sliding window searches, showed that the Cry1A.105 and Cry2Ab2 proteins do not share any amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins that have adverse effects to mammals. Assessment of the in vitro digestibility in simulated gastric fluid (SGF) showed that the Cry1A.105 and Cry2Ab2 proteins are rapidly digested, with greater than 95% to 99% of the proteins, respectively, being digested in less than 30 seconds. Proteins that are rapidly digestible have a lower risk of causing allergic reactions or resulting in toxicity when consumed. Mice acute oral toxicity studies demonstrate that the Cry1A.105 and Cry2Ab2 proteins are not acutely toxic and do not cause any adverse effects even at maximum attainable dose levels of 2072 and 2198 mg/kg body weight for the Cry1A.105 and Cry2Ab2 proteins, respectively. The independent safety assessment for each of the Cry proteins in mice was considered appropriate and adequate based on the extensive history of safe use of mixtures of Cry proteins present in Bt microbial pesticides.

The levels of the Cry1A.105 and Cry2Ab2 proteins estimated in tissues of MON 89034 showed trends that were consistent for exposure calculations and intended uses. Tissues of MON 89034 were collected from field trials conducted at five sites in the U.S. during 2005. Tissues from the different growth stages of the corn plant were collected throughout the growing season and analyzed by enzyme-linked immunosorbent assay (ELISA). The mean Cry1A.105 levels across sites were 520 μg/g dwt in young leaf, 42 μg/g dwt in forage, and 5.9 μg/g dwt in grain. The mean Cry2Ab2 levels across sites were in 180 μg/g dwt in young leaf, 38 μg/g dwt in forage, and 1.3 μg/g dwt in grain. In general, the levels of the two Cry proteins declined over the growing season.

A dietary safety assessment based on Cry1A.105 and Cry2Ab2 protein levels, expected dietary exposure, and the results of acute toxicology tests showed large MOEs for humans and animals. The MOEs for the overall U.S. population were greater than or equal to 199,000 and 981,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively. For children aged 3-5 years old, an age group with the highest corn consumption (body weight basis), the MOEs were greater than or equal to 79,400 and 390,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively. For poultry and livestock, the MOEs ranged between 1,930 – 13,500 and 2,160 – 47,600 for the Cry1A.105 and Cry2Ab2 proteins, respectively. These results are consistent with the extensive safety testing previously conducted for the Cry1A and Cry2A class of proteins.

The EPA further confirmed the safety of the Cry1A.105 and Cry2Ab2 proteins recently when they established temporary exemptions from the requirement of a tolerance for the Cry1A.105 and Cry2Ab2 proteins and the genetic material for their production in corn (40 CFR §174.453 and 40 CFR §174.454, respectively).

Compositional assessment of the grain and forage from MON 89034 demonstrated that it is nutritionally and biologically equivalent to its conventional counterpart, LH198 x LH172. Compositional data on key nutrients, anti-nutrients and other components were collected for the forage and grain from MON 89034 and conventional control corn, grown at five field sites in the U.S. during 2004. Three different conventional, commercial corn reference hybrids were also grown at each site, for a total of 15 references. Composition data from the references was used to establish a range of variability described by a 99% tolerance interval for each component analyzed. Statistical comparisons of 61 components from MON 89034 and the control were conducted for the combination of all five sites (i.e., the combined-site) and for each individual site. The overall data set was examined for evidence of biologically relevant changes. Evaluation of the data, including the results of statistical analysis, leads to the conclusion that MON 89034 is compositionally and nutritionally equivalent to conventional corn.

No statistical differences were observed in 58 of 61 combined-site site comparisons made between MON 89034 and the conventional control. The three differences observed were generally small (3.4 – 19.2%), considering the range of natural variability, and the mean levels and ranges of MON 89034 were well within the 99% tolerance intervals for commercial corn. For the individual site analyses, there were no statistical differences that were consistently observed across all sites. Furthermore, the means and ranges of all components from MON 89034 showing a statistical difference were within the 99% tolerance intervals of conventional corn and/or within the International Life Sciences Institute Crop Composition Database.

In conclusion, the data and information presented in this summary demonstrate that the foods and feeds derived from MON 89034 are as safe and nutritious as the comparable foods and feeds derived from conventional corn. This conclusion is based on several lines of evidence including:

1. The detailed molecular characterization of the inserted DNA, which confirmed the presence of single functional copies of the *cry1A.105* and *cry2Ab2* cassettes, stably integrated at a single locus of the genome;
2. The biochemical characterization of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034, which confirmed their identity and equivalence to the same Cry1A.105 and Cry2Ab2 proteins produced by recombinant strains of *Escherichia coli* used in the various safety assessment studies;
3. A safety assessment of the Cry1A.105 and Cry2Ab2 proteins, which shows the lack of allergenic potential and acute toxicity when ingested;
4. A dietary safety assessment, which showed and no meaningful risks to human or animal health from dietary exposure to the Cry1A.105 and Cry2Ab2 proteins; and
5. Compositional and nutritional assessments, which demonstrate that the MON 89034 is compositionally equivalent to and as safe as conventional corn.

Therefore it is concluded that the consumption of MON 89034 and the food and feed derived from it will be as safe and nutritious as that of conventional corn.

PART II: SYNOPSIS OF CONSULTATION SUMMARY

SECTION 1. Name and Address of the Submitter

The submitter of this safety and nutritional assessment summary for corn MON 89034 is:

Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Communications with regard to this submission should be directed to [redacted] Regulatory Affairs Manager, at the above address. She can also be contacted by telephone at [redacted].

SECTION 2. The subject of this Summary and the Plant Species from which They were Derived

The subject of this summary is MON 89034, a corn product derived from a Monsanto proprietary corn inbred.

SECTION 3. Distinctive Designations Given to the Subject of This Summary

The lepidopteran-protected corn that is the subject of this summary has been designated MON 89034. In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants," MON 89034 has been assigned the unique identifier MON-89Ø34-3.

SECTION 4. Identity and Sources of the Genetic Material Introduced into MON 89034

MON 89034 was developed through *Agrobacterium*-mediated transformation of corn using the binary plasmid vector, PV-ZMIR245 (Figure IV.1). *Agrobacterium*-mediated transformation is a well-documented process for the transfer and integration of exogenous DNA into a plant's nuclear genome (Ishida et al., 1996). PV-ZMIR245 contains two separate T-DNAs (therein referred to as 2 T-DNA system). The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette that encodes the neomycin phosphotransferase enzyme that confers tolerance to certain antibiotics such as neomycin and paromomycin. Each T-DNA was independently integrated into the corn genome. Plants containing both T-DNA's I and II were identified

and then subjected to conventional plant breeding to identify segregating plants that only contained T-DNA I, which includes the *cry1A.105* and *cry2Ab2* genes. Only plants containing T-DNA I, but lacking T-DNA II, were developed further by breeding. A detailed description of the transformation and selection process is provided in Figure IV.2. The genetic elements present in PV-ZMIR245 are listed in Table IV.2.

The expression cassette for the coding sequence of the Cry1A.105 protein consists of the promoter (P-e35S) and leader sequence for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing a duplicated enhancer region (Kay et al., 1987). It contains the 5' untranslated leader of the wheat chlorophyll a/b/ binding protein (L-*Cab*) (Lamppa et al., 1985), the intron from the rice actin gene (*I-Ract1*) (McElroy et al., 1991), the *cry1A.105* coding sequence that was optimized for expression in monocots, and the 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3 (T-*Hsp17*), which terminates transcription and provides the signal for mRNA polyadenylation (McElwain and Spiker, 1989).

The *cry2Ab2* gene expression cassette that produces the Cry2Ab2 protein consists of the 35S promoter from figwort mosaic virus (P-*FMV;* Rogers, 2000) and the first intron from the corn heat shock protein 70 gene (I-*Hsp 70*; Brown and Santino, 1995). It contains a *cry2Ab2* coding sequence with a modified codon usage (CS-*cry2Ab2;* Widner and Whitely, 1989, Donovan, 1991) fused to a chloroplast transit peptide region of corn ribulose 1,5-biphosphate carboxylase small subunit including the first intron (TS-*SSU-CTP*; Matsuoka et al., 1987). The 3' nontranslated region of the nopaline synthase (T-*nos*) coding region from *Agrobacterium tumefaciens* T-DNA terminates transcription and directs polyadenylation (Bevan et al., 1983).

The molecular analyses described in Part IV demonstrate that MON 89034 contains a single copy of introduced T-DNA I (~9.3 kb) inserted at a single locus. This insert contains one intact copy each of the *cry1A.105* and *cry2Ab2* gene expression cassettes. There are no detectable plasmid backbone sequences, except for sequences common with the T-DNA I, and no additional elements, linked or unlinked to intact cassettes, from transformation vector PV-ZMIR245.

These results support the conclusion that the insert in MON 89034 only encodes the two expected full-length proteins, Cry1A.105 and Cry2Ab2.

SECTION 5. The Intended Technical Effect of MON 89034

MON 89034 produces two structurally different Bt proteins, Cry1A.105 and Cry2Ab2, which are both highly efficacious against a variety of lepidopteran insect pests. MON 89034 provides a level of protection from feeding damage caused by lepidopteran larvae that is superior to that offered by currently available conventional chemical insecticides or insect-protected, biotechnology-derived corn products. Further details regarding the benefits of MON 89034 are discussed in Section 6 below.

Cry1A.105 is a chimeric protein comprised of domains I, II from Cry1Ab and Cry1Ac, domain III from Cry1F (Bt subsp. *aizawai*), and the C-terminal portion from Cry1Ac (Bt subsp. *kurstaki*). Domains I and II of Cry1A.105 are 100% identical in amino acid sequence to domains I and II of both Cry1Ab and Cry1Ac, domain III is 99% identifical to domain III of Cry1F, and the C-terminal portion is 100% identical to the C-terminal portion of Cry1Ac. Cry1A.105 has an overall amino acid sequence identity of 93.6%, 90.0%, and 76.7 % to the Cry1Ac, Cry1Ab, and Cry1F proteins, respectively. Cry1Ac, Cry1Ab, and Cry1F are well known Cry insecticidal proteins that have been used in a number of microbial Bt insect control products and biotechnology-derived crops. As with other Cry1A proteins, Cry1A.105 is active against major lepidopteran insect pests. The spectrum of activity includes corn borers from the genera *Ostrinia* and *Diatraea* (such as European corn borer, Asian corn borer, sugarcane borer, southwestern corn borer), armyworms (*Spodoptera* spp. including fall armyworm), earworms (*Helicoverpa* spp. including corn earworm), and cutworms (e.g., *Agrotis ipsilon*, black cutworm).

The Cry2Ab2 protein produced in MON 89034 is derived from the Bt subspecies *kurstaki* and its amino acid sequence differs from that of the wild-type protein by a single amino acid. The Cry2Ab2 protein has 88% amino acid sequence identity to the Cry2Aa protein which is present in commercial microbial pest control products such as Dipel and Crymax. The Cry2Ab2 proteins produced in MON 89034 and Bollgard II cotton have an identical amino acid sequence. Bollgard II cotton has been on the market since 2003 and there have been no concerns regarding its safety to date. The Cry2Ab2 protein is active against lepidopteran insects and protects plants from damage due to larval feeding of *Ostrinia, Spodoptera,* and *Diptera* species.

Further details regarding the identity and function of the Cry1A.105 and Cry2Ab2 proteins are provided in Part VI.

SECTION 6. The Applications and Uses of MON 89034

Corn (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2005, 81.8 million acres of corn were planted in the U.S. The U.S. production yielded 11 billion bushels with an average national yield of 148 bushels per acre (NCGA, 2006). The majority of corn acres (52% or 42.5 million acres) were planted using seed developed via biotechnology methods (USDA-NASS, 2006). In 2006, biotechnology-derived corn plantings increased to approximately 48 million acres (or 61% of total corn acres), with approximately 32 million acres planted with insect-protected Bt corn products (USDA-NASS, 2006). This rapidly expanding trend in the use of Bt corn also is seen globally, with 17.8 million hectares of insect-protected corn planted in 2005, up from 16.9 million hectares in 2004 (James, 2005).

In the U.S., insect pests cause significant damage to the corn crop resulting in poor grain quality and reduced yields (James, 2003). In 1997, Monsanto commercialized the

biotechnology-derived product, YieldGard® Corn Borer corn (hereafter referred to as MON 810) that contains the *cry1Ab* gene from *Bacillus thuringiensis* (Bt), which encodes the Cry1Ab protein that provides effective protection against damage caused by lepidopteran insect pests, especially the European corn borer (ECB, *Ostrinia nubilalis*) and the corn earworm (CEW, *Helicoverpa zea*). At that time in the U.S., the combination of crop losses and management costs resulting from lepidopteran pests were estimated to be over $1 billion per year (Mason et al., 1996). The introduction of MON 810 and other Bt corn products provided corn growers with a more effective solution for the control of lepidopteran larval pests such as ECB and CEW. In addition to the benefits of MON 810 as an effective pest management tool, the use of this product has also led to lower levels of harmful mycotoxins in Bt corn leading to improved food and feed safety. This results from the control of insects that can cause damage to the ear leading to fungal infections. The reduction in mycotoxin levels has been consistently demonstrated in countries around the world where corn borers are the predominant insect pests (Clements et al., 2003; Dowd, 2000 and 2001; Hammond et al., 2002 and 2004; de la Campa et al., 2005; Bakan et al., 2002; Magg et al. 2002; Munkvold et al., 1999; Munkvold, 2003; Papst et al., 2005; Pietri and Piva 2000; Wu, 2006). Furthermore, the use of MON 810 and other Bt corn products has reduced the use of chemical insecticides (Carpenter et al., 2004). Within ten years of the first Bt corn product introduction, the safe and effective use of Bt corn has been adopted globally on over 17 million hectares (James, 2005) to control several primary insect pests of corn in 12 countries. In 2005, insect protected corn was used on over 40% of the U.S corn acres (USDA-NAAS, 2006; Monsanto, 2006).

Recently, Monsanto has developed MON 89034 as a second-generation product to provide enhanced benefits for the control of lepidopteran pests of corn. MON 89034 will effectively address a corn grower's need to control a wider spectrum of lepidopteran pests, strengthen insect resistance management, further reduce the potential for mycotoxins in grain, enable more efficient plant breeding of this multi-genic trait into superior hybrids compared to MON 810, and potentially reduce the refuge acreage required for resistance management purposes. By producing effective levels of two insecticidal proteins, Cry1A.105 and Cry2Ab2, MON 89034 increases the durability of the product against the primary lepidopteran pests of corn. In addition, the individual proteins extend the spectrum of control against lepidopteran insects commonly present in corn fields. Specifically, the Cry1A.105 protein provides increased activity against fall armyworm (FAW, *Spodoptera sp*.) and black cutworm (BCW, *Agrotis ipsilon*) compared to Cry1Ab. The Cry2Ab2 protein provides improved control over Cry1Ab-containing products from damage caused by corn earworm. This wider spectrum of activity also will potentially contribute to the further reduction of mycotoxins in grain that result from fungal invasion after insect feeding damage.

Taken together, adoption of MON 89034 is likely to enhance the economic and other benefits to farmers and improve the quality of grain and the safety of derived food and feed products. In addition, MON 89034 was developed to allow the simultaneous and efficient introgression of two insect protection traits into improved corn germplasm,

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which will reduce the time and costs for new improved variety introductions into the marketplace. MON 89034 was developed using a single transformation vector containing both the *cry1A.105* and *cry2Ab2* genes. This approach, known as vector stacking, increases the efficiency of breeding multiple traits into new corn hybrids, thereby providing growers an earlier access to improved germplasm containing these traits rather than through conventional inbred stacking.

The benefits of MON 89034 are multi-faceted and interact in several ways to provide four key advantages, which are more specifically described below:

- *Extended Spectrum:* A major benefit of MON 89034 is the protection of corn plants from feeding damage caused by lepidopteran insect pest larvae. MON 89034 provides outstanding control of *Ostrinia* species such as European corn borer and Asian corn borer (ACB), and *Diatraea* species such as southwestern corn borer (SWCB) and sugarcane borer (SCB). Control of these insects provided by MON 89034 is comparable to MON 810. MON 89034 also provides a high level of control of fall armyworm throughout the season, whereas MON 810 principally controls damage caused by FAW larvae during vegetative growth. In addition, MON 89034 provides significant protection from damage caused by corn earworm. Although earworm control is not complete, it is superior to that provided by MON 810 and other Cry1-containing corn products. Introduction of MON 89034 will offer farmers a safe and effective alternative to the use of chemical insecticides for the control of the lepidopteran pests.

- *Improved IRM*: MON 89034 produces two structurally different Bt proteins, Cry2Ab2 and Cry1A.105, which are both highly efficacious against a variety of lepidopteran pests, and act by different mechanisms. The mechanism of insecticidal activity or mode of action of Bt proteins consists of a number of steps e.g., proteolysis, binding, and pore formation (English and Slatin, 1992; Gill et al., 1992; Schnepf et al., 1998; Zhuang and Gill, 2003). There are important differences in each step of the mechanism of activity that influence the interactions of these proteins with susceptible organisms without qualitatively influencing their host range. Several lines of evidence establish that Cry1A.105 and Cry2Ab2 have important differences in their mode of action, particularly in the way in which they bind to the lepidopteran midgut. These proteins have different structures, share only ~14% of amino acid sequence identity, and bind to distinct proteins in the midgut of target species at different rates with different affinities. Therefore, the probability of cross-resistance between these proteins is very low. Furthermore, *in vitro* and *in planta* studies of the Cry1A.105 and Cry2Ab proteins demonstrate that both proteins are highly active against the primary lepidopteran pests of corn: ECB, SWCB, CEW, and FAW. In view of the dual effective dose and the distinct mode of action of the two proteins produced in MON 89034, the likelihood for the evolution of resistance is significantly reduced compared to products containing a single introduced protein. Therefore, MON 89034 is expected to be sustainable using a reduced structured refuge. Based on conservative mathematical models, a 5% structured refuge in the U.S. Corn Belt and a 20% structured refuge in cotton growing regions will ensure the durability of MON 89034.

- *Mycotoxin Reduction:* Lepidopteran pests such as ECB, CEW and FAW cause substantial damage to stalks, ears, and leaves of developing corn plants resulting in reduced yields. MON 89034 has been shown to provide protection against a wider variety of lepidopteran pests than MON 810 and in addition to greater yield protection, this is expected to result in a reduced potential for mycotoxin contamination in diverse environments. Corn ears that are protected from feeding damage caused by ECB, CEW, and FAW would have fewer ports of entry for invasion by fungi that produce mycotoxins. As regulations are implemented globally that limit mycotoxin levels in food and feed, the economic impacts of mycotoxin contamination in grain will become increasingly important. In the U.S., the FDA has set guidelines for acceptable levels of the mycotoxin, fumonisin, in corn used for food and feed. In the recent study by Wu (2006), the economic benefit of mycotoxin reduction in Bt corn grain to meet this FDA standard resulted in an annual benefit in the U.S. of $23 million. With the expanded spectrum of MON 89034 compared to MON 810, it is expected that there will be a greater benefit to food and feed consumed globally.

- *Efficient Trait Integration:* The rapid development of elite corn hybrids containing the *cry1A.105* and *cry2Ab2* genes is made possible by vector stack technology, i.e., the incorporation of multiple genes into a single transformation event. This approach increases the efficiency of introduction of both genes into new corn germplasm by conventional breeding, thereby providing growers early access to a variety of elite corn germplasm containing both insecticidal proteins. Corn hybrids containing multiple Cry proteins conferring insect protection traits have been developed previously using traditional breeding techniques, i.e., two inbreds containing individual traits were crossed to produce the combined trait product. However, such breeding programs are generally more costly because of duplicated work to introgress two transformation events into new germplasms. MON 89034 reduces the time and cost factors in typical breeding programs, which rely on the sequential introgression of events containing single traits.

In summary, the introduction of MON 89034 will provide superior corn hybrids with higher yields, enhanced breeding efficiencies, better quality grain, and reduced potential for the development of insect resistance to Cry proteins.

SECTION 7. Applications for which MON 89034 is not Suitable

Monsanto Company is not aware of food or feed uses of conventional corn that are not applicable to MON 89034.

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PART III: STATUS OF SUBMISSIONS TO OTHER REGULATORY AGENCIES

SECTION 1. Status of Submission to USDA-APHIS

Monsanto will be requesting a Determination of Nonregulated Status for MON 89034, including all progenies derived from crosses between MON 89034 and other corn, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in 2006. Under regulations administered by USDA-APHIS (7 CFR 340), MON 89034 is currently considered a "regulated article." Monsanto will continue to conduct all field tests for MON 89034 in strict compliance with USDA field regulations until a Determination of Nonregulated Status is obtained for MON 89034.

SECTION 2. Status of Submission to U.S. EPA

The U.S. Environmental Protection Agency (EPA) has authority over the use of pesticidal substances under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), as amended (7 U.S.C. § 136 *et seq.*). An application for the registration of Bt Cry1A.105 and Cry2Ab2 proteins and the genetic material (vector PV-ZMIR245) necessary for their production in corn was submitted to the EPA in September 2006.

Pursuant to §408(d) of the Federal Food Drug and Cosmetic Act [21 U.S.C. 346 a(d)] Monsanto petitioned EPA for temporary exemptions from the requirement of a tolerance for Bt Cry1A.105 and Cry2Ab2 proteins in 2005. In July 2006, EPA established temporary exemptions from the requirement of a tolerance for: a) the Cry1A.105 protein and the genetic material necessary for its production in the food and feed commodities of field corn, sweet corn and popcorn (40 CFR §174.453), and b) for the Cry2Ab2 protein and the genetic material necessary for its production in the food and feed commodities of field corn, sweet corn and popcorn (40 CFR §174.454).

SECTION 3. Status of Submissions to Foreign Governments

Regulatory submissions for import and/or production approvals will be made to countries that import significant U.S. corn grain or derived food and feed products and have regulatory approval processes in place. These will include submissions to a number of foreign government regulatory agencies including, but not limited to, Japan's Ministry of Agriculture, Forestry and Fisheries (MAFF), the Ministry of Health, Labor and Welfare (MHLW), the Canadian Food Inspection Agency (CFIA) and Health Canada, and the European Commission of the European Union. As appropriate, notifications of import will be made to importing countries that do not have a formal approval process.

PART IV: DEVELOPMENT OF MON 89034

SECTION 1. Corn as a Crop

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. In the U.S., corn is a highly productive crop, yielding an average of 148 bushels per acre in the U.S. during 2005 (NCGA, 2006). Its high yield makes it one of the most economical sources of metabolizable energy for feeds, and of starch and sugar for food and industrial products. In 2005, approximately 55% of the corn grain produced in the U.S. was used as animal feed (NCGA, 2006). Therefore, indirect consumption is much greater than direct consumption for humans.

Corn, the host plant, has been a staple of the human diet for centuries. Corn grain and its processed fractions are consumed in a multitude of food and animal feed products. Corn forage is extensively consumed as animal feed by ruminants.

A thorough description of the anti-nutrients present in corn has been presented in an OECD consensus document (OECD, 2002). These anti-nutrients include phytic acid, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), raffinose, and trypsin and chymotrypsin inhibitors. Phytic acid is considered an important anti-nutrient for animals, especially nonruminants, since it reduces the bioavailability of phosphorus in corn tissues to levels below 15%. Feed formulators add the enzyme phytase to swine and poultry diets to improve the utilization of phosphorus. The OECD does not recommend analysis of DIMBOA due to the variable levels found across corn hybrids. Raffinose is a low molecular weight carbohydrate present in corn grain that is considered an anti-nutrient due to the gas production and resulting flatulence caused by consumption. Trypsin and chymotrypsin inhibitors occur at low levels in corn and are not considered nutritionally significant for human health (White and Pollak, 1995)

Corn is not a common allergenic food and there have been few reports of allergenic reactions to the consumption of corn products (OECD, 2002). In the U.S. there have been only two reported cases of documented allergy to the ingestion of corn (Pauls and Cross, 1998; Tanaka et al., 2001) over the past seven years. Jones et al. (1995) found that many individuals with apparent grain allergy are really sensitized to pollens and that a food challenge in ~80% of these individuals did not provoke clinical symptoms. Further, those with bona fide grain allergy are typically allergic to wheat proteins (~75%) and that allergies to corn (corn) are rare (less than six in over one hundred patients referred to a pediatric allergy specialty center for grain allergy).

Two recent reports from Italy (Pasterollo, 2000; Pasini et al., 2002) indicate that at least a few food allergic individuals have reactions consistent with allergies resulting from the consumption of corn products, including polenta, with mild food challenge positive results in six individuals from Naples (Pasini et al., 2002). However, all of the patients in the Italian studies have multiple sensitivities, usually to grass pollen, other grains, spices

and/or from members of the prunidae. The multiple allergies make clinical histories less reliable in identifying the causative agent of a particular episode, and also make diagnosis by skin prick test and *in vitro* IgE binding more complicated due to potentially weak and irrelevant cross-reactivity. At this time there is not enough evidence to understand the prevalence of allergies to corn products in Italy or the Mediterranean region. There have been no other reported instances of food allergic reactions to corn products from other parts of the world.

This evaluation suggests that the toxic or allergenic risk to humans from the consumption of corn and derived products is likely to be very low.

1.1. Scientific name and taxonomic classification of corn

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

Table IV.1. Taxonomic Classification of Corn and Its Close 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*
A. Section – *Tripsacum*
Species
1. *T. andersomii* (2n = 64)
2. *T. australe* (2n = 36)
Varieties
a) *T. australe* var. *australe*
b) *T. australe* var. *hirstum*
3. *T. bravum* (2n = 36, 72)
4. *T. cundinamarce* (2n = 36)
5. *T. dactyloides* (2n = 72)
Varieties
a) *T. dactyloides* var. *hispidum*
b) *T. dactyloides* var. *dactyloides*
c) *T. dactyloides* var. *meridonale*
d) *T. dactyloides* var. *mexicanum*
6. *T. floridanum* (2n = 36)
7. *T. intermedium* (2n = 72
8. *T. manisuroides* (2n = 72)
9. *T. latifolium* (2n = 36)
10. *T. percuvianum* (2n = 72, 90, 108)
11. *T. zopilotense* (2n = 36, 72)

B. Section – *Fasciculata*
Species
1. *T. jalapense* (2n = 72)
2. *T. lanceolatum* (2n = 72)
3. *T. fasciculatum* (2n = 36)
4. *T. maizar* (2n = 36, 72)
5. *T. pilosum* (2n = 72)
Varieties
a). *T. pilosum* var. *guatemalense*
b). *T. pilosum* var. *pilosum*

Asia:
I. Genera—
Chionachne (2n = 20)
Coix (2n = 10, 20)
Polytoca (2n = 20)
Schlerachne (2n = 20)
Trilobachne (2n = 20)

Tribe—Andropogoneae
I. Genus - *Manisuris*

1.2. Growth and Reproductive Characteristics 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, the large number of seeds produced on the female inflorescence, the ease in handling (growing and hand pollinating), the nature of the chromosomes, and the low basic chromosome number (n = 10), corn has been accessible for study at all levels of genetics.

Corn evolved as an open-pollinated (cross-fertilizing) crop species and, until the 20th century, 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 using simple mass selection by the indigenous native people prior to the arrival of Columbus. Their methods of selection were simple compared to 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. 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, 1909). Because corn naturally cross-fertilizes, the genetic composition of each plant is not known. Continuous self-pollination of individuals for seven to ten 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 pure-line parents to reproduce the superior hybrid and distribute it for use by growers (Shull, 1909).

1.3. History of corn development

Corn likely originated in the highlands of Mexico 7,000 to 10,000 years ago where it was first domesticated as a crop. Recent genotyping studies suggest that corn originated from a single domestication in southern Mexico 9000 years ago (Matsuoka et al., 2002). The putative parents of corn have not been recovered, but it is likely that 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.

By the time Columbus visited the Western Hemisphere, corn was being grown by indigenous civilizations from Chile to southern Canada. Columbus noted the presence of corn on the north coast of Cuba in 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 Northcentral region (U.S. Corn Belt) of the United States. The highly productive U.S. Corn Belt dent corn was 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 corn evolved from the gradual mingling of those settlements that spread north and west from the 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 corn grown in the southeast U.S. seemed to have originated from the southeast coast of Mexico. Southern dent corn is 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 eventually led to the highly productive U.S. Corn Belt dent corn that is used extensively throughout the world today.

The origin of corn has been studied extensively and four main hypotheses have been suggested (OECD, 2003):

1. *Descent from teosinte* - corn originated by direct selection from teosinte;
2. *The tripartite hypothesis*: 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);
3. *The common origin hypothesis* - corn, teosinte, and *Tripsacum* descended independently from a common, unknown ancestor; and,
4. *The catastrophic sexual transmutation hypothesis* – that modern corn originated from teosinte by an epigenetic sexual mutation causing ear development.

Other suggestions have included *Coix* and species of the genus *Manisuris* in the tribe *Andropogoneae* as 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 the 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 one to three 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).

SECTION 2. Characterization of the Vector Used in Transformation

Molecular analyses are an integral part of the characterization of crop products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product.

This section describes the plasmid vector PV-ZMIR245 and the method of transformation that was used to produce MON 89034. The next section describes the molecular analyses of the insert in MON 89034.

2.1. Method of transformation

MON 89034 was developed through *Agrobacterium*-mediated transformation of corn using the binary plasmid vector, PV-ZMIR245 (Figure IV.2). *Agrobacterium*-mediated transformation is a well-documented process for the transfer and integration of exogenous DNA into a plant's nuclear genome (Ishida et al., 1996). PV-ZMIR245 contains two separate T-DNAs (herein referred to as 2 T-DNA system). The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette that encodes the neomycin phosphotransferase enzyme that confers tolerance to certain antibiotics such as neomycin and paromomycin.

The use of the 2 T-DNAs system is the basis for an effective approach to generate marker-free plants. It allows for insertion of the T-DNA with the traits of interest (e.g., T-

DNA I) and the T-DNA encoding the selectable marker (e.g., *nptII*, T-DNA II) into two independent loci within the genome of the plant. Following selection of the transformants that contain both T-DNAs, the inserted T-DNA encoding the selectable marker (e.g., T-DNA II) can be segregated from progeny through subsequent conventional breeding and genetic selection, while the inserted T-DNA containing the trait(s) of interest is maintained (e.g., T-DNA I). This 2T-DNA binary vector approach has been successfully used in tobacco (Komari et al., 1996); soybean (Xing et al., 2000), barley (Matthews et al., 2001), corn (Miller et al., 2002); and rice (Komari et al., 1996; Breitler et al., 2004). MON 89034 was developed using such 2T-DNA vector transformation and selection techniques.

Freshly isolated immature corn embryos were used in the transformation (Ishida et al., 1996; Rout and Armstrong, 1997) that resulted in production of MON 89034. *Agrobacterium tumefaciens* strain ABI, containing plasmid PV-ZMIR245 was induced to be virulent by the use of acetosyringone. Strain ABI also contains a helper plasmid that does not contain any T-DNA but allows for the transfer of T-DNAs I and II into the plant cells. Following inoculation with *Agrobacterium*, the immature corn embryos were transferred to a co-culture medium for one to three days to ensure transformation of individual cells. This process of *Agrobacterium*-mediated transformation of corn involves the attachment of the bacterium to the corn cells, which leads to transfer of the region of DNA between the Left and Right Borders of the binary plasmid (i.e., the T-DNA) into the corn genomic DNA. Each T-DNA was integrated into the plant genome at separate loci.

Following the incubation period on the co-culture medium, the immature embryos were transferred to selection medium containing carbenicillin to eliminate *Agrobacterium*, and paromomycin to eliminate cells that were not transformed, so that only cells containing T-DNA II and/or T-DNA I + T-DNA II survived. The resulting transformed cells were then subcultured several times on a selection medium and regenerated into the R_0 plants containing both T-DNA's I and II, according to the protocol described by Armstrong and Phillips (1988).

During subsequent breeding at the F_1 generation the unlinked insertions of T-DNA I or T-DNA II were segregated. The plants containing only the insert that contains the *cry1A.105* and *cry2Ab2* gene cassettes were selected using molecular analysis, while the plants containing the nptII cassette (T-DNA II) were eliminated from subsequent breeding. The absence of the *nptII* gene and the NPTII protein was further confirmed by both Southern blot and ELISA analyses. Figure IV.2 displays the process map of the major steps involved in the transformation, selection, and development of MON 89034.

2.2. Plasmid PV-ZMIR245

The section describes the different genetic elements present in plasmid PV-ZMIR245.

2.2.1. T-DNA I

This section describes the elements contained on T-DNA I that were integrated into the corn genome to produce MON 89034.

2.1.1.1. The *cry1A.105* gene and Cry1A.105 protein

The *cry1A.105* coding sequence encodes the 133 kDa Cry1A.105 insecticidal protein that provides protection against feeding damage by lepidopteran insect pests. Cry1A.105 is a chimeric protein comprised of domains I, II from Cry1Ab and Cry1Ac, domain III from Cry1F (Bt subsp. *aizawai*), and the C-terminal portion from Cry1Ac (Bt subsp. *kurstaki*). Domains I and II of Cry1A.105 are 100% identical in amino acid sequence to domains I and II of both Cry1Ab and Cry1Ac, domain III is 99% identifical to domain III of Cry1F, and the C-terminal portion is 100% identical to the C-terminal portion of Cry1Ac. The Cry1A.105 is a modified Cry1A protein with overall amino acid sequence identity to Cry1Ab, Cry1Ac and Cry1F proteins of 90.0%, 93.6% and 76.7%, respectively (Crickmore et al., 1998). The deduced amino acid sequence of the Cry1A.105 protein produced in MON 89034 is presented in Figure IV.3.

2.1.1.2. The *cry1A.105* regulatory sequences

The expression cassette for the coding sequence of the Cry1A.105 protein consists of the promoter (P-e35S) and leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing a duplicated enhancer region (Kay et al., 1987). It contains the 5' untranslated leader of the wheat chlorophyll a/b/ binding protein (L-*Cab*) (Lamppa et al., 1985), the intron from the rice actin gene (*I-Ract1*) (McElroy et al., 1991), the *cry1A.105* coding sequence that was optimized for expression in monocots, and the 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3 (T-*Hsp17*), which terminates transcription and provides the signal for mRNA polyadenylation (McElwain and Spiker, 1989).

2.1.1.3. The *cry2Ab2* gene and Cry2Ab2 protein

The Cry2Ab2 protein present in MON 89034 is a member of the Cry2Ab class of proteins that share >95% amino sequence homology (Crickmore et al., 1998). It has an amino acid sequence identity of >99% with the wild-type Cry2Ab2 protein derived from Bt subsp. *kurstaki*, differing by a single amino acid. The deduced amino acid sequence of the Cry2Ab2 protein produced in MON 89034 is presented in Figure IV.4.

2.1.1.4. The *cry2Ab2* regulatory sequences

The *cry2Ab2* gene expression cassette that produces the Cry2Ab2 protein consists of the 35S promoter from figwort mosaic virus (P-*FMV;* Rogers, 2000), the first intron from the corn heat shock protein 70 gene (I-*Hsp* 70; Brown and Santino, 1995). It contains a *cry2Ab2* coding sequence with a modified codon usage (CS-*cry2Ab2*; Widner and Whitely, 1989, Donovan, 1991) fused to a chloroplast transit peptide region of corn ribulose 1,5-biphosphate carboxylase small subunit including the first intron (TS-*SSU-CTP*; Matsuoka et al., 1987). The 3' nontranslated region of the nopaline synthase (T-*nos*) coding region from *Agrobacterium tumefaciens* T-DNA terminates transcription and directs polyadenylation (Bevan et al., 1983).

2.1.1.5. T-DNA borders

Plasmid vector PV-ZMIR245 contains sequences that are necessary for transfer of T-DNA into the plant cell. These sequences are termed the Right and Left Border regions. The Right and Left Border regions each contains a border sequence that is a 24-26 bp sequence that defines the extent of the DNA that should be transferred into the plant genome. They flank both T-DNAs I and II, allowing for independent transfer and integration of each T-DNA into the plant genome during transformation. The Right Borders present in PV-ZMIR245 are made of a 24 bp nucleotide sequence that was originally derived from plasmid pTiT37, which was isolated from *A. tumefaciens* (Depicker et al., 1982). The Left Borders present in PV-ZMIR245 are made of a 25 bp nucleotide sequence from the *A. tumefaciens* plasmid pTi5955, a derivative of plasmid pTiA6 (Barker et al., 1983).

2.2.2. T-DNA II

This section describes the elements contained on T-DNA II that were not integrated into the corn genome to produce MON 89034.

2.2.2.1. *nptII* gene and NPTII protein

The *nptII* genes encodes the neomycin phosphotransferase II enzyme (NPTII) that inactivates certain aminoglycoside antibiotics such as kanamycin, neomycin and paromomycin. The use of selectable marker genes, such as *nptII*, is essential to select transformed cells under selective growth conditions. In the presence of paromomycin; cells transformed with *nptII* survive, while those that do not contain *npt II* are removed due to the action of the paromomycin. The T-DNA II, and therefore the *nptII* gene, is segregated out at the F1 generation.

2.2.2.2. *nptII* regulatory sequences

The *nptII* gene cassette that produces the NPTII protein consists of the promoter (P-*e35S*) from the the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985). The sequence coding for the NPTII protein (Beck et al., 1982) is followed by the 3' nontranslated region of the nopaline synthase (T-*nos*) coding region from *Agrobacterium tumefaciens* T-DNA that ends transcription and directs polyadenylation (Bevan et al., 1983).

2.2.2.3. T-DNA borders

The right and left T-DNA borders are described under Section 2.1.1.5.

2.2.3. Genetic elements outside the T-DNA borders

The backbone region outside of the inserted DNA, contains two origins of replication necessary for replication and maintenance of the plasmid in bacteria. In addition, it contains a bacterial selectable marker gene, *aad*, which encodes an aminoglycoside-modifying enzyme that confers resistance to the action of the antibiotics spectinomycin

and streptomycin. Detailed descriptions of all elements in the plasmid backbone region are presented in Table IV.2.

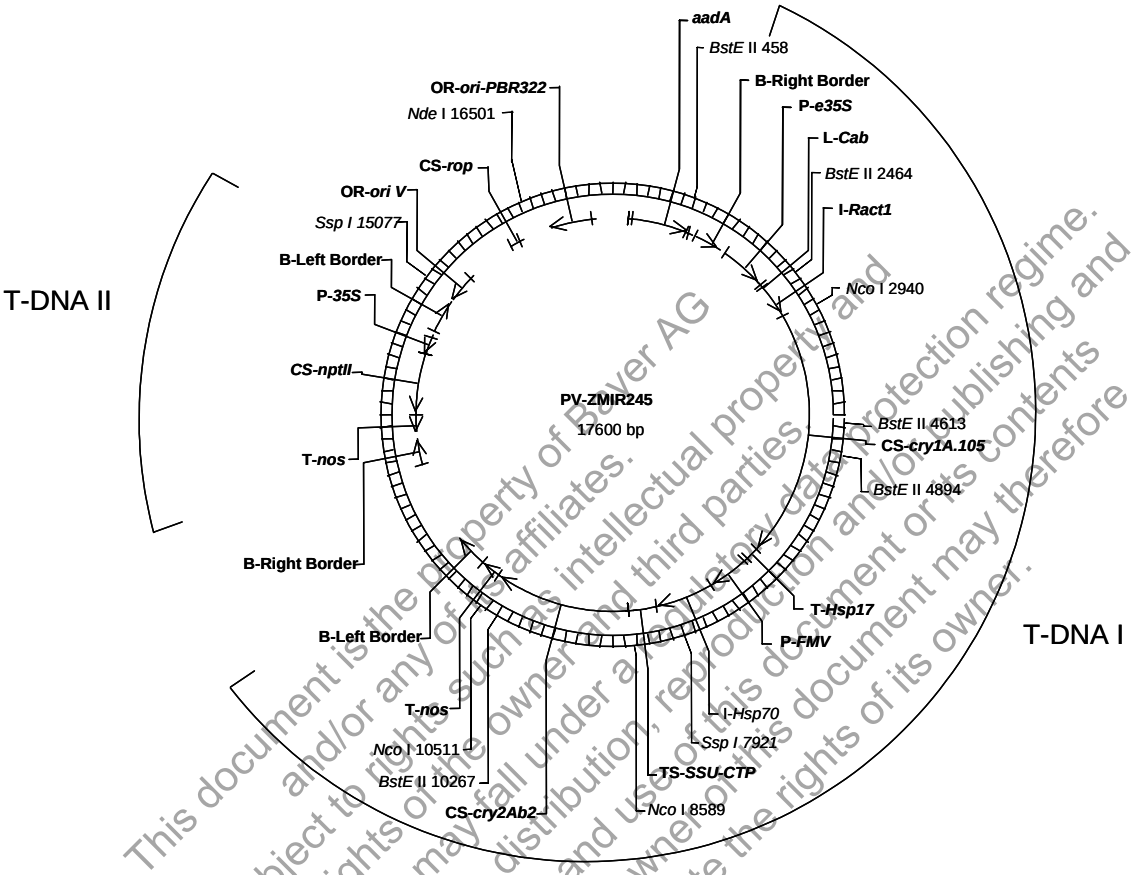


Figure IV.1. Plasmid Map of Vector PV-ZMIR245

A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown. PV-ZMIR245 contains two T-DNA regions designated as T-DNAs I and II. The genetic elements and restriction sites used in Southern blot analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map.

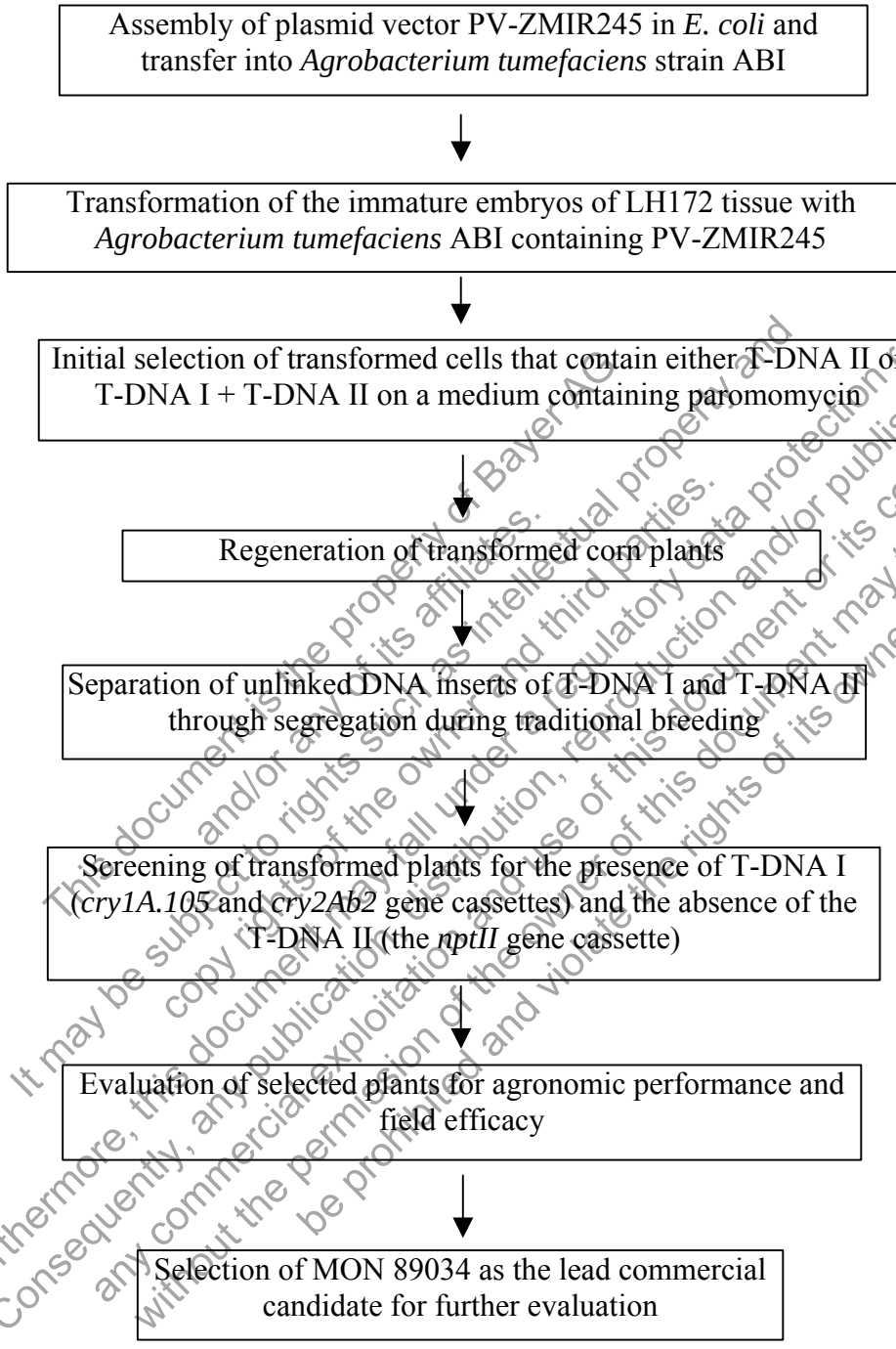


Figure IV.2. Process Map for Transformation, Selection, Regeneration and Evaluation of MON 89034

Table IV.2. Summary of Genetic Elements in Vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Vector Backbone		
Intervening Sequence	1-257	Sequences used in DNA cloning
aadA	258-1146	Bacterial promoter, coding sequence, and terminator sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al. 1985) (GenBank accession X03043). *aad*A confers resistance to streptomycin and spectinomycin
Intervening Sequence	1147-1261	Sequences used in DNA cloning
T-DNA I		
B[1]-Right Border	1262-1618	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al. 1982)
Intervening Sequence	1619-1728	Sequences used in DNA cloning
P[2]-*e35S*	1729-2349	The promoter and 9 bp leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al. 1987)
Intervening Sequence	2350-2375	Sequences used in DNA cloning
L[3]-*Cab*	2376-2436	The 5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al. 1985)
Intervening Sequence	2437-2452	Sequences used in DNA cloning
I[4]-*Ract1*	2453-2932	Intron from the rice actin gene (McElroy et al. 1991)
Intervening Sequence	2933-2941	Sequences used in DNA cloning
CS[5]-*cry1A.105*[6]	2942-6475	Coding sequences for the *Bacillus thuringiensis* Cry1A.105 protein (Monsanto unpublished data)

[1] B – border region
[2] P - promoter
[3] L - leader
[4] I - intron
[5] CS – coding sequence
[6] The *cry1A.105* coding sequence includes one stop codon, which accounts for three base pairs.

Table IV.2 (cont.). Summary of Genetic Elements in Vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	6476-6506	Sequences used in DNA cloning
T[1]-*Hsp17*	6507-6716	The 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation (McElwain and Spiker, 1989)
Intervening Sequence	6717-6783	Sequences used in DNA cloning
P-*FMV*	6784-7347	The Figwort Mosaic Virus 35S promoter (Rogers, 2000)
Intervening Sequence	7348-7369	Sequences used in DNA cloning
I-*Hsp70*	7370-8173	The first intron from the maize heat shock protein 70 gene (Brown and Santino, 1995)
Intervening Sequence	8174-8189	Sequences used in DNA cloning
TS[2]-SSU-CTP	8190-8590	DNA region containing the targeting sequence for the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit and the first intron (Matsuoka et al. 1987)
CS-*cry2Ab2*[3]	8591-10498	Coding sequence for a Cry2Ab2 protein from *Bacillus thuringiensis* (Widner and Whitely, 1989; Donovan, 1991). This coding sequence uses a modified codon usage.
Intervening Sequence	10499-10524	Sequences used in DNA cloning
T-*nos*	10525-10777	3' transcript termination sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983)
Intervening Sequence	10778-10844	Sequence used in DNA cloning
B-Left Border	10845-11286	DNA region from *Agrobacterium tumefaciens* containing the 25 bp left border sequence used for transfer of the T-DNA (Barker et al. 1983)

[1] T – transcript termination sequence
[2] TS – targeting sequence
[3] The *cry2Ab2* coding sequence includes two stop codons, which accounts for six base pairs.

Table IV.2 (cont.). Summary of Genetic Elements in Vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Vector Backbone		
Intervening Sequence	11287-12489	Sequences used in DNA cloning
T-DNA II		
B-Right Border	12490-12846	DNA region from *Agrobacterium tumefaciens* containing the 24 bp right border sequence used for transfer of the T-DNA (Depicker et al. 1982)
Intervening Sequence	12847-12971	Sequences used in DNA cloning
T-*nos*	12972-13224	3' transcript termination sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983)
Intervening Sequence	13225-13255	Sequences used in DNA cloning
CS-*nptII*	13256-14050	Coding sequence for neomycin phosphotransferase II protein that confers resistance to neomycin and kanamycin (Beck et al., 1982)
Intervening Sequence	14051-14083	Sequence used in DNA cloning
P-*35S*	14084-14407	Promoter and 31 bp leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al. 1985)
Intervening Sequence	14408-14457	Sequences used in DNA cloning
B-Left Border	14458-14899	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al. 1983)
Vector Backbone		
Intervening Sequence	14900-14985	Sequences used in DNA cloning
OR[1]-*ori V*	14986-15382	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker et al. 1981)

[1] OR – origin of replication.

Table IV.2 (cont.). Summary of Genetic Elements in Plasmid Vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	15383-16119	Sequence used in DNA cloning
CS-*rop*	16120-16311	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang 1989)
Intervening Sequence	16312-16738	Sequence used in DNA cloning
OR-*ori-PBR322*	16739-17327	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Suteliffe 1978)
Intervening Sequence	17328-17600	Sequence used in DNA cloning

```
0001  MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
0051  VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
0101  EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
0151  QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
0201  GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
0251  DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
0301  NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
0351  VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
0401  YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
0451  PMFSWIHRSA EFNNIIASDS ITQIPLVKAH TLQSGTTVVR GPGFTGGDIL
0501  RRTSGGPFAY TIVNINGQLP QRYRARIRYA STTNLRIYVT VAGERIFAGQ
0551  FNKTMDTGDP LTFQSFSYAT INTAFTFPMS QSSFTVGADT FSSGNEVYID
0601  RFELIPVTAT LEAEYNLERA QKAVNALFTS TNQLGLKTNV TDYHIDQVSN
0651  LVTYLSDEFC LDEKRELSEK VKHAKRLSDE RNLLQDSNFK DINRQPERGW
0701  GGSTGITIQG GDDVFKENYV TLSGTFDECY PTYLYQKIDE SKLKAFTRYQ
0751  LRGYIEDSQD LEIYSIRYNA KHETVNVPGT GSLWPLSAQS PIGKCGEPNR
0801  CAPHLEWNPD LDCSCRDGEK CAHHSHHFSL DIDVGCTDLN EDLGVWVIFK
0851  IKTQDGHARL GNLEFLEEKP LVGEALARVK RAEKKWRDKR EKLEWETNIV
0901  YKEAKESVDA LFVNSQYDQL QADTNIAMIH AADKRVHSIR EAYLPELSVI
0951  PGVNAAIFEE LEGRIFTAFS LYDARNVIKN GDFNNGLSCW NVKGHVDVEE
1001  QNNQRSVLVV PEWEAEVSQE VRVCPGRGYI LRVTAYKEGY GEGCVTIHEI
1051  ENNTDELKFS NCVEEEIYPN NTVTCNDYTV NQEEYGGAYT SRNRGYNEAP
1101  SVPADYASVY EEKSYTDGRR ENPCEFNRGY RDYTPLPVGY VTKELEYFPE
1151  TDKVWIEIGE TEGTFIVDSV ELLLMEE
```

Figure IV.3. Deduced Amino Acid Sequence of the Cry1A.105 Protein Produced in MON 89034


```
001 MAPTVMMASS ATAVAPFQGL KSTASLPVAR RSSRSLGNVS NGGRIRCMQV WPAYGNKKFE
061 IRTLSYLPPL STGGRCMQAM DNSVLNSGRT TICDAYNVAA HDPFSFQHKS LDTVQKEWTE
121 WKKNNHSLYL DPIVGTVASF LLKKVGSLVG KRILSELRNL IFPSGSTNLM QDILRETEKF
181 LNQRLNTDTL ARVNAELTGL QANVEEFNRQ VDNFLNPNRN AVPLSITSSV NTMQQLFLNR
241 LPQFQMQGYQ LLLLPLFAQA ANLHLSFIRD VILNADEWGI SAATLRTYRD YLKNYTRDYS
301 NYCINTYQSA FKGLNTRLHD MLEFRTYMFL NVFEYVSIWS LFKYQSLLVS SGANLYASGS
361 GPQQTQSFTS QDWPFLYSLF QVNSNYVLNG FSGARLSNTF PNIVGLPGST TTHALLAARV
421 NYSGGISSGD IGASPFNQNF NCSTFLPPLL TPFVRSWLDS GSDREGVATV TNWQTESFET
481 TLGLRSGAFT ARGNSNYFPD YFIRNISGVP LVVRNEDLRR PLHYNEIRNI ASPSGTPGGA
521 RAYMVSVHNR KNNIHAVHEN GSMIHLAPND YTGFTISPIH ATQVNNQTRT FISEKFGNQG
681 DSLRFEQNNT TARYTLRGNG NSYNLYLRVS SIGNSTIRVT INGRVYTATN VNTTTNNDGV
701 NDNGARFSDI NIGNVVASSN SDVPLDINVT LNSGTQFDLM NIMLVPTNIS PLY
```

Figure IV.4. Deduced Amino Acid Sequence of the Cry2Ab2 Protein Produced in MON 89034

The chloroplast transit peptide (CTP) region is underlined.

SECTION 3. Characteriztion of the Introduced Genetic Material

Molecular analysis was performed to characterize the inserted DNA in MON 89034. This analysis was performed to confirm that only the DNA associated with the desired trait is present in the final product.

Specifically Southern blot analyses were performed to assess:

a) Number of inserts in the genome,
b) Number of copies of insert,
c) Intactness of the genetic elements within the insert,
d) Absence of backbone sequences,
e) Absence of the T-DNA II encoding the selectable marker,
f) Stability of the inserted DNA across multiple generations, and
g) Organization of the insert

Genomic DNA from corn MON 89034 was digested using restriction enzymes and subjected to Southern blot analyses. Maps of plasmid vector PV-ZMIR245 annotated with the probes used in the Southern analysis are presented in Figures IV.6 and IV.7. Figure IV.8 provides a schematic of the insert present in MON 89034. The Southern blot analyses are presented in Figures IV.9 – IV.20 and the breeding generations used for analysis are depicted in Figure IV.21. For estimating the sizes of bands present in the long-run lanes of Southern blots, the molecular size markers on the left of the figure were used. For estimating the sizes of bands present in the short-run lanes, the molecular size markers on the right of the figure were used. Materials and methods for the molecular analysis are provided in Appendix A.

Molecular analyses confirmed that MON 89034 contains one insert derived from T-DNA I at a single locus of the nuclear genome. The insert contains one functional copy of the *cry1A.105* and of the *cry2Ab2* expression cassettes. All genetic elements from T-DNA I are intact and present in the expected order, except for the 5' end of the insert as discussed below. These analyses also confirmed the absence of plasmid backbone sequence, *nptII* and T-DNA II derived elements, other those in common with the T-DNA I (i.e., *35S* promoter, *nos 3´* end sequence, and the Left Border sequence). The Southern blot fingerprint of MON 89034 was maintained through seven generations of conventional breeding, thereby confirming the stability of the insert over multiple generations. These generations did not contain any detectable T-DNA II elements other than those in common with T-DNA I, nor did they contain any detectable backbone sequence from plasmid PV-ZMIR245. PCR and DNA sequence analyses confirmed the organization of the elements within the insert, the 5' and 3' insert-to-plant junctions, and the complete DNA sequence of the insert.

DNA sequence analyses of the MON 89034 insert showed that the *e35S* promoter that regulates expression of the *cry1A.105* gene has been modified and that the Right Border sequence present in PV-ZMIR245 is replaced by a Left Border sequence (Part IV,

Section 3.5). This molecular rearrangement can be explained by a recombination, either prior to or during the process of T-DNA transfer to the plant cell, between the shared DNA sequences of the 35S promoters in T-DNA's I and II (see Figure IV.5). Due to fidelity of this recombination event, the reconstituted *35S* promoter, *e35S*[89], was found to be identical to the *e35S* promoter, except that there is a deletion in the 5' portion from the duplicated enhancer elements (see Figure IV.5). The enhancer elements are not critical for expression of the Cry1A.105 protein in MON 89034 and the levels of this protein are sufficient to provide the expected efficacy against target lepidopteran pests.

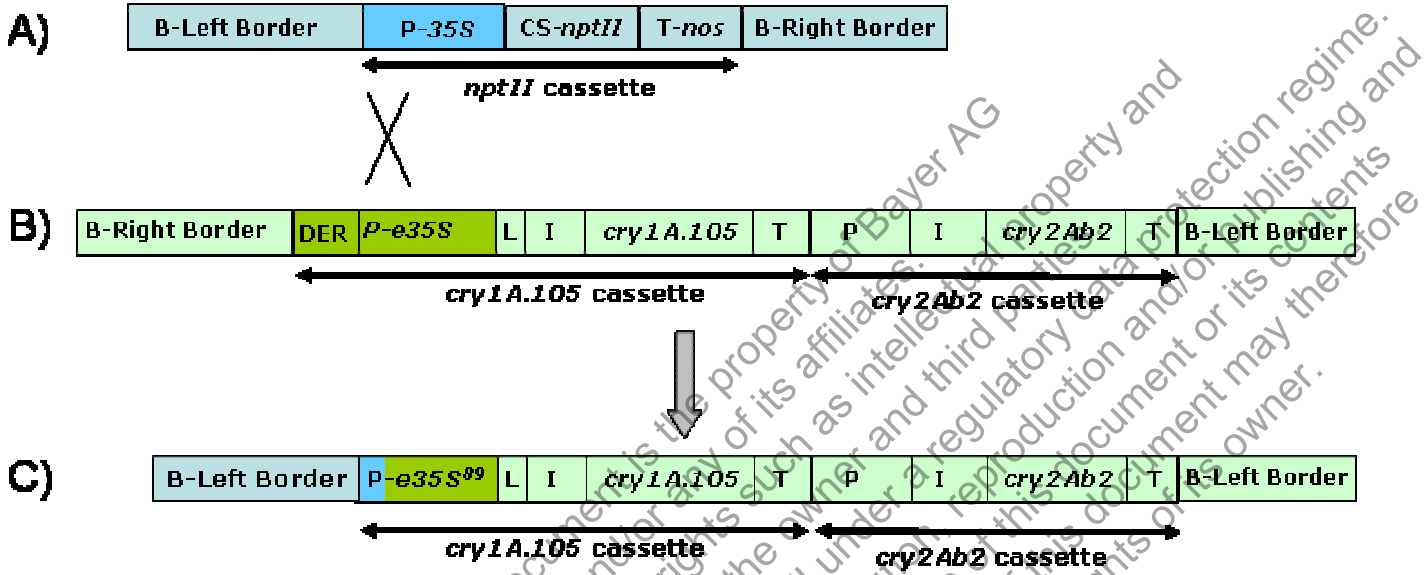


Figure IV.5. Description of the Recombination Process that Explains the Modified 5'End of the Insert

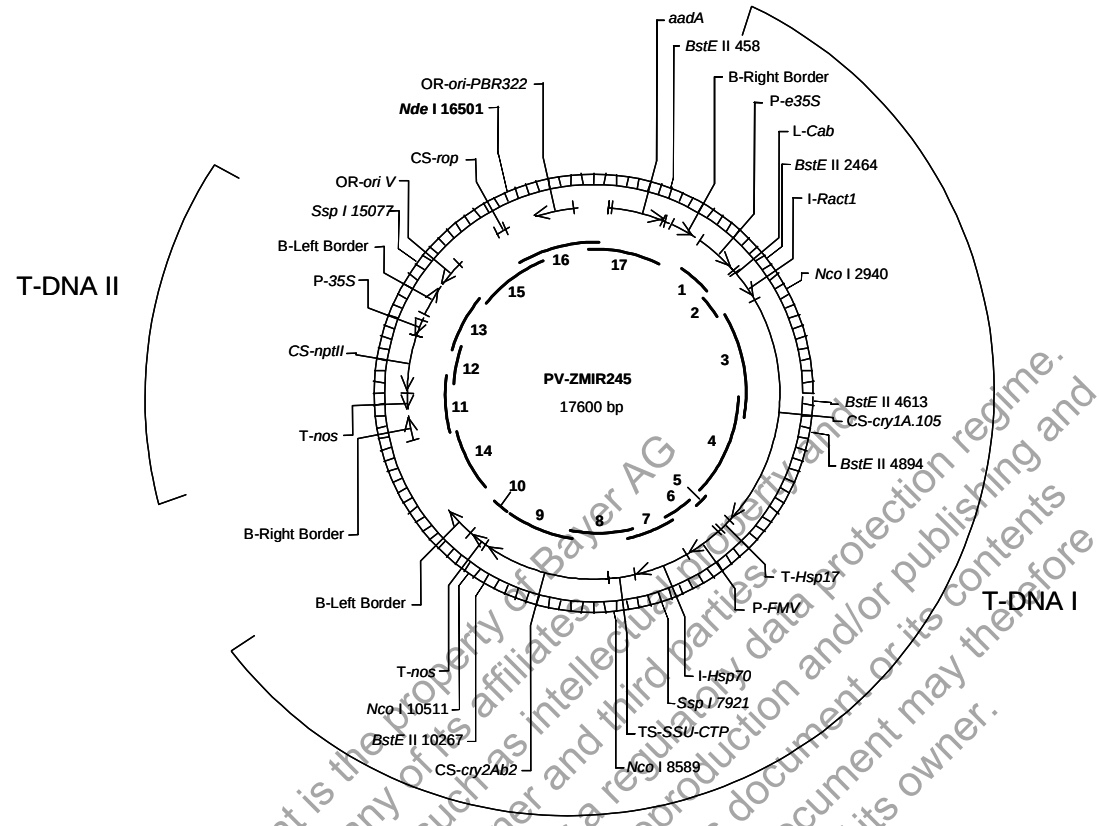
A) Illustration of the T-DNA II of plasmid PV-ZMIR245.

B) Illustration of the T-DNA I of plasmid PV-ZMIR245.

C) Illustration of the modified T-DNA I in MON 89034.

Abbreviations and symbols: DER = duplicated enhancer region; L = leader sequence; I = intron sequence; P = promoter; T = termination sequence. Detailed description of all the genetic elements are described in Tables IV.2 and IV.3.

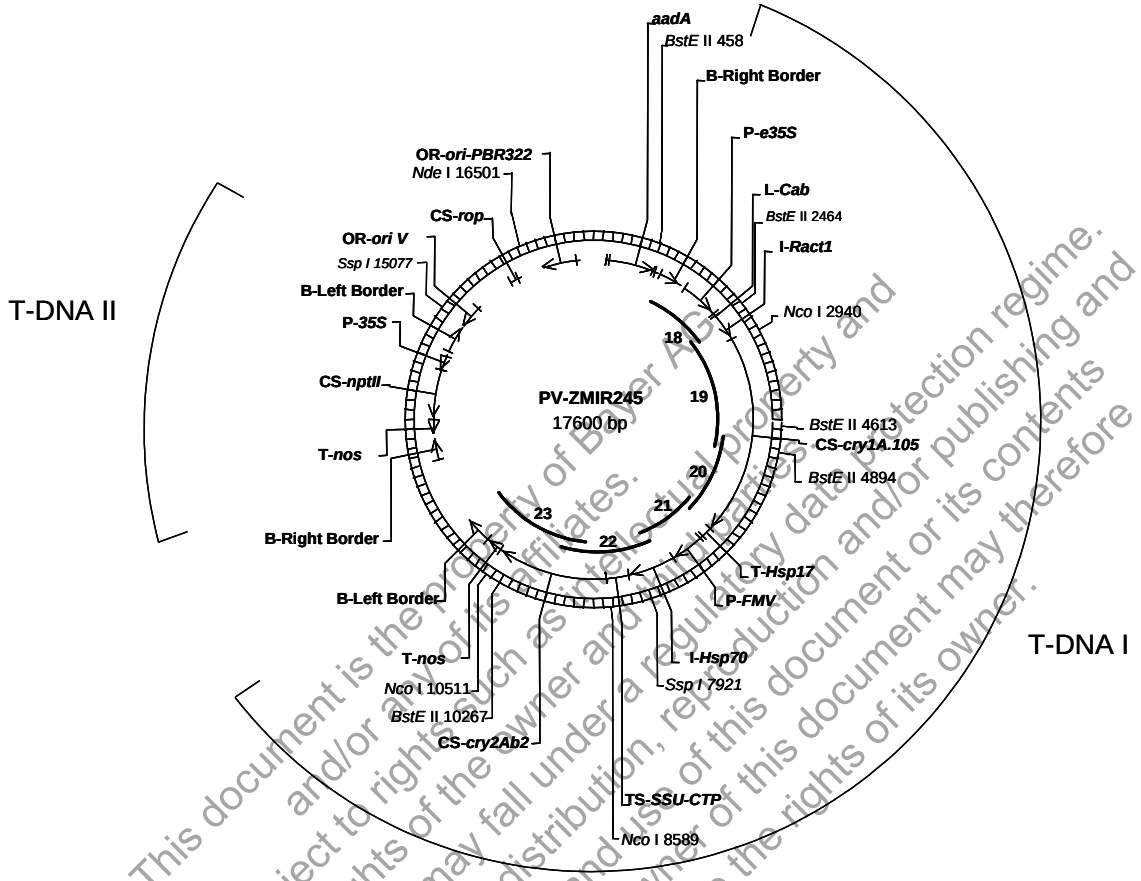
The diagram illustrates a recombination event, which likely occurred prior to or during the process of T-DNA transfer to the plant cells, between the DNA sequences near the *35S* promoters in T-DNAs I and II. Due to this recombination event, the reconstituted *e35S* promoter in MON 89034 (referred to as modified *e35S* or *e35S*^{89}) no longer has the duplicated enhancer elements compared to the original *e35S* promoter in PV-ZMIR245. Despite the deletion of the enhance elements, the Cry1A.105 protein expression levels in MON 89034 are still sufficiently high under the regulation of the modified *e35S* promoter to deliver the required efficacy against target insect pests.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	P-*e35S*/L-*Cab*	1714	2447	0.7
2	I-*Ract1*	2427	2941	0.5
3	CS-*cry1A.105* probe 1	2942	4923	2.0
4	CS-*cry1A.105* probe 2	4726	6505	1.8
5	T-*Hsp17*	6490	6797	0.3
6	P-*FMV*	6755	7366	0.6
7	I-*Hsp70*	7347	8179	0.8
8	TS-*SSU-CTP*/CS-*cry2Ab2* probe 1	8173	9516	1.3
9	TS-*SSU-CTP*/CS-*cry2Ab2* probe 2	9296	10509	1.2
10	T-*nos*	10525	10778	0.3
11	T-DNA II probe 1	12458	13391	0.9
12	T-DNA II probe 2/CS-*nptII* probe	13256	14050	0.8
13	T-DNA II probe 3	13973	14916	0.9
14	Backbone 1	11287	12489	1.2
15	Backbone 2	14900	16511	1.6
16	Backbone 3	16289	136	1.4
17	Backbone 4	48	1261	1.2

Figure IV.6. Genetic Elements and Restriction Sites of Vector PV-ZMIR245 Used in Southern Blot Analyses (Probes 1-17)

A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map. PV-ZMIR245 contains two T-DNA regions designated as T-DNA's I and II.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
18	T-DNA I probe 1	1210	2753	1.5
19	T-DNA I probe 2	2649	4676	2.0
20	T-DNA I probe 3	4518	6505	2.0
21	T-DNA I probe 4	6371	8179	1.8
22	T-DNA I probe 5	8004	9863	1.9
23	T-DNA I probe 6	9780	11354	1.6

Figure IV.7. Genetic Elements and Restriction Sites of Vector PV-ZMIR245 Used in Southern blot Analyses (Probes 18 - 23)

A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The overlapping T-DNA I probes used in the Southern analyses are shown on the interior of the map.

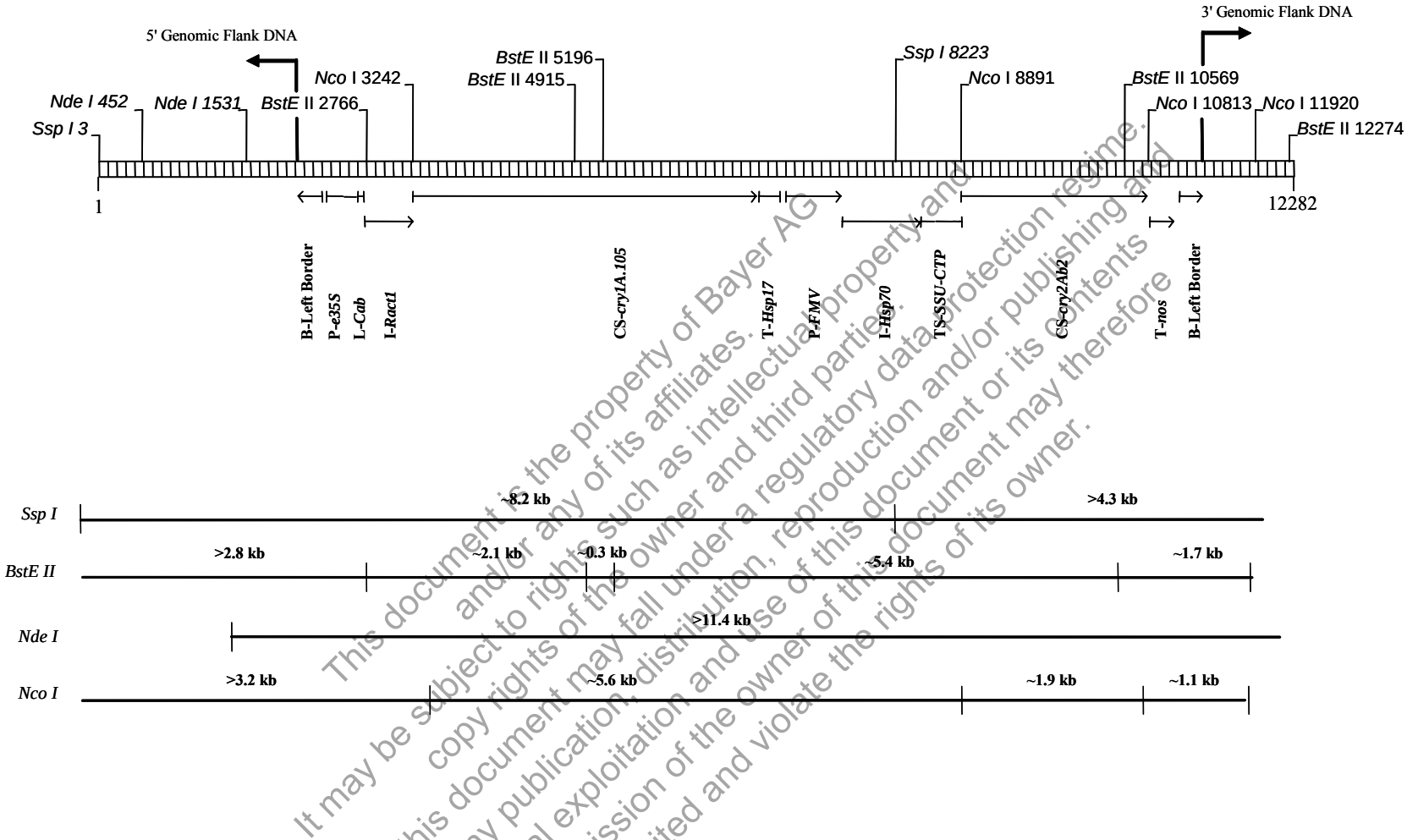


Figure IV.8. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 89034

The linear DNA derived from T-DNA I of vector PV-ZMIR245, which was incorporated into MON 89034 is shown. Arrows indicate the end of the insert and the beginning of corn genomic flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern blot analyses. A portion of Left Border sequence and a modified *e35S* promoter sequence is present at the 5' insert-to-flank junction in MON 89034.

Table IV.3. Summary of Genetic Elements in MON 89034

Genetic Element	Location in Sequence	Function (Reference)
Sequence flanking the 5′ end of the insert	1-2060	Corn genomic DNA
B[1]-Left Border[r1]	2061-2299	239 bp DNA region from the B-left Border region remaining after integration
Intervening Sequence	2300-2349	Sequence used in DNA cloning
P-*e35S*[89]	2350-2651	Modified e35s promoter and 9 bp leader resulting from a recombination between the P-*e35s* and P-*35s* promoters
Intervening Sequence	2652-2677	Sequence used in DNA cloning
L[2]-*Cab*	2678-2738	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al., 1985)
Intervening Sequence	2739-2754	Sequence used in DNA cloning
I[3]-*Ract1*	2755-3234	Intron from the rice actin gene (McElroy et al. 1991)
Intervening Sequence	3235-3243	Sequence used in DNA cloning
CS[4]-*cry1A.105*[5]	3244-6777	Coding sequence for the *Bacillus thuringiensis* Cry1A.105 protein (Monsanto unpublished data)
Intervening Sequence	6778-6808	Sequence used in DNA cloning
T[6]-*Hsp17*	6809-7018	3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation (McElwain and Spiker, 1989)
Intervening Sequence	7019-7085	Sequence used in DNA cloning

[1] B – border region
[2] L - leader
[3] I - intron
[4] CS – coding sequence
[5] Coding sequence of the *cry1A.105* including stop codon
[6] T – transcript termination sequence

Table 3 (cont.). Summary of Genetic Elements in MON 89034

Genetic Element	Location in Sequence	Function (Reference)
P[1]-*FMV*	7086-7649	Figwort Mosaic Virus 35S promoter (Rogers, 2000)
Intervening Sequence	7650-7671	Sequence used in DNA cloning
I-*Hsp70*	7672-8475	The first intron from the maize heat shock protein 70 gene (Brown and Santino, 1995)
Intervening Sequence	8476-8491	Sequence used in DNA cloning
TS[2]-SSU-CTP	8492-8892	DNA region containing the targeting sequence for the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit and the first intron. (Matsuoka et al., 1987)
CS-*cry2Ab2*	8893-10800	Coding sequence for a Cry2Ab2 protein from *Bacillus thuringiensis* (Widner and Whitely, 1989; Donovan, 1991) This coding sequence uses a modified codon usage
Intervening Sequence	10801-10826	Sequence used in DNA cloning
T-*nos*	10827-11079	3' termination sequence of the nopaline synthase (*nos*) coding sequence from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983)
Intervening Sequence	11080-11146	Sequence used in DNA cloning
B-Left Border[r2]	11147-11377	230 bp DNA region from the B-Left Border region remaining after integration
Sequence flanking the 3′ end of the insert	11378-12282	Corn genomic DNA

[1] P – promoter
[2] TS – targeting sequence

3.1. Insert and copy number

Southern blot analyses were performed to assess copy number and insert number of the DNA inserted in MON 89034. Results of these analyses are presented in Figure IV.9. The insert number (the number of integration sites of T-DNA I in the corn genome) was evaluated by digesting the test and control DNA with *Nde* I, a restriction enzyme that does not cleave within T-DNA I. This enzyme generates a restriction fragment containing T-DNA I and adjacent, plant genomic DNA. Thus, the number of restriction fragments detected indicates the number of inserts present in MON 89034. It is noted that the long run (overnight runs) enabled greater separation of the higher molecular weight restriction fragments while the short run (runs for a few hours) allowed the smaller molecular weight restriction fragments to be retained on the gel.

The number of copies of the T-DNA present was determined by digesting test and control genomic DNA samples with *Ssp* I, which cleaves once within the insert. If MON 89034 contains one copy of the insert, probing with overlapping T-DNA I [probes 18 – 23 in Figure IV.7] should result in two bands, each representing a portion of the insert along with adjacent, plant genomic DNA.

Genomic DNA isolated from conventional corn digested with *Nde* I (lanes 2 and 6) or *Ssp* I (lanes 4 and 8) produced several hybridization signals. This was expected because several of the genetic elements comprising T-DNA I were originally derived from corn (see Table IV.2). These hybridization signals result from the probes hybridizing to endogenous targets residing in the conventional corn genome and are not specific to the inserted DNA. These signals were produced in all lanes, including those containing the conventional corn control DNA material, and therefore they are considered to be endogenous background. Plasmid PV-ZMIR245 DNA mixed with conventional corn control DNA and digested with *Ssp I* (lanes 9 and 10) produced the expected bands at ~10.4 and ~7.2 kb in addition to the endogenous background hybridization produced by the conventional corn control DNA (lane 8).

MON 89034 DNA digested with *Nde* I (lanes 1 and 5) produced a single unique band of ~13 kb in addition to the endogenous background hybridization observed in the conventional corn control DNA (lanes 2 and 6). This result confirms that MON 89034 contains one insert located on ~13 kb *Nde* I restriction fragment.

The MON 89034 DNA digested with *Ssp* I (lanes 3 and 7) produced two bands in addition to the endogenous background hybridization observed in the conventional corn control DNA (lanes 4 and 8). The ~8.2 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (corresponding to T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure IV.8). The ~7.4 kb band, which was expected to be >4.3 kb, represents the 3' border fragment containing the 3' end of the inserted DNA along with the adjacent genomic DNA flanking the 3' end of the insert.

The results presented in Figure IV.9 indicate that MON 89034 contains one copy of T-DNA I that resides at a single locus of integration on ~13 kb *Nde* I restriction fragment.

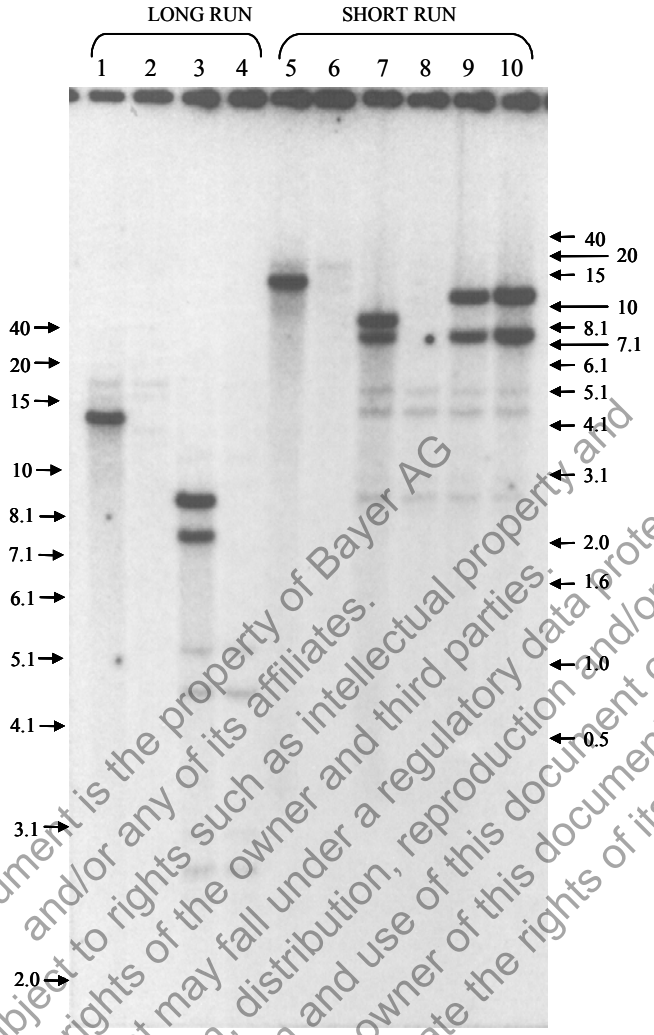


Figure IV.9. Southern Blot Analysis of MON 89034: Insert and Copy Number
The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 - 23, Figure IV.7). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Nde* I)
2: Conventional corn (*Nde* I)
3: MON 89034 (*Ssp* I)
4: Conventional corn (*Ssp* I)
5: MON 89034 (*Nde* I)
6: Conventional corn (*Nde* I)
7: MON 89034 (*Ssp I*)
8: Conventional corn (*Ssp I)*
9: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.2. Intactness of the *cry1A.105* and *cry2Ab2* expression cassettes

The presence and intactness of all the elements of the inserted *cry1A.105* and *cry2Ab2* expression cassettes was assessed by digestion of MON 89034 genomic DNA with the restriction enzyme *Ssp* I, *Nco* I, or *Bst*E II. This is necessary to ensure that only expected proteins are produced in MON 89034.

Digestion with *Ssp* I releases two border fragments with the expected size of ~8.2 and >4.3 kb (Figure IV.8). The ~8.2 kb fragment contains genomic DNA flanking the 5' end of the insert, Left Border sequence, modified *e35S* promoter sequence, *Cab* leader, *Ract1* intron, *cry1A.105* coding sequence, *Hsp17* 3' end sequence, *FMV* promoter, and a portion of the *Hsp70* intron. The border fragment >4.3 kb contains the remaining portion of the *Hsp70* intron, *SSU-CTP* targeting sequence, *cry2Ab2* coding sequence, *nos* 3' end sequence, Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA (Figure IV.8).

Digestion of MON 89034 genomic DNA with *Nco* I releases two internal restriction fragments and two border fragments (Figure IV.8). The 5' border fragment is expected to be >3.2 kb and contains genomic DNA flanking the 5' end of the insert, the Left Border[1] sequence, modified *e35S* promoter sequence, the *Cab* leader, and the *Ract1* intron. The ~5.6 kb internal fragment contains the *cry1A.105* coding sequence, *Hsp17* 3' end sequence, *FMV* promoter, *Hsp70* intron, and the *SSU-CTP* targeting sequence. The ~1.9 kb internal fragment contains the *cry2Ab2* coding sequence. The 3' border fragment is expected to be ~1.1 kb and contains the *nos* 3' end sequence, a second Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA.

Digestion of MON 89034 genomic DNA with *Bst*E II generates two border fragments and three internal fragments (Figure IV.8). The 5' border fragment is expected to be >2.8 kb and contains genomic DNA flanking the 5' end of the insert, Left Border[1] sequence, modified *e35S* promoter sequence, and the *Cab* leader sequence. The 3' border fragment is expected to be ~1.7 kb and contains a portion of the *cry2Ab2* coding sequence, the *nos* 3' end, Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA.

Plasmid PV-ZMIR245 DNA was combined with conventional corn control DNA and digested with *Nco* I or *Bst*E II (*Bst*E II was used for the T-*nos* and T-DNA II Southern blots) and loaded on the gel to serve as a positive hybridization control. Individual Southern blots were examined with the following probes: *e35S* promoter including the *Cab* leader, the *Ract1* intron, the *cry1A.105* coding sequence, the *Hsp17* 3' end sequence, the *FMV* promoter, the *Hsp70* intron, the *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence, and the *nos* 3' end sequence (probes 1-10, Figure IV.6).

[1] See section 3.1.

3.2.1. *e35S* promoter/*Cab* leader

The results of this analysis are presented in Figure IV.10. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) probed with the *e35S* promoter and *Cab* leader probe showed no hybridization bands, as expected for the negative control. Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~10 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the single unique band of ~5.4 kb. This is consistent with the expected band being >3.2 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *e35S* promoter and *Cab* leader elements other than those associated with the *cry1A.105* cassette.

3.2.2. *Ract1* intron

The results of this analysis are presented in Figure IV.11. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) probed with the *Ract1* intron probe showed no hybridization bands, as expected for the negative control.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~10 kb. The migration of the ~10 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the single unique ~ 5.4 kb band. This is consistent with the expected band being >3.2 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *Ract1* intron elements other than those associated with the *cry1A.105* cassette.

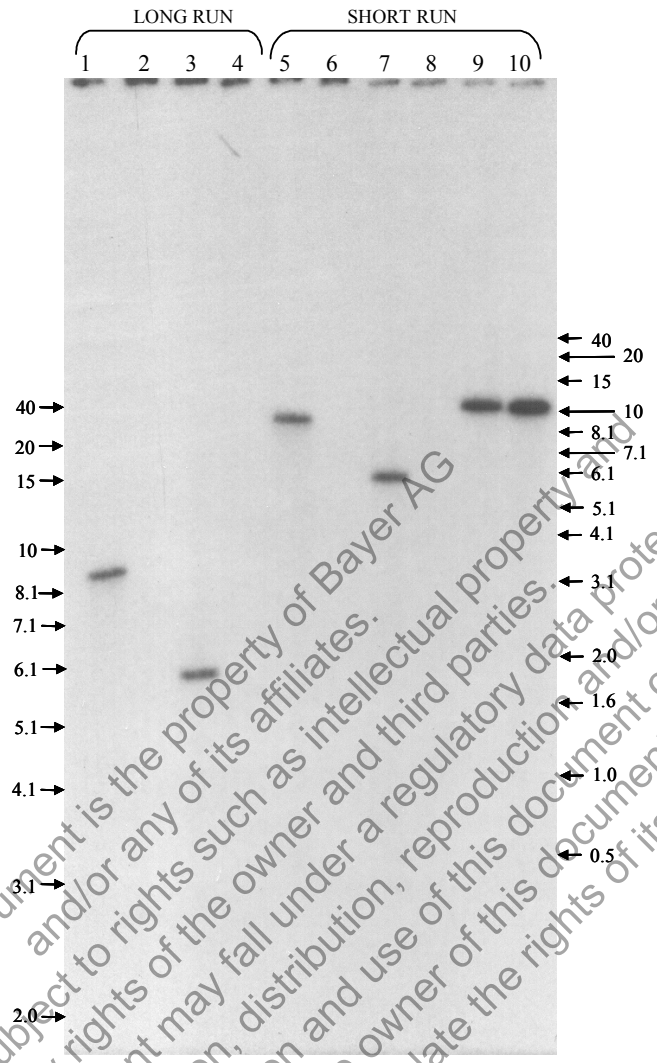


Figure IV.10. Southern Blot Analysis of MON 89034: *e35S* Promoter/*Cab* Leader
The blot was examined with a ^{32}P-labeled probe that spanned the *e35S* promoter and *Cab* leader region (probe 1, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

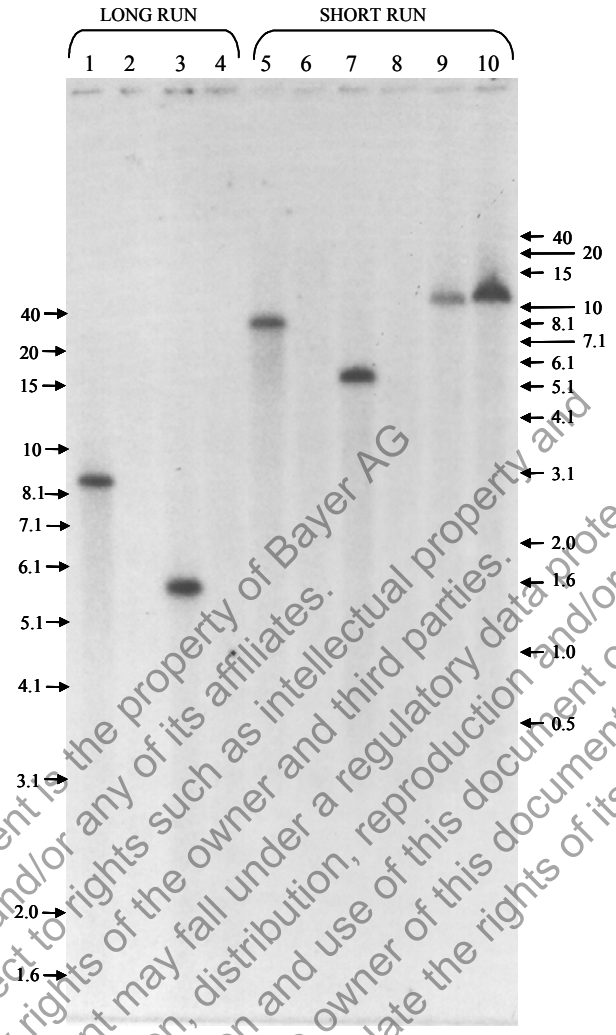


Figure IV.11. Southern Blot Analysis of MON 89034: *Ract1* Intron

The blot was examined with a ^{32}P-labeled probe that spanned the *Ract1* intron (probe 2, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.2.3. *cry1A.105* coding sequence

The results of this analysis are presented in Figure IV.12. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) hybridized simultaneously with overlapping probes spanning the *cry1A.105* coding sequence showed no hybridization bands as expected for the negative control. Conventional DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb. The migration of the ~ 5.6 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected single unique band of ~5.6 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *cry1A.105* elements other than those associated with the *cry1A.105* cassette.

3.2.4. *Hsp17* 3' end sequence

The results of this analysis are presented in Figure IV.13. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *Hsp17* 3' end sequence probe (probe 5, Figure 5.IV) showed no hybridization bands, as expected for the negative control. Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected single unique band of ~5.6 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *Hsp17* 3' end elements other than those associated with the *cry1A.105* cassette.

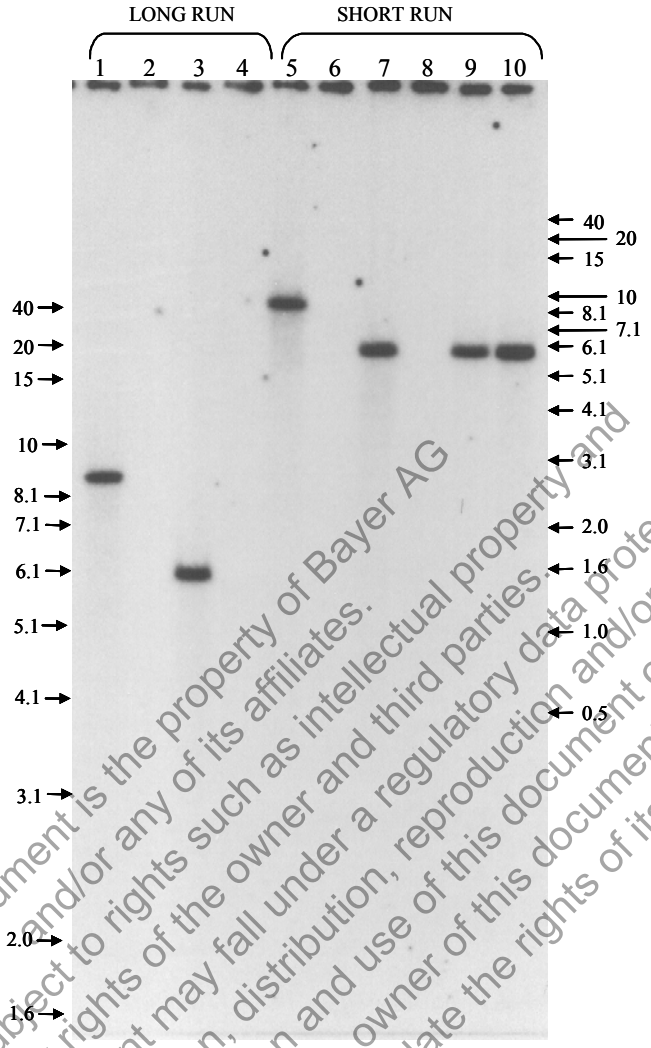


Figure IV.12. Southern Blot Analysis of MON 89034: *cry1A.105* Coding Sequence
The blot was examined with overlapping ^{32}P-labeled probes that spanned the *cry1A.105* coding sequence (probes 3 and 4, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

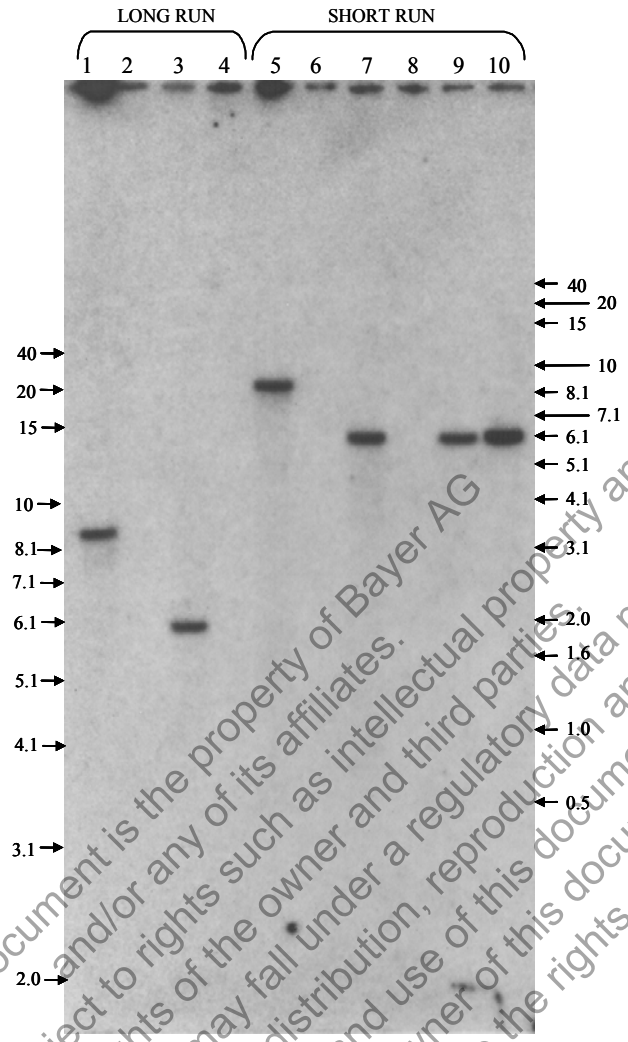


Figure IV.13. Southern Blot Analysis of MON 89034: *Hsp17*3' End Sequence
The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp17* 3' end sequence (probe 5, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.2.5. *FMV* promoter

The results of this analysis are presented in Figure IV.14. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *FMV* promoter probe showed no hybridization bands, as expected for the negative control.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb. The migration of the ~5.6 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced the expected single unique band of ~8.2 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected single unique band of ~5.6 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *FMV* elements other than those associated with the *cry2Ab2* cassette.

3.2.6. *Hsp70* intron

The results of this analysis are presented in Figure IV.15. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *Hsp70* intron probe produced several hybridization signals. This is not unexpected because the *Hsp70* intron was originally derived from corn. These hybridization signals result from the probes hybridizing to endogenous sequences residing in the corn genome and are not specific to the inserted DNA. These signals were produced in both test and control lanes, and therefore the bands are considered to be endogenous background.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at ~5.6 kb in addition to the endogenous bands. The migration of the ~5.6 kb fragments is slightly higher than indicated by the molecular marker band sizes. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the molecular marker (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced two expected bands of ~8.2 and ~7.4 kb in addition to the endogenous bands. The ~8.2 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (see Figure IV.8). The ~7.4 kb band represents the 3' border fragment containing the 3' end of the inserted DNA along with the adjacent genomic DNA flanking the 3' end of the insert which was expected to be >4.3 kb. MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced the expected band of ~5.6 kb in addition to the endogenous bands that resulted from non-specific hybridization as shown on lanes 4 and 8. No unexpected bands were detected, indicating that MON 89034 contains no additional *Hsp70* intron elements other than those associated with the *cry2Ab2* cassette.

3.2.7. *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence

The results of this analysis are presented in Figure IV.16. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) and examined with overlapping probes spanning the *SSU-CTP*/*cry2Ab2* coding sequence probe (probes 8 and 9, Figure IV.6) produced several hybridization signals. This is expected because the *SSU-CTP* targeting sequence was originally derived from corn. These hybridization signals result from the probes hybridizing to endogenous targets residing in the corn genome and are not specific to the inserted DNA. These signals were produced with both test and control material, therefore they are considered to be endogenous background. Endogenous bands were not detected in the long runs of the *Nco* I digests because they ran off the gel, as expected.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size bands at ~1.9 and ~5.6 kb in addition to the endogenous bands.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced one expected band of ~7.4 kb in addition to the endogenous bands. The ~7.4 kb band is consistent with the expected band of >4.3 kb (see Figure IV.8). MON 89034 DNA digested with *Nco* I (lanes 3 and 7) produced two bands in addition to the endogenous bands that are consistent with the expected sizes of ~5.6 and ~1.9 kb. The position of the these bands was slightly higher than indicated by the molecular marker band sizes in the long run (lane 3) but was concurrent with the bands produced by PV-ZMIR245 in the short run (lanes 7, 9, and 10). The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the molecular marker (Sambrook and Russell, 2001). No unexpected bands were detected, indicating that MON 89034 contains no additional *SSU-CTP*/*cry2Ab2* elements other than those associated with the *cry2Ab2* cassette.

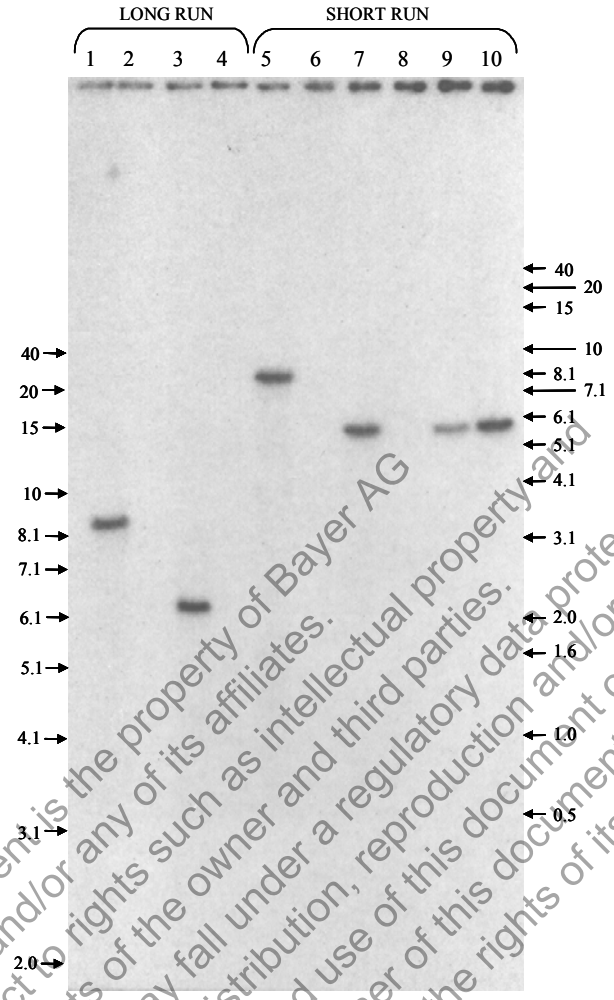


Figure IV.14. Southern Blot Analysis of MON 89034: *FMV* Promoter

The blot was examined with a ^{32}P-labeled probe that spanned the *FMV* promoter (probe 6, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

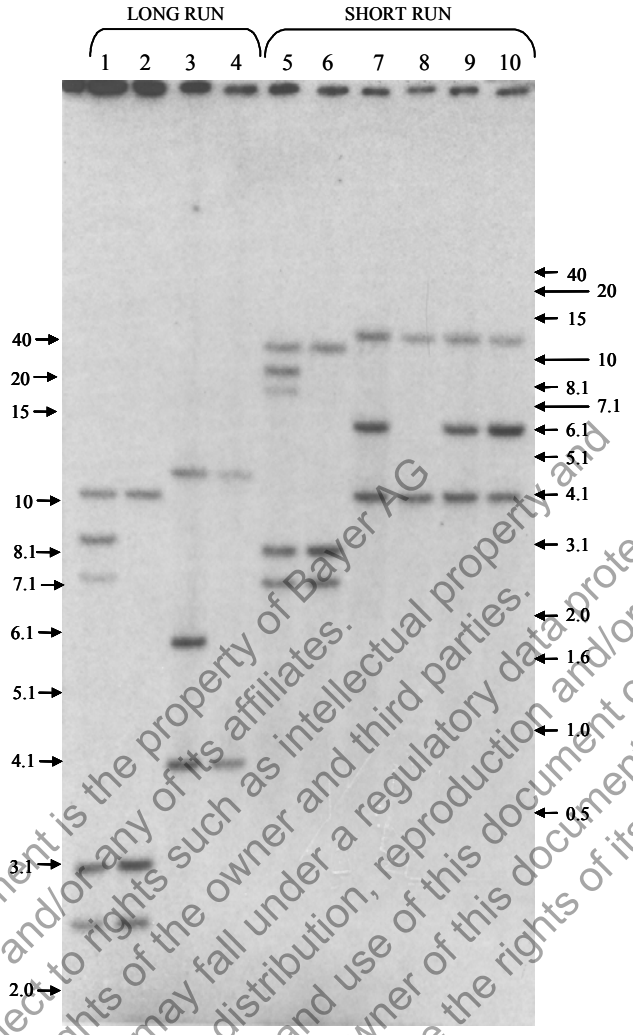


Figure IV.15. Southern Blot Analysis of MON 89034: *Hsp70* Intron

The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp70* intron (probe 7, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

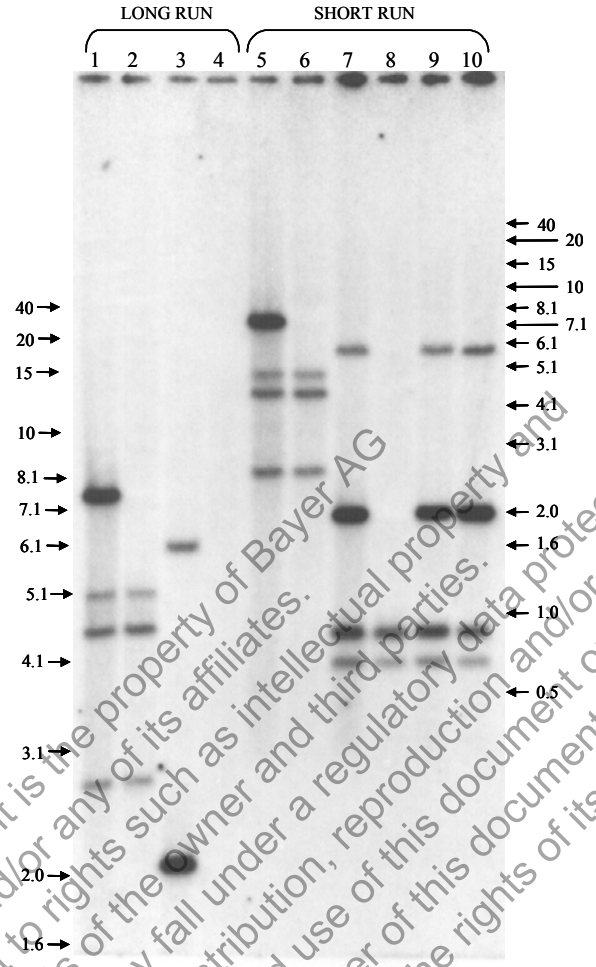


Figure IV.16. Southern Blot Analysis of MON 89034: *SSU-CTP* Targeting Sequence/*cry2Ab2* Coding Sequence

The blot was examined with overlapping ^{32}P-labeled probes that spanned the *SSU-CTP* targeting sequence and *cry2Ab2* coding sequence (probes 8 and 9, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.2.8. *nos* 3' end sequence

The results of this analysis are presented in Figure IV.17. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Bst*E II (lanes 4 and 8) examined with the *nos* 3' end sequence probe showed no detectable hybridization bands, as expected for the negative control. Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Bst*E II (lanes 9 and 10) produced the expected size band at ~7.8 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced a single, unique band of ~7.4 kb that is consistent with the expected band >4.3 kb (see Figure IV.8). MON 89034 DNA digested with *Bst*E II (lanes 3 and 7) produced the expected single unique band of ~1.7 kb. No unexpected bands were detected, indicating that MON 89034 contains no additional *nos* 3'end elements other than those associated with the *cry2Ab2* cassette.

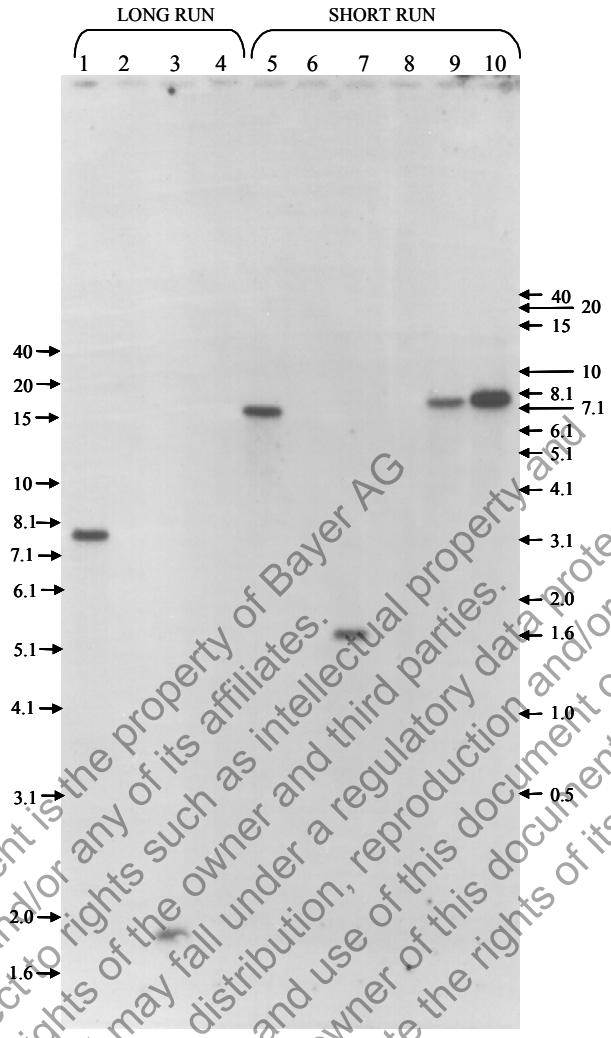


Figure IV.17. Southern Blot Analysis of MON 89034: *nos* 3' end Sequence

The blot was examined with a ^{32}P-labeled probe that spanned the *nos* 3' end sequence (probe 10, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*BstE* II)
4: Conventional corn (*BstE* II)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*BstE II*)
8: Conventional corn (*BstE II)*
9: Conventional corn spiked with PV-ZMIR245 (*BstE* II) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*BstE* II) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.2.9. Analysis to confirm the absence of plasmid PV-ZMIR245 backbone

Southern blot analysis was used to confirm that the plasmid backbone sequences necessary for transformation are no longer present in MON 89034. This is important to ensure that only the desired proteins are produced in MON 89034.

The results of this analysis are presented in Figure IV.18. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with overlapping probes spanning the vector backbone of PV-ZMIR245 (probes 14-17, Figure IV.6) showed no hybridization bands as expected for the negative control.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of ~10 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) or *Nco* I (lanes 3 and 7) produced no detectable hybridization bands, indicating that MON 89034 contains no PV-ZMIR245 backbone elements.

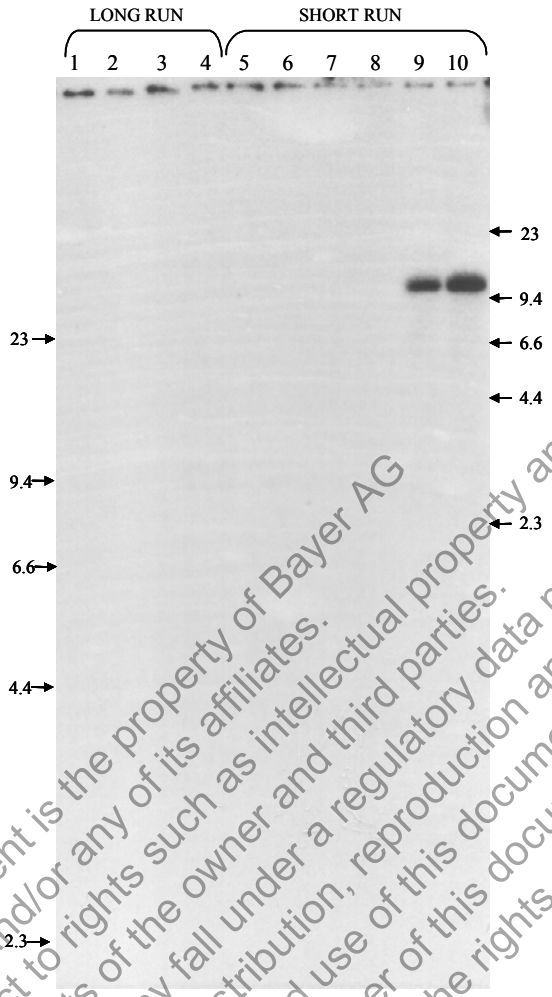


Figure IV.18. Southern Blot Analysis of MON 89034: PV-ZMIR245 Backbone Sequence

The blot was examined with ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.3. Analysis to confirm the absence of T-DNA II

3.3.1. Analysis to confirm the absence of the nptII coding sequence

Southern blot analysis was used to confirm that the *nptII* sequence necessary for transformation is no longer present in MON 89034. This is important to ensure that only the desired proteins are produced in MON 89034.

The results of this analysis are presented in Figure IV.19. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *nptII* coding sequence probe (probe 12, Figure IV.6) showed no detectable hybridization bands, as expected for the negative control.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of ~10 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) or *Nco* I (lanes 3 and 7) produced no detectable hybridization bands, indicating that MON 89034 contains no *nptII*-derived elements.

3.3.2. Analysis to confirm the absence of the overall T-DNA II region

This Southern blot analysis confirms the absence of the *nptII* coding sequence and demonstrates the absence of any additional T-DNA II sequences except for those elements that are in common with the T-DNA I expression cassette (i.e., *35S* promoter, *nos* 3´ end sequence, Left Border sequence). This analysis confirms that the F_1 plants that contained T-DNA II elements were segregated out and that the plants selected for further development only contained T-DNA I.

The results of this analysis are presented in Figure IV.20. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) and *Bst*E II (lanes 4 and 8) examined with overlapping probes spanning T-DNA II (probes 11-13, Figure IV.6) showed no hybridization bands.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Bst*E II (lanes 9 and 10) produced the two expected size bands at ~7.8 and ~2 kb. The overlapping probes spanning T-DNA II contain the *35S* promoter, *nos* 3' end, and Left Border sequences, which are contained on T-DNA I. Therefore, the T-DNA II probe is expected to hybridize to bands derived from T-DNA I.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced two bands of ~8.2 and ~7.4 kb The presence and size of these bands is consistent with the 8.2 kb band detected during the analysis of the *e35S* promoter/*Cab* leader region (Figures IV.10, lanes 1 and 5), and the 7.4 kb band detected during the analysis of the *nos* 3' end sequences (Figure IV.17, lanes 1 and 5).

MON 89034 DNA digested with *Bst*E II (lanes 3 and 7) produced two bands of ~4.2 and ~1.7 kb. The ~4.2 kb band is consistent with the >2.8 kb expected band for sequence

from T-DNA I digested with *Bst*E II (Figure IV.8), and the ~1.7 kb band is consistent with the T-DNA I specific band observed in Figure IV.17, lanes 3 and 7. No unexpected bands were detected, indicating that MON 89034 contains no additional elements other than those in common with T-DNA I.

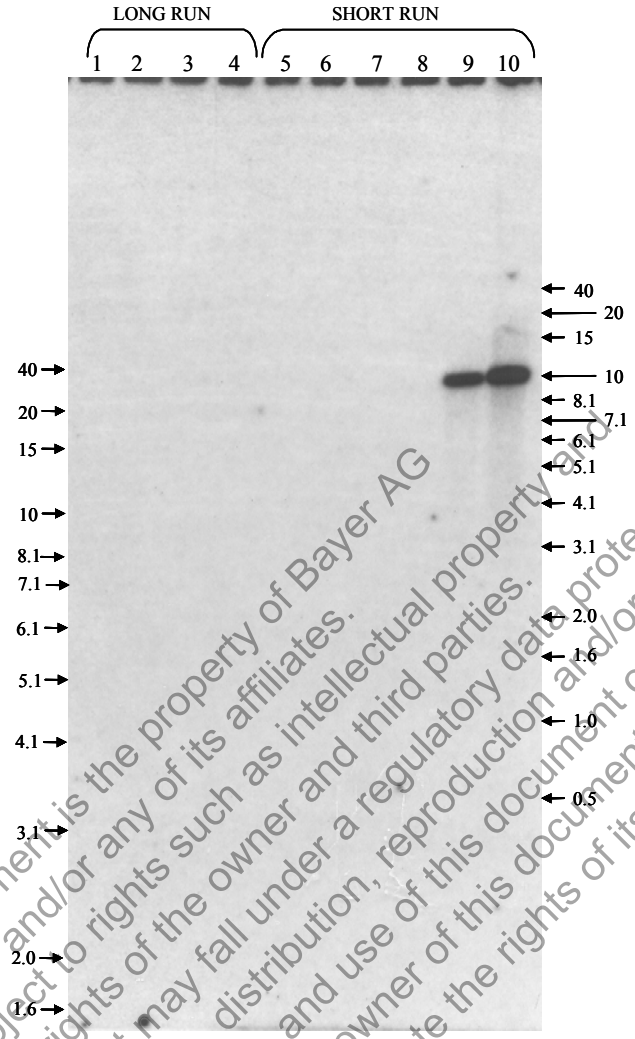


Figure IV.19. Southern Blot Analysis of MON 89034: *npt II* Coding Sequence
The blot was examined with a ^{32}P-labeled probe that spanned the *npt II* coding sequence (probe 12, Figure IV.6). Each lane contains ~10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*Nco* I)
4: Conventional corn (*Nco* I)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*Nco I*)
8: Conventional corn (*Nco I)*
9: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*Nco* I) [1.0 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

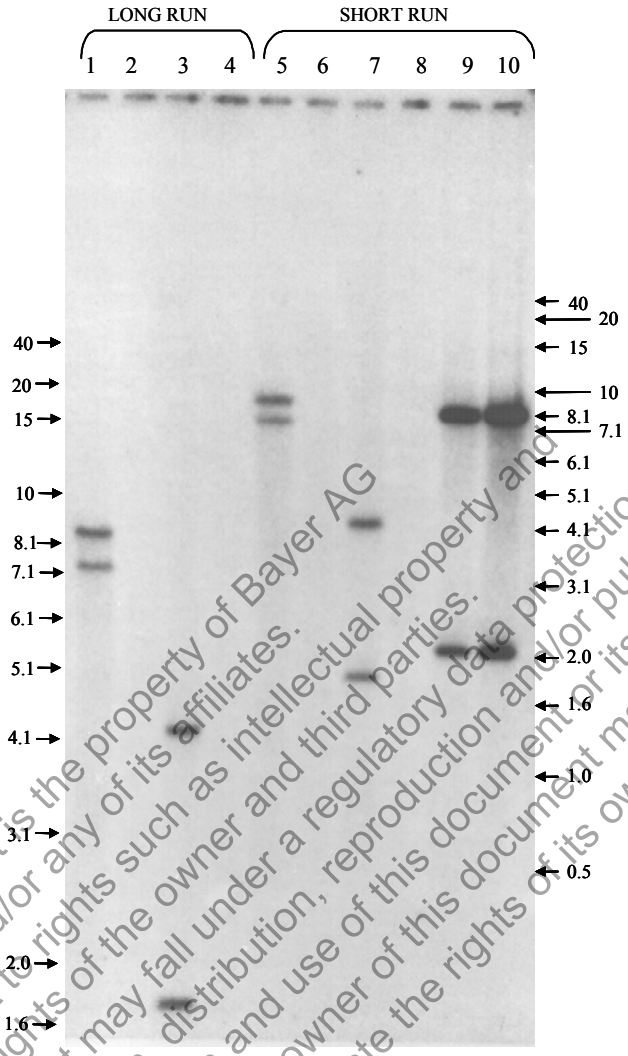


Figure IV.20. Southern Blot Analysis of MON 89034: T-DNA II

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (*Ssp* I)
2: Conventional corn (*Ssp* I)
3: MON 89034 (*BstE* II)
4: Conventional corn (*BstE* II)
5: MON 89034 (*Ssp* I)
6: Conventional corn (*Ssp* I)
7: MON 89034 (*BstE II*)
8: Conventional corn (*BstE II)*
9: Conventional corn spiked with PV-ZMIR245 (*BstE* II) [0.5 copy]
10: Conventional corn spiked with PV-ZMIR245 (*BstE* II) [1.0 copy]

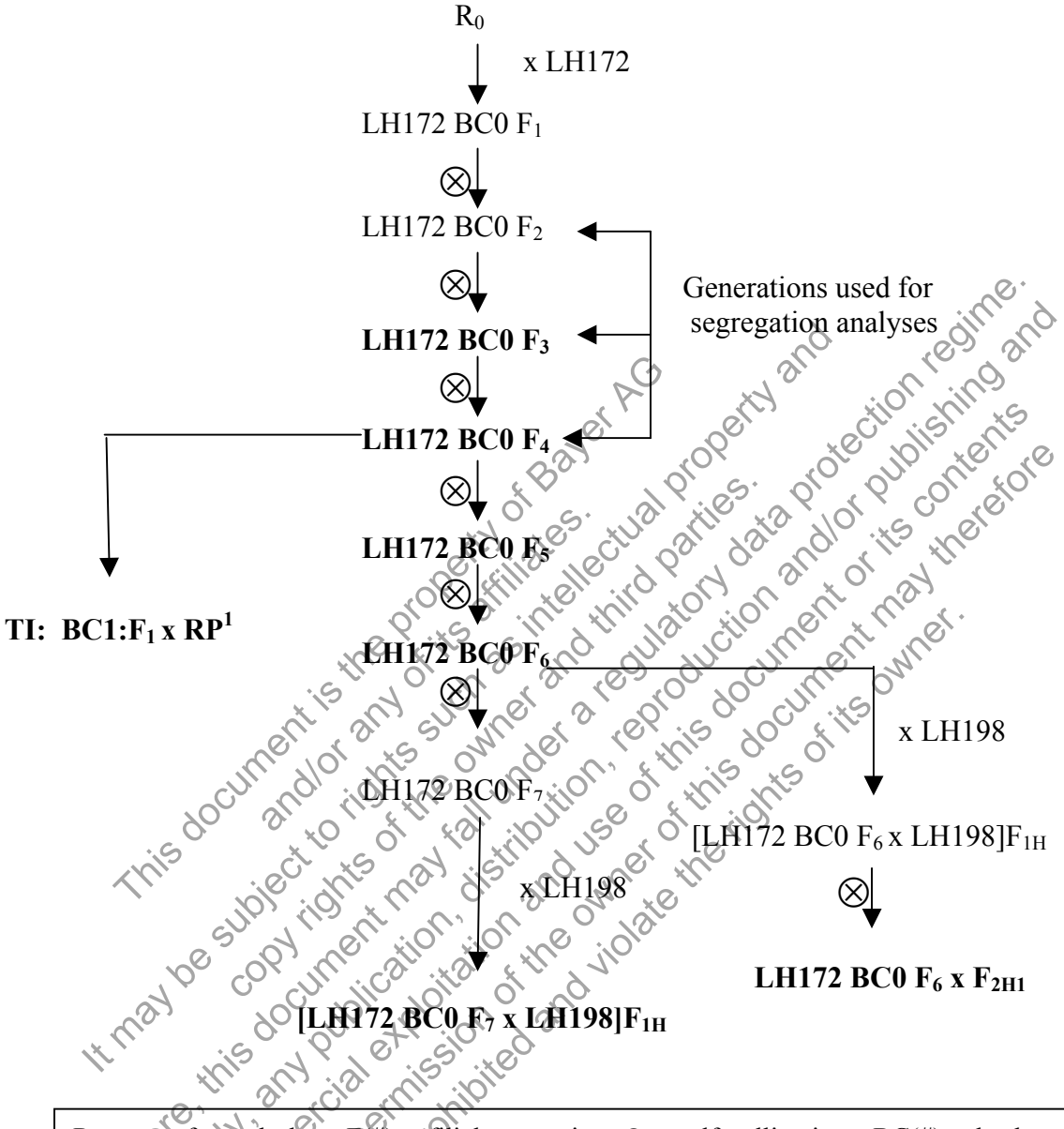
→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

3.4. Insert stability across generations of MON 89034

3.4.1. Summary

To demonstrate the stability of the MON 89034 insert during breeding, additional Southern blot analyses were performed using DNA samples obtained from multiple generations of the MON 89034 breeding history. For reference, the breeding history of MON 89034 is presented in Figure IV.21. The specific generations tested are indicated in the legends of Figures IV.22-24. For these analyses, DNA samples were digested with the restriction enzyme *Ssp* I, which cleaves once within the inserted DNA and in both the 5' and 3' genomic flanking sequences of MON 89034. This produces two DNA fragments of ~8.2 and >4.3 kb (Figure IV.8). The ~8.2 kb fragment contains genomic DNA flanking the 5' end of the insert, Left Border sequence, modified *e35S* promoter sequence, *Cab* leader, *Ract1* intron, *cry1A.105* coding sequence, *Hsp17* 3' end sequence, *FMV* promoter, and a portion of the *Hsp70* intron. The >4.3 kb fragment contains the remaining portion of the *Hsp70* intron, *SSU-CTP* targeting sequence, *cry2Ab2* coding sequence, *nos* 3' end sequence, Left Border sequence, and genomic DNA flanking the 3' end of the inserted DNA (Figure IV.8).

Plasmid PV-ZMIR245 DNA was spiked into the conventional corn control DNA, digested with *Ssp* I, and loaded on the gel to serve as a positive hybridization control. Individual Southern blots were examined with three-probe sets. The stability of the MON 89034 insert across generations was confirmed using overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18 – 23 in Figure IV.7). The absence of the *nptII* selectable marker and unique T-DNA II genetic elements not contained in T-DNA I was confirmed using overlapping probes spanning T-DNA II (probes 11, 12, and 13, Figure IV.6). The absence of plasmid PV-ZMIR245 backbone sequence across generations was confirmed using overlapping probes spanning the vector backbone of PV-ZMIR245 (probes 14 - 17, Figure IV.6). A second conventional corn control (referred to as conventional corn A) was used in these Southern blots to ensure that the genetic backgrounds of all the generations were accurately represented.



R_0 = transformed plant; F(#) = filial generation; ⊗ = self-pollination; BC(#) = backcross generation; RP = recurrent parent; H = hybrid; TI - Trait Integration

Figure IV.21. Breeding History of MON 89034
The LH172 BC0 F_6 x F_{2H1} generation was used for all molecular analyses. Generations used for stability analysis are shown in bold in the breeding tree.

[1] Recurrent parent (RP) that was used in the analyses depicted in Figures IV.22 and IV.23 is referred to as Conventional corn A.

3.4.2. Stability of the T-DNA I insert across generations of MON 89034

The results of this analysis are presented in Figure IV.22. Conventional corn control DNA digested with *Ssp* I (lane 7 and 9) and examined with overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18 - 23, Figure IV.7) showed several faint hybridization bands. This was expected because T-DNA I contain several genetic elements originally derived from corn. These endogenous bands were only detected in conventional corn or upon longer exposure and were seen previously when blots were probed with the overlapping T-DNA I probe (see Figure IV.9), the *Hsp 70* intron probe (Figure IV.15), and the *SSU-CTP*/*cry2Ab2* probe (Figure IV.16).

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at ~10.4 and ~7.2 kb in addition to the expected endogenous bands. The migration of the ~10.4 kb fragment is slightly higher than indicated by the molecular marker band sizes in the lanes 10 and 11. The altered migration may be due to the difference in salt concentration between the corn DNA samples and the molecular marker (Sambrook and Russell, 2001).

DNA extracted from seven MON 89034 generations digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) each produced two bands of ~8.2 and ~7.4 kb in addition to the expected endogenous hybridization. The ~8.2 kb band is the expected size for the 5' border fragment and the ~7.4 kb band is consistent with the expected band size of >4.3 kb for the 3' border fragment. These bands are consistent with the bands detected in Figure IV.9 (lanes 3 and 7).

Two faint bands of ~15.6 and ~12 kb were observed in lanes 3 and 4 (Figure IV.22). These bands likely are the result of partial digestion because they are not seen in prior or subsequent generations that were produced by self pollination. In support of this conclusion the presence of the ~15.6 kb band is consistent with the lack of digestion at the internal *Ssp* I site. This band would be expected as a combination of the 7.4 and 8.2 kb bands observed for *Ssp* I digestion. The ~12 kb band is consistent with the partial digestion of the *Ssp* I site in the 5' flanking genomic sequence which would result in an ~11.8 kb band.

No additional unexpected bands were detected, indicating that the single copy of T-DNA I in MON 89034 is stable in the selected generations.

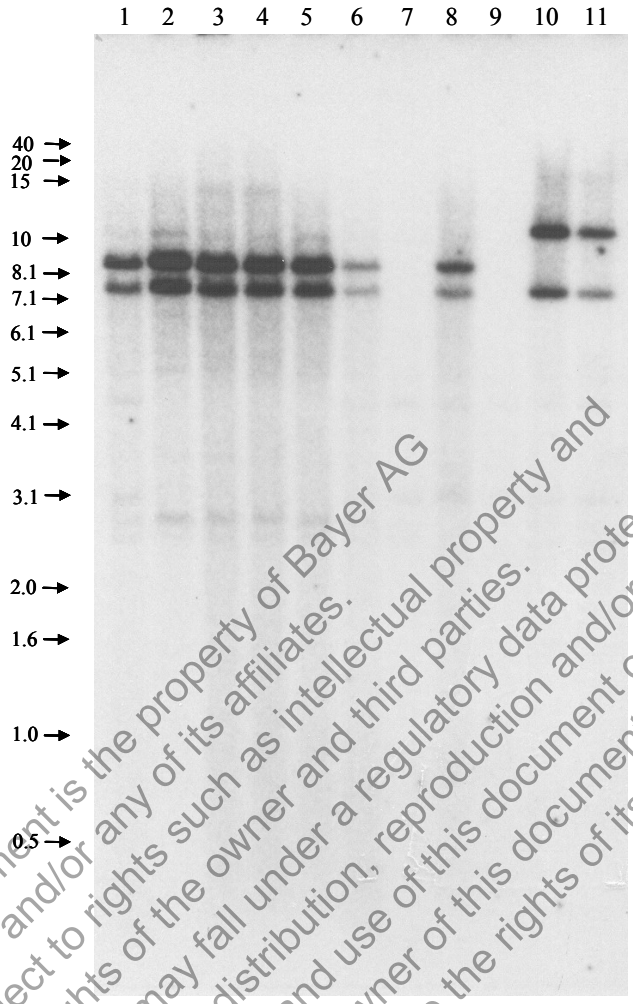


Figure IV.22. Insert Stability of MON 89034: T-DNA I

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 - 23, Figure IV.7). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0F$_6$ x F$_{2H1,}$ *Ssp* I)
2: MON 89034 (LH172 BC0 F$_3$, *Ssp* I)
3: MON 89034 (LH172 BC0 F$_4$, *Ssp* I)
4: MON 89034 (LH172 BC0 F$_5$, *Ssp* I)
5: MON 89034 (LH172 BC0 F$_6$, *Ssp* I)
6: MON 89034 ([LH172 BC0 F$_7$ x LH198]F$_{1H}$, *Ssp* I)
7: Conventional corn (LH172, *Ssp* I)
8: MON 89034 (TI: BC1: F$_1$ x RP, *Ssp* I)
9: Conventional corn A[1] (*Ssp* I)
10: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
11: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

[1] Monsanto proprietary conventional corn hybrid

3.4.3. Confirmation of the absence of T-DNA II in multiple generations of MON 89034

The results of this analysis are presented in Figure IV.23. Conventional corn control DNA digested with *Ssp* I (lane 7 and 9) and examined with three overlapping probes spanning T-DNA II (probes 11-13, Figure IV.6) showed no detectable hybridization bands. Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at ~10 and ~7.2 kb. The migration of the ~10 kb fragment is slightly higher than indicated by the molecular marker band sizes in the lanes 10 and 11. The altered migration may be due to the difference in salt concentration between the corn DNA samples and the molecular marker (Sambrook and Russell, 2001). The overlapping probes spanning T-DNA II contains the *35S* promoter, *nos* 3' end, and the Left Border which are contained on T-DNA I. Therefore, the T-DNA II probe is expected to hybridize to fragments derived from T-DNA I.

DNA extracted from seven generations of MON 89034 digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) each produced two bands of ~8.2 and ~7.4 kb which are consistent with those observed with the overlapping T DNA I probes. The ~ 8.2 kb band is the expected size for the 5' border fragment and the ~7.4 kb band is consistent with the expected band size of > 4.3 kb for the 3' border fragment. On longer exposures, two faint bands of ~15.6 and ~12 kb were observed in lanes 3 and 4 (Figure IV.23). These bands are consistent with the bands observed in lanes 3 and 4 of Figure IV.22 and are likely the result of partial digestion because they are not seen in prior generations produced by self-pollination. The presence of the 15.6 kb band is consistent with the possibility that the internal *Ssp* I site was not digested and a combination of the 7.4 and 8.2 kb bands produced the 15.6 kb band. The 12.0 kb band is consistent with the partial digestion of the *Ssp* I site in the 5' flanking genomic sequence which would result in an approximately 11.8 kb band.

No additional bands were detected, indicating that the seven generation tested do not contain any additional T-DNA II elements other than those associated with T-DNA I and that therefore those T-DNA II elements segregated as expected.

3.4.4. Confirmation of the absence of plasmid PV-ZMIR245 backbone sequence in multiple generations of MON 89034

The results of this analysis are presented in Figure IV.24. Conventional corn control DNA obtained from LH172, digested with *Ssp* I (lanes 7 and 9), and examined with four overlapping probes spanning the PV-ZMIR245 backbone sequence (probes 14-17, Figure IV.6) showed no detectable hybridization bands. Although difficult to observe in Figure IV.24, overexposures of Southern blots showed that conventional corn A control DNA, digested with *Ssp* I (lanes 7 and 9), produced two faint hybridization bands at 6.0 and 3.5 kb. These are likely the result of endogenous hybridization to corn genetic elements specific to this background. Conventional control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at ~10.4 and ~7.2 kb. DNA samples extracted from the seven generation digested

with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) produced no hybridization bands. This confirmed that the backbone sequences from PV-ZMIR245 are not present in MON 89034.

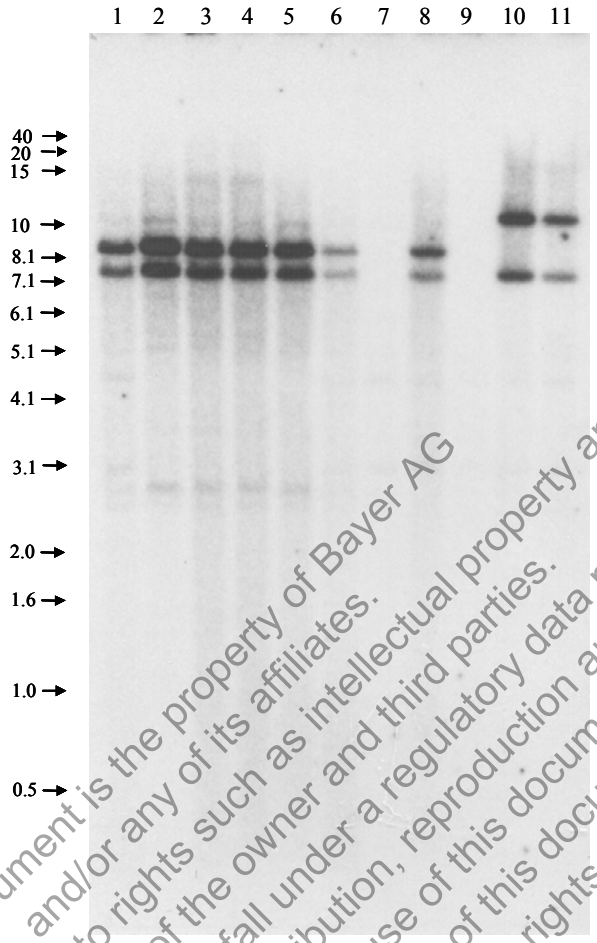


Figure IV.23. Analysis of Multiple Generations of MON 89034: T-DNA II
The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0F_6 x $F_{2H1,}$ *Ssp* I)
2: MON 89034 (LH172 BC0 F_3, *Ssp* I)
3: MON 89034 (LH172 BC0 F_4, *Ssp* I)
4: MON 89034 (LH172 BC0 F_5, *Ssp* I)
5: MON 89034 (LH172 BC0 F_6, *Ssp* I)
6: MON 89034 ([LH172 BC0 F_7 x LH198]F_{1H}, *Ssp* I)
7: Conventional corn (LH172, *Ssp* I)
8: MON 89034 (TI: BC1: F_1 x RP, *Ssp* I)
9: Conventional corn A[1] (*Ssp* I*)*
10: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
11: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on ethidium bromide stained gel.

[1] Monsanto proprietary conventional corn hybrid

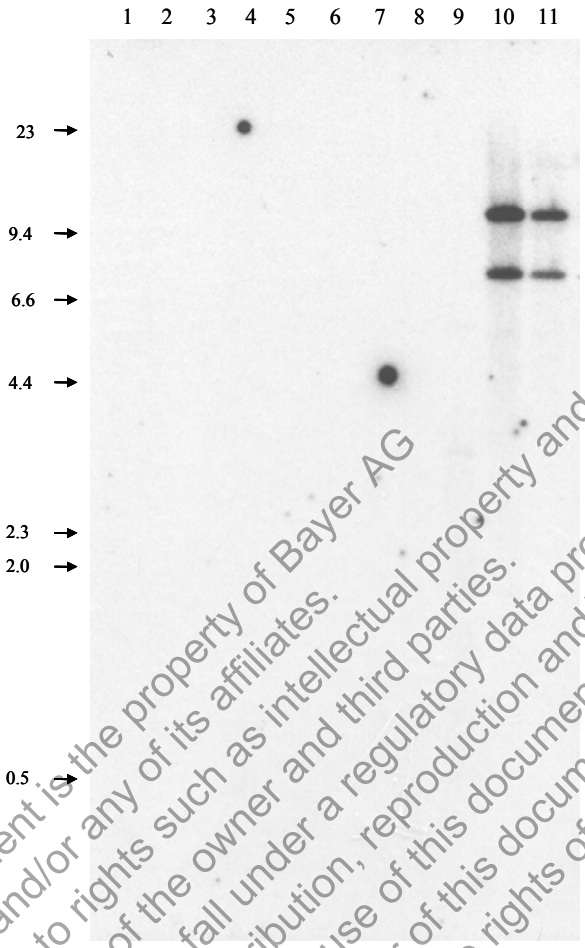


Figure IV.24. Backbone Analysis of Multiple Generations of MON 89034: PV-ZMIR245 Backbone Sequence

The blot was examined with overlapping ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17, Figure IV.6). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0F_6 x $F_{2H1,}$ *Ssp* I)
2: MON 89034 (LH172 BC0 F_3, *Ssp* I)
3: MON 89034 (LH172 BC0 F_4, *Ssp* I)
4: MON 89034 (LH172 BC0 F_5, *Ssp* I)
5: MON 89034 (LH172 BC0 F_6, *Ssp* I)
6: MON 89034 ([LH172 BC0 F_7 x LH198]F_{1H}, *Ssp* I)
7: Conventional corn (LH172, *Ssp* I)
8: MON 89034 (TI: BC1: F_1 x RP, *Ssp* I)
9: Conventional corn A[1] (*Ssp* I)
10: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
11: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from molecular markers on the ethidium bromide stained gel.

[1] Monsanto proprietary conventional corn hybrid

3.5. Organization and sequence of the insert DNA in MON 89034

The reported organization of the elements within the insert in MON 89034 was confirmed by DNA sequencing analyses. PCR primers were designed to amplify seven overlapping regions of DNA that span the entire length of the insert (9.3 kb). The amplified DNA fragments were subjected to DNA sequencing analyses to further confirm the organization of the elements within the insert. The results confirmed that the sequence of the DNA insert in MON 89034 matched the designed, corresponding sequences in PV-ZMIR245 with one exception. The *e35S* promoter that regulates expression of the *cry1A.105* gene has been modified and that the Right Border sequence present in PV-ZMIR245 was replaced by a Left Border sequence in MON 89034. This molecular rearrangement can be explained by a recombination event, which occurred either prior to or during the process of T-DNA transfer to the plant cell, between the DNA sequences near the *35S* promoters in T-DNA I and T-DNA II (Figure IV.5). Due to this recombination event, the reconstituted *e35S* promoter in MON 89034 (referred to as modified *e35S* or *e35S*[89]) no longer has the duplicated enhancer elements compared to the original *e35S* promoter in PV-ZMIR245. Despite the deletion of the enhancer elements, the modified *e35S* promoter in MON 89034 regulates Cry1A.105 protein expression to provide sufficiently high levels for efficacious control of target insect pests.

3.6. Inheritance of the lepidopteran protection trait in MON 89034

Significance of the segregation pattern (positives/negatives) was assessed by Chi square analysis over four generations of MON 89034 to determine the heritability and stability of the trait (*cry1A.105* and *cry2Ab2* genes, and Cry1A.105 and Cry2Ab2 proteins) in (Table IV.4). The confirmation of the presence of the gene and stability of the trait was based on one of several assays: 1) ELISA to detect the Cry2Ab2 protein; 2) ELISA to detect the Cry1A.105 protein; 3) PCR assay to detect presence of the *cry* genes; and 4) GeneCheck® immunoassays to detect Cry2Ab2 protein (Cry2A QuickStix Lateral Flow bulk strips, Envirologix Inc).

As described in Part IV, Section 2.1, MON 89034 was developed using a 2 T-DNA vector transformation system that employed two separate T-DNA regions. One T-DNA (T-DNA I) contained the *cry2Ab2* and *cry1A.105* genes while the other T-DNA (T-DNA II) contained the *npt*II expression cassette. F_1 plants were generated in a LH172 germplasm by making crosses of the R_0 plant with the LH172[1] inbred. From the population of produced F_1 plants, selections were made for the absence of *nptII* and plants were screened for copy number of the *cry1A.105* and *cry2Ab2* inserted cassettes using Southern blot analysis. Plants selected in the F_1 generation were either used to make BC_1F_1 seed, or were self-pollinated to generate F_2 seed (Figure IV.21). The overall goal in the F_1 population of plants was to select single copy, marker–free plants. A final plant was selected from the F_1 generation, designated as event MON 89034, and progeny derived from this plant showed the expected patterns for genetic segregation (Table IV.4).

® GeneCheckTM is an Envirologix Inc Trademark.

[1] LH172 is a commercial corn line produced by Holdens Company, a Monsanto affiliate.

The Chi-square analysis is based on testing the observed segregation ratio of the Cry proteins to the ratio that is expected according to Mendelian principles as shown below.

Generation	Expected Ratio[a]	Comment
LH172 $BC0F_1$	n.a.	screened for copy number and absence of *nptII* (segregation data not shown)
LH172 $BC0F_2$	3:1	positive:negative (product of self pollination)
LH172 $BC0F_3$	1:0	positive:negative (homozygous plant selection)
LH172 $BC0F_4$	1:0	positive:negative (homozygous plant selection)
LH172 $BC1F_1$[b]	1:1	positive:negative (product of backcrossing)
LH172 $BC1F_2$[c]	3:1	positive:negative (product of self pollination)
LH172 $BC1F_2$[c]	3:1	positive:negative (product of self pollination)

[a]n.a. = not applicable.
[b]To confirm segregation, LH172 $BC0F_1$ plants were backcrossed to the recurrent parent (LH172) to produce this generation (not shown on the breeding tree, Figure IV.21).
[c]To confirm segregation, The LH172 $BC1F_1$ plants were selfed to produce two different plant populations for this generation (not shown on the breeding tree, Figure IV.21).

The Chi-square test was computed as:

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

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 (Little and Hills, 1978).

Results of the Chi-square test are summarized in Table IV.4. All χ^2 values indicated no significant differences between observed and expected genetic ratios across all tested generations of MON 89034. These results are consistent with molecular characterization data indicating a single insertion site for the *cry1A.105* and *cry2Ab2* expression cassettes.

Table IV.4. Segregation Analyses of MON 89034[a]

Generation	Number of plants	Observed Positives[1]	Observed Negatives	Expected Positives	Expected Negatives	Chi-Square	Probability
LH172 $BC0F_2$	11	7	4	8.25	2.75	0.2727	**>0.05**
LH172 $BC0F_3$	24	24	0	24	0	**Fixed +**	n.a.
LH172 $BC0F_4$	30	30	0	30	0	**Fixed +**	n.a.
LH172 $BC1F_1$	28	13	15	14	14	0.0357	**>0.05**
LH172 $BC1F_2$	24	20	4	18	6	0.5000	**>0.05**
LH172 $BC1F_2$	24	17	7	18	6	0.0556	**>0.05**

[a]The confirmation of the trait was based on one of several assays: 1) ELISA to detect the Cry2Ab2 protein; 2) ELISA to detect the Cry1A.105 protein; 3) PCR assay to detect presence of the *cry* genes; and, 4) GeneCheck immunoassays to detect the Cry2Ab2 protein.

3.7. Conclusion

Molecular analyses confirmed that MON 89034 contains one insert derived from T-DNA I at a single locus of the nuclear genome. The insert contains one functional copy of the *cry1A.105* and of the *cry2Ab2* expression cassettes. All genetic elements from T-DNA I are intact and present in the expected order. These analyses also confirmed the absence of plasmid backbone sequence, *nptII* and T-DNA II derived elements, other those in common with the T-DNA I (i.e., *35S* promoter, *nos* 3´ end sequence, and the Left Border sequence). The Southern blot fingerprint of MON 89034 was maintained through seven generations of conventional breeding, thereby confirming the stability of the insert over multiple generations. These generations did not contain any detectable T-DNA II elements other than those in common with T-DNA I, nor did they contain any detectable backbone sequence from plasmid PV-ZMIR245. PCR and DNA sequence analyses confirmed the organization of the elements within the insert, the 5' and 3' insert-to-plant junctions, and the complete DNA sequence of the insert.

SECTION 4. Other data and information about the development of MON 89034

All relevant information regarding development of MON 89034 is described in Parts II – VII of this summary.

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PART V: PRESENCE OF GENES THAT ENCODE RESISTANCE TO ANTIBIOTICS

No genes that encode resistance to an antibiotic were inserted into the genome of MON 89034. Molecular characterization data presented in Part IV, Section 3 confirms the absence of the *add* and *nptII* genes encoding antibiotic resistance markers that were used in cloning and transformation processes.

PART VI: CHARACTERIZATION OF THE PROTEINS INTRODUCED INTO MON 89034

SECTION 1. Identity and Characterization of the Cry1A.105 and Cry2Ab2 Proteins Produced in MON 89034

Safety assessment of biotechnology-derived crops includes characterization of introduced proteins, confirmation of their functions and physicochemical properties, and confirmation of the safety of each protein produced from the inserted DNA. Expression of introduced proteins *in planta* is usually too low to allow purification of sufficient quantities for use in safety assessment studies. Therefore, it is necessary to produce the proteins in high-expressing recombinant host systems (such as bacteria) in order to obtain larger quantities of the protein(s) of interest. Proteins produced by the bacterial systems are engineered to have exactly the same amino acid sequences as their counterparts expressed in the biotechnology-derived plants. Thus, physicochemical and functional equivalence of plant- and bacteria-produced proteins need to be examined as part of the protein characterization and safety assessment.

The lepidopteran-protected corn MON 89034 produces the Cry1A.105 and Cry2Ab2 proteins. The bacteria-derived Cry1A.105 and Cry2Ab2 proteins were produced in *Escherichia coli* and subsequently purified. Small quantities of the Cry1A.105 and Cry2Ab2 proteins were purified from the grain of MON 89034. The identities of the MON 89034-derived proteins were confirmed and the physicochemical properties and functional activities were compared to those of the bacteria-produced protein standards. The characteristics that were analyzed to establish the equivalence between the recombinant *E. coli*- and the MON 89034-produced Cry1A.105 and Cry2Ab2 proteins included:

1. SDS-PAGE analysis to estimate molecular weight;
2. Western blot analysis to demonstrate identity and immunoreactivity;
3. Confirmation of intactness of N-terminus of the protein with an antibody specific to the N-terminal peptide;
4. N-terminal sequencing by Edman degradation chemistry;
5. MALDI-TOF MS to establish protein identity by peptide mapping;
6. Glycosylation analysis to examine the presence or absence of covalently linked carbohydrates; and
7. Insect activity bioassay to assess functional equivalence.

Results obtained for the characterization of the Cry1A.105 and Cry2Ab2 proteins are discussed in the sections below.

1.1. Identity and function of the Cry1A.105 protein

Cry1A.105 is a full-length, insecticidal protein consisting of 1,177 amino acids with a molecular weight (MW) of 133 kDa. Cry1A.105 is a chimeric protein that consists of domains I and II from Cry1Ab or Cry1Ac[1], domain III from Cry1F, and the C-terminal portion from Cry1Ac (Figure VI.1). Cry1Ac, Cry1Ab and Cry1F are all well known and well characterized insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt), that have been used both in microbial insect control products and biotechnology-derived crops. Cry1A.105 was designed using domain exchange strategy to achieve high levels of activity against the target lepidopteran insect pests. Data in Table VI.1 shows the extremely high homology of the various domains of the Cry1A.105 protein to the respective domains in the Cry1Ab, Cry1Ac and Cry1F proteins. Domains I and II of Cry1A.105 are 100% identical in amino acid sequence to Domains I and II of both Cry1Ab and Cry1Ac. Domain III of Cry1A.105 is 99% identical in amino acid sequence to Domain III of Cry1F. The C-terminal portion has 100% homology to the C-terminal portion of the Cry1Ac protein The overall amino acid sequence identity of Cry1A.105 to Cry1Ac, Cry1Ab, and Cry1F is 93.6%, 90.0%, and 76.7 %, respectively (Table VI.1). According to an accepted phylogram (Crickmore et al., 1998) for Bt crystal proteins, Cry1A.105 can be clustered with Cry1Ac and Cry1Ab due the high degree of homology (Crickmore, 2004; see Part VI, Section 5.4.2 and Figure VI.1).

Domain exchange is a well-known naturally occurring mechanism that increases Cry protein diversities (De Maagd et al., 2001; Masson 2002; De Maagd et al., 2003). Domain exchange strategies with modern molecular biological tools have been used to switch the functional domains of Cry1 proteins to develop commercial microbial biopesticides with improved specificity to lepidopteran insect pests. Microbial pesticides that contain a Cry1Ac/Cry1F chimeric protein have been used for control of lepidopteran pests since 1997 (Baum, 1998; Baum et al., 1999), and a biotechnology-derived cotton product expressing a chimeric protein consisting of domains or sequences from Cry1F, Cry1C, and Cry1Ab has been commercialized (Gao et al., 2006).

The general mechanism of insecticidal activity of Cry proteins is well understood (Gill et al., 1992; Schnepf et al., 1998; Zhuang and Gill, 2003). Cry proteins are comprised of several functional domains that have highly conserved regions among the classes. For example, the amino acid sequence of Cry1A proteins is highly conserved in domains I, II and III. These functional domains have been shown to determine the activity and specificity of the Cry proteins. Domain I is involved in membrane insertion and pore formation. Domain II is involved in specific receptor recognition and binding. Domain III maintains the structural integrity of the protein molecule (Li et al., 1991) and also contributes to specificity (De Maagd et al., 2001). The C-terminal domain is implicated in crystal formation, which does not directly contribute to the insecticidal activity (De Maagd et al., 2001). The C-terminal domain is cleaved upon entry into the insect midgut or by certain proteases *in vitro*. Only insects with specific receptors are affected and no toxicity is observed in species that lack these receptors (Crickmore et al., 1998; De Maagd et al., 2001). As with other Cry1A proteins, Cry1A.105 is active against major

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[1] Cry1Ab and Cry1Ac share 100% amino acid sequence identity in domains I and II.

lepidopteran insect pests. The spectrum of activity includes corn borers from the genera *Ostrinia* and *Diatraea* (such as European corn borer, Asian corn borer, sugarcane borer, southwestern corn borer), armyworms (*Spodoptera* spp. including fall armyworm), earworms (*Helicoverpa* spp. including corn earworm), and cutworms (e.g., *Agrotis ipsilon*, black cutworm).

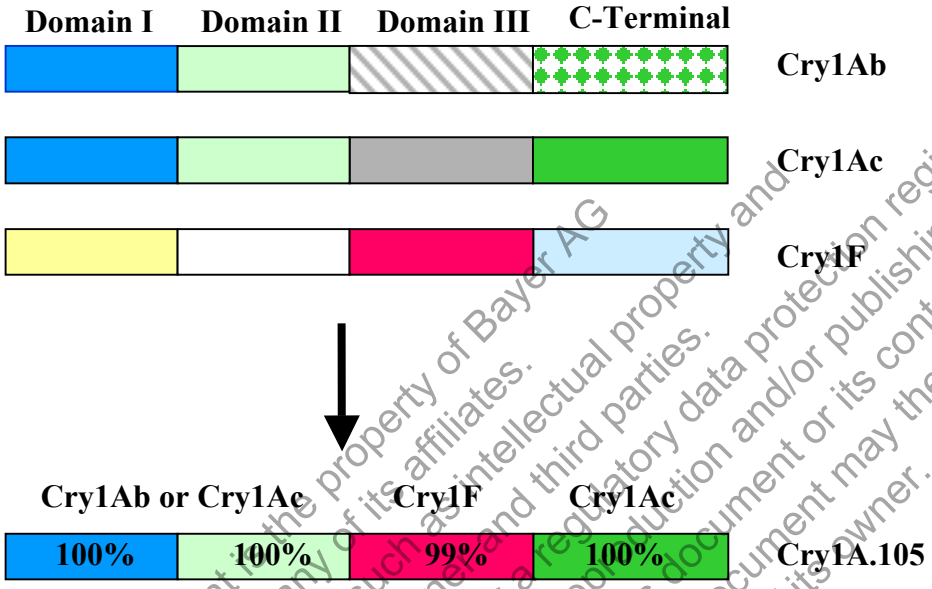


Figure VI.1. Schematic Representation of the Origin of Cry1A.105 Protein Domains

Different color and patterns are used to differentiate the origin of domains. For simplicity, the lengths of domains in this illustration are not in proportion to the lengths of amino acid sequence of the respective domains.

Table VI.1. Amino Acid Sequence Identity Between the Cry1A.105 and Cry1Ac, Cry1Ab, and Cry1F Proteins

Domain	Amino acid identity to Cry1A.105 (%)		
	Cry1Ac	Cry1Ab	Cry1F
I	100	100	57
II	100	100	37
III	57	46	99
C-terminal	100	92	93
Overall	93.6	90	76.7

1.2. Characterization of the Cry1A.105 protein

The Cry1A.105 protein was purified from the grain of MON 89034 as well as from a fermentation of a recombinant *E. coli* strain. A panel of analytical tests was used to identify, characterize and compare the MON 89034-produced and the *E. coli*-produced Cry1A.105 proteins including: (1) SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) to examine molecular weight equivalence, (2) western blot analysis to demonstrate immunoreactivity, (3) confirmation of N-terminus intactness with an antibody specific to the N-terminal peptide, (4) MALDI-TOF MS analysis to establish protein identity by tryptic peptide mapping, (5) glycosylation analysis to examine the presence or absence of covalently linked carbohydrates, and (6) activity in an insect bioassay to assess functional equivalence.

1.2.1. Cry1A.105 protein molecular weight equivalence

The equivalence in molecular weight of the purified MON 89034- and *E. coli*-produced Cry1A.105 proteins was demonstrated using SDS-PAGE. The full-length MON 89034-produced Cry1A.105 protein migrated to a similar position compared to that of the *E. coli*-produced protein standard which was analyzed concurrently (Figure VI.2.).

Based on the comparable electrophoretic mobility, the MON 89034- and *E. coli*-produced Cry1A.105 proteins were determined to have equivalent molecular weights.

1.2.2. Cry1A.105 protein immunoreactivity

Western blot analysis using a polyclonal anti-Cry1A.105 antibody was conducted to determine the relative immunoreactivity of the purified MON 89034-produced Cry1A.105 protein and the *E. coli*-produced Cry1A.105 reference standard. The results demonstrated that the anti-Cry1A.105 antibody recognized the full-length MON 89034-produced Cry1A.105 protein that migrated similarly to the full-length *E. coli*-produced reference Cry1A.105 protein (Figure VI.3). As expected, the immunoreactive signal increased with increasing levels of loading for both MON 89034- and *E. coli*-produced proteins. The immunoreactive bands with lower molecular weight were present in both protein samples. These bands most likely represent proteolytic fragments of the Cry1A.105 protein. It is common to observe such proteolytic fragments of Cry1 proteins due to the cleavage by proteases in vivo or in vitro. A faint immunoreactive band with molecular weight above 250 kDa was observed in the samples of the MON 89034-produced and *E. coli*-produced Cry1A.105 protein. This band most likely represents the aggregate of the Cry1A.105 protein.

The western blot analysis confirmed the identity of the MON 89034-produced Cry1A.105 protein and demonstrated that the MON 89034- and *E. coli*-produced Cry1A.105 protein have equivalent immunoreactivity with the anti-Cry1A.105 antibody.

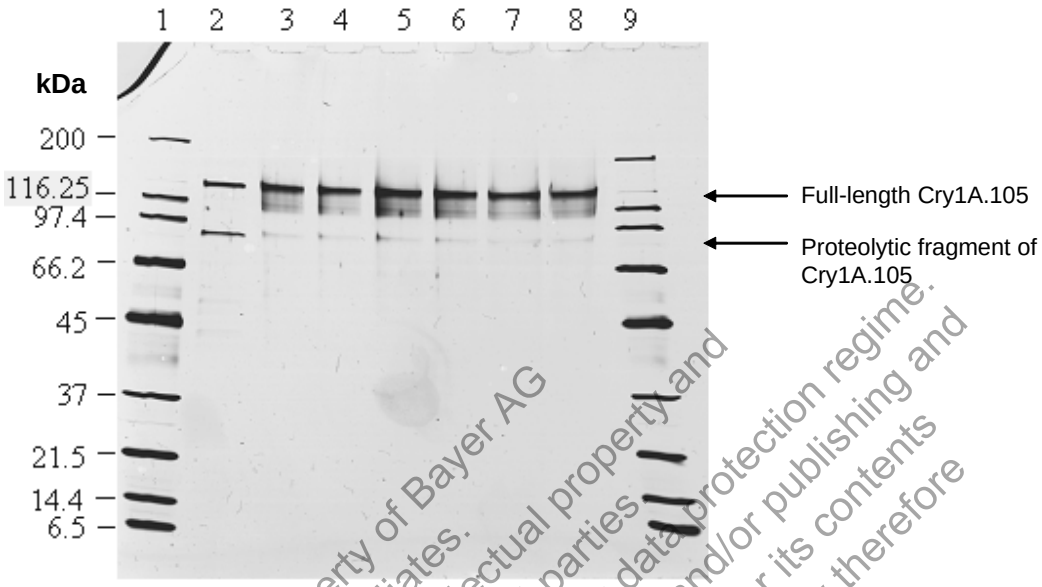


Figure VI.2. SDS-PAGE of the MON 89034- and *E. coli*-produced Cry1A.105 Proteins

Aliquots of the MON 89034-produced Cry1A.105 protein and the *E. coli*-produced Cry1A.105 reference standard were separated by a Tris-glycine 4→20% polyacrylamide gradient gel and stained with an Invitrogen SilverXpress silver staining kit. Approximate molecular weights (kDa) are shown on the left side of the gel and correspond to the markers loaded in lanes 1 and 9.

Lane	Sample	Amount (ng)
1	Broad Range molecular weight markers (Bio-Rad)	—
2	*E. coli*-produced Cry1A.105 reference standard	96
3	MON 89034-produced Cry1A.105 protein	48
4	MON 89034-produced Cry1A.105 protein	48
5	MON 89034-produced Cry1A.105 protein	72
6	MON 89034-produced Cry1A.105 protein	72
7	MON 89034-produced Cry1A.105 protein	96
8	MON 89034-produced Cry1A.105 protein	96
9	Broad Range molecular weight markers (Bio-Rad)	—

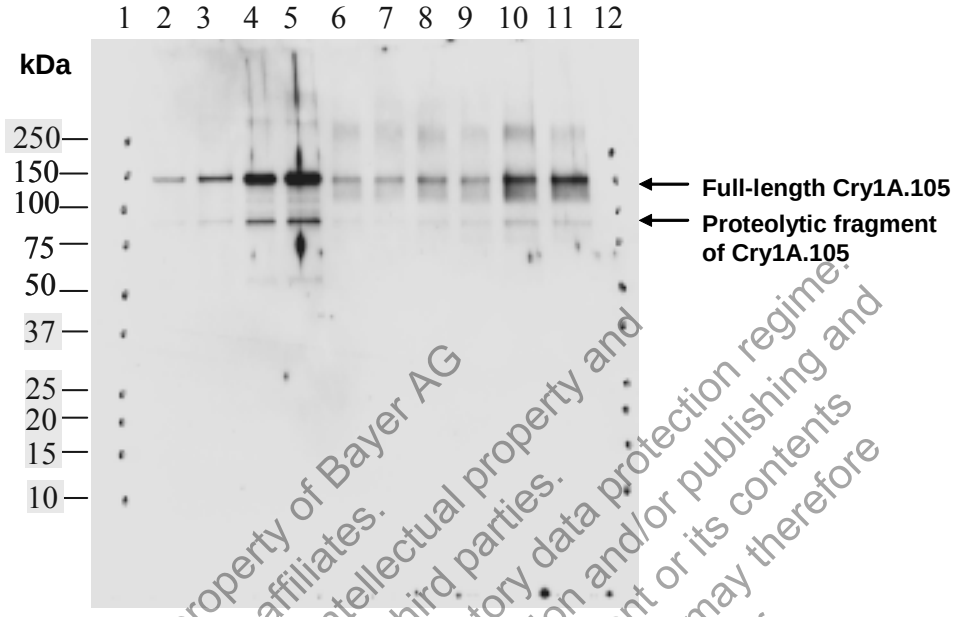


Figure VI.3. Western Blot Analysis of MON 89034- and *E. coli*-produced Cry1A.105 Proteins

Aliquots of the purified, MON 89034-produced and *E. coli*-produced Cry1A.105 protein were separated by SDS-PAGE, and electrotransferred to a PVDF membrane. The membrane was probed with rabbit polyclonal anti-Cry1A.105 antibody and developed using an ECL system (Amersham Pharmacia). Approximate molecular weights (kDa) are shown on the left side of the blot, which correspond to the markers loaded in lanes 1 and 12.

Lane	Sample	Amount Loaded (ng)	Amount Loaded (µl)
1	Precision Plus Dual Color molecular weight markers	—	—
2	*E. coli*-produced Cry1A.105 standard	1	—
3	*E. coli*-produced Cry1A.105 standard	2	—
4	*E. coli*-produced Cry1A.105 standard	4	—
5	*E. coli*-produced Cry1A.105 standard	6	—
6	MON 89034-produced Cry1A.105 protein	—	7.5
7	MON 89034-produced Cry1A.105 protein	—	7.5
8	MON 89034-produced Cry1A.105 protein	—	10
9	MON 89034-produced Cry1A.105 protein	—	10
10	MON 89034-produced Cry1A.105 protein	—	20
11	MON 89034-produced Cry1A.105 protein	—	20
12	Precision Plus Dual Color molecular weight markers	—	—

1.2.3. N-terminus intactness of the Cry1A.105 protein

The N-terminus of the plant-produced proteins could be blocked by chemical modifications, which would not allow N-terminal analysis by the typical Edman degradation method (Brown and Roberts, 1976; Arfin and Bradshaw, 1988). Therefore, the intactness of the N-terminus of the MON 89034-produced Cry1A.105 was examined using western blot analysis with an N-terminal peptide-specific antibody. The anti-N-terminal peptide antibody was raised against a synthetic peptide consisting of the first 14 amino acids (MDNNPNINECIPYN) at the N-terminus of the Cry1A.105 protein (see Figure IV.3).

The *E. coli*-produced Cry1A.105 containing the intact N-terminal sequence, and the Cry1A.105 trypsin-resistant core lacking the N-terminus of the full-length protein were used as positive and negative reference standards, respectively. As expected, no immunoreactive bands were observed in the lanes loaded with Cry1A.105 trypsin-resistant core (Figure VI.4, lanes 6 and 7). The band corresponding to the full-length Cry1A.105 protein (approximately 130 kDa) was observed in the lanes loaded with both the MON 89034- and *E. coli*-produced Cry1A.105 proteins. As expected, the intensity of the bands increased in a manner dependent of the loading quantities. Additionally, a fragment of lower molecular weight (approximately 85 kDa) was observed in both protein preparations (Figure VI.4, lanes 2-5). This fragment likely represents a proteolytic product of C-terminal degradation of Cry1A.105 protein. These results are consistent with published literature that the intermediate proteolytic fragments of Cry1 proteins have intact N-termini, while the trypsin-resistant core is formed by the cleavage of the N-terminal peptide (approximately 25-30 amino acids; Gao et al., 2006).

In conclusion, the intactness of the N-terminus of the MON 89034- and the *E.coli*-produced Cry1A.105 proteins was confirmed.

1.2.4. Cry1A.105 tryptic peptide mapping by MALDI-TOF MS

The MON 89034-produced, full-length Cry1A.105 protein was further identified by tryptic peptide mapping analysis using MALDI-TOF MS. The protein sample was heat-denatured, chemically reduced, alkylated and digested with trypsin, and the masses of the tryptic peptides were measured.

There were 52 protein peptide masses identified that matched the expected tryptic peptides generated *in silico* based on the predicted cleavage sites in the sequence. The identified masses were used to assemble a coverage map that displays those matched peptide sequences for the protein (Figure VI.5). Overall, the confirmed sequence accounts for 43.8% (516 out of 1,177 amino acids) of the full-length sequence of Cry1A.105 protein. In general, a protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). In the current case, the detected peptide coverage was 43.8% with 52 matched peptides, therefore, the protein identity of the MON 89034-produced Cry1A.105 was confirmed.

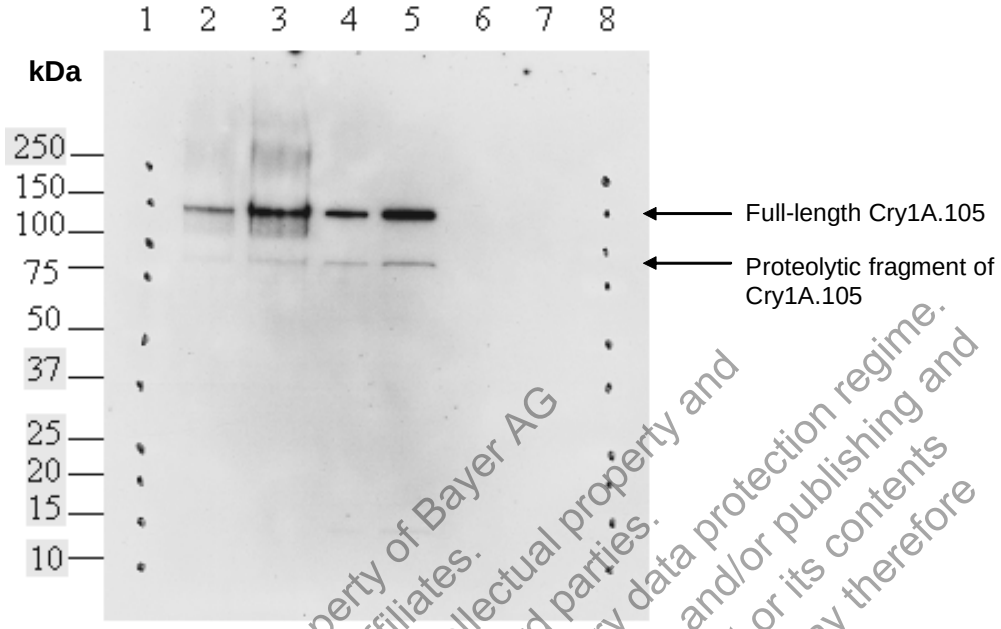


Figure VI.4. N-terminus Intactness by Immunoblot Analysis Using Anti-N-terminal Peptide Antibody

Aliquots of the MON 89034–produced Cry1A.105 protein, *E. coli*-produced Cry1A.105 reference standard, and Cry1A.105 trypsin-resistant core standard were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was probed with the anti-N-terminal peptide antibody, and immunoreactive bands were visualized using an ECL system (5 min exposure). Approximate molecular weights (kDa) are shown on the left side of the blot and correspond to the markers loaded in lanes 1 and 8.

Lane	Sample	Amount (ng)
1	Precision Plus Dual Color molecular weight markers	—
2	MON 89034-produced Cry1A.105 protein	20
3	MON 89034-produced Cry1A.105 protein	40
4	*E. coli*-produced Cry1A.105 reference standard	20
5	*E. coli*-produced Cry1A.105 reference standard	40
6	Cry1A.105 trypsin-resistant core standard	20
7	Cry1A.105 trypsin-resistant core standard	40
8	Precision Plus Dual Color molecular weight markers	—

```
0001  MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
0051  VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
0101  EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
0151  QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
0201  GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
0251  DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
0301  NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
0351  VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
0401  YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
0451  PMFSWIHRSA EFNNIIASDS ITQIPLVKAH TLQSGTTVVR GPGFTGGDIL
0501  RRTSGGPFAY TIVNINGQLP QRYRARIRYA STTNLRIYVT VAGERIFAGQ
0551  FNKTMDTGDP LTFQSFSYAT INTAFTFPMS QSSFTVGADT FSSGNEVYID
0601  RFELIPVTAT LEAEYNLERA QKAVNALFTS TNQLGLKTNV TDYHIDQVSN
0651  LVTYLSDEFC LDEKRELSEK VKHAKRLSDE RNLLQDSNFK DINRQPERGW
0701  GGSTGITIQG GDDVFKENYV TLSGTFDECY PTYLYQKIDE SKLKAFTRYQ
0751  LRGYIEDSQD LEIYSIRYNA KHETVNVPGT GSLWPLSAQS PIGKCGEPNR
0801  CAPHLEWNPD LDCSCRDGEK CAHHSHHFSL DIDVGCTDLN EDLGVWVIFK
0851  IKTQDGHARL GNLEFLEEKP LVGEALARVK RAEKKWRDKR EKLEWETNIV
0901  YKEAKESVDA LFVNSQYDQL QADTNIAMIH AADKRVHSIR EAYLPELSVI
0951  PGVNAAIFEE LEGRIFTAFS LYDARNVIKN GDFNNGLSCW NVKGHVDVEE
1001  QNNQRSVLVV PEWEAEVSQE VRVCPGRGYI LRVTAYKEGY GEGCVTIHEI
1051  ENNTDELKFS NCVEEEIYPN NTVTCNDYTV NQEEYGGAYT SRNRGYNEAP
1101  SVPADYASVY EEKSYTDGRR ENPCEFNRGY RDYTPLPVGY VTKELEYFPE
1151  TDKVWIEIGE TEGTFIVDSV ELLLMEE
```

Figure VI.5. Sequence Coverage Map of the MON 89034-produced Cry1A.105 Protein by MALDI-TOF MS Analysis

Shaded regions correspond to 52 fragments of tryptic peptide masses that were identified from the full-length protein band. Overall, 43.8% of the complete protein sequence was identified.

1.2.5. Lack of glycosylation for Cry1A.105 protein

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple oligosaccharides to monosaccharides. In contrast, prokaryotic organisms such as non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical synthetic capacity required for protein glycosylation. Therefore, determining whether the MON 89034-produced Cry1A.105 protein is equivalent to the *E. coli*-produced Cry1A.105 protein requires an investigation of its glycosylation status.

To assess whether potential post-translational glycosylation of the MON 89034-produced Cry1A.105 protein occurred, the purified protein sample was subjected to glycosylation analysis. The *E.* coli-produced Cry1A.105 reference standard represented a negative control. The positive controls were the transferrin and horseradish peroxidase (HRP) proteins which are known to have multiple covalently-linked carbohydrate modifications. The transferrin protein and HRP, as well as the purified Cry1A.105 protein isolated from MON 89034 and *E. coli* were separated on SDS-PAGE, and glycosylation analysis was performed to detect oxidized carbohydrate moieties on the proteins. The results of these analyses are shown in Figure VI.6. The positive controls were detected at the expected molecular weights in a concentration-dependent manner (Figure VI.6, lanes 2-5). No detectable signal was observed for the MON 89034-produced and *E. coli*-produced Cry1A.105 protein (Figure VI.6, lanes 6-9).

These results indicate that the MON 89034-produced protein is not glycosylated and, thus is equivalent to the *E. coli*-produced Cry1A.105 reference standard with respect to the lack of glycosylation.

1.2.6. Cry1A.105 functional activity

The biological activities of *E. coli*- and MON 89034-produced Cry1A.105 proteins were estimated by determining EC_{50} values in a corn earworm (CEW) diet-incorporation bioassay. The EC_{50} value is defined as the level of Cry1A.105 protein in the diet that results in 50% inhibition of larval growth.

The EC_{50} values for each replicate bioassay are summarized in Table VI.2, and the dose response relationships for MON 89034- and *E. coli*-produced Cry1A.105 are illustrated in Figure VI.7. The ranges of the estimated EC_{50} values overlapped for the protein from the two host sources. The EC_{50} values for the MON 89034-produced protein ranged from 0.0055 to 0.0089 µg Cry1A.105/ml diet and the EC_{50} values for the *E. coli*-produced protein ranged from 0.0053 to 0.0170 µg Cry1A.105/ml diet. Figure VI.7 shows an equivalent slope for the dose-response relationship for the *E. coli*- and MON 89034-produced Cry1A.105 proteins in the CEW bioassay, which demonstrates that the they have equivalent functional activities.

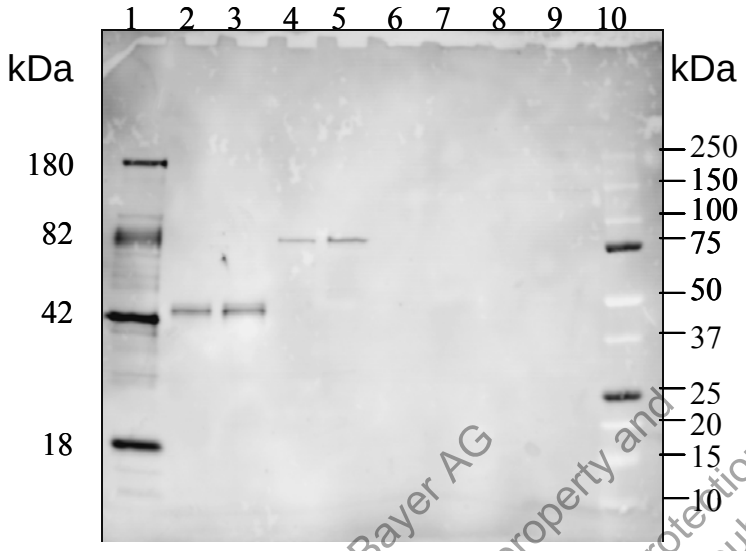


Figure VI.6. Glycosylation Analysis of the MON 89034-produced Cry1A.105 Protein

Aliquots of the MON 89034-produced Cry1A.105 protein, *E. coli*-produced Cry1A.105 reference standard (negative control), horseradish peroxidase (positive control) and transferrin (positive control) were separated by SDS-PAGE (4→20% gradient) and electrotransferred to a PVDF membrane. Where present, periodate-oxidized protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain and emitted a fluorescent signal at 488 nm. The signal was captured using a Bio-Rad Molecular Imager FX. Approximate molecular weights (kDa) correspond to the CandyCane glycoprotein markers loaded in lane 1 and the Precision Dual Color markers in lane 10.

Lane	Sample	Amount (ng)
1	CandyCane glycoprotein molecular weight standards	—
2	Horseradish Peroxidase (positive control)	48
3	Horseradish Peroxidase (positive control)	96
4	Transferrin (positive control)	48
5	Transferrin (positive control)	96
6	MON 89034-produced Cry1A.105	48
7	MON 89034-produced Cry1A.105	96
8	*E. coli*-produced Cry1A.105 (negative control)	48
9	*E. coli*-produced Cry1A.105 (negative control)	96
10	Precision Plus Dual Color molecular weight markers	—

Table VI.2. EC_{50} Values of *E. coli*- and MON 89034-produced Cry1A.105 Proteins in a Corn Earworm Diet-incorporation Bioassay

		EC_{50} (µg Cry1A.105/ml diet)[1]	
		***E. coli*-produced**	**MON 89034-produced**
	1	0.0150 ± 0.0025	0.0055 ± 0.0014
Replicate[2]	2	0.0053 ± 0.0022	0.0089 ± 0.0018
	3	0.0170 ± 0.0021	0.0077 ± 0.0012
Overall		0.0120 ± 0.0062	0.0074 ± 0.0017

[1] EC_{50} (mean ± standard error) represents the concentration needed to inhibit the growth of the target insect by 50%.

[2] Each bioassay replicate consisted of a series of five protein levels yielding a dose series ranging from 0.00048 – 0.039 µg Cry1A.105 protein/ml diet with a 3-fold separation factor between dose levels. Insect larvae were placed on the diets with 16 insects per treatment. The combined weight of the surviving insects at each dose level was recorded at the end of the 6-7 day incubation period. EC_{50} was calculated with SAS software.

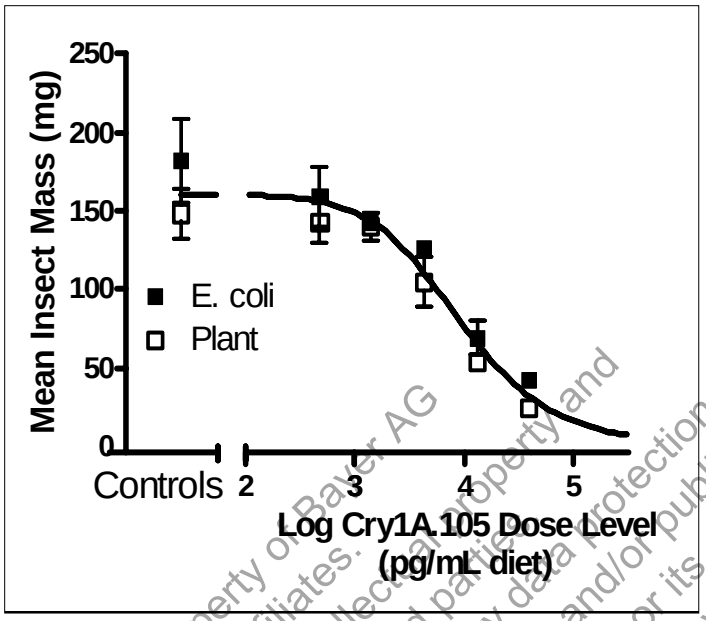


Figure VI.7. Functional Equivalence of the MON 89034- and *E. coli*-produced Cry1A.105 Proteins Against Corn Earworm (CEW)

Bioassay results from three replicates have been combined for illustration purpose only and are shown on a log concentration scale. Each data point represents the mean of the three bioassays along with the standard error of the mean. Equivalent slope was seen for dose-response relationships for the MON 89034-produced and *E. coli*-produced Cry1A.105 protein in the CEW bioassay. The dose response curve was modeled with SAS software. The data demonstrate that the test insect body weight decreases with the increase of the Cry1A.105 dose level, indicating the greater growth inhibitory effect on the test insects at higher doses.

1.2.7. Conclusions for characterization of the Cry1A.105 protein

The Cry1A.105 protein isolated from MON 89034 was purified and characterized, and results confirmed the equivalence of the MON 89034- and *E. coli*-produced Cry1A.105 proteins. SDS-PAGE demonstrated that the MON 89034-produced Cry1A.105 co-migrated with the *E. coli*-produced Cry1A.105 protein on the gel, indicating the protein from both sources have equivalent molecular weights. On the basis of western blot analysis with a polyclonal antibody against Cry1A.105, the electrophoretic mobility and immunoreactivity of the MON 89034-produced Cry1A.105 protein were shown to be equivalent to that of the *E. coli*-produced Cry1A.105 reference standard. Tryptic peptide mapping by MALDI-TOF MS yielded peptide masses consistent with the expected tryptic peptides generated *in silico* based on the predicted trypsin cleavage sites in the Cry1A.105 sequences. In addition, the MON 89034- and the *E. coli*-produced Cry1A.105 proteins were found to be equivalent in terms of functional activity and the lack of glycosylation. Taken together, these data provide a detailed characterization of the Cry1A.105 protein isolated from MON 89034 and establish its equivalence to the *E. coli*-produced Cry1A.105 protein.

2.1. Identity and function of the Cry2Ab2 protein

The Cry2Ab2 protein produced in MON 89034 is derived from the Bt subspecies *kurstaki* and its amino acid sequence differs from that of the wild-type protein by a single amino acid. The Cry2Ab2 protein has 88% amino acid sequence identity to the Cry2Aa protein, which is present in commercial microbial pest control products such as Dipel and Crymax. The Cry2Ab2 proteins produced in MON 89034 and Bollgard II cotton share an identical amino acid sequence. Bollgard II cotton has been on the market since 2003 and there have been no concerns regarding its safety to date.

Like the Cry2Ab2 protein produced in the biotechnology-derived cotton Bollgard II, the Cry2Ab2 protein produced in corn MON 89034 is a variant of the wild-type Cry2Ab2 protein from Bt. Accumulation of the Cry2Ab2 protein in MON 89034 is targeted to the chloroplast using a chloroplast transit peptide (CTP), which facilitates the intracellular transport of proteins from the cytoplasm to the plastids (Bruce, 2000). CTPs are typically cleaved from the mature protein upon translocation into the chloroplast, and then rapidly degraded. To allow targeting of the Cry2Ab2 protein to the plastids in MON 89034, the DNA sequence encoding the CTP region of corn ribulose 1,5-biosphosphate carboxylase small subunit was fused to the *cry2Ab2* coding sequence.

The Cry2Ab2 protein expressed in cotton Bollgard II is also targeted to a plastid through a CTP. Attempts to determine the N-terminal sequence of the Cry2Ab2 protein in cotton Bollgard II (after the CTP was cleaved) indicate that the N-terminus was blocked, and therefore the exact excision site of the CTP is not known. Attempts to determine the N-terminal sequence of the full-length MON 89034-produced Cry2Ab2 also indicated that the protein was also blocked at the N-terminus, and as a consequence, the cleavage site in the CTP that is processed in the chloroplast could not be determined. The CTP used in MON 89034 has a potential cleavage site (methionine), located three amino acids upstream from the start of the Cry2Ab2 protein sequence (Figure VI.8). As such, the

three additional amino acids from the CTP were included at the N-terminus for the *E. coli*-produced Cry2Ab2 protein used in the safety assessment studies for MON 89034 (Figure VI.8). This resulted in the production of a Cry2Ab2 protein of 637 amino acids (634 from Cry2Ab2 and 3 from the CTP) with a theoretical molecular weight of 71 kDa.

MON 89034	**M-Q-A**[1]-M-**D**[2]- N-S-V-L-N
Recombinant *E. coli*	**M-Q-A**[1]-M-**D**[2]- N-S-V-L-N
B. thuringiensis	-M- - N-S-V-L-N

[1] **M-Q-A** – predicted amino acids from chloroplast transit peptides (CTP)
[2] **D** – an additional amino acid included for the ease of cloning

Figure VI.8. Comparison of the Putative N-terminal Sequences of the Cry2Ab2 Proteins Produced in MON 89034, Recombinant *E. coli*, and *Bacillus thuringiensis*

2.2. Characterization of the Cry2Ab2 protein

The Cry2Ab2 protein was purified from the grain of MON 89034 corn as well as from an *E. coli* culture. A panel of analytical tests was used to identify, characterize and compare the MON 89034- and *E. coli*-produced Cry2Ab2 proteins including: (1) western blot analysis; (2) SDS-PAGE; (3) MALDI-TOF MS; (4) N-terminal sequence analysis with Edman degradation chemistry; (5) glycosylation analysis; and (6) insect activity bioassay.

2.2.1. Cry2Ab2 protein molecular weight equivalence

The equivalence in molecular weight of the purified MON 89034- and the *E. coli*-produced Cry2Ab2 protein was demonstrated using SDS-PAGE stained with Brilliant Blue G-Colloidal stain. The MON 89034-produced, full-length Cry2Ab2 protein migrated to a position comparable to that of the *E. coli*-produced protein standard, which was analyzed concurrently (Figure VI.9, band-1). Based on the comparable electrophoretic mobility, the MON 89034- and *E. coli*-produced, full-length Cry2Ab2 proteins were determined to have equivalent molecular weight. Band-2 observed below the full-length band is a proteolytic fragment (see Section 2.2.2 below).

2.2.2. Cry2Ab2 protein immunoreactivity

Western blot analysis using a polyclonal anti-Cry2Ab2 antibody was conducted to confirm the identity and determine the relative immunoreactivity of the MON 89034-produced Cry2Ab2 protein and the *E. coli*-produced Cry2Ab2 reference standard. Results indicated that the anti-Cry2Ab2 antibody recognized the MON 89034-produced Cry2Ab2 protein, which migrated comparably to the *E. coli*-produced reference standard protein (Figure VI.10, band-1). The immunoreactive signal increased with increasing levels of the Cry2Ab2 protein. Besides the expected band, an immunoreactive band with

lower molecular weight (Figure VI.10, band-2) was observed in the partially purified MON 89034-produced Cry2Ab2 sample, which represents a proteolytic fragment of the Cry2Ab2 protein.

These western blot results confirm the identity of Cry2Ab2 protein produced by MON 89034, and demonstrate that MON 89034- and *E. coli*-produced Cry2Ab2 proteins have equivalent immunoreactivity with anti-Cry2Ab2 antibody.

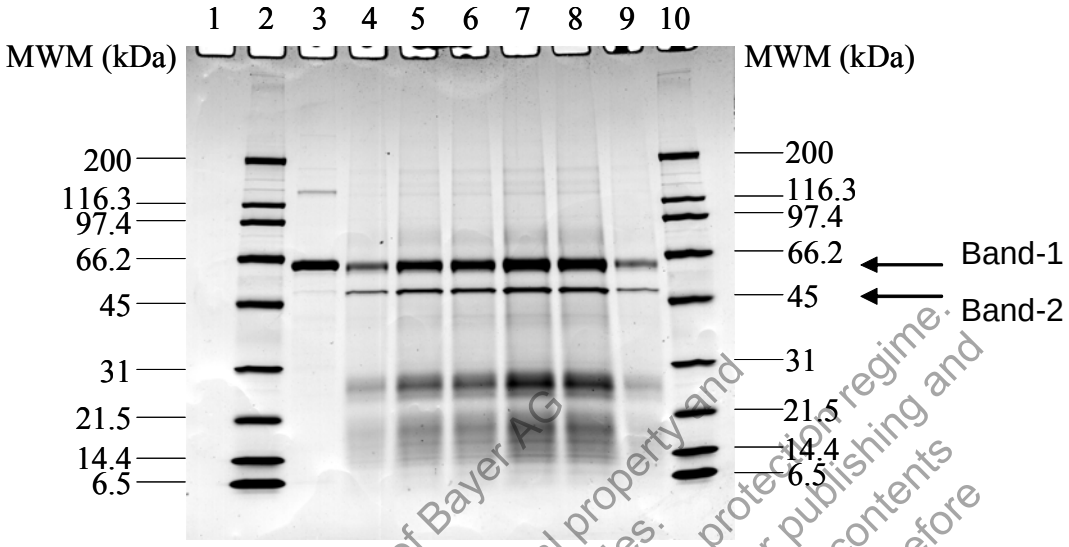


Figure VI.9. SDS-PAGE of the MON 89034- and *E. coli*-produced Cry2Ab2 Proteins

Samples of the partially purified MON 89034-produced Cry2Ab2 protein, and the *E. coli*-produced Cry2Ab2 reference standard were separated by a Tris-glycine 4→20% SDS polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Amounts loaded correspond to total protein loaded per lane. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.

Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MWM (molecular weight markers, Bio-Rad, broad range)	4.5
3	*E. coli*-produced Cry2Ab2 reference standard	1
4	MON 89034-produced Cry2Ab2 protein	1
5	MON 89034-produced Cry2Ab2 protein	2
6	MON 89034-produced Cry2Ab2 protein	2
7	MON 89034-produced Cry2Ab2 protein	3
8	MON 89034-produced Cry2Ab2 protein	3
9	MON 89034-produced Cry2Ab2 protein	1
10	MWM (molecular weight markers, Bio-Rad, broad range)	4.5

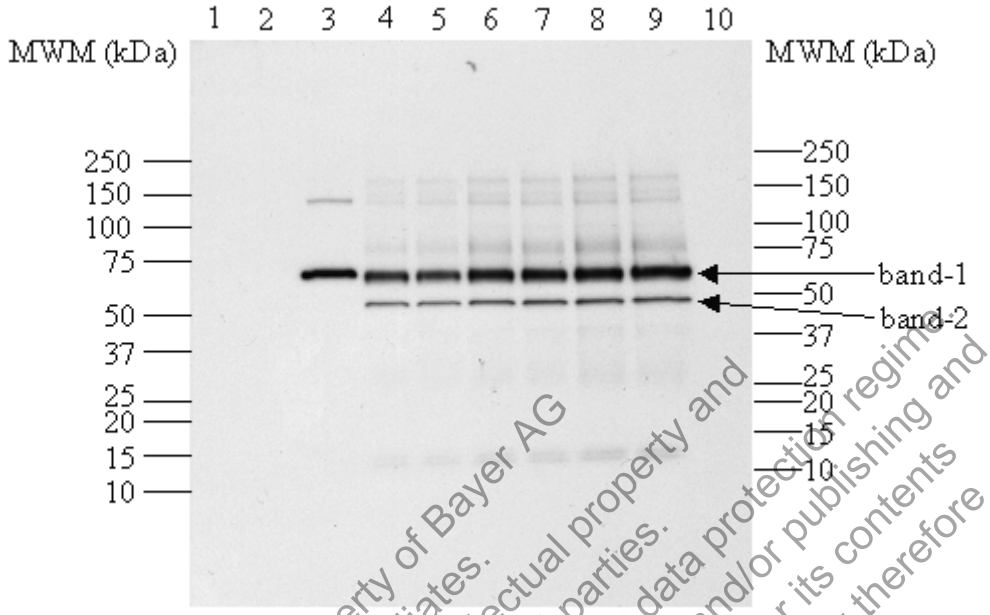


Figure VI.10. Western Blot Analysis of MON 89034- and *E. coli*-produced Cry2Ab2 Proteins

Samples of the partially purified MON 89034- and *E. coli*-produced Cry2Ab2 proteins were separated by SDS-PAGE (4→20% gradient), electrotransferred to a PVDF membrane. The membrane was then detected using goat anti-Cry2Ab2 antibody and developed using an ECL system. Amounts loaded correspond to subject protein after normalization with purity. The approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.

Lane	Sample	Amount of Cry2Ab2 (ng)
1	Empty Lane	N/A
2	Precision Plus Dual Color molecular weight markers (MWM)	N/A
3	*E. coli*-produced Cry2Ab2 protein	20
4	MON 89034-produced Cry2Ab2 protein	20
5	MON 89034-produced Cry2Ab2 protein	20
6	MON 89034-produced Cry2Ab2 protein	30
7	MON 89034-produced Cry2Ab2 protein	30
8	MON 89034-produced Cry2Ab2 protein	40
9	MON 89034-produced Cry2Ab2 protein	40
10	Precision Plus Dual Color molecular weight markers (MWM)	N/A

2.2.3. Analysis of the N-terminal sequence

N-terminal sequencing analysis demonstrated that the MON 89034-produced, full-length Cry2Ab2 (Figure VI.10, band-1) was blocked at the N-terminus with no definitive sequence obtained in the sequencing analysis using Edman degradation chemistry. A minor portion of the protein co-migrating with the full-length protein was proteolytically degraded and the sequence was determined to start from amino acid residue 24. N-terminal sequence analysis of the lower molecular weight proteolytic fragment (Figure VI.10, band-2) revealed that this its sequence starts from amino acid residue 145. With *E. coli*-produced Cry2Ab2 (band-1) the N-terminus sequence was determined as MQAMDN, as expected (Figure VI.8). This result further confirms that band-1 is the full-length Cry2Ab2 protein.

In summary, the N-terminal sequencing results indicate that MON 89034-produced, full-length Cry2Ab2 protein is blocked at its N-terminus, but the N-terminal sequencing result of the lower molecular weight proteolytic fragment (band-2) confirms the Cry2Ab2 identity. In addition, the N-terminus of *E. coli*-produced, full-length Cry2Ab2 was confirmed.

2.2.4. Cry2Ab2 tryptic peptide mapping by MALDI-TOF MS

The MON 89034-produced, full-length Cry2Ab2 (band-1, in Figure VI.10) and the proteolytic fragment (band-2, in Figure VI.10) were characterized by tryptic peptide mapping analysis with MALDI-TOF MS to further confirm their identity. For band-1, a total of 32 observed peptide masses matched the theoretical tryptic peptide masses of Cry2Ab2 protein (Appendix B). These identified peptides were used to assemble a coverage map in the Cry2Ab2 protein sequence (Figure VI.11). The overall peptide sequence coverage was 44.4% out of the 637 amino acid residues of the full-length Cry2Ab2 protein. For band-2, a total of 24 observed peptide masses matched the expected tryptic peptide masses of Cry2Ab2 protein, which yielded a coverage map equal to 47.7% out of the 493 amino acid residues of this proteolytic fragment (Figure VI.12).

In general, a protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). In the current case, the detected peptide coverage was 44.4% with 32 matched peptides for the full-length Cry2Ab2 protein, and 47.7% with 24 matched peptides for the proteolytic fragment of Cry2Ab2; therefore, the identity of the MON 89034-produced Cry2Ab2 protein is confirmed.

```
  1 MQAMDNSVLN SGRTTICDAY NVAAHDPFSF QHKSLDTVQK EWTEWKKNNH SLYLDPIVGT
 61 VASFLLKKVG SLVGKRILSE LRNLIFPSGS TNLMQDILRE TEKFLNQRLN TDTLARVNAE
121 LTGLQANVEE FNRQVDNFLN PNRNAVPLSI TSSVNTMQQL FLNRLPQFQM QGYQLLLLPL
181 FAQAANLHLS FIRDVILNAD EWGISAATLR TYRDYLKNYT RDYSNYCINT YQSAFKGLNT
241 RLHDMLEFRT YMFLNVFEYV SIWSLFKYQS LLVSSGANLY ASGSGPQQTQ SFTSQDWPFL
301 YSLFQVNSNY VLNGFSGARL SNTFPNIVGL PGSTTTHALL AARVNYSGGI SSGDIGASPF
361 NQNFNCSTFL PPLLTPFVRS WLDSGSDREG VATVTNWQTE SFETTLGLRS GAFTARGNSN
421 YFPDYFIRNI SGVPLVVRNE DLRRPLHYNE IRNIASPSGT PGGARAYMVS VHNRKNNIHA
481 VHENGSMIHL APNDYTGFTI SPIHATQVNN QTRTFISEKF GNQGDSLRFE QNNTARYTL
541 RGNGNSYNLY LRVSSIGNST IRVTINGRVY TATNVNTTTN NDGVNDNGAR FSDINIGNVV
601 ASSNSDVPLD INVTLNSGTQ FDLMNIMLVP TNISPLY
```

Figure VI.11. Peptide Sequence Coverage Map of the MON 89034-Produced Cry2Ab2 Protein by MALDI-TOF MS Analysis

Shaded regions correspond to 32 fragments of tryptic peptide masses that were identified from the band-1 (Figure VI.10.) of the isolated Cry2Ab2 protein from MON 89034. MQA sequence (underlined) originates from the chloroplast transit peptide (CTP).

```
145 AVPLSITSSV NTMQQLFLNR LPQFQMQGYQ LLLLPLFAQA ANLHLSFIRD VILNADEWGI
205 SAATLRTYRD YLKNYTRDYS NYCINTYQSA FKGLNTRLHD MLEFRTYMFL NVFEYVSIWS
265 LFKYQSLLVS SGANLYASGS GPQQTQSFTS QDWPFLYSLF QVNSNYVLNG FSGARLSNTF
325 PNIVGLPGST TTHALLAARV NYSGGISSGD IGASPFNQNF NCSTFLPPLL TPFVRSWLDS
385 GSDREGVATV TNWQTESFET TLGLRSGAFT ARGNSNYFPD YFIRNISGVP LVVRNEDLRR
445 PLHYNEIRNI ASPSGTPGGA RAYMVSVHNR KNNIHAVHEN GSMIHLAPND YTGFTISPIH
505 ATQVNNQTRT FISEKFGNQG DSLRFEQNNT TARYTLRGNG NSYNLYLRVS SIGNSTIRVT
565 INGRVYTATN VNTTTNNDGV NDNGARFSDI NIGNVVASSN SDVPLDINVT LNSGTQFDLM
625 NIMLVPTNIS PLY
```

Figure VI.12. Peptide Sequence Coverage Map of the MON 89034-derived Cry2Ab2 Proteolytic Fragment by MALDI-TOF MS Analysis

Shaded regions correspond to 24 tryptic peptide masses that were identified from the band-2 proteolytic fragment of Cry2Ab2 (Figure VI.10) from MON 89034. The amino acid residue number was assigned based on the respective position in the full-length sequence of Cry2Ab2 protein. N-terminal sequencing result showed that the sequence of band-2 started from amino acid residue 145.

2.2.5. Lack of glycosylation of MON 89034-produced Cry2Ab2

Some eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple oligosaccharides to monosaccharides. In contrast, prokaryotic organisms such as non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical "machinery" required for protein glycosylation. Therefore, determining whether the MON 89034-produced Cry2Ab2 protein is equivalent to the *E.coli*-produced Cry2Ab2 protein requires an investigation of its glycosylation status.

To determine whether post-translational glycosylation of the MON 89034-produced Cry2Ab2 protein occurred, the isolated MON 89034-produced Cry2Ab2 protein was analyzed for the presence of covalently bound carbohydrate moieties. The *E. coli*-produced Cry2Ab2 reference standard (negative control) and transferrin (positive control) were analyzed concurrently with the MON 89034-produced Cry2Ab2 protein.

The result showed that positive glycoprotein transferrin was detected, as expected, in a concentration-dependent manner at loadings of 0.5 and 1.0 µg/lane (Figure VI.13**,** lanes 3-4). No detectable signal was observed for the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein at the positions of the expected molecular weights between 50 to 75 kDa (Figure VI.13**,** lanes 5-8).

These results indicate that the MON 89034-produced Cry2Ab2 protein is not glycosylated and, thus is equivalent to the *E. coli*-produced Cry2Ab2 reference standard with respect to the lack of glycosylation.

2.2.6. Functional activity of the Cry2Ab2 protein

The functional activities of the MON 89034- and *E. coli*-produced Cry2Ab2 proteins were compared by determining EC_{50} values in a CEW diet-incorporation bioassay. The EC_{50} value is defined as the level of Cry2Ab2 protein in the diet that results in 50% inhibition to larval growth.

The EC_{50} values for each replicate bioassay are summarized in Table VI.3 and the dose response relationships for the Cry2Ab2 protein from the two sources are illustrated in Figure VI.14. The mean EC_{50} values for the *E. coli*- and the MON 89034-produced proteins were similar and estimated to be 0.16 µg Cry2Ab2/ml diet, with standard errors of 0.04 and 0.01 µg Cry2Ab2/ml diet, respectively. Figure VI.14 shows an equivalent slope for the dose-response relationship for the *E. coli*- and MON 89034-produced Cry2Ab2 protein in the CEW bioassay. These results clearly showed that the MON 89034- and *E. coli*-produced Cry2Ab2 proteins have equivalent functional activities.

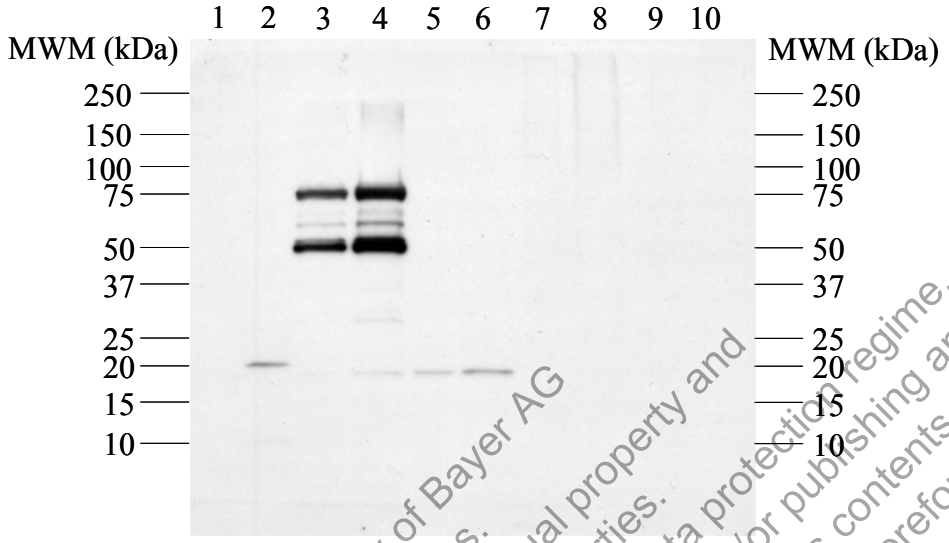


Figure VI.13. Glycosylation Analysis of the MON-89034- and *E.coli*-produced Cry2Ab2 Proteins

Samples of the MON 89034-produced Cry2Ab2 protein, *E. coli*-produced Cry2Ab2 reference standard (negative control) and transferrin (positive control) were separated by a Tris-glycine 4→20% SDS polyacrylamide gel, and electrotransferred to PVDF membrane. Where present, protein-bound carbohydrate moieties were labeled with biotin, and detected with streptavidin-horseradish peroxidase and enhanced chemiluminescence. Amount refers to total protein loaded per lane, except for the *E. coli* and the MON 89034-produced proteins whose concentrations were normalized based on Cry2Ab2 purity.

Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MWM (molecular weight markers, Precision Plus Dual Color)	N/A
3	Transferrin (positive control)[1]	0.5
4	Transferrin (positive control)[1]	1
5	*E. coli*-produced Cry2Ab2 protein (negative control)	0.5
6	*E. coli*-produced Cry2Ab2 protein (negative control)	1
7	MON 89034-produced Cry2Ab2 protein	0.5
8	MON 89034-produced Cry2Ab2 protein	1
9	Empty Lane	N/A
10	Empty Lane	N/A

[1] Part of the transferrin appeared to be degraded. But this did not affect the utility of this protein as a positive control for glycoproteins in this test.

Table VI.3. EC_{50} Values of the MON 89034- and *E.coli*-produced Cry2Ab2 Proteins in a Corn Earworm Diet-incorporation Bioassay

		EC_{50} (µg Cry2Ab2/ml diet)[1]	
		***E. coli*-produced**	**MON 89034-produced**
	1	0.13 ± 0.03	0.17 ± 0.03
Replicate[2]	2	0.16 ± 0.02	0.16 ± 0.03
	3	0.20 ± 0.02	0.16 ± 0.02
Overall		0.16 ± 0.04	0.16 ± 0.01

[1] EC_{50} (mean ± standard error) represents the concentration needed to inhibit the growth of the target insect by 50%.

[2] Each bioassay replicate for the *E. coli*-produced and MON 89034-produced Cry2Ab2 proteins consisted of a series of seven dilutions yielding a dose series with a 2-fold separation factor ranging from 0.016 – 1.0 µg Cry2Ab2 protein/ml diet. Insect larvae were placed on the diets with 16 insects per treatment. The combined weight of the surviving insects at each dose level was recorded at the end of the 7 day incubation period. EC_{50} was calculated with SAS software.

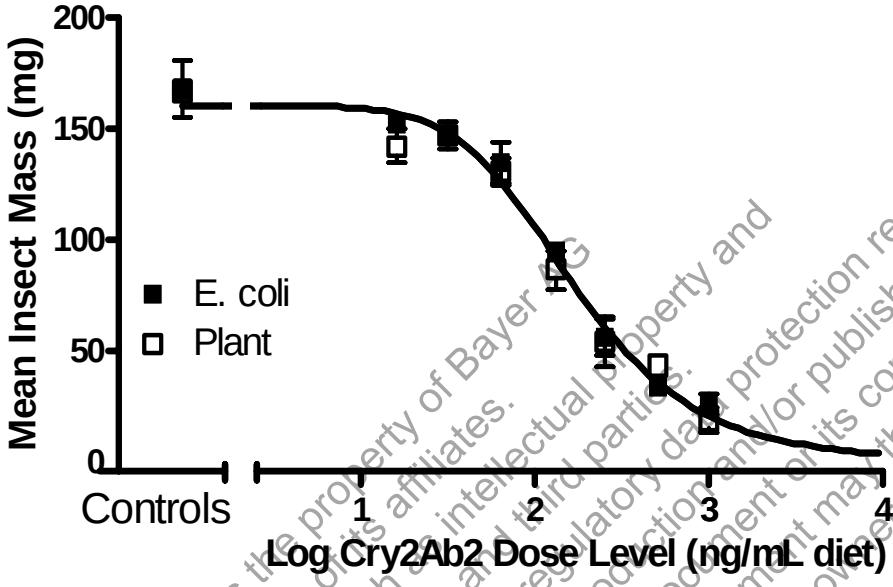


Figure VI.14. Functional Equivalence of the MON 89034- and *E. coli*-produced Cry2Ab2 Proteins Against Corn Earworm (CEW)

Bioassay results from three replicates have been combined for illustration purpose only and are shown on a log concentration scale. Each data point represents the mean of the three bioassays along with the standard error of the mean. Equivalent slope was seen for dose-response relationships for the *E. coli*- and MON 89034-produced Cry2Ab2 protein in the CEW bioassay. The dose response curve was modeled with SAS software. The data demonstrate that the test insect body weight decreases with the increase of the Cry2Ab2 dose level, indicating the greater growth inhibitory effect on the test insects at higher doses.

2.2.7. Conclusions of the characterization of the Cry2Ab2 protein

The Cry2Ab2 protein from MON 89034 was purified and characterized, and the results confirmed the equivalence between MON 89034- and *E. coli*-produced Cry2Ab2 proteins. The apparent molecular weight was estimated by SDS-PAGE. Since the MON 89034-derived Cry2Ab2 migrated comparably to the *E. coli*-produced protein on SDS-PAGE, the apparent molecular weight of the Cry2Ab2 protein from both MON 89034 and *E. coli* was determined to be equivalent. On the basis of western blot analysis, the electrophoretic mobility and immunoreactive properties of the MON 89034-produced Cry2Ab2 protein were demonstrated to be comparable to those of the *E. coli*-produced Cry2Ab2 reference standard. The N-terminus of the Cry2Ab2 from MON 89034 was blocked but the peptide mapping analysis by MALDI-TOF MS yielded the expected peptide masses based on the trypsin cleavage sites in the amino acid sequence of the Cry2Ab2 protein, thereby confirming its identity. In addition, the MON 89034- and the *E. coli*-produced Cry2Ab2 reference standard were found to be equivalent based on functional activities and the lack of glycosylation. Taken together, these data provide a detailed characterization of the Cry2Ab2 protein isolated from MON 89034 and established its equivalence to the *E. coli*-produced Cry2Ab2 protein.

SECTION 2. Levels of the Cry1A.105 and Cry2Ab2 Proteins Produced in MON 89034

The levels of the Cry1A.105 and Cry2Ab2 proteins in various tissues of MON 89034 that are relevant to the risk assessment were assessed by validated enzyme-linked immunosorbent assay (ELISA). Tissue samples for analysis were collected from five field trials conducted in the U.S. during 2005. The trial locations were in the states of Iowa, Illinois, Ohio, and Nebraska, which represent the major corn-growing region of the U.S. and provide a range of environmental conditions that would be encountered in the commercial production of corn. At each site, three replicated plots of MON 89034 and a conventional control hybrid were planted using a randomized complete block field design. Overseason leaf, overseason whole plant, overseason root, pollen, silk, forage, forage root, grain, stover, and senescent root tissues were collected from each replicated plot at all field sites. A description of the tissues collected is provided below.

Leaf. The youngest immature whorl leaf was collected from 15 plants in each plot at each site and combined to form the leaf sample. Overseason leaf samples were collected as follows:

Overseason leaf (OSL)	*Corn development stage*	*Days after planting (DAP)*
OSL-1	V2-V4	21-29
OSL-2	V6-V8	28-43
OSL-3	V10-V12	41-53
OSL-4	pre-VT (pre-tasseling)	56-68

Whole plant. The aerial portion of the plant without the root was collected from four plants in each plot at each site at the V2-V4 stage and combined to form the whole plant sample. Two plants were collected and combined to form the whole plant samples for the later growth stages. Overseason whole plant samples were collected as follows:

Overseason whole plant (OSWP)	*Corn development stage*	*DAP*
OSWP-1	V2-V4	21-29
OSWP-2	V6-V8	28-43
OSWP-3	V10-V12	41-53
OSWP-4	pre-VT (pre-tasseling)	56-68

Root. Roots remaining after collection of whole plants from each plot were combined to form the root sample. Overseason root samples were collected as follows.

Overseason root (OSR)	*Corn development stage*	*DAP*
OSR-1	V2-V4	21-29
OSR-2	V6-V8	28-43
OSR-3	V10-V12	41-53
OSR-4	pre-VT (pre-tasseling)	56-68
Forage root	early dent stage (R4-R6)	100-120
Senescent root	after harvest	130-160

Pollen. Approximately 10 ml of pollen was collected from multiple tassels in each plot at each site at pollination, approximately 60-74 days after planting.

Silks. Silks were collected from the ears of five plants in each plot at each site, approximately 60-74 days after planting. Silks were only collected from ears of plants that were covered with shoot bags to preserve their genetic identity.

Forage. Two whole plants in each plot at each site were cut above the soil surface at an early dent stage, at approximately 100-120 days after planting, and then combined to form the forage sample.

Grain. Grain was harvested at maturity from all plants in each plot at each site and dried to a moisture content of 12-15%.

Stover. Following harvest, approximately 130-160 days after planting, two whole plants in each plot at each site were cut above the soil surface and combined to form the stover sample.

All tissue samples, except grain, were stored and shipped on dry ice to Monsanto's facility for processing and analysis. Grain was stored and shipped at room temperature. All tissue samples were stored in a –80ºC freezer upon receipt. Tissue samples were extracted and analyzed by ELISA according to applicable Monsanto SOPs (standard operating procedures). Further details regarding sample collection and ELISA analysis are provided in Appendix E.

The results obtained from ELISA analysis are summarized in Table VI.4 for the various tissue types and in Tables VI.5 and VI.6 for the tissues collected throughout the growing season. Cry1A.105 and Cry2Ab2 proteins levels are provided in µg/g fresh weight tissue (fwt) and µg/g dry weight tissue (dwt).

The levels of the Cry1A.105 and Cry2Ab2 proteins estimated in tissues of MON 89034 showed trends that were consistent for exposure calculations and intended uses. The mean Cry1A.105 levels across sites were highest in young leaf (520 µg/g dwt), followed by stover (50 µg/g dwt), forage (42 µg/g dwt), silk (26 µg/g dwt), pollen (12 µg/g dwt), forage root (12 µg/g dwt), senescent root (11 µg/g dwt), and grain (5.9 µg/g dwt). The mean Cry2Ab2 levels across sites were highest in young leaf (180 µg/g dwt), followed by silk (71 µg/g dwt), stover (62 µg/g dwt), forage (38 µg/g dwt), senescent root (26 µg/g dwt), forage root (21 µg/g dwt), and grain (1.3 µg/g dwt). In general, the levels of the two Cry proteins declined over the growing season.

Table VI.4. Cry1A.105 and Cry2Ab2 Protein Levels in MON 89034 Tissues

Tissue Type	Growth Stage	Cry1A.105[2] Mean (SD) [Range], n=15		Cry2Ab2[2] Mean (SD) [Range], n=15	
		µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt
Young leaf	V2-V4	85 (21) 56 - 130	520 (130) 380 - 850	29 (6.8) 19 - 43	180 (59) 94 - 270
Pollen	R1	6.4 (1.5) 3.8 – 8.8	12 (1.7) 8.5 - 16	0.34 (0.084) 0.21 – 0.47	0.64 (0.091) 0.49 - 0.79
Silk	R1	3.0 (0.57) 2.0 – 3.8	26 (3.9) 20 - 31	8.2 (3.6) 3.3 - 16	71 (35) 33 - 160
Forage	R4-R6 (early dent)	14 (3.6) 8.3 - 24	42 (9.4) 20 - 56	12 (4.0) 6.5 - 18	38 (14) 15 - 55
Forage root	R4-R6 (early dent)	2.2 (0.35) 1.3 – 2.7	12 (3.1) 6.2 - 16	4.1 (1.4) 2.2 – 6.5	21 (5.9) 14 - 33
Grain	R6	5.1 (0.67) 4.1 – 6.0	5.9 (0.77) 4.7 - 7.0	1.1 (0.31) 0.67 – 1.8	1.3 (0.36) 0.77 – 2.1
Stover	R6 (after harvest)	17 (4.4) 9.5 - 26	50 (17) 26 - 85	22 (3.6) 17 - 29	62 (15) 46 - 97
Senescent root	R6 (after harvest)	2.2 (0.36) 1.7 – 3.1	11 (1.4) 9.4 - 15	5.3 (2.0) 2.4 – 9.1	26 (8.8) 13 - 43

[1] Young leaf = overseason leaf 1 (OSL-1); n = number of samples; SD = standard deviation; fwt = fresh weight tissue; dwt = dry weight tissue.

[2] Limits of detection (LOD) and limits of quantitation (LOQ) in the various tissues were as follows:

	Cry1A.105 (µg/g fwt)		Cry2Ab2 (µg/g fwt)	
Tissue	LOD	LOQ	LOD	LOQ
Forage	0.372	0.44	0.191	0.44
Leaf	0.568	0.66	0.081	0.44
Pollen	0.412	1.1	0.055	0.11
Root	0.254	0.33	0.056	0.22
Silk	0.275	0.44	0.040	0.22
Grain	0.262	1.1	0.123	0.22

Whole plant and stover were analyzed in the same manner as forage and senescent root was analyzed in the same manner as root.

Table VI.5. Cry1A.105 Proteins Levels in Overseason Tissues of MON 89034

Over-Season Tissue (n = 15) Tissues	Units[2]	Plant growth stages[1]											
		V2-V4 (21-29 DAP)		V6-V8 (28-43 DAP)		V10-V12 (41-53 DAP)		pre-VT (56-68 DAP)		R4-R6 (100-120 DAP)		R6 (130-160 DAP)	
		µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt
Leaf	Mean (SD)	520 (130)	85 (21)	140 (36)	28 (8.7)	72 (14)	16 (4.3)	120 (77)	30 (20)	N/A	N/A	N/A	N/A
	Range	380-850	56-130	80-200	12-45	47-89	9.4-24	27-240	6.3-59	N/A	N/A	N/A	N/A
Whole plant	Mean (SD)	380 (90)	40 (5.7)	260 (52)	24 (3.7)	100 (26)	11 (2.4)	120 (29)	17 (3.7)	42 (9.4)	14 (3.6)	50 (17)	17 (4.4)
	Range	230-570	30-52	170-350	16-31	58-160	7.0-15	58-170	9.3-22	20-56	8.3-24	26-85	9.5-26
Root	Mean (SD)	79 (17)	8.9 (1.3)	48 (11)	5.8 (1.6)	45 (10)	6.4 (1.8)	42 (10)	6.7 (0.63)	12 (3.1)	2.2 (0.35)	11 (14)	2.2 (0.36)
	Range	52-110	7.3-12	30-63	3.0-8.5	26-64	4.4-10	30-63	5.6-8.1	6.2-16	1.3-2.7	9.4-15	1.7-3.1

[1] Growth stages V2-V4, V6-V8, V10-V12 and pre-VT (tasseling) correspond to overseason tissue samples –1, -2, -3 and –4, respectively (e.g., OSWP-1, etc.). The whole plant and root samples collected at the R4-R6 (early dent) and R6 (after harvest) stages correspond to forage plant/root and stover/senescent root, respectively. N/A - not applicable; VT-vegetative stage at tasseling; DAP - days after planting; dwt - dry weight tissue; fwt - fresh weight tissue.

[2] The LODs and LOQs for Cry1A.105 can be found in Table VI.4. SD = standard deviation.

Table VI.6. Cry2Ab2 Proteins Levels in Overseason Tissues of MON 89034

Over-Season Tissue (n = 15)	Units[2]	Plant growth stages[1]											
		V2-V4 (21-29 DAP)		V6-V8 (28-43 DAP)		V10-V12 (41-53 DAP)		pre-VT (56-68 DAP)		R4-R6 (100-120 DAP)		R6 (130-160 DAP)	
		µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt	µg/g fwt
Leaf	Mean (SD)	180 (59)	29 (6.8)	170 (34)	32 (5.3)	130 (34)	29 (5.4)	160 (44)	37 (12)	N/A	N/A	N/A	N/A
	Range	94-270	19-43	110-230	23-44	85-200	23-41	48-210	11-56	N/A	N/A	N/A	N/A
Whole plant	Mean (SD)	130 (51)	13 (4.6)	79 (18)	7.5 (1.8)	40 (9.9)	4.2 (0.94)	39 (16)	5.9 (2.6)	38 (14)	12 (4.0)	62 (15)	22 (3.6)
	Range	52-230	5.2-21	45-110	4.0-9.7	22-61	2.4-5.8	5.0-67	0.7-11	15-55	6.5-18	46-97	17-29
Root	Mean (SD)	56 (17)	6.4 (1.6)	58 (18)	7.6 (4.2)	35 (17)	5.0 (7.7)	26 (5.9)	4.2 (1.2)	21 (5.9)	4.1 (1.4)	26 (8.8)	5.3 (2.0)
	Range	33-100	4.4-10	25-86	2.5-15	15-74	2.2-12	15-45	3.2-7.6	14-33	2.2-6.5	13-43	2.4-9.1

[1] Growth stages V2-V4, V6-V8, V10-V12 and pre-VT (tasseling) correspond to overseason tissue samples –1, -2, -3 and –4, respectively (e.g., OSWP-1, etc.). The whole plant and root samples collected at the R4-R6 (early dent) and R6 (after harvest) stages correspond to forage plant/root and stover/senescent root, respectively. N/A - not applicable; VT-vegetative stage at tasseling; DAP - days after planting; dwt - dry weight tissue; fwt - fresh weight tissue.

[2] The LODs and LOQs for Cry1A.105 can be found in TableVI.4. SD = standard deviation.

SECTION 3. Dietary Exposure Assessment

A dietary safety assessment was conducted to assess the potential risks to humans and animals from dietary exposure to the Cry1A.105 and Cry2Ab2 proteins from the consumption of foods and feeds derived from MON 89034. Potential risks were evaluated by calculating a margin of exposure (MOE), which was defined as the ratio of the no observed effect level (NOEL) from the acute mouse gavage study to the estimates of the dietary intake of the respective Cry protein. The MOEs for the overall U.S. population were greater than or equal to 199,000 and 981,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively. For children aged 3-5 years old, the age group with the highest corn consumption (body weight basis), the MOEs were greater than or equal to 79,400 and 390,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively. For poultry and livestock, the MOEs ranged between 1,930 – 13,500 and 2,160 – 47,600 for the Cry1A.105 and Cry2Ab2 proteins, respectively.

These large MOEs indicate that there are no meaningful risks to human or animal health from dietary exposure to the Cry1A.105 and Cry2Ab2 proteins. Details are provided below.

3.1. Human dietary safety assessment

An acute dietary safety assessment was conducted to assess the potential human health risks from dietary exposure to Cry1A.105 and Cry2Ab2 from consumption of MON 89034. Details are discussed below and the results of this assessment are summarized in Table VI.7.

3.1.1. Risk assessment of the Cry1A.105 and Cry2Ab2 proteins to mammals

Cry proteins have been used as components of topical Bt microbial pesticides for over 45 years. Numerous animal safety studies have demonstrated that Bt microbial insecticides containing mixtures of Cry proteins are non-toxic when fed to mammals at high does (Betz et al., 2000; EPA, 1988, 2000). Collectively, these studies demonstrate the absence of acute, subchronic, and chronic oral toxicity in mice, rats and humans (Betz et al., 2000). As discussed previously (Part VI, Section 1), the Cry1A.105 and Cry2Ab2 proteins are structurally and functionally related to Cry proteins that have a history of safe use as the active ingredients either in Bt microbial pesticides and/or in biotechnology-derived food and feed crops.

Most known protein toxins, including the insecticidal Cry proteins, act through acute mechanisms to exert toxicity (Sjoblad et al., 1992; Pariza and Johnson, 2001; Hammond and Fuchs, 1998). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which typically require a short-term (2-4 week) feeding study to manifest toxicity (Leiner, 1994). The amino acid sequences of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 are not similar to any of these anti-nutritional proteins or to any other known protein toxin (see Part VI, Sections 4 and 5). In addition, since Cry proteins act through acute mechanisms to control insect pests, and have no activity against non-target organisms such as mammals, the U.S. EPA

has determined that a high dose acute test is sufficient to confirm their absence of toxicity towards mammals (McClintock et al., 1995). Therefore, an acute oral mouse toxicity study was considered appropriate and adequate to confirm the lack of mammalian toxicity of the Cry1A.105 and Cry2Ab2 proteins. Results from these tests showed that no adverse effects were observed, and that the NOEL for Cry1A.105 was equal to or greater than 2072 mg/kg bw, the highest dose tested. For Cry2Ab2, the NOEL was equal to or greater than 2198 mg/kg bw, the highest dose tested (Part VI, Section 5).

The potential for synergistic interaction between the Cry1A.105 and Cry2Ab2 proteins was also considered for human dietary safety assessment. Synergism is generally defined as an interaction that occurs when the combined effect of two substances is much greater than the sum of the effects of each substance administered alone. In order for the two Cry proteins to act synergistically, they must be capable of producing toxicity in the species of interest. The mode of action for Cry proteins involves binding of the Cry protein to Cry-specific receptors in the intestinal tissues of susceptible insect species. However, Cry proteins do not show binding to the intestinal tissues of mammalian species such as mouse, rat, monkey and human (Hoffmann et al., 1988; Noteborn et al., 1993). This indicates that the mode of action by which Cry proteins produce toxicity in susceptible insects would be inoperative in humans and other mammalian species, and that no toxic response let alone a synergistic interaction would occur. Furthermore, studies have shown that the Cry1A.105 and Cry2Ab2 proteins do not act synergistically even in target species (*e.g.*, lepidopteran) that have specific receptors and are thus susceptible to the toxic effects of Cry proteins (see Part VI, Section 5.1, for further discussion). Therefore it is reasonable to conclude that the Cry1A.105 and Cry2Ab2 proteins would not have synergistic activity in mammals and it is appropriate to evaluate the toxicity of the two proteins separately.

3.1.2. Corn consumption

The amount of corn-derived food consumed by humans in the U.S. that could potentially contain the Cry1A.105 and Cry2Ab2 proteins from MON 89034 was estimated using the Dietary Exposure Evaluation Model (DEEM-FCID version 2.03, Exponent Inc.) and food consumption data from the 1994-1996 and 1998 USDA Continuing Surveys of Food Intakes by Individuals (CSFII). MON 89034 is intended for use in field corn, sweet corn and popcorn. Therefore, all three commodities were included in this assessment. DEEM-FCID separates field corn into six fractions: flour, meal, bran, starch, oil and syrup. However, corn oil and corn syrup were excluded from this analysis since these food items are essentially devoid of protein. The remaining corn-derived food items were considered potential sources of the two Cry proteins, although, because of the very low protein content, any contribution from corn starch is expected to be minimal. No adjustments were made for anticipated market share; *i.e.*, for the purposes of this assessment, all corn-derived food products consumed were assumed to be derived from MON 89034. This is a very conservative assumption since both field corn and popcorn are blended commodities. Thus, MON 89034 field corn would be mixed with other field corn, and MON 89034 popcorn would be mixed with other popcorn, before these commodities are processed and consumed.

According to the DEEM-FCID analysis, 95% of the overall U.S. population consumes no more than 2.04 g/kg bw of corn (flour, meal, bran, starch, sweet corn and popcorn) on any one day. The highest value was for children 3-5 years of age, for whom the 95th percentile estimate of consumption was 5.12 g/kg bw.

3.1.3. Intake of the Cry1A.105 and Cry2Ab2 proteins

For the purposes of this assessment, the concentration of the Cry1A.105 and Cry2Ab2 proteins in all consumed corn products was assumed to be equal to the mean level of the protein in the whole grain [5.1 µg/g fwt for Cry1A.105 and 1.1 µg/g fwt for Cry2Ab2; from Part VI, Section 2]. The dietary intake of each protein from consumption of MON 89034 can then be estimated by multiplying these levels by the estimates of corn consumption. These estimates conservatively assume that there is no loss of the Cry1A.105 and Cry2Ab2 proteins during storage, processing and/or cooking of the grain or food items. Utilizing these assumptions, the 95th percentile estimates for acute dietary intake of Cry1A.105 are 10.4 and 26.1 µg/kg bw for the overall U.S. population and children aged 3-5 years, respectively. For Cry2Ab2, the 95th percentile estimates for acute dietary intake are 2.24 and 5.63 µg/kg bw for the overall U.S. population and children aged 3-5 years, respectively.

3.1.4. Margins of exposure

As discussed above, no adverse health effects were observed when mice were administered a total of 2072 mg/kg bw of the Cry1A.105 protein or 2198 mg/kg bw of the Cry2Ab2 protein in one day. Therefore, potential health risks from acute dietary intake of these proteins from consumption of MON 89034 were evaluated by calculating the MOEs based on the acute mouse oral NOELs and the 95th percentile estimates of acute dietary exposure. Utilizing these values, the MOEs for Cry1A.105 were determined to be approximately greater than or equal to 199,000 for the overall U.S. population and greater than or equal to 79,400 for children aged 3-5 years old, an age group with the highest corn consumption (body weight basis). The corresponding MOEs for Cry2Ab2 were approximately greater than or equal to 981,000 and 390,000, respectively. The very large MOEs for both proteins indicate that there are no meaningful risks to human health from dietary exposure to either Cry1A.105 or Cry2Ab2 from consumption of MON 89034.

Table VI.7. Acute Dietary Intake and Margin of Exposure (MOE) for the Cry1A.105 and Cry2Ab2 Proteins from Human Consumption of MON 89034 in the U.S.

Population	Corn Consumption (g/kg bw)[a]	Acute Dietary Intake (mg/kg bw x 10^{-3})[b]		MOE[c]	
		Cry1A.105	Cry2Ab2	Cry1A.105	Cry2Ab2
Overall U.S. population	2.04	10.4	2.24	≥ 199,000	≥ 981,000
Children aged 3-5 y	5.12	26.1	5.63	≥ 79,400	≥ 390,000

[a] 95th percentile estimate by DEEM-FCID of acute consumption of flour, meal, bran, starch, sweet corn and popcorn.

[b] Assuming mean levels of Cry1A.105 and Cry2Ab2 in corn grain of 5.1 and 1.1 µg/g fw, respectively.

[c] MOE = NOEL/Acute Dietary Intake; values rounded to three significant figures. Based on NOELs of 2072 and 2198 mg/kg for Cry1A.105 and Cry2Ab2, respectively, in the acute mouse gavage studies.

3.2. Animal dietary safety assessment

Since corn is a major ingredient in the diets of poultry and livestock, a dietary safety assessment was conducted for the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034. The quantity of corn consumed on a daily basis by poultry and livestock, as well as the levels of Cry1A.105 and Cry2Ab2 proteins in corn, is necessary to derive an estimate of daily dietary intake. Livestock may consume both corn grain and forage (as corn silage). Daily dietary intake is computed as follows:

Daily dietary intake = daily corn consumption (g) x Cry1A.105 or Cry2Ab2 protein level [µg/g dry weight (dwt)][1]

A MOE, defined as the ratio of the NOEL derived from toxicology tests to the estimate of the daily dietary intake, can then be calculated to provide a measure of potential risks to animals from dietary exposure to the Cry1A.105 and Cry2Ab2 proteins in MON 89034.

The exposure calculations make the conservative assumption that there is no loss of the Cry1A.105 and Cry2Ab2 proteins during the processing of corn grain or forage into animal feed. It also assumes that 100% of the corn grain or forage used in animal feed is derived from the MON 89034. This would be the case if a farmer were to feed all

[1] In contrast to the human dietary safety assessment, corn consumption and protein levels are expressed on a 100% dry weight basis for the dietary safety assessment in animals. This is the convention of choice for animal nutritionists when there is variability in dry matter content among dietary ingredients.

harvested corn to livestock on farm. However, larger livestock operations purchase commodity corn that is a blend of many different hybrids.

3.2.1. Corn consumption

The daily U.S. consumption of corn grain is ~ 36 g/kg bw/day (assuming 60% dietary inclusion rate) for the young pig and ~ 22 g/kg bw/day for the finishing pig (assuming 80% dietary inclusion rate) (NRC, 1998). The four-week old broiler consumes ~51 g/kg bw/day of corn grain when the inclusion rate of corn is 60% of the diet (NRC, 1994). The lactating dairy cow consumes about 7.7 g/kg bw/day of corn grain and about 18.2 g/kg bw/day of forage (as corn silage), respectively (Ouellet et al., 2003).

3.2.2. Dietary intake of the Cry1A.105 and Cry2Ab2 proteins

The dietary intakes of the Cry1A.105 and Cry2Ab2 proteins for the broiler chicken, young pig, and finishing pig were estimated using the daily corn grain consumption and the highest levels of the Cry1A.105 (7.0 µg/g dwt) and Cry2Ab2 (2.1 µg/g dwt) proteins in grain (Table VI.4). The highest expression levels were used since there are situations where farmers grow corn for feeding their livestock directly on farm. For the lactating dairy cow, dietary intake was estimated based on the daily corn grain and forage (corn silage) consumption. In forage, the highest levels of Cry1A.105 and Cry2Ab2 were 56 and 55 µg/g dwt, respectively (Table VI.4). Table VI.8 provides estimates of the daily dietary intakes of the Cry1A.105 and Cry2Ab2 proteins by poultry and livestock.

3.2.3. Margins of exposure

The MOE, defined as the ratio of the NOEL to the daily dietary intake, was calculated for the Cry1A.105 and Cry2Ab2 proteins for the broiler chicken, young pig, finishing pig and lactating dairy cow (See Table VI.8). For poultry and livestock, the MOEs ranged between 1,930 – 13,500 and 2,160 – 47,600 for the Cry1A.105 and Cry2Ab2 proteins, respectively. These large MOEs indicate that there is negligible risk to poultry and livestock from dietary exposure to animal feed containing MON 89034.

Table VI.8. Daily Dietary Intake and Margin of Exposure (MOE) for the Cry1A.105 and Cry2Ab2 Proteins from Consumption of MON 89034 by Poultry and Livestock

	Acute Dietary Intake (mg/kg bw x 10^{-3})		MOE[a]	
Animal	**Cry1A.105**	**Cry2Ab2**	**Cry1A.105**	**Cry2Ab2**
Broiler chicken	357	107	5,800	20,500
Young pig	252	76	8,220	29,100
Finishing pig	154	46	13,500	47,600
Lactating dairy cow	1073	1017	1,930	2,160

[a] MOE (for poultry and livestock) = NOEL/daily dietary intake; values rounded to three significant figures. Based on NOELs of 2072 mg/kg for Cry1A.105 and 2198 mg/kg for Cry2Ab2, in the acute mouse gavage studies.

SECTION 4. Assessment of the Potential for Allergenicity of the Cry1A.105 and Cry2Ab2 Proteins Produced in MON 89034

4.1. Approach to the assessment of allergenicity

This assessment of the allergenic potential of the Cry1A.105 and Cry2Ab2 proteins compares the biochemical characteristics of these proteins to characteristics of known allergens. A protein is not likely to be an allergen if:

a) The protein is from a non-allergenic source;
b) The protein does not share structural similarities to known allergens based on the amino acid sequence;
c) The protein is rapidly digested in simulated gastric fluid;
d) The protein represents only a very small portion of the total protein in the grain.

In the following sections, these four characteristics are discussed in detail for the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034. General information on the methods used to assess the structural similarity to known allergens and stability in simulated digestive fluids is provided below.

4.1.1. Rational for studying structural similarity to known allergens

In 2003, the Codex Alimentarius Commission published guidelines for the evaluation of the potential allergenicity of novel proteins (Codex, 2003). The guideline is based on the comparison of amino acid sequences between introduced proteins and known protein allergens. The potential allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with a known allergen over any segment of at least 80 amino acids. The Codex guideline also recommended that a sliding window search with a scientifically justified peptide size, such as eight amino acids, could be used to identify immunologically relevant peptides in otherwise unrelated proteins.

Bioinformatic analyses were performed on Cry1A.105 and Cry2Ab2 proteins expressed in MON 89034 to assess potential similarity to allergens and identify immunologically relevant peptides. The comparisons were performed using the AD6 database, which is an allergen, gliadin, and glutenin sequence database assembled from sequences found on the FARRP allergen database (FARRP, 2006).

The bioinformatics analyses of potential simililarity of Cry1A.105 and Cry2Ab2 amino acid sequences to known allergens reveal no significant matches to known allergens.

4.1.2. Rational for studying stability in simulated digestive fluids

A factor that increases the likelihood of allergic oral sensitization to proteins is the stability of the proteins to gastrointestinal digestion. Protein allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response (Astwood et al., 1996; Metcalfe et al., 1996). Proteins that are rapidly digestible are highly correlated with a

significantly decreased likelihod to cause sensitization or allergic reaction when consumed.

One aspect of this assessment includes analysis of the digestibility of the protein in a simulated gastric fluid (SGF) assay containing pepsin. A relationship between digestibility in SGF and the likelihood of being an allergen has been previously reported with a group of proteins consisting of both allergens and non-allergens (Astwood et al., 1996). Recently, the International Life Science Institute (ILSI) standardized the SGF assay protocol based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). This test showed that the results of *in vitro* pepsin digestion assays are reproducible when standard protocols were followed. Using these protocols, the pepsin digestion assay was used to assess the susceptibility of the Cry1A.105 and Cry2Ab2 proteins to pepsin digestion in vitro.

In addition to SGF, simulated intestinal fluid (SIF) is also used for in vitro studies to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002). SIF is an in vitro digestion model where proteins undergo digestion at neutral pH by a mixture of enzymes known as pancreatin. The relationship between protein allergenicity and protein stability in the in vitro SIF study is limited, because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case in vivo (FAO/WHO, 2001). In vitro susceptibility of Cry1A.105 and Cry2Ab2 proteins to pancreatin was assessed for digestibility in SIF according to methods described in the United States Pharmacopeia (1995).

4.2. Assessment of the potential for allergenicity of the Cry1A.105 protein

4.2.1. Source of the Cry1A.105 protein

Cry1A.105 is a Bt Cry1A chimeric protein with overall amino acid sequence identity of 93.6%, 90.0%, and 76.7% to Cry1Ac, Cry1Ab, and Cry1F proteins, respectively. Data presented in Table VI.1 shows the extremely high homology of the various domains of the Cry1A.105 protein to the respective domains in the Cry1Ab, Cry1Ac and Cry1F proteins. Domains I and II of Cry1A.105 are 100% identical in amino acied sequence to Domains I and II of both Cry1Ab and Cry1Ac. Domain III of Cry1A.105 is 99% identical in amino acid sequence to Domain III of Cry1F. The C-terminal portion of the Cry1A.105 protein is identical to the C-terminal portion of the Cry1Ac protein. Bt is a spore-forming, gram-positive bacterium that is found naturally in soil. Bt strains have been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). There are no known reports of allergies to Bt species or to the proteins produced by these species.

4.2.2. Bioinformatics analyses of sequence similarity of the Cry1A.105 protein produced in MON 89034 to allergens

Using the methods described in Part VI, Section 4.1.1, bioinformatics analyses were performed to assess the potential for allergenicity of the Cry1A.105 protein sequence.

The data generated from these analyses confirm that the Cry1A.105 protein does not share any amino acid sequence similarities with known allergens, gliadins, or glutenins.

The allergen database 6 (AD6) was used for the evaluation of sequence similarities shared between the Cry1A.105 protein and all proteins. Using the FASTA sequence alignment tool, proteins were ranked according to their degree of similarity to Cry1A.105. None of the proteins in the AD6 database met or exceeded the threshold of 35% identity over 80 amino acids. One low quality alignment between *Actinidia deliciosa* kiwifruit actinidin protein was identified, where a stretch of 24.2% identity over a 318 aa (amino acids) window was identified. This alignment had an *E*-score of 2.3. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of 1×10^{-5} or smaller to be considered to have potentially significant homology in sequence. This *E*-score of 2.3 is not reflective of homology between Cry 1A.105 and the kiwifruit protein, as *E*-scores of ~1 or larger are expected to occur for alignments between random, non-homologous sequences (Pearson, 2000). Therefore, this low quality alignment is considered not relevant from an allergenic assessment perspective. Inspection of the remaining alignments also did not show any significant similarities between the Cry1A.105 protein and other allergens.

A second bioinformatics tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known or suspected allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain smaller immunologically significant epitopes. An amino acid sequence may be considered to have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Metcalfe et al., 1996; Hileman et al., 2002). Using a sliding window of less than eight amino acids can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005).

An eight-mer search was performed using an algorithm (ALLERGENSEARCH) that was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the Cry1A.105 sequence and amino acid sequences within AD6. This program compares the Cry1A.105 sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven-amino acid overlap relative to the preceding window. No alignments of eight contiguous amino acid identities were detected when the Cry1A.105 protein sequence was compared to all sequences in the AD6 database.

Together, these data demonstrate that the Cry1A.105 protein does not share any relevant amino acid sequence similarities with known allergens, gliadins, or glutenins.

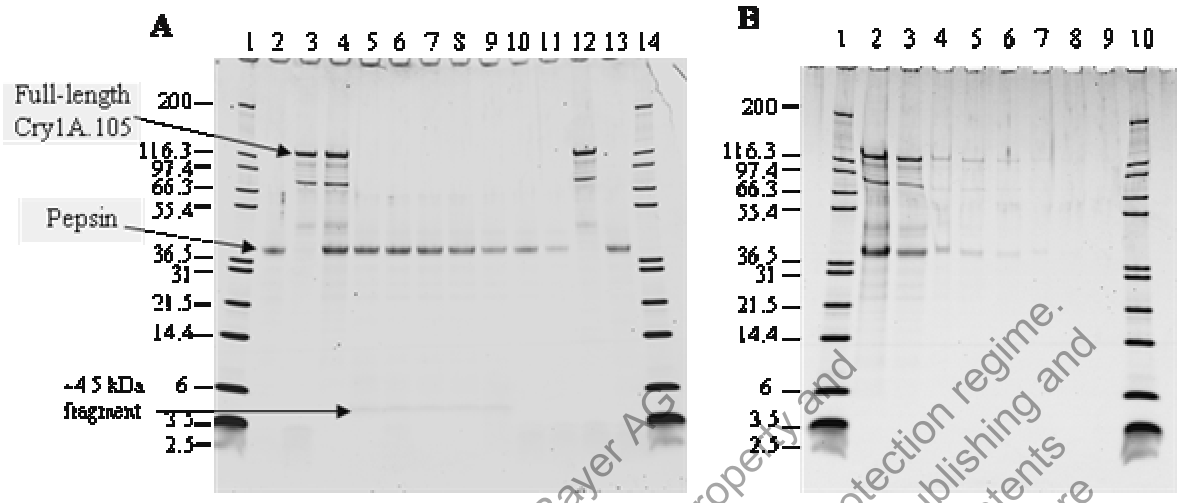
4.2.3. Digestibility of the Cry1A.105 in simulated gastric digestive fluid

Digestibility of the Cry1A.105 protein in simulated gastric fluid (SGF) was assessed by SDS-PAGE and western blot methods. The extent of digestion of the Cry1A.105 protein was evaluated by visual analysis of stained polyacrylamide gels (Figure VI.15) or by visual analysis of developed X-ray film (Figure VI.16). A separate gel or blot was performed concurrently to determine the limit of detection (LOD) of each assay (Figure VI.15, panel B; Figure VI.16, panel B). The limit of detection of the full-length Cry1A.105 protein by Colloidal Brilliant Blue G staining was 0.005 µg or approximately 0.7% of the total Cry1A.105 protein loaded (0.005 µg divided by 0.7 µg of the loaded protein in the test). The limit of detection of the full-length Cry1A.105 protein by western blotting was 1.0 ng or approximately 5% of the total Cry1A.105 protein loaded (1 ng divided by 20 ng of the loaded protein in the test). In both methods, in addition to the full-length Cry1A.105 protein, some bands with lower molecular weight were observed. These bands represent proteolytic fragments of the Cry1A.105 protein that result from proteolysis during the purification procedure.

The gel used to assess the digestibility of the Cry1A.105 protein to pepsin (Figure VI.15, panel A) by Colloidal Brilliant Blue G staining was loaded with 0.7 µg (based on pre-digestion concentrations) for each of the digestion time points. Visual examination of the stained gel showed that the full-length Cry1A.105 protein was digested below LOD within 30 seconds of digestion in SGF (Figure VI.15, panel A, lane 5). Therefore, at least 99.3% (100% – 0.7% = 99.3%) of the full-length Cry1A.105 protein was digested within 30 seconds of incubation based on this analysis. A faint band with a molecular weight of approximately 4.5 kDa was observed at a very low level between the 30-second and 20-minute digestion time points (Figure VI.15, panel A, lanes 5-9). No protein band was visible at the 30-minute digestion time point (Figure VI.15, panel A, lane 10). Since there were only trace amounts of the ~4.5 kDa fragment present in the first 20 minutes of digestion and this fragment was undetectable in the 30 minute time point and beyond, it is unlikely to pose a human health risk.

The gel used to assess the Cry1A.105 protein *in vitro* digestibility by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that the Cry1A.105 protein was digested below the LOD within 30 seconds of incubation in SGF (Figure VI.16, panel A, lane 5). Based on the western blot LOD for the Cry1A.105 protein in SGF and the observation that no full-length protein or immunoreactive bands were observed on the western blot at the 30-second digestion time point, it was concluded that greater than 95% $\left(100\% - 5\% = 95\%\right)$ of the full-length Cry1A.105 protein was digested within 30 seconds of incubation with SGFs.

The results of this study demonstrated that the full-length Cry1A.105 protein was rapidly digested after incubation in SGF. The full-length Cry1A.105 protein was digested below the LOD within 30 seconds when analyzed using Colloidal Brilliant Blue G staining or by western blotting. There were no stable proteolytic degradation products, with the exception of a very faint band detected by Colloidal Brilliant Blue G staining with a MW of ~4.5 kDa; this band was not observed in the 30-minute time point or beyond.



Lane assignment for Panel A

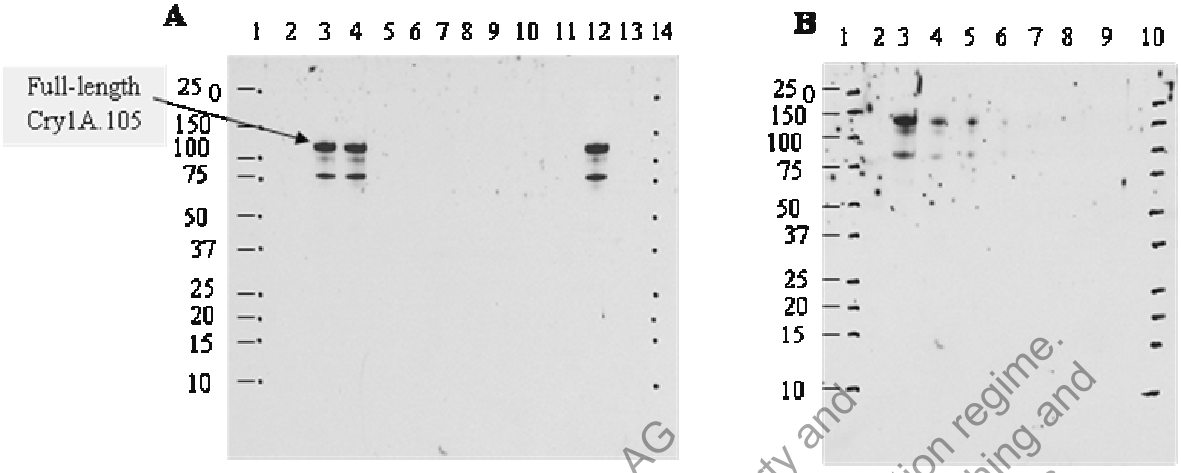
Lane	Sample	Incubation Time (min)
1	MW marker	—
2	N0, SGF only	0
3	P0, protein only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	MW marker	—

Lane assignment for Panel B

Lane	Sample	Amount (µg)
1	MW marker	—
2	T0, protein+SGF	0.7
3	T0, protein+SGF	0.35
4	T0, protein+SGF	0.1
5	T0, protein+SGF	0.05
6	T0, protein+SGF	0.02
7	T0, protein+SGF	0.01
8	T0, protein+SGF	0.005
9	T0, protein+SGF	0.0025
10	MW marker	—

Figure VI.15. Colloidal Brilliant Blue G Stained SDS-gels of Cry1A.105 Protein Digestion in SGF

Panel A corresponds to Cry1A.105 protein digestion in SGF. Based on pre-digestion protein concentrations, 0.7 µg (total Cry1A.105 protein) was loaded in lanes containing Cry1A.105 protein. The incubation times are indicated. Panel B corresponds to the limit of detection (LOD) of Cry1A.105 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.



Lane assignment for Panel A

Lane	Sample	Incubation Time (min)
1	MW marker	—
2	N0, SGF only	0
3	P0, protein only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	MW marker	—

Lane assignment for Panel B

Lane	Sample	Amount (ng)
1	MW marker	—
2	T0, protein+SGF	7
3	T0, protein+SGF	3.5
4	T0, protein+SGF	2
5	T0, protein+SGF	1
6	T0, protein+SGF	0.5
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	MW marker	—

Figure VI.16. Western blot Analysis of Cry1A.105 Protein Digestion in SGF

Panel A corresponds to Cry1A.105 protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in lanes containing Cry1A.105 protein. Panel B corresponds to the limit of detection (LOD) of the Cry1A.105 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.

4.2.4. Digestibility of the Cry1A.105 protein in simulated intestinal fluid

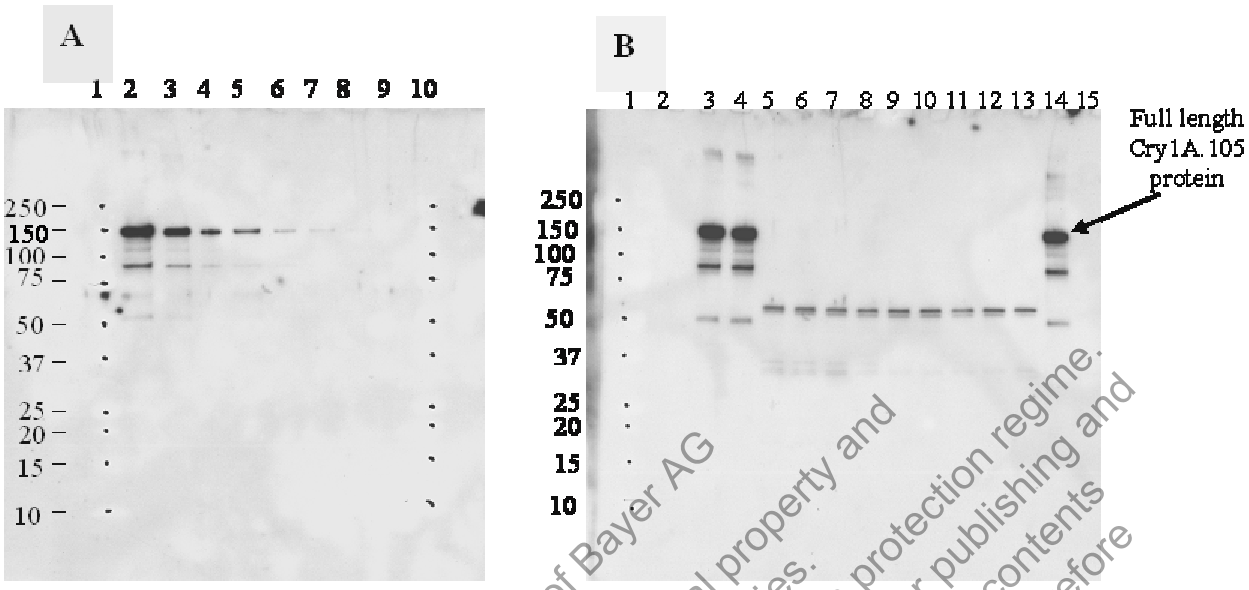
The digestibility of the Cry1A.105 protein in SIF, which contains a mixture of enzymes called pancreatin.

The digestion of the Cry1A.105 protein was evaluated by western blot method (Figure VI.17). A western blot to determine the LOD (Figure VI.17, panel A) of the Cry1A.105 protein was performed concurrently with the western blot used to assess the Cry1A.105 protein digestibility in SIF (Figure VI.17, panel B). The LOD was estimated to be 0.1 ng, which represented 0.5% of the total protein loaded in this experiment (0.1 ng divided by 20 ng of loaded protein).

The gel to assess the Cry1A.105 protein SIF digestibility by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that the full-length Cry1A.105 protein was digested below the LOD within 5 minutes of incubation in SIF (Figure VI.17, panel B, lane 5). Therefore, at least 99.5% (100% - 0.5% = 99.5%) of the full-length Cry1A.105 protein was broken down within 5 minutes. Proteolytic fragments with approximate molecular weight of ~60, 32 and 30 kDa were observed at the 5 minute digestion time point and were stable for the various times up to 24 hours of digestion in SIF.

The results of this study demonstrate that the full-length Cry1A.105 protein was digested within 5 min of incubation in SIF, yielding fragments with molecular weights of approximately 60, 32, and 30 kDa. At least 99.5% of the full-length Cry1A.105 protein was digested in SIF within 5 minutes. The major proteolytic fragment at approximately 60 kDa migrated as a doublet, which represents the tryptic core of the Cry1A.105 protein, was observed for up to 24 hours (the longest time point tested). These results are consistent with observations for other Cry proteins with demonstrated safety.

Overall, the results for digestibility of Cry1A.105 are consistent with proteins with demonstarted safety. The fact that Cry1A.105 is readily digestible in simulated gastric fluid makes it unlikely be a food allergen.



Lane assignment for Panel A

Lane	Sample	Amount (ng)
1	Molecular weight marker	—
2	T0, protein+SIF	10
3	T0, protein+SIF	5
4	T0, protein+SIF	2
5	T0, protein+SIF	1
6	T0, protein+SIF	0.5
7	T0, protein+SIF	0.2
8	T0, protein+SIF	0.1
9	T0, protein+SIF	0.05
10	Molecular weight marker	—

Lane assignment for Panel B

Lane	Sample	Incubation Time
1	Molecular weight marker	—
2	N0, SIF only	0
3	P0, protein only	0
4	T0, protein+SIF	0
5	T1, protein+SIF	5 min
6	T2, protein+SIF	15 min
7	T3, protein+SIF	30 min
8	T4, protein+SIF	1 h
9	T5, protein+SIF	2 h
10	T6, protein+SIF	4 h
11	T7, protein+SIF	8 h
12	T8, protein+SIF	12 h
13	T9, protein+SIF	24 h
14	P9, protein only	24 h
15	N9, SIF only	24 h

Figure VI.17. Western Blot Analysis of Cry1A.105 protein Digestion in SIF

Panel A corresponds to the limit of detection of Cry1A.105 protein. Panel B corresponds to Cry1A.105 protein digestion in SIF. Based on the pre-digestion protein concentration, 20 ng (total protein) was loaded in lanes containing Cry1A.105 protein. The digestion times are indicated. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel.

4.2.5. Proportion of Cry1A.105 protein to the total protein in MON 89034 grain

The overall mean level of Cry1A.105 protein in MON 89034 grain is 5.9 µg/g (dwt) (Part VI, Section 2). The mean % dry weight of total protein in MON 89034 grain is 12.51% (or 125,100 µg/g). The percent of Cry1A.105 protein in MON 89034 grain is calculated as follows:

(5.9 µg/g ÷ 125,100 µg/g) x 100% = 0.0047%

Therefore, the Cry1A.105 protein represents a very small portion of the total protein in MON 89034 grain.

4.3. Assessment of the potential for allergenicity of the Cry2Ab2 protein

4.3.1. Source of the Cry2Ab2 protein

The Cry2Ab2 protein is a variant of the wild-type Cry2Ab2 protein isolated from Bt subsp. *kurstaki*, which is a spore-forming, gram-positive bacterium found naturally in soil. Bt strains have been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). There are no known reports of allergies to Bt species or the proteins produced from these species.

4.3.2. Bioinformatics analysis of sequence similarity of the Cry2Ab2 protein produced in MON 89034 to allergens

Using the methods described in Part VI, Section 4.1.1, potential structural similarities shared between the Cry2Ab2 protein and proteins in the allergen database were evaluated using the bioinformatic tool.

FASTA comparision results showed that the greatest similarity to the Cry2Ab2 protein was to the *Coprinus comatus* protein Cop c1, demonstrating only 32.7% identity over a 52 aa (amino acids) window with an E score of 0.89. This protein did not meet or exceeded the threshold of 35% identity over 80 amino acids, and based on the low percent-identity and the small alignment window relative to the length of the Cry2Ab2 protein (637 aa), this FASTA alignment does not represent a *bona fide* homology (Doolittle, 1990). Therefore, this low quality alignment is considered not relevant from an allergenic assessment perspective. Inspection of the remaining alignments also did not show any significant similarities between the Cry2Ab2 protein and other allergens. The pair-wise comparison of eight-mer search results showed that no immunologically relevant sequences (eight contiguous amino acid identities) were detected when the Cry2Ab2 protein sequence was compared to the sequence database.

Together these data demonstrate that the Cry2Ab2 protein is unlikely to share structurally relevant or immunologically relevant sequence similarities with known allergens, gliadins, or glutenins.

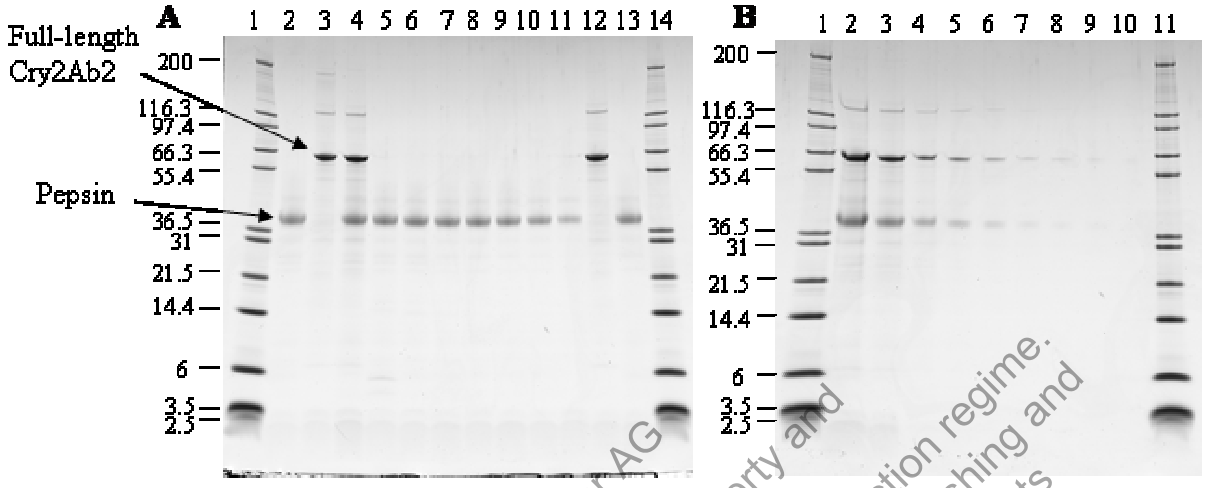
4.3.3. Digestibility of the Cry2Ab2 protein in simulated gastric fluid

Digestibility of the Cry2Ab2 protein in SGF was assessed by SDS-PAGE and western blot methods. The extent of digestion of the Cry2Ab2 protein was evaluated by visual analysis of stained polyacrylamide gels (Figure VI.18) or by visual analysis of developed X-ray film (Figure VI.19). A separate gel or blot was performed concurrently to determine the limit of detection (LOD) of each assay (Figure VI.18, panel B; Figure VI.19, panel B). The limit of detection of the full-length Cry2Ab2 protein by Colloidal Brilliant Blue G staining was 0.005 µg or approximately 0.6% of the total Cry2Ab2 protein loaded (0.005 µg divided by 0.8 µg of the loaded protein in the test). The limit of detection of the full-length Cry2Ab2 protein by western blotting was 0.2 ng or approximately 1% of the total Cry2Ab2 protein loaded (0.2 ng divided by 20 ng of the loaded protein in the test).

Digestibility of the Cry2Ab2 protein in SGF assessed using stained SDS-polyacrylamide gels showed that the full-length Cry2Ab2 protein was rapidly digested. At least 99.4% of the full-length Cry2Ab2 protein was digested within 30 seconds when analyzed using Colloidal Brilliant Blue G stained polyacrylamide gels (Figure VI.18). A very faint band with molecular weight of ~5 kDa was observed at the 30-second digestion time point. No Cry2Ab2 bands were visible at the 2-minute digestion time point (Figure VI.18, panel A, lane 6).

The extent of digestion of the Cry2Ab2 protein was also evaluated by western blot method (Figure VI.19). At least 99% of the Cry2Ab2 protein was digested in SGF within 30 seconds when analyzed using western blot analysis (Figure VI.19, panel A). No stable proteolytic bands were observed at any time points by western blot analysis.

The results of this study demonstrated that Cry2Ab2 protein was rapidly digested after incubation in SGF. The Cry2Ab2 protein was digested below the LOD within 30 seconds when analyzed using SDS-PAGE by Colloidal Brilliant Blue G staining or by western blotting. There were no stable proteolytic degradation products detected.



Lane assignment for Panel A

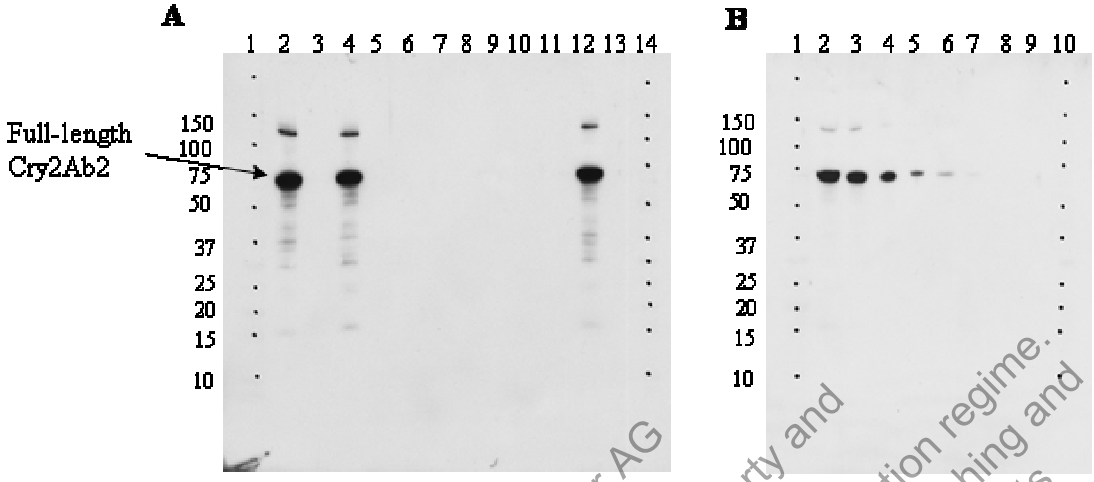
Lane	Sample	Incubation Time (min)
1	Molecular weight marker	n/a
2	N0, SGF only	0
3	P0, protein only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	Molecular weight marker	n/a

Lane assignment for Panel B

Lane	Sample	Amount (µg)
1	Molecular weight marker	n/a
2	T0, protein+SGF	1
3	T0, protein+SGF	0.5
4	T0, protein+SGF	0.2
5	T0, protein+SGF	0.1
6	T0, protein+SGF	0.05
7	T0, protein+SGF	0.02
8	T0, protein+SGF	0.01
9	T0, protein+SGF	0.005
10	T0, protein+SGF	0.0025
11	Molecular weight marker	n/a

Figure VI.18. Colloidal Brilliant Blue G Stained SDS-PAGE Gels of Cry2Ab2 Protein Digestion in SGF

Panel A corresponds to Cry2Ab2 protein digestion in SGF. Based on the pre-digestion protein concentration, ~0.8 µg (total protein) was loaded in lanes containing Cry2Ab2 protein. Panel B corresponds to the limit of detection (LOD) of Cry2Ab2 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.



Lane assignment for Panel A

Lane	Sample	Incubation Time (min)
1	Molecular weight marker	n/a
2	P0, protein only	0
3	N0, SGF only	0
4	T0, protein+SGF	0
5	T1, protein+SGF	0.5
6	T2, protein+SGF	2
7	T3, protein+SGF	5
8	T4, protein+SGF	10
9	T5, protein+SGF	20
10	T6, protein+SGF	30
11	T7, protein+SGF	60
12	P7, protein only	60
13	N7, SGF only	60
14	Molecular weight marker	n/a

Lane assignment for Panel B

Lane	Sample	Amount (ng)
1	Molecular weight marker	n/a
2	T0, protein+SGF	10
3	T0, protein+SGF	5
4	T0, protein+SGF	2
5	T0, protein+SGF	1
6	T0, protein+SGF	0.5
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	Molecular weight marker	n/a

Figure VI.19. Western Blot Analysis of Cry2Ab2 Protein Digestion in SGF

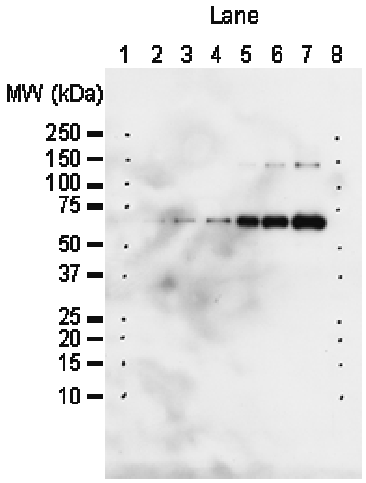
Panel A corresponds to Cry2Ab2 protein digestion in SGF. Based on the pre-digestion protein concentration, 20 ng (total protein) was loaded in lanes containing Cry2Ab2 protein. Panel B corresponds to the limit of detection of the Cry2Ab2 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.

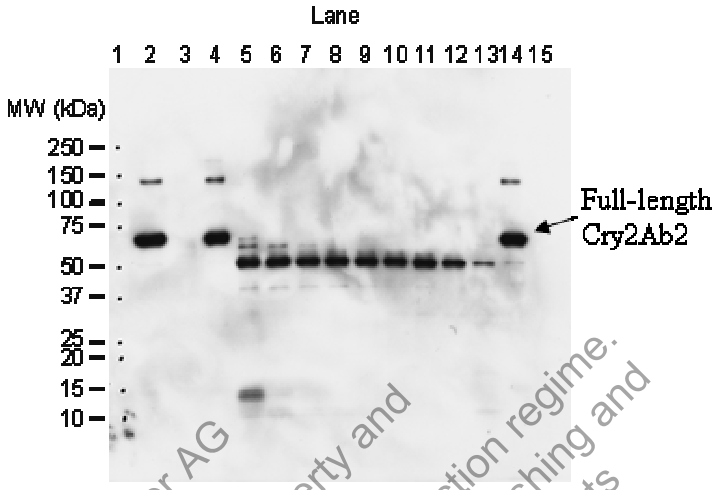
4.3.4. Digestibility of the Cry2Ab2 protein in Simulated Intestinal Fluid

The *in vitro* digestibility of the Cry2Ab2 protein in SIF was assessed by western blot analysis. A western blot to determine the LOD (Figure VI.20, panel A) of the Cry2Ab2 protein was run concurrently with the western blot used to assess the Cry2Ab2 protein digestibility in SIF (Figure VI.20, panel B). The LOD was determined by the lowest amount of Cry2Ab2 protein observed on the X-ray film under visual inspection. The LOD was estimated to be 0.5 ng, which represents 2.5% of the total protein (0.5 ng divided by 20 ng of loaded protein in the test).

The gel used to assess the Cry2Ab2 protein digestibility by western blot was loaded with 20 ng total protein (based on pre-digestion concentrations) for each of the incubation time points. Western blot analysis demonstrated that a band corresponding to the full-length Cry2Ab2 protein was digested below the LOD within 15 minutes of incubation in SIF (Figure VI.20, panel B, lane 6). Therefore, at least 97.5% (100% - 2.5% = 97.5%) of the full-length Cry2Ab2 protein was digested within 15 minutes. Proteolytic bands with approximate molecular weight of 60, 55, 50, 40, 12 and 10 kDa were observed at the 5-minute time point. Several new bands with molecular weights smaller than 50 kDa were detectable beginning at the 4-hour digestion time point. These bands, which were transient in nature and displayed a weak immunoreactive signal, were detectable at the 24-hour incubation time point.

Overall, the results for digestibility of Cry2Ab2 are consistent with other Cry proteins with demonstrated safety. The fact that Cry2ABb2 is readily digestible in simulated gastric fluid makes it unlikely be a food allergen





Lane assignment for Panel A

Lane	Sample	Amount (ng)
1	Molecular weight marker	—
2	T0, protein + SIF	0.5
3	T0, protein + SIF	1
4	T0, protein + SIF	2
5	T0, protein + SIF	5
6	T0, protein + SIF	10
7	T0, protein + SIF	20
8	MW marker	—

Lane assignment for Panel B

Lane	Sample	Incubation Time
1	Molecular weight marker	—
2	P0, protein only	0
3	N0, SIF only	0
4	T0, protein + SIF	0
5	T1, protein + SIF	5 min
6	T2, protein + SIF	15 min
7	T3, protein + SIF	30 min
8	T4, protein + SIF	1 h
9	T5, protein + SIF	2 h
10	T6, protein + SIF	4 h
11	T7, protein + SIF	8 h
12	T8, protein + SIF	12 h
13	T9, protein + SIF	24 h
14	P9, protein only	24 h
15	N9, SIF only	24 h

Figure VI.20. Western Blot Analysis of Cry2Ab2 Protein Digestion in SIF

Panel A corresponds to the LOD of Cry2Ab2 protein (5 min exposure). Panel B corresponds to Cry2Ab2 protein digestion in SIF (5 min exposure). Based on the pre-digestion concentration, 20 ng (total protein) was loaded in lanes containing Cry2Ab2 protein. The incubation times are indicated. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel.

4.3.5. Proportion of Cry2Ab2 protein to the total protein in MON 89034 grain

The mean level of Cry2Ab2 protein in corn grain is 1.3 µg/g (dwt) (Part VI, Section 2). The mean % dry weight of total protein in MON 89034 grain is 12.51% (or 125,100 µg/g). The percent of Cry2Ab2 protein in MON 89034 grain is calculated as follows:

(1.3 µg/g ÷ 125,100 µg/g) x 100% = 0.0010 %

Therefore, the Cry2Ab2 protein represents a very small portion of the total protein in MON 89034 grain.

4.4. Conclusions

The Cry1A.105 and Cry2Ab2 proteins have been assessed for their potential allergenicity according to the recommendations of Codex Alimentarius Commission. The proteins are from non-allergenic sources, lack structural similarity to known allergens, are rapidly digested in simulated gastric fluid, and constitute a very small portion of the total protein present in the grain of MON 89034.

The Cry1A.105 and Cry2Ab2 proteins are derived from *Bacillus thuringiensis*, an organism that is not a commonly allergenic source, and the Cry class of proteins has a long history of safe use. Bioinformatics analyses demonstrated that the proteins do not share structurally or immunologically relevant amino acid sequence similarities with known allergens. Thus, the Cry1A.105 and Cry2Ab2 proteins are highly unlikely to contain immunologically cross-reactive allergenic epitopes. Digestive fate experiments conducted with Cry1A.105 or Cry2Ab2 proteins demonstrated that both proteins are rapidly digested in simulated gastric fluid, a characteristic shared among many proteins with a history of safe consumption. Finally, the Cry1A.105 and Cry2Ab2 proteins represent no more than 0.005% and 0.001% of the total protein in the grain of MON 89034, respectively. Taken together these data lead to the conclusion that the Cry1A.105 and Cry2Ab2 proteins are unlikely to be allergenic, and MON 89034 is as safe as conventional corn regarding the risk for allergenicity.

SECTION 5. Assessment of the Potential for Toxicity of the Cry1A.105 and Cry2Ab2 Proteins

5.1. Approach to the assessment of toxicity

The previous section described an assessment of the potential for allergenicity of the Cry1A.105 and Cry2Ab2 proteins in MON 89034 based on the sources of the proteins, a comparison of their sequence to known allergens, their digestibility in simulated digestive fluids, and finally their proportion to the total protein in MON 89034. In this section, an assessment of a potential for toxicity of the Cry1A.105 and Cry2Ab2 proteins is conducted. This assessment is based on the established premise that a protein is not likely to have a toxic effect if:

a) The protein has a demonstrated history of safe use;
b) The protein has no structural similarity to known toxins or other biologically active proteins that could cause adverse effects in humans or animals;
c) The protein does not exert any acute toxic effects to mammals.

In addition, the low concentration of the introduced proteins in tissues that are consumed and the rapid digestibility in simulated digestive fluids provide further assurance for their safety.

The potential for synergistic or antagonistic effects between the Cry1A.105 and Cry2Ab2 proteins was considered for assessment of potential toxicity. To date, there have been only a few compelling examples of interactive effects between Cry proteins that have either decreased (antagonism) or increased (synergism) activity towards target pests when combined (Tabashnik 1992, Schnepf et al. 1998). Demonstrating the lack of interaction between the two Cry proteins allows for each of them to be tested independently in safety assessment studies. The principle of independent assessment has been used for many years for food additives and microbial risk assessments (EPA, 2004). The Cry1A.105 and Cry2Ab2 proteins were tested alone and in combination against two sensitive lepidopteran species – the European corn borer (ECB) and corn earworm (CEW). Two species were tested to demonstrate reproducibility thereby strengthening the validity of the study. The insects were exposed to purified Cry1A.105 and Cry2Ab2 proteins in diet-incorporation bioassays. Results demonstrated that when tested in combination, the insecticidal activities of the two proteins were additive - no synergistic or antagonistic effects were observed. These results are consistent with the results from a study that demonstrated no interaction between the Cry1Ac and Cry2Ab2 proteins (Greenplate et al, 2003). Therefore, it is appropriate that the safety of each protein be assessed independently in the safety assessments.

5.2. Safety of the donor organism: *Bacillus thuringiensis*

Bacillus thuringiensis is a spore-forming, gram-positive bacterium that is found naturally in soil. Bt strains have been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). Many Bt microbial

strains have been shown to produce protein crystals or inclusion bodies that are selectively toxic to certain orders and species of insect pests. The current nomenclature, based on amino acid identity, allows closely related proteins to be ranked together. Each protein is assigned a unique name incorporating four hierarchical ranks consisting (in order) of an Arabic numeral, upper case letter, lower case letter, and an Arabic numeral (e.g., Cry2Ab2 protein). Thus, proteins with identity of:

a) <45% differ in a primary rank (e.g., Cry2, Cry3, etc.),
b) >45% but <78% differ in secondary rank (e.g., Cry3A, Cry3B),
c) >78% but <95% differ in tertiary rank (e.g., Cry3Ba, Cry3Bb), and
d) >95% differ in quaternary rank (e.g., Cry2Ab1, Cry2Ab2) and are considered allelic variants.

In general, the primary rank of the Cry proteins denotes its specific insecticidal activity; for example, Cry1, Cry2, Cry3, and Cry4 proteins are toxic to lepidopteran, lepidopteran/dipteran, coleopteran, and dipteran pests, respectively (Bravo, 1997; Höfte and Whitely, 1989). It has been established that Cry proteins have a narrow range of insecticidal activity against one or, less commonly, two orders of insects (Crickmore et al., 1998).

In the U.S., the FDA granted an exemption from the requirement of a tolerance for the first microbial Bt product in 1960 after an extensive toxicity and infectivity evaluation program. The testing program consisted of acute, subchronic, and chronic studies, which resembled the testing required for conventional chemical pesticides. In 1971, EPA assumed responsibility for all pesticide tolerance exemptions. Since then, a variety of naturally occurring and genetically modified microbial Bt products have been registered and included under this tolerance exemption. EPA has established separate tolerance exemptions by amendment for various Cry proteins (e.g., Cry1Ab, Cry1Ac, Cry2Ab2, Cry3Bb1) expressed in biotechnology-derived crops (EPA 1996, 1997, 2001). The conclusion of reasonable certainty of no harm and the resultant tolerance exemptions for this wide array of Bt mixtures and Cry proteins in food or feed were based on the lack of adverse effects to mammals in numerous toxicological studies. This conclusion is supported by a history of safe use in agriculture for over 40 years (McClintock et al., 1995). There are no adverse effects known to have occurred in humans during this prolonged period of use (EPA, 1998). An extensive review of the safety of the various Cry proteins that have been expressed in biotechnology-derived plants is available (Betz et al., 2000).

5.3. Assessment of the potential for toxicity of the Cry1A.105 protein

In this section the potential toxicity of the Cry1A.105 protein is discussed. The assessment includes comparing the similarity of Cry1A.105 to proteins with an established history of safe use and consumption, bioinformatics analyses of structural similarity of the Cry1A.105 protein to known toxins and other proteins that may adversely affect animal or human health, and testing of acute oral toxicity.

5.4. Similarity of Cry1A.105 to Cry1A proteins with a history of safe use and consumption

5.4.1. Structural similarity of the Cry1A.105 protein with Cry1A class of Bt proteins

As previously described (Part VI, Section 4.2.1), Cry1A.105 is a chimeric protein with overall amino acid sequence identity of Cry1A.105 to the Cry1Ac, Cry1Ab, and Cry1F proteins is 93.6%, 90.0%, and 76.7%, respectively. To better understand the structural relatedness of the Cry1A.105 protein to other Cry proteins, a phylogenetic tree (Figure VI.20) was produced using the Cry1A.105 amino acid sequence and the methods employed to define the Bt protein nomenclature (Crickmore, 2004; Crickmore et al., 1998). The analysis demonstrates that Cry1A.105 belongs to the Cry1A class of Bt proteins and is most closely related (93.6% identical) to the Cry1Ac protein.

Recombinant DNA techniques have been used to generate Bt strains with enhanced insecticidal activity for use as biopesticides (Baum, 1998 and 1999). For example, the microbial pesticide Lepinox that contains a Cry1Ac/Cry1F chimeric protein has been approved and used for control of lepidopteran pests since 1997 (Baum, 1998; Baum et al., 1999).

Cry1A proteins are widely used as topical bioinsecticides and in biotechnology-derived crops. The US FDA completed consultations for the safety assessment of corn that produces the Cry1Ab (YieldGard – MON 810 and Bt11) and Cry1F (Herculex® I) proteins, as well as cotton producing the Cry1Ac (Bollgard and Bollgard II, WideStrike®) and Cry1F (WideStrike) proteins. The U.S. EPA, which has responsibility of assessment the safety of pesticidal proteins, issued exemptions from the requirement of a tolerance for the Cry1Ab protein in corn (YieldGard – MON 810 and Bt11), the Cry1Ac protein in cotton Bollgard and WideStrike, and the Cry1F protein in corn Herculex I and cotton WideStrike.

The detailed human and animal safety assessments conducted on the Cry1Ab, Cry1Ac, and Cry1F proteins, combined with a history of safe use, confirm their safety. Due to the chimeric nature of Cry1A.105, which was derived from domains of Cry1Ab, Cry1Ac, and Cry1F, a similar safety profile is expected for Cry1A.105.

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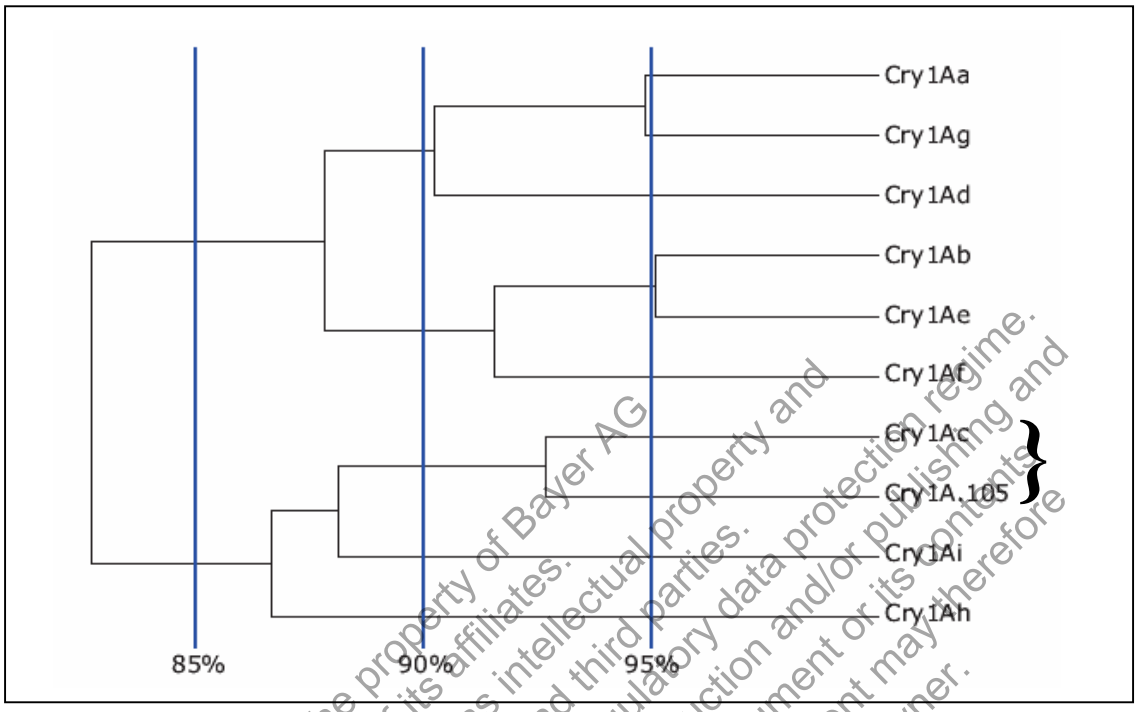

Figure VI.21. Phylogram Establishing that the Cry1A.105 and Cry1Ac Proteins are Located in the Same Cluster Based on Amino Acid Sequence Identity

5.4.2. Functional similarity of the Cry1A.105 Protein to the Cry1A class of Bt proteins

Having established the high structural similarity between the Cry1A.105 protein and the Cry1A class of proteins, it is important also to establish the functional similarity. To assess the function of the Cry1A.105 protein, its biological activity was compared to that of the related proteins Cry1Ab, Cry1Ac, and Cry1F. Amino acid sequence identity for insecticidal proteins predict similarity in biological function, i.e., activity towards a similar spectrum of insects.

It has been established that Cry proteins have a defined spectrum of insecticidal activity within a particular insect order (Crickmore et al., 1998; De Maagd et al., 2001). This high degree of specificity is governed by four levels of selectivity, which collectively lead to intoxication (Federici, 2002). The levels of selectivity include: 1) the route by which the insect is exposed to Cry proteins (i.e., ingestion of plant tissues); 2) protein toxin activation by specific proteolytic enzymes (determined by differences in gut physiology between insects); 3) toxin binding to available specific midgut receptors; and 4) changes in the protein configuration. The reconfigured protein then is able to enter the midgut membrane and form channels. This activity affects larval ability to feed and develop, and eventually leads to death of the susceptible insect.

As a consequence, only insects with specific receptors are affected and no toxicity is observed in species that lack these receptors. For example, the Cry1Ab, Cry1Ac and Cry1F proteins are active against lepidopteran but not coleopteran insects, and the Cry3Bb1 protein is active against coleopteran insects such as corn rootworms but not against lepidopteran insects.

Studies were conducted to evaluate the insecticidal activity of the Cry1A.105 protein against a variety of agronomically important insects from three major taxa. Insect species tested included four representative lepidopterans: black cutworm, corn earworm, fall armyworm, and European corn borer; two representative coleopterans: boll weevil (*Anthonomus grandis grandis*) and southern corn rootworm (*Diabrotica undecimpunctata howardi*); and two representative hemipterans: western tarnished plant bug (*Lygus hesperus*) and green peach aphid (*Myzus persicae*). The insects were exposed to high concentrations of Cry1A.105 protein (up to 80 or 100 μg of protein per ml of insect diet in diet-incorporation bioassays). The results showed that Cry1A.105 had activities against all four representative lepidopteran insects. However, there was no indication of activity by Cry1A.105 against the two coleopteran or two hemipteran representatives. This activity spectrum of Cry1A.105 is similar to the activities of the Cry1Ac, Cry1Ab, and Cry1F proteins described in the literature (De Maagd et al., 2001 and 2003). Similarity in biochemical and functional characteristics between proteins with established safety is a good indication of the general safety of that particular class.

5.4.3. Structural similarity of Cry1A.105 to known toxins or other biologically active proteins

The safety assessment of proteins expressed in biotechnology-derived crops evaluates potential health effects through a comprehensive approach, which includes bioinformatic analysis of the amino acid sequence of the introduced protein to ensure the protein is not similar to proteins that are known to cause adverse health effects.

The FASTA sequence alignment tool was used to assess structural similarity. Although the FASTA program directly compares amino acid sequences (i.e., primary protein structure), the alignment data may be used to infer secondary and tertiary protein structural similarities. Proteins that share a high degree of similarity throughout their entire lengths are often homologous. Homologous proteins share secondary structure and common three-dimensional configuration. Structural similarities between the Cry1A.105 protein sequence and the toxin (TOXIN5) and public domain (ALLPEPTIDES) database sequences were examined. The extent of each similarity was evaluated by visual inspection of the alignment, the calculated percent identity, and the *E* score value for that alignment.

The results showed that no biologically relevant structural similarities to human and animal toxins were observed for the Cry1A.105 protein sequence.

5.4.4. Acute oral toxicity study with the Cry1A.105 protein

An oral acute toxicity study was conducted with *E. coli*-produced Cry1A.105 protein. The *E. coli*-produced Cry1A.105 protein was shown to be equivalent to the MON 89034-

produced Cry1A.105 (see Part VI, Section 1.2). Acute administration was considered appropriate to assess the potential toxicity of Cry1A.105 protein because toxic proteins generally act via acute mechanisms (Pariza and Foster, 1983; Sjoblad et al., 1992; Pariza and Johnson, 2001).

Two groups of ten male and ten female young adult CD1 mice received an acute high dose of the Cry1A.105 protein by oral gavage. The target dose of 2072 mg/kg body weight was based on the maximum attainable Cry1A.105 concentration of the dosing solution (estimated at 34.1 mg/mL) and a total dose volume of 66.6 mL/kg body weight. The limited solubility of the Cry1A.105 protein precluded its administration as a single dose. Therefore, dosing was subdivided into two doses of 33.3 mL/kg body weight (66.6 mL/kg total) to achieve the target dose of 2072 mg/kg body weight. On the day of dosing (Day 0) the two individual doses of 33.3 mg/mL body weight were administered about four hours apart. A separate group of ten male and ten female animals served as protein control group and received bovine serum albumin (BSA) at a dose of 1998 mg/kg body weight. In addition, the vehicle control (buffer) groups of ten males and ten females received the dosing vehicle substance only.

Following dosing, all mice were observed daily, and body weights and food consumption were measured weekly. A gross necropsy examination was performed on all animals at the time of death or the end of the study (day 14).

No test article related mortality or clinical observations were recorded. There were no significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and the Cry1A.105 treated group. No treatment–related gross pathological findings were observed at necropsy.

Under the conditions of this test, no test article related mortality or other toxicity was observed in the Cry1A.105 treated group. Therefore, the acute oral LD_{50} of the Cry1A.105 protein in mice is greater than 2072 mg/kg body weight, which was determined to be NOEL for this protein.

5.4.5. Exposure to the Cry1A.105 protein

Cry1A.105 is present at low levels of approximately 0.005% of the total protein in the grain of MON 89043 (Part VI, Section 4.2.5). A dietary safety assessment based on these levels indicates that the MOEs for Cry1A.105 are equal to or greater than 199,000 for the overall U.S. population and 79,400 for children aged 3-5 years old, an age group with the highest corn consumption on a body weight basis (see Part VI, Section 3). Also, greater than 95% of Cry1A.105 is digested after 30 seconds incubation in SGF (Part VI, Section 4.2.3). These results suggest that there are no meaningful risks to human and animal health from the dietary exposure to the Cry1A.105 protein.

5.5. Assessment of the potential for toxicity of the Cry2Ab2 protein

The safety assessment of the Cry2Ab2 protein includes an assessment of the similarity of the Cry2Ab2 protein to proteins with an established history of safe use and consumption, bioinformatics analyses of structural similarity of the Cry2Ab2 to known toxins and other proteins that may adversely affect animal or human health, and acute oral toxicity.

5.5.1. Similarity of the Cry2Ab2 protein with proteins with a history of safe use and consumption

The Cry2Ab2 protein produced in MON 89034 is derived from the Bt subspecies *kurstaki* and its amino acid sequence differs from that of the wild-type protein by a single amino acid. The Cry2Ab2 protein has 88% amino acid sequence identity to the Cry2Aa protein, which is present in commercial microbial pest control products such as Dipel and Crymax. The Cry2Ab2 proteins produced in MON 89034 and Bollgard II cotton share an identical amino acid sequence. Bollgard II cotton has been on the market since 2003 and there have been no reports of adverse effects on human or animal health.

5.5.2. Structural similarity of the Cry2Ab2 to known toxins or other biologically active proteins

The safety assessment of proteins produced in genetically modified crops evaluates potential health effects through a comprehensive approach, which includes bioinformatic analysis of the amino acid sequence of the newly expressed protein to ensure that the protein is not similar to toxic proteins that are known to cause adverse health effects. The comparison of the Cry2Ab2 sequence was performed with the toxin (TOXIN5), and public domain (ALLPEPTIDES) database sequences using bioinformatic tools. Sequence identity and amino acid similarity to allergens, toxins, and other proteins was assessed using the sequence alignment tool FASTA. Identified proteins were ranked according to their degree of similarity. The highest similarity observed was to pesticidial crystal protein Cry2Ab, demonstrating 100% identity over 632 amino acids with and an E score of zero. All remaining alignments with significant *E* scores are to Cry protein homologues derived from *B. thuringiensis*, *Paenibacillus popilliae* or *Paenibacillus lentimorbus*. Based on these data, the Cry2Ab2 protein does not share structural congruence with any proteins that may cause adverse effects in humans and animals.

5.5.3. Acute oral toxicity study with Cry2Ab2 protein

An acute oral toxicity assessment was conducted to evaluate potential adverse effects on mice as a result from exposure to *E. coli*-produced Cry2Ab2 protein. The Cry2Ab2 protein produced in *E. coli* was shown to be equivalent to the MON 89034-produced Cry2Ab2.

Two groups of ten male and ten female CD1 mice received an acute high dose of the Cry2Ab2 protein by gavage. The target dose 2,198 mg/kg body weight was based on the maximum attainable Cry2Ab2 concentration of the dosing solution (estimated at 37 mg/mL) and a total dose volume of 66.6 mL/kg body weight. The limited solubility of the Cry2Ab2 protein precluded its administration as a single dose. Therefore, dosing was

subdivided into two doses of 33.3 mL/kg body weight (66.6 mL/kg total) to achieve the target dose of 2,198 mg/kg. On the day of dosing (Day 0) the two individual doses of 33.3 mg/mL body weight were separated by approximately four hours. A separate control group of ten male and ten female animals received BSA at a dose of 2,442 mg/kg.

Following dosing, all mice were observed daily, and body weights and food consumption were measured weekly. A gross necropsy examination was performed on all animals at the time of death or the end of the study (day 14).

No mortality or treatment-related clinical observations were observed during the study. Likewise, there were no statistically significant differences in food consumption, body weight, or body weight changes. No gross pathological findings related to consumption of Cry2Ab2 protein were observed at necropsy.

Under the conditions of this test, no mortality or other evidence of toxicity was observed following Cry2Ab2 administration at the maximum attainable dose. Therefore, the acute oral LD_{50} of the Cry2Ab2 protein in mice is greater than 2,198 mg/kg body weight, which was was determined to be the NOEL for this protein.

5.5.4. Exposure to the Cry2Ab2 protein

Cry2Ab2 is present at levels of approximately 0.001% of the total protein in the grain of MON 89043 (Part VI, Section 4.3.5). A dietary safety assessment based on these levels indicates that the MOEs for Cry2Ab2 are greater than or equal to 981,000 for the overall U.S. population and greater than or equal to 390,000 for children aged 3-5 years old, an age group with the highest corn consumption on a body weight basis (see Part VI, Section 3). Also, greater than 99% of Cry2Ab2 is digested after 30 seconds incubation in SGF (Part VI, Section 4.3.3). These results suggest that there are no meaningful risks to human and animal health from the dietary exposure to the Cry2Ab2 protein.

5.6. Conclusions

The Cry1A.105 and Cry2Ab2 proteins have been assessed for their potential toxicity according to the recommendations of Codex. The proteins have a long history of safe use, lack structural similarity to known toxins or biologically active proteins known to have adverse effects on mammals, do not show acute oral toxicity in mice, and constitute a very small portion of the total protein present in feed and food derived from MON 89034.

The Cry1A.105 and Cry2Ab2 proteins are from Bt, an organism that has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity and whose safety has been demonstrated by over 45 years of use. Bioinformatics analyses demonstrated that the proteins do not share structural or sequence similarities to known toxins or biologically active proteins that are known to cause adverse health effects in humans or animals. Results from acute oral toxicity studies with mice demonstrated that the Cry1A.105 and Cry2Ab2 proteins are not acutely toxic and do not cause any adverse effects even at maximum attainable dose levels.

Finally, the Cry1A.105 and Cry2Ab2 proteins represent no more than 0.005% and 0.001% of the total protein in the grain of MON 89034, respectively. Taken together these data lead to the conclusion that the Cry1A.105 and Cry2Ab2 proteins are unlikely to have any toxic effect on animals or humans.

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Part VII. FOOD AND FEED SAFETY AND NUTRITIONAL ASSESSMENT OF MON 89034

SECTION 1. Corn as the Comparable Food and Feed

Corn is widely used for a variety of food and feed purposes, and it is intended that MON 89034 will be utilized in the same manner and for the same uses as conventional corn. Corn grain and its processed products are consumed in a multitude of human food and animal feed products. Corn forage (as silage) is extensively consumed as an animal feed by ruminants. Further discussion of the food and feed uses of corn are provided in Section 2 below.

SECTION 2. Historical Uses of Corn

Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2005, corn was planted on 81.8 million acres and grain harvested from 75.1 million acres (NCGA, 2006). The corn grain harvested had an average yield of 148 bushels per acre, with a total production of 11 billion bushels valued at $21 billion (NCGA, 2006). In 2005, more than 42 million acres (52% of the total) were planted with biotechnology-derived corn seed (USDA-NASS, 2006a).

The low price and ready availability of corn has resulted in the development of large volume food, feed and industrial uses. In 2005, the major uses of corn grain in the U.S. were: feed/residual (56%); food, seed, industrial (27%); and, export (17%; NCGA, 2006). The food, seed and industrial uses include the production of ethanol (14.6%), high fructose corn syrup (4.9%), and other food ingredients (8%) including starch, sweetners, and cereals. Only a small proportion (<0.2%) of the overall corn produced is used as seed.

2.1. History and utilization of corn

In addition to the description below regarding the history and uses of corn as food and feed, OECD consensus document on corn compositional considerations (OECD, 2002) provides an overview of whole and processed corn fractions for food and feed uses.

Corn (*Zea mays* L.) originated in Mexico and was grown as a food crop as early as 2700 B.C. (Salvador, 1997). The history of corn has been studied extensively and multiple hypotheses for its origin and parentage have been advanced (Mangelsdorf, 1974). The preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). The history and development of corn has been discussed previously (see Part IV, Section 1). Corn has been a staple in the human diet for centuries. The native peoples of the Americas domesticated and improved corn after realizing its potential for food, feed, and fuel. By the time European settlers came to America, corn was the main cultivated crop (Kastner, 1980). The settlers rapidly adopted corn as the main staple

crop, and by the late 19th century, nearly 90% of Americans depended on mainly on corn for their survival (Hardeman, 1981).

Today, the high yield of corn makes it one of the most economical sources of metabolizable energy for food, feed and industrial products. Little whole kernel corn is consumed by humans when compared to corn-based food ingredients, in spite of its great value as a source of energy (OECD, 2002). Therefore, indirect consumption is much greater than direct consumption by humans. Approximately two-thirds of the corn produced in the U.S. is fed to livestock, either as silage, grain, or by the use of processed feeds in the animal diet. Corn is valued for feed and food uses as it does not produce significant quantities of toxins or anti-nutritional factors that warrant analytical or toxicological tests (Watson, 1982; White and Pollak, 1995).

2.2. Corn as a food source

Corn is the leading cereal in the U.S. Most of the human consumption is in the form of corn-based ingredients produced by the wet mill process including high fructose corn syrup, starch, sweetners, cereals, oil and alcohol. Other food-based ingredients are derived from the dry mill process, and include, corn meal, flour, grits and oil. Corn grain is also used for the production of tortillas and other ethnic Mexican prepared foods by the alkaline cooking process. Fresh-cooked corn provides macronutrients, vitamins and minerals in the human diet. Corn is a significant source of the nutritionally essential sulfur-containing amino acids, methionine and cystine. Corn contains colored pigments called carotenoids, which are primarily responsible for the yellow color of corn grain. The carotenes are precursors for the production of vitamin A and also function as antioxidants. Corn grain is a significant source of Vitamin E (tocopherol), which also serves as an antioxidant (White and Webber, 2003).

Corn is an excellent raw material for the manufacture of starch, not only because of price and availability, but also because the starch is easily recovered in high yield and purity. Approximately 6.7 billion pounds of starch were produced in the U.S. and sold into food and industrial markets in 2004 (CRA, 2005). Starch can be converted to a variety of sweetener and fermentation products including high fructose corn syrup and ethanol. Starch is used as a food ingredient in: dairy and ice cream; batters and breading; baked goods; soups, sauces and gravies; salad dressings; meat, poultry, and fish analogues; confections; and, in drinks.

Corn oil, commercially processed from the germ, is another important food ingredient derived from corn grain. In 2004, approximately one billion pounds of corn oil were produced in the U.S. (CRA, 2005). Although a minor component of overall vegetable oil market, corn oil's high polyunsaturated fatty acid content has important nutritional and health benefits. Corn oil is considered a premium vegetable oil because of its flavor, color, stability, and clarity at refrigerator temperatures. The nutritional benefits, primarily related to its linoleic fatty acid and vitamin E content, have made it a premium oil for the consumer and for the production of margarine. About 50% of the refined oil is used for frying and salad oil, 25% is used in margarine production, and 25% is used for other purposes (Orthoefer et al., 2003).

2.3. Corn as a feed source

Animal feeding represents the largest use of corn in the U.S. In 2005, approximately 55% of the grain harvested (or 6 million bushels) was used as animal feed (NCGA, 2006). In addition, corn silage harvested from 5.9 million acres (approx. 7% of total acres planted) was fed to livestock (USDA-NASS, 2006b). Corn gluten meal, corn gluten feed, and distillers dried grains, derived as co-products by wet and dry milling, are also important components of livestock feed. In 2004, approximately 10 and 2.6 billion pounds of corn gluten feed and meal, respectively, were produced for the animal feed industry in the U.S. (CRA, 2005).

Corn grain contains the most metabolizable energy of all the grains used in livestock feed (Ensminger et al., 1990). The corn kernel contains about 83% carbohydrate in the form of starch, pentosans, dextrins, sugars, cellulose, and hemicellulose. Starch is the biggest component in the carbohydrate fraction and provides most of the energy. The fiber portion includes cellulose and hemicellulose, which are generally available to ruminants but not to nonruminants. Corn grain contains approximately 4% (w/w) oil (White and Weber, 2003), which has a high content of 18:2 linoleic acid, one of the essential polyunsaturated fatty acids needed by swine and poultry. Although corn grain has a relatively low protein content (10% dw; ILSI-CCD, 2006) compared to other cereal grains, it is a major source of essential amino acids due to the high percentage incorporated in animal diets. Corn grain is a good source of methionine, but a poor source for lysine and tryptophan. Methionine and lysine are the two most limiting amino acids for poultry, swine and other livestock fed corn-based diets (NRC, 2001).

Calcium and phosphorus are important minerals in animal nutrition. Corn grain has low levels of calcium, and thus, is not a big contributor to calcium in the animal diet. On the other hand, corn grain is a source of phosphorus in the animal diet. However, the phosphorus is bound up in the form of phytic acid, which reduces its bioavailability to nonruminants such as swine and poultry (Ensminger et al., 1990). Many producers now add the enzyme phytase to the diet to release the phosphorus bound in phytic acid. Nutritionists incorporate supplemental sources of calcium, phosphorus, sodium, magnesium, iron, zinc, copper, manganese, iodine, and selenium as needed to balance animal diets. Corn grain is a source of a number of vitamins in animal feed, which include: vitamins A, B1 (thiamin), B2 (riboflavin), B6 (pyridioxine), C (ascorbic acid), E, folate, niacin and pantothenic acid. While the content of niacin in corn grain is relatively high, it exists in a bound form (niacytin) that is not biologically available to monogastric animals. Nutritionists supplement animal diets with vitamins, since their levels in corn grain are insufficient to meet dietary needs.

Corn silage is a major forage ingredient for feedlot and dairy cattle due its importance as a palatable energy source (Newcomb, 1995). Corn gluten feed and meal are byproducts of the wet milling process and are incorporated into animal diets. Gluten meal contains high levels of protein (~60%) and is an important source of carotenoids. It is commonly used in feed for cattle, fish, poultry, pets, and other animals but primarily in poultry diets. Corn gluten feed (wet or dry) is an excellent feed that is a significant source of protein

(~20%), low in starch, high in digestible fiber, and low in oil and is used mainly in dairy and beef cattle diets. In addition, with the increasing use of U.S. corn in dry mill plants to produce ethanol, the distillers dried grains co-product will be in greater supply and is expected to replace small amounts of corn grain in livestock and poultry diets.

SECTION 3. Comparison of the Composition and Characteristics of MON 89034 to Conventional Corn

Analyses of the composition of crops produced by methods of biotechnology, and their comparison to conventional varieties, are an integral part of the nutritional and safety assessment of these products. These assessments are conducted according to the principles of comparative risk assessment or substantial equivalence, which has been adopted by a number of international organizations including the World Health Organization, United Nations Food and Agricultural Organization, and the OECD. According to this principle, the food or feed from a biotechnology-derived crop are compared with its conventional counterpart as an initial step in the safety assessment process. The goal is to determine whether the nutrient and anti-nutrient levels in grain and forage tissues from biotechnology-derived corn are comparable to those of conventional corn. Further safety assessment of these components, if necessary, should focus only on those components of the biotechnology-derived crop that are shown to be significantly and biologically different to those of conventional control.

To assess whether the introduction or expression of the insect protection traits in MON 89034 caused any unintended effects, compositional analyses were conducted on the corn grain and forage of MON 89034. These tissues were produced in replicated field trials, conducted at five sites in the U.S. during the 2004 growing season. The levels of the various analytes in MON 89034 were compared to those of conventional control corn, which has the background genetics representative of MON 89034 but does not contain the *cry1A.105* and *cry2Ab2* genes or produce the Cry1A.105 and Cry2Ab2 proteins. Additionally, the grain and forage from 15 conventional corn hybrids produced in the same field trials alongside MON 89034 and control corn, were also subjected to compositional analyses. Values derived from these conventional hybrids were used as references to generate a 99% tolerance interval for each of the analytes for conventional corn. MON 89034 was found to be compositionally equivalent to conventional corn, and thus is considered as nutritious as conventional corn for food and feed uses. Further details of this assessment are provided below.

3.1. Assessment of significant nutrients, antinutrients, and key secondary metabolites in corn forage and grain

Compositional analyses were conducted, following OECD recommendations, to assess whether the nutrient, antinutrient and secondary metabolite levels in the grain and forage tissues derived from MON 89034 are comparable to those in the conventional control, LH198 x LH172. The conventional control has background genetics similar to MON 89034, but does not contain the *cry1A.105* and *cry2Ab2* genes. Additional conventional

corn hybrids currently in the marketplace were also included in the analysis to establish a range of natural variability for each analyte, which is defined by a 99% tolerance interval for that particular analyte (Ridley et al., 2002). Results of the comparisons indicate that MON 89034 is compositionally and nutritionally equivalent to conventional corn hybrids currently in commerce.

Grain and forage tissues of MON 89034 and control corn were harvested from plants grown at each of five field sites during 2004. The field sites were located in regions of the U.S. that are conducive to the growth of corn, and representative of commercial corn production. Seed was planted in a randomized complete block design with three replicates per block. In addition, 15 conventional, commercial corn hybrids were also included as references by growing three different hybrids at each of five sites for a total of 15 references. The 15 conventional corn reference hybrids were included to provide data for the development of a 99% tolerance interval for each component analyzed. For each compositional component, a 99% tolerance interval was calculated. This interval is expected to contain, with 95% confidence, 99% of the values obtained from the population of commercial references. It is important to establish the 99% tolerance interval from representative conventional corn hybrids for each of the analytes, because such data illustrate the compositional variability that naturally occurs in commercially grown varieties. By comparison to the 99% tolerance interval, any statistically significant difference between MON 89034 and the control may be put into perspective, and can be assessed for biological relevance in the context of the natural variability in corn.

The compositional analyses were conducted on a total of 77 components - nine in forage and 68 in grain. Components were selected based on recommendations of the OECD (OECD, 2002). Compositional analyses of the forage samples included proximates (protein, fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), minerals (calcium and phosphorus), and carbohydrates by calculation. Compositional analyses of the grain samples included proximates (protein, fat, ash, and moisture), ADF, NDF, total dietary fiber (TDF), amino acids, fatty acids (C8-C22), vitamins (B1, B2, B6, E, niacin, and folic acid), antinutrients (phytic acid and raffinose), secondary metabolites (furfural, ferulic acid, and p-coumaric acid), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and carbohydrates by calculation. Methods for analysis were based on internationally recognized procedures and literature publications (see Appendix F).

Statistical analyses of the compositional data were conducted using a mixed model analysis of variance. Each individual analyte for MON 89034 was compared to that of the conventional control, for the combination of all five sites (i.e., the combined-site) and for each individual site. The statistical significance was defined at the level of $p<0.05$. Of the 77 components analyzed, 16 components had greater than 50% of the analytical values that were below the limit of quantitation, and therefore, were not included in the statistical analyses.

Statistical analyses of the remaining 61 components (77 minus the 16) were conducted for comparison of MON 89034 with control corn. The overall data set was examined for evidence of biologically relevant changes. Based on this evaluation and the results of statistical analyses, analytes for which the levels were not statistically different were deemed to be present at equivalent levels between MON 89034 and the control. Analyses using data from the combined sites indicated that there were no statistical differences in the levels of 95% of the analytes (58 of the 61). Statistical analyses for the combined-site data are presented in Table VII.1 for forage and Tables VII.2 - 7 for grain. Analyses using data from the five single sites indicated that there were no statistically significant differences in analytes for 261 of 305 comparisons made between MON 89034 and the control. Table VII.8 summarizes the statistical differences observed between MON 89034 and the control for the combined-site and individual sites. Appendix G provides compostion data from the individual sites and Appendix H provides supplementary composition data, where the amino acid and fatty acid values are expressed in alternative units.

3.2. Levels of nutrients in corn forage and grain

For the combined-site analyses, statistical differences between MON 89034 and control corn were observed for three analytes, which included phosphorus in forage, and 18:0 stearic and 20:0 arachidic acids in grain (Table VII.8). The differences observed are generally small (3.4 – 19.2%), considering the range of natural variability, and the mean levels and ranges of MON 89034 are well within the 99% tolerance intervals for commercial corn. The mean levels and ranges of phosphorus in forage, and 18:0 stearic and 20:0 arachidic acids in grain, were also within the ranges in the International Life Sciences Institute Crop Composition Database (ILSI-CCD, 2006), as well as within published literature ranges. Therefore, it is concluded that MON 89034 and control corn are compositionally equivalent based on analyses of the combined-site data. The reported ILSI and published literature ranges for the analytical components present in corn are summarized in Table VII.9.

The reproducibility and trends across sites were also examined, and comparisons made to conventional corn hybrids using the 99% tolerance intervals. Of the 44 statistical differences observed in the individual site analyses, 33 were only observed at one site. There were no consistent trends and, except in two cases, the mean and ranges of the analytes in MON 89034 were well within the 99% tolerance interval for conventional corn. The mean levels and ranges for calcium and methionine in grain were slightly outside the 99% tolerance interval but within the ILSI database. It is concluded that these differences are not biologically significant. Of the remaining 11 differences observed at more than one site, there were no analytes that were consistently and statistically different across five or four sites. In addition, there were no analytes that showed statistically significant differences in three sites that had not been previously observed (i.e., 20:0 arachidic acid) to be different in the combined-site analysis. Statistically significant differences were observed in as many as two sites for three analytes (carbohydrates, copper, iron), which were not previously found to be different (i.e., 18:0 stearic acid) in the combined-site analysis. For carbohydrates and iron, the observed differences from the control were small and lower at one site and higher at the other site (Table VII.8). As

there is no evidence of any reproducibility across sites, it is concluded that the statistical differences are not biologically relevant for carbohydrates and iron. For copper, the values for MON 89034 were higher than the control at both sites (Table VII.8). The observed differences are small in magnitude, and the mean levels and ranges of MON 89034 are well within the 99% tolerance interval. Therefore, it is concluded that the statistical differences for copper are not biologically relevant.

Based on the data and information presented above, it was concluded that corn grain and forage derived from MON 89034 are compositionally and nutritionally equivalent to those of conventional corn. The few statistical differences between MON 89034 and control corn likely reflect the natural variability of the components since the mean levels of analytes for MON 89034 are well within the 99% tolerance intervals for conventional corn, and/or within the ranges in the ILSI database (ILSI-CCD, 2006) and the scientific literature.

3.3. Levels of key anti-nutrients and secondary metabolites in corn forage and grain

A description of the anti-nutrients and key secondary metabolites present in corn is provided in the OECD consensus document on compositional considerations (OECD, 2002). The anti-nutrients include phytic acid, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), raffinose, and trypsin and chymotrypsin inhibitors. The secondary metabolites include furfural, ferulic acid, and p-coumaric acid. The OECD does not recommend analysis of DIMBOA due to the variable levels found across corn hybrids. Similarly, analysis of trypsin and chymotrypsin inhibitors is not recommended because they occur at low levels in corn and are not considered nutritionally significant. According to the OECD (2002), "in considering the anti-nutrients and natural toxins in maize, only phytic acid is significant to the animal feed". The anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid and p-coumaric acid were analyzed in the grain of MON 89034 and compared to those of control corn. Furfural was excluded from further consideration since the levels present in MON 89034 and control corn were at or below the limit of quantitation of the assay.

The overall data set was examined for evidence of biologically relevant changes. In addition, statistical analyses were applied to assess significant differences of each analyte at $p<0.05$. As described in Section 3.1, 58 of 61 comparisons in the combined-site analysis and 261 of 305 comparisons in the single site analysis showed no statistically significant differences between MON 89034 and control corn. The few observed statistical differences were not considered biologically relevant since these differences were not reproducible across sites and no consistent trends were observed. Furthermore, the mean levels and ranges of MON 89034 analytes were within the 99% tolerance intervals, and/or within the ILSI database and literature ranges. The statistical summaries for the anti-nutrients are described under the grain analyses, where the combined-site data are presented in Table VII.7. The analytes that are statistically different between MON 89034 and control corn are presented in Table VII.8. The ILSI and literature ranges for corn are provided in Table VII.9.

Phytic acid is considered an important anti-nutrient for animals, especially non-ruminants, since it can significantly reduce the bioavailability of phosphorus in corn tissues. Feed formulators add the enzyme phytase to swine and poultry diets to improve the utilization of phosphorus. Ruminants are more efficient in utilizing phytic acid since microbes in the rumen produce phytase that breaks down phytate and releases phosphorus (Ensminger et al., 1990). Compositional analyses of the grain indicated that phytic acid was present at similar levels in MON 89034 and control corn, and no statistical differences were observed for all comparisons.

Raffinose is a low molecular weight carbohydrate present in corn grain that is considered an anti-nutrient due to the gas production and resulting flatulence caused by consumption (Maynard et al., 1979). Composition analyses of the grain indicated that raffinose was present at similar levels in MON 89034 and control corn, and no statistical differences were observed for all comparisons.

The phenolic acids, ferulic acid and p-coumaric acid, are structural and functional components of plant cells (Kroon and Williamson, 1999). They are found in vegetables, fruit and cereals and act as natural pesticides against insect and fungal pests. Composition analyses of the grain indicated that ferulic acid was present at similar levels in MON 89034 and control corn, and no statistical differences were observed for all comparisons. A statistical significant difference was observed for p-coumaric acid at one site but not at the other sites. Since there was no trend, the statistically significant difference for p-coumaric acid is not considered reproducible and hence not biologically significant.

Based on these results, it is concluded that the levels of anti-nutrients and key secondary metabolites (as defined by the OECD) in MON 89034 are comparable to those found in conventional corn.

Table VII.1. Comparison of Proximates, Fiber, and Mineral Content in Forage from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Fiber						
Acid Detergent Fiber (% DW)	28.95 ± 1.69 (22.60 - 35.85)	27.26 ± 1.69 (19.93 - 35.59)	1.69 ± 1.18 (-6.22 - 10.45)	-0.81,4.19	0.170	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fiber (% DW)	39.69 ± 1.32 (33.99 - 46.82)	37.60 ± 1.32 (31.44 - 43.96)	2.09 ± 1.40 (-3.47 - 7.47)	-0.88,5.05	0.155	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.20 ± 0.019 (0.16 - 0.24)	0.19 ± 0.019 (0.13 - 0.28)	0.0066 ± 0.011 (-0.036 - 0.063)	-0.017,0.031	0.569	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.25 ± 0.011 (0.22 - 0.32)	0.21 ± 0.011 (0.15 - 0.25)	0.040 ± 0.014 (-0.0019 - 0.13)	0.011,0.069	0.010	(0.14 - 0.25) [0.071,0.32]
Proximate						
Ash (% DW)	3.70 ± 0.27 (2.51 - 4.67)	3.90 ± 0.27 (2.59 - 5.10)	-0.20 ± 0.21 (-1.72 - 0.97)	-0.65,0.25	0.356	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	86.90 ± 0.43 (84.93 - 89.13)	86.69 ± 0.43 (84.36 - 89.57)	0.21 ± 0.53 (-4.23 - 4.41)	-0.91,1.33	0.697	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	72.20 ± 1.35 (68.50 - 75.40)	71.53 ± 1.35 (65.90 - 76.80)	0.67 ± 0.52 (-3.50 - 4.20)	-0.44,1.77	0.220	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	7.82 ± 0.27 (6.34 - 8.98)	7.70 ± 0.27 (6.06 - 8.87)	0.13 ± 0.26 (-2.32 - 2.35)	-0.43,0.68	0.635	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	1.57 ± 0.24 (0.63 - 3.17)	1.71 ± 0.24 (0.77 - 2.91)	-0.13 ± 0.23 (-2.28 - 1.95)	-0.59,0.32	0.558	(0.58 - 3.11) [0,4.54]

[1] DW = dry weight; FW = fresh weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.2. Comparison of the Amino Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E. [1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Amino Acid (% DW)						
Alanine (% DW)	0.77 ± 0.039 (0.64 - 0.89)	0.78 ± 0.039 (0.67 - 0.89)	-0.0070 ± 0.019 (-0.13 - 0.089)	-0.046,0.032	0.709	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.48 ± 0.013 (0.38 - 0.52)	0.47 ± 0.013 (0.41 - 0.51)	0.011 ± 0.012 (-0.090 - 0.062)	-0.014,0.036	0.361	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.68 ± 0.029 (0.56 - 0.78)	0.67 ± 0.029 (0.60 - 0.76)	0.0038 ± 0.015 (-0.11 - 0.078)	-0.028,0.036	0.804	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.23 ± 0.0057 (0.20 - 0.26)	0.23 ± 0.0057 (0.21 - 0.25)	0.0023 ± 0.0038 (-0.022 - 0.023)	-0.0057,0.010	0.554	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	1.97 ± 0.097 (1.63 - 2.29)	1.99 ± 0.097 (1.70 - 2.26)	-0.012 ± 0.049 (-0.33 - 0.24)	-0.11,0.091	0.809	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.38 ± 0.0087 (0.32 - 0.41)	0.38 ± 0.0087 (0.36 - 0.41)	0.0042 ± 0.0071 (-0.067 - 0.035)	-0.011,0.019	0.566	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.31 ± 0.011 (0.25 - 0.35)	0.31 ± 0.011 (0.28 - 0.34)	0.0027 ± 0.0055 (-0.050 - 0.030)	-0.0090,0.014	0.632	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.36 ± 0.018 (0.30 - 0.43)	0.36 ± 0.018 (0.30 - 0.42)	-0.00003 ± 0.0088 (-0.056 - 0.041)	-0.019,0.019	0.997	(0.32 - 0.45) [0.23,0.51]

Table VII.2 (cont). Comparison of the Amino Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Amino Acid (% DW)						
Leucine (% DW)	1.31 ± 0.077 (1.09 - 1.57)	1.32 ± 0.077 (1.08 - 1.55)	-0.014 ± 0.036 (-0.21 - 0.16)	-0.089,0.062	0.700	(1.14 - 1.68) [0.77,1.92]
Lysine (% DW)	0.33 ± 0.0097 (0.26 - 0.36)	0.32 ± 0.0097 (0.29 - 0.36)	0.0088 ± 0.0078 (-0.056 - 0.033)	-0.0077,0.025	0.273	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.23 ± 0.0064 (0.20 - 0.27)	0.22 ± 0.0064 (0.20 - 0.24)	0.0038 ± 0.0047 (-0.017 - 0.028)	-0.0061,0.014	0.427	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.51 ± 0.028 (0.43 - 0.61)	0.52 ± 0.028 (0.43 - 0.60)	-0.0012 ± 0.013 (-0.080 - 0.067)	-0.029,0.026	0.925	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	0.93 ± 0.030 (0.79 - 1.05)	0.93 ± 0.030 (0.83 - 1.01)	0.0034 ± 0.019 (-0.15 - 0.10)	-0.037,0.044	0.861	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.52 ± 0.022 (0.44 - 0.61)	0.52 ± 0.022 (0.46 - 0.60)	-0.0046 ± 0.012 (-0.087 - 0.058)	-0.030,0.021	0.703	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.33 ± 0.010 (0.27 - 0.37)	0.33 ± 0.010 (0.29 - 0.36)	0.00063 ± 0.0074 (-0.052 - 0.039)	-0.015,0.016	0.933	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.056 ± 0.0018 (0.048 - 0.064)	0.056 ± 0.0018 (0.045 - 0.063)	0.00031 ± 0.0013 (-0.0055 - 0.0072)	-0.0025,0.0031	0.817	(0.043 - 0.059) [0.032,0.072]

Table VII.2 (cont). Comparison of the Amino Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Amino Acid (% DW)						
Tyrosine (% DW)	0.37 ± 0.015 (0.22 - 0.43)	0.36 ± 0.015 (0.24 - 0.42)	0.0088 ± 0.016 (-0.21 - 0.14)	-0.026,0.043	0.596	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.49 ± 0.020 (0.40 - 0.55)	0.49 ± 0.020 (0.43 - 0.55)	0.0034 ± 0.010 (-0.084 - 0.055)	-0.019,0.026	0.748	(0.42 - 0.55) [0.35,0.62]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.3. Comparison of the Fatty Acid Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.19 ± 0.060 (8.98 - 9.46)	9.12 ± 0.060 (8.91 - 9.34)	0.071 ± 0.049 (-0.14 - 0.33)	-0.034,0.18	0.171	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.13 ± 0.0058 (0.11 - 0.14)	0.12 ± 0.0058 (0.048 - 0.14)	0.0022 ± 0.0054 (-0.012 - 0.079)	-0.0093,0.014	0.696	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.89 ± 0.021 (1.79 - 2.03)	1.82 ± 0.021 (1.76 - 1.87)	0.072 ± 0.021 (-0.055 - 0.18)	0.028,0.12	0.002	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	24.96 ± 0.34 (23.38 - 25.75)	24.84 ± 0.34 (23.62 - 26.66)	0.12 ± 0.20 (-1.48 - 1.15)	-0.32,0.55	0.574	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	61.82 ± 0.40 (60.85 - 63.61)	62.07 ± 0.40 (60.51 - 63.41)	-0.25 ± 0.23 (-1.62 - 1.24)	-0.73,0.24	0.292	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.19 ± 0.027 (1.12 - 1.23)	1.22 ± 0.027 (1.15 - 1.43)	-0.028 ± 0.016 (-0.23 - 0.036)	-0.063,0.0061	0.099	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.39 ± 0.0062 (0.36 - 0.42)	0.38 ± 0.0062 (0.36 - 0.40)	0.013 ± 0.0031 (-0.019 - 0.032)	0.0063,0.019	<0.001	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.28 ± 0.0040 (0.26 - 0.29)	0.28 ± 0.0040 (0.25 - 0.29)	0 ± 0.0024 (-0.014 - 0.011)	-0.0051,0.0051	0.999	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.16 ± 0.0050 (0.13 - 0.20)	0.15 ± 0.0050 (0.13 - 0.18)	0.0027 ± 0.0062 (-0.019 - 0.029)	-0.010,0.016	0.665	(0.12 - 0.19) [0.081,0.23]

[1] FA = fatty acid; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.4. Comparison of the Mineral Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Mineral						
Calcium (% DW)	0.0050 ± 0.00034 (0.0038 - 0.0066)	0.0049 ± 0.00034 (0.0040 - 0.0059)	0.00016 ± 0.00011 (-0.00027 - 0.00090)	-0.00008,0.00040	0.180	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	1.74 ± 0.38 (1.33 - 2.38)	2.07 ± 0.37 (1.26 - 4.54)	-0.33 ± 0.53 (-2.96 - 0.78)	-1.45,0.79	0.547	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	21.40 ± 1.00 (19.23 - 25.23)	22.20 ± 0.99 (19.03 - 28.26)	-0.80 ± 0.67 (-6.50 - 5.90)	-2.22,0.62	0.250	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.12 ± 0.0043 (0.10 - 0.14)	0.12 ± 0.0043 (0.11 - 0.14)	-0.00028 ± 0.0021 (-0.018 - 0.011)	-0.0047,0.0041	0.893	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	6.79 ± 0.29 (5.43 - 9.32)	6.51 ± 0.29 (5.57 - 8.00)	0.28 ± 0.21 (-1.54 - 2.36)	-0.18,0.73	0.213	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.33 ± 0.0095 (0.27 - 0.36)	0.33 ± 0.0095 (0.29 - 0.36)	0.00039 ± 0.0043 (-0.038 - 0.026)	-0.0087,0.0095	0.929	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.36 ± 0.0065 (0.32 - 0.40)	0.36 ± 0.0065 (0.34 - 0.40)	0.0032 ± 0.0042 (-0.030 - 0.035)	-0.0052,0.012	0.450	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	22.05 ± 1.14 (18.91 - 26.89)	21.91 ± 1.14 (18.81 - 26.04)	0.14 ± 0.51 (-3.37 - 3.19)	-0.94,1.22	0.788	(16.72 - 34.04) [7.16,38.55]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.5. Comparison of the Proximates and Fiber Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E. (Range)	Control Mean ± S.E. [1] (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Ash (% DW)	1.41 ± 0.036 (1.25 - 1.56)	1.39 ± 0.036 (1.28 - 1.51)	0.014 ± 0.041 (-0.11 - 0.13)	-0.072,0.10	0.734	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	84.85 ± 0.42 (83.29 - 86.52)	84.96 ± 0.42 (83.58 - 86.22)	-0.11 ± 0.18 (-1.42 - 0.84)	-0.50,0.28	0.562	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	9.52 ± 0.77 (7.89 - 12.80)	9.50 ± 0.77 (7.86 - 13.10)	0.021 ± 0.22 (-1.00 - 0.87)	-0.44,0.48	0.923	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	10.43 ± 0.42 (8.54 - 11.98)	10.36 ± 0.42 (9.22 - 11.52)	0.070 ± 0.19 (-1.26 - 1.28)	-0.34,0.48	0.725	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.32 ± 0.069 (3.05 - 3.89)	3.29 ± 0.069 (3.05 - 3.75)	0.025 ± 0.089 (-0.50 - 0.29)	-0.16,0.21	0.784	(2.66 - 3.71) [2.20,4.55]
Fiber						
Acid Detergent Fiber (% DW)	5.48 ± 0.19 (3.82 - 7.24)	5.27 ± 0.19 (4.17 - 7.00)	0.21 ± 0.25 (-3.18 - 3.07)	-0.30,0.72	0.410	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fiber (% DW)	10.06 ± 0.37 (8.59 - 12.08)	9.75 ± 0.37 (8.48 - 11.75)	0.31 ± 0.34 (-2.26 - 2.05)	-0.41,1.03	0.370	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fiber (% DW)	15.17 ± 0.47 (13.39 - 17.02)	14.67 ± 0.47 (12.82 - 17.62)	0.50 ± 0.54 (-3.61 - 4.20)	-0.66,1.65	0.375	(12.99 - 18.03) [9.20,20.27]

[1] DW = dry weight; FW = fresh weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.6. Comparison of the Vitamin Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Vitamin						
Folic Acid (mg/kg DW)	0.35 ± 0.037 (0.26 - 0.48)	0.36 ± 0.037 (0.23 - 0.53)	-0.0080 ± 0.022 (-0.11 - 0.11)	-0.054,0.038	0.717	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	30.08 ± 1.11 (25.72 - 34.84)	29.59 ± 1.11 (24.93 - 35.75)	0.48 ± 0.65 (-4.44 - 5.64)	-0.82,1.79	0.461	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	3.07 ± 0.13 (2.39 - 3.44)	2.94 ± 0.13 (2.39 - 3.36)	0.13 ± 0.17 (-0.66 - 0.68)	-0.24,0.49	0.474	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.42 ± 0.046 (1.24 - 1.65)	1.42 ± 0.046 (1.16 - 1.61)	0.0015 ± 0.050 (-0.30 - 0.45)	-0.099,0.10	0.976	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	6.22 ± 0.23 (5.28 - 6.99)	6.26 ± 0.23 (5.37 - 6.80)	-0.036 ± 0.18 (-0.72 - 1.10)	-0.41,0.34	0.838	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	6.77 ± 0.42 (5.55 - 8.62)	6.63 ± 0.42 (2.72 - 9.02)	0.14 ± 0.36 (-2.35 - 3.83)	-0.64,0.91	0.714	(2.71 - 13.94) [0,20.49]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.
[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.7. Comparison of the Anti-nutrients and Secondary Metabolites Content in Grain from Test (MON 89034) and Conventional Control (LH198 x LH172) Corn for Combined Sites

Analytical Component[1]	Test Mean ± S.E.[1] (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI[1] (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[2]]
Antinutrient						
Phytic Acid (% DW)	0.75 ± 0.050 (0.53 - 0.87)	0.73 ± 0.050 (0.56 - 0.88)	0.016 ± 0.027 (-0.15 - 0.18)	-0.037,0.069	0.537	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	2131.38 ± 108.09 (1790.25 - 2525.31)	2148.05 ± 108.09 (1878.66 - 2669.85)	-16.67 ± 50.08 (-330.17 - 264.79)	-116.98,83.65	0.740	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	194.25 ± 7.12 (166.11 - 253.04)	183.96 ± 7.12 (167.76 - 210.13)	10.28 ± 7.08 (-24.37 - 70.84)	-4.73,25.30	0.165	(99.30 - 285.75) [0,378.57]

[1] DW = dry weight; S.E. = standard error; CI = confidence interval.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.8. Summary of Differences (p<0.05) for the Comparison of Analyte Levels for MON 89034 vs. the Conventional Control (LH198 x LH172) and Commercial References

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (MON 89034 minus Control) % of Control	Mean Difference (MON 89034 minus Control) Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Statistical Differences Observed in Combined Site Analyses						
Mineral						
Forage Phosphorus (% DW)	0.25	0.21	19.24	0.010	(0.22 - 0.32)	[0.071,0.32]
Fatty Acid						
Grain 18:0 Stearic (% Total FA)	1.89	1.82	3.97	0.002	(1.79 - 2.03)	[0.86,2.98]
Grain 20:0 Arachidic (% Total FA)	0.39	0.38	3.43	<0.001	(0.36 - 0.42)	[0.23,0.54]
Statistical Differences in More Than One Site						
Proximate						
Site IA Grain Carbohydrates (% DW)	83.38	84.52	-1.34	0.008	(83.29 - 83.55)	[81.08,88.80]
Site OH Grain Carbohydrates (% DW)	84.26	83.80	0.55	0.009	(83.99 - 84.59)	[81.08,88.80]
Mineral						
Site IL-1 Grain Copper (mg/kg DW)	1.76	1.36	29.35	0.023	(1.51 - 2.21)	[0,4.20]
Site NE Grain Copper (mg/kg DW)	2.15	1.67	28.66	0.023	(1.92 - 2.38)	[0,4.20]
Site IL-1 Grain Iron (mg/kg DW)	20.86	19.48	7.11	0.048	(19.23 - 21.79)	[8.88,34.51]
Site OH Grain Iron (mg/kg DW)	21.37	25.74	-17.00	0.006	(20.59 - 21.76)	[8.88,34.51]
Fatty Acid						
Site IL-1 Grain 18:0 Stearic (% Total FA)	1.96	1.82	7.94	<0.001	(1.89 - 2.02)	[0.86,2.98]
Site IL-2 Grain 18:0 Stearic (% Total FA)	1.98	1.82	9.05	<0.001	(1.93 - 2.03)	[0.86,2.98]
Site IL-1 Grain 20:0 Arachidic (% Total FA)	0.41	0.39	5.23	0.007	(0.40 - 0.42)	[0.23,0.54]
Fatty Acid (cont)						

Table VII.8. Summary of Differences (p<0.05) for the Comparison of Analyte Levels for MON 89034 vs. the Conventional Control (LH198 x LH172) and Commercial References

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (MON 89034 minus Control) % of Control	Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Site IL-2 Grain 20:0 Arachidic (% Total FA)	0.39	0.37	6.83	0.021	(0.38 - 0.40)	[0.23,0.54]
Site OH Grain 20:0 Arachidic (% Total FA)	0.38	0.37	3.12	0.035	(0.38 - 0.39)	[0.23,0.54]
Statistical Differences Observed in One Site Only						
Amino Acid						
Site IA Grain Alanine (% DW)	0.88	0.81	7.83	0.030	(0.87 - 0.88)	[0.48,1.08]
Site IA Grain Arginine (% DW)	0.51	0.46	10.83	0.005	(0.50 - 0.52)	[0.33,0.56]
Site IA Grain Aspartic acid (% DW)	0.77	0.71	8.66	0.003	(0.77 - 0.78)	[0.43,0.90]
Site IA Grain Cystine (% DW)	0.25	0.23	7.54	0.014	(0.24 - 0.26)	[0.18,0.27]
Site IA Grain Glutamic acid (% DW)	2.27	2.09	8.66	0.011	(2.26 - 2.28)	[1.25,2.75]
Site IA Grain Glycine (% DW)	0.41	0.38	6.94	0.020	(0.40 - 0.41)	[0.28,0.46]
Site IA Grain Histidine (% DW)	0.34	0.32	7.16	0.022	(0.34 - 0.34)	[0.22,0.38]
Site IA Grain Leucine (% DW)	1.49	1.37	8.96	0.032	(1.48 - 1.51)	[0.77,1.92]
Site IA Grain Lysine (% DW)	0.35	0.32	6.66	0.028	(0.33 - 0.36)	[0.20,0.40]
Site IA Grain Methionine (% DW)	0.25	0.23	11.20	0.003	(0.25 - 0.27)	[0.14,0.25]
Site IA Grain Phenylalanine (% DW)	0.58	0.53	9.45	0.028	(0.57 - 0.59)	[0.32,0.73]
Amino Acid (cont)						
Site IA Grain Proline (% DW)	1.05	0.98	7.29	0.028	(1.04 - 1.05)	[0.68,1.21]

Table VII.8. Summary of Differences (p<0.05) for the Comparison of Analyte Levels for MON 89034 vs. the Conventional Control (LH198 x LH172) and Commercial References

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (MON 89034 minus Control) % of Control	Mean Difference (MON 89034 minus Control) Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Site IA Grain Serine (% DW)	0.60	0.56	8.28	0.004	(0.60 - 0.61)	[0.34,0.71]
Site IA Grain Threonine (% DW)	0.37	0.34	8.45	0.004	(0.37 - 0.37)	[0.24,0.41]
Site IA Grain Tyrosine (% DW)	0.43	0.36	17.50	0.006	(0.42 - 0.43)	[0.17,0.52]
Proximate						
Site IA Grain Protein (% DW)	11.89	10.85	9.59	0.005	(11.73 - 11.98)	[7.54,13.13]
Site IL-1 Forage Moisture (% FW)	69.03	66.53	3.76	0.031	(68.50 - 69.40)	[57.62,86.45]
Site NE Forage Ash (% DW)	3.20	4.39	-27.12	0.021	(2.93 - 3.38)	[1.93,6.31]
Site NE Forage Carbohydrates (% DW)	88.16	84.98	3.74	0.004	(86.86 - 88.84)	[83.05,90.74]
Fiber						
Site NE Grain Neutral Detergent Fiber (% DW)	10.52	9.05	16.27	0.028	(10.43 - 10.69)	[5.93,13.63]
Site OH Forage Acid Detergent Fiber (% DW)	31.31	23.58	32.78	0.012	(26.92 - 34.93)	[16.76,43.76]
Site OH Forage Neutral Detergent Fiber (% DW)	43.21	37.87	14.11	0.027	(40.07 - 46.82)	[25.94,55.67]

Table VII.8. Summary of Differences (p<0.05) for the Comparison of Analyte Levels for MON 89034 vs. the Conventional Control (LH198 x LH172) and Commercial References

Analytical Component (Units)[1]	MON 89034 Mean	Control Mean	Mean Difference (MON 89034 minus Control) % of Control	Signif. (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Fatty Acid						
Site IA Grain 18:3 Linolenic (% Total FA)	1.21	1.34	-9.40	0.009	(1.20 - 1.23)	[0.63,1.77]
Site IL-1 Grain 16:1 Palmitoleic (% Total FA)	0.13	0.14	-6.87	0.012	(0.12 - 0.13)	[0,0.28]
Site IL-2 Grain 18:1 Oleic (% Total FA)	24.75	23.82	3.93	0.003	(24.14 - 25.25)	[7.51,46.46]
Site IL-2 Grain 18:2 Linoleic (% Total FA)	61.87	63.17	-2.07	0.001	(61.19 - 62.42)	[39.41,76.74]
Site NE Grain 20:1 Eicosenoic (% Total FA)	0.28	0.29	-1.50	0.030	(0.28 - 0.28)	[0.15,0.39]
Mineral						
Site IA Grain Calcium (% DW)	0.0064	0.0058	10.96	0.012	(0.0062 - 0.0066)	[0.0016,0.0059]
Site IA Grain Manganese (mg/kg DW)	8.34	6.99	19.32	0.017	(7.62 - 9.32)	[3.17,9.99]
Site IA Forage Calcium (% DW)	0.24	0.26	-8.77	0.033	(0.24 - 0.24)	[0.016,0.38]
Site NE Forage Phosphorus (% DW)	0.25	0.17	46.95	0.036	(0.23 - 0.28)	[0.071,0.32]
Vitamin						
Site IL-2 Grain Folic Acid (mg/kg DW)	0.37	0.32	13.81	<0.001	(0.35 - 0.38)	[0.012,0.69]
Secondary Metabolite						
Site OH Grain p-Coumaric Acid (µg/g DW)	218.38	185.63	17.64	0.032	(187.79 - 253.04)	[0,378.57]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; Combined Site = analyses of the combined data from each of the five replicated field trials.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table VII.9. Literature and ILSI Database Ranges of Components of Corn Forage and Grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
Forage		
Proximates (% dw)		
Ash	2.43-9.64[a]; 2-6.6[b]	1.527 – 9.638
Carbohydrates	83.2-91.6[b]; 76.5-87.3[a]	76.4 – 92.1
Fat, total	0.35-3.62[b]; 1.42-4.57[a]	0.296 – 4.570
Moisture (% fw)	56.5-80.4[a];55.3-75.3[b]	49.1 – 81.3
Protein	4.98-11.56[a]	3.14 – 11.57
Fiber (% dw)		
Acid detergent fiber (ADF)	18.3-41.0[b]; 17.5-38.3[a]	16.13 – 47.39
Neutral detergent fiber (NDF)	26.4-54.5[b]; 27.9-54.8[a]	20.29 – 63.71
Minerals (% dw)		
Calcium	0.0969-0.3184[b]	0.0714 – 0.5768
Phosphorous	0.1367-0.2914[b]	0.0936 – 0.3704
Grain		
Proximates (% dw)		
Ash	1.1-3.9[d]; 0.89-6.28[b]	0.616 – 6.282
Carbohydrates	77.4-87.2[b]; 82.2-88.1[a]	77.4 – 89.5
Fat, total	3.1-5.7[d]; 2.48-4.81[b]	1.742 – 5.823
Moisture (% fw)	7-23[d]; 8.18-26.2[b]	6.1 – 40.5
Protein	6-12[d]; 9.7-16.1[c]	6.15 – 17.26
Fiber (% dw)		
Acid detergent fiber (ADF)	3.3-4.3[d]; 2.46-11.34[a,b]	1.82 – 11.34
Neutral detergent fiber (NDF)	8.3-11.9[d]; 7.58-15.91[b]	5.59 – 22.64
Total dietary fiber (TDF)	10.99-11.41[h]	8.82 – 35.31
Minerals		
Calcium (% dw)	0.01-0.1[d]	0.00127 – 0.02084
Copper (mg/kg dw)	0.9-10[d]	0.73 – 18.50
Iron (mg/kg dw)	1-100[d]	10.42 – 49.07
Magnesium (% dw)	0.09-1[d]	0.0594 – 0.194
Manganese (mg/kg dw)	0.7-54[d]	1.69 – 14.30
Phosphorous (% dw)	0.26-0.75[d]	0.147 – 0.533
Potassium (% dw)	0.32-0.72[d]	0.181 – 0.603
Zinc (mg/kg dw)	12-30[d]	6.5 – 37.2

Table VII.9 (cont.). Literature and ILSI Database Ranges of Components of Corn Forage and Grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
<u>Grain</u>		
Amino Acids (% dw)		
Alanine	n.a.	0.439 – 1.393
Arginine	n.a.	0.119 – 0.639
Aspartic acid	n.a.	0.335 – 1.208
Cystine	n.a.	0.125 – 0.514
Glutamic acid	n.a.	0.965 – 3.536
Glycine	n.a.	0.184 – 0.539
Histidine	n.a.	0.137 – 0.434
Isoleucine	n.a.	0.179 – 0.692
Leucine	n.a.	0.642 – 2.492
Lysine	n.a.	0.172 – 0.668
Methionine	n.a.	0.124 – 0.468
Phenylalanine	n.a.	0.244 – 0.930
Proline	n.a.	0.462 – 1.632
Serine	n.a.	0.235 – 0.769
Threonine	n.a.	0.224 – 0.666
Tryptophan	n.a.	0.0271 – 0.215
Tyrosine	n.a.	0.103 – 0.642
Valine	n.a.	0.266 – 0.855
Fatty Acids	(% total fat)	(% total fatty acid)
16:0 Palmitic	7-19[e]	7.94 – 20.71
16:1 Palmitoleic	1[e]	0.095 – 0.447
18:0 Stearic	1-3[e]	1.02 – 3.40
18:1 Oleic	20-46[e]	17.4 – 40.2
18:2 Linoleic	35-70[e]	36.2 – 66.5
18:3 Linolenic	0.8-2[e]	0.57 – 2.25
20:0 Arachidic	0.1-2[e]	0.279 – 0.965
20:1 Eicosenoic	n.a.	0.170 – 1.917
22:0 Behenic	n.a.	0.110 – 0.349
Vitamins (mg/kg dw)		
Folic acid	0.3[d]	0.147 – 1.464
Niacin	9.3-70[d]	10.37 – 46.94
Vitamin B_1	3-8.6[e]	1.26 – 40.00
Vitamin B_2	0.25-5.6[e]	0.50 – 2.36
Vitamin B_6	5.3[d]; 9.6[e]	3.68 – 11.32
Vitamin E	3-12.1[e]; 17-47[d]	1.5 – 68.7

Table VII.9 (cont.). Literature and ILSI Ranges of Components of Corn Forage and Grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
Grain		
Antinutrients (% dw)		
Phytic acid	0.48-1.12[a]	0.111 – 1.570
Raffinose	0.08-0.30[e]	0.020 – 0.320
Secondary Metabolites (µg/g dw)		
Ferulic acid	113-1194[f]; 3000[g]	291.9 – 3885.8
p-Coumaric acid	22-75[f]	53.4 – 576.2

[1]fw=fresh weight; dw=dry weight; Niacin =Vitamin B_3; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine ; n.a. = not available as percent dry wt.
[2]Literature range references: [a]Ridley et al., 2002. [b]Sidhu et al., 2000. [c]Jugenheimer, 1976. [d]Watson, 1987. [e]Watson, 1982. [f]Classen *et al.*, 1990. [g]Dowd and Vega, 1996. [h]Choi et al., 1999.
[3]ILSI range is from ILSI Crop Composition Database, 2006.

Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw

SECTION 4. Other Information Relevant to the Safety and Nutritional Assessment of MON 89034

The long history of safe consumption of corn as food and feed, combined with the demonstration of the compositional equivalence of MON 89034 to conventional corn, is considered sufficient to support the safety and nutritional assessment of MON 89034.

SECTION 5. Substantial Equivalence of MON 89034 to Conventional Corn

A detailed compositional assessment of corn grain and forage was presented in Part VII, Section 3, where the levels of key nutrients, anti-nutrients and other components in MON 89034 were examined and compared to that of the conventional control, LH198 x LH172. Additionally, tolerance intervals representing 99% of the values of each analyte from a conventional corn population were established. Results demonstrate that the levels of key nutrients, anti-nutrients and other components in the forage and grain of MON 89034 are equivalent to those of conventional corn. Based on the data and information presented, Monsanto concludes that MON 89034 is substantially equivalent to conventional corn, and this conclusion extends to the foods and feeds produced from MON 89034.

Collectively, these data and a history of safe use of the host organism, corn, as a common source of human food and animal feed, support a conclusion of "no concerns" for every criterion specified in the flowcharts outlined in the FDA's Food Policy document (FigureVII.1; FDA, 1992). Corn MON 89034 is not materially different in composition, safety or agronomic characteristics from conventional corn other than its protection from lepidopteran feeding damage. Sales and consumption of corn grain and processed products derived from MON 89034 would be fully consistent with the FDA's Food Policy, the Federal Food, Drug and Cosmetic Act, and current practices for the development and introduction of new corn varieties.

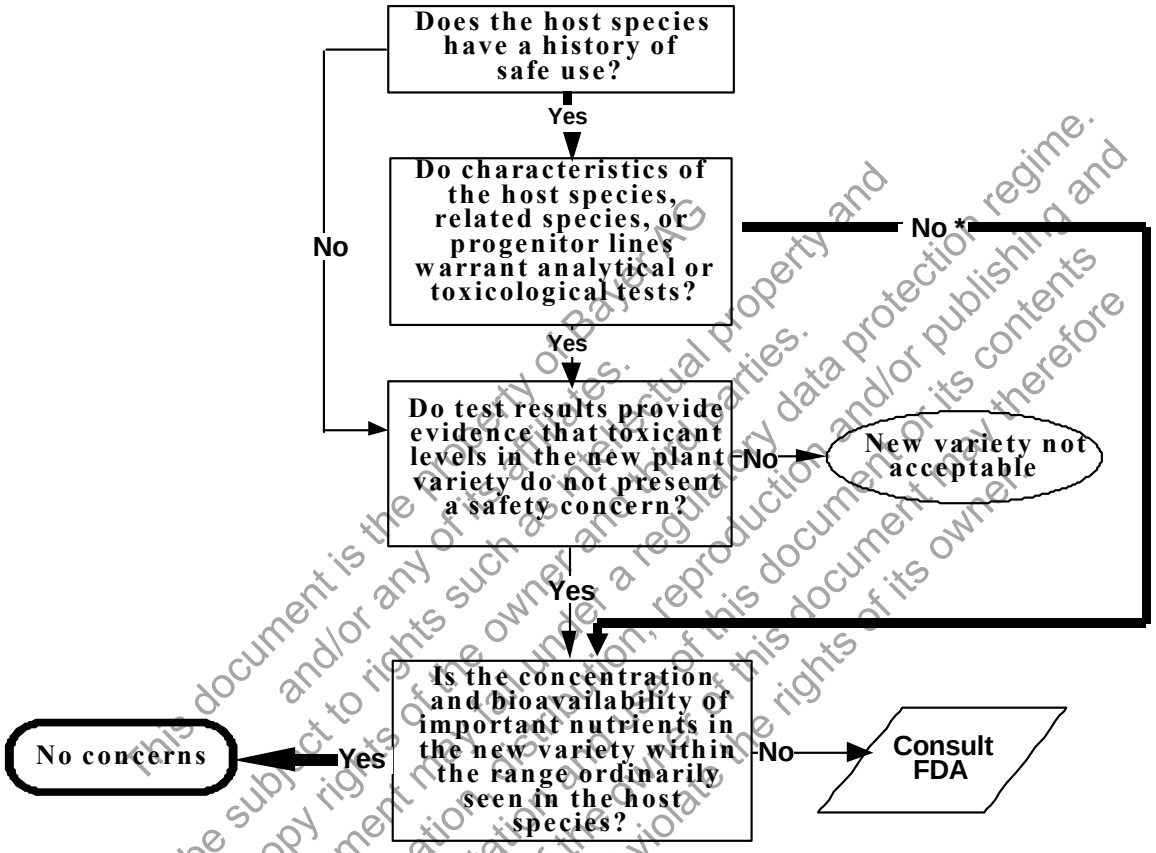

Figure VII.1. Safety Assessment of New Varieties: the Host Plant

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APPENDICES

APPENDIX A: Materials and Methods for the Molecular Analysis of MON 89034

Molecular analysis was performed using genomic DNA isolated from MON 89034 in order to characterize the integrated DNA. MON 89034 genomic DNA was analyzed by Southern blot analysis for the number of integration sites, the number of copies of the integrated DNA at each locus, the integrity of the inserted gene cassettes, the presence or absence of plasmid backbone sequence and the stability of the introduced DNA across multiple generations. Additionally, PCR amplification and DNA sequencing were used to confirm the 5' and 3' insert-to-plant junctions, confirm the organization of the elements within the insert, and determine the complete DNA sequence of the integrated DNA.

1. Test Substance

The test substance was grain, leaf and seed from corn MON 89034.

2. Control Substance

The control substance was conventional corn with genetic background similar to MON 89034.

3. Reference Substances

PV-ZMIR245 was used as a positive hybridization control for Southern blots and as a template control for PCR analyses. The plasmid was isolated and its identity confirmed by restriction enzyme digestion.

The 1 kb DNA extension ladder and λ DNA/*Hin*d III fragments from Invitrogen (Carlsbad, CA) were used for size estimations on Southern blots and agarose gels. Additionally, low DNA mass ladder and high DNA mass ladder from Invitrogen were used for size estimations and quantitative estimations on agarose gels.

4. Characterization of Test, Control and Reference Substances

Event specific PCR assays were used to confirm the identity of these test substances and the absence of MON 89034 for control substances. The test and control substances were considered stable during storage if they did not appear visibly degraded on ethidium-stained gels and/or yielded interpretable signals on the Southern blot.

5. Genomic DNA Isolation for Southern Blot Analyses

Grain and seed samples were processed and genomic DNA was extracted using a CTAB (hexadecyltrimethylammonium bromide)-based method (Rogers and Bendich, 1985). For a single DNA extraction, chloroform was used rather than chloroform:isoamyl alcohol.

Genomic DNA was extracted from leaf tissue using a Sarkosyl DNA extraction method (Fulton T. M. et al, 1995). For two Sarkosyl DNA extractions DNA was hooked out rather than using centrifugation. Extracted DNA solutions were stored in a 4°C refrigerator and/or a -20°C freezer.

For purification of plasmid DNA Qiagen QIA filter Plasmid Mini Kit was used.

6. Quantification of Genomic DNA

Extracted DNA was quantified using Hoefer's DyNA Quant 200 Fluorometer. Molecular size marker IX (Roche, Indianapolis, IN) was used as the calibration standard.

7. Restriction Enzyme Digestion of Genomic DNA

Approximately 10 or 20 µg of genomic DNA extracted from the test and control substances were digested overnight in a total volume of approximately 500 µl of buffer using 100 units of the restriction enzyme. For positive hybridization controls, approximately one or one-half genomic equivalent of PV-ZMIR245 was spiked into conventional corn DNA prior to digestion. For positive hybridization controls, approximately one or one-half genomic equivalent of PV-ZMIR245 was spiked into conventional corn DNA prior to digestion.

8. Agarose Gel Electrophoresis

Approximately 10 µg of digested DNA were separated using 0.8% agarose gels. For insert number, copy number, and cassette intactness experiments, a 'long run' and 'short run' were performed during the gel electrophoresis. Approximately 20 µg of digested test and control substance DNA was divided in half to load approximately 10 µg on the long run and approximately 10 µg on the short run. The long run enabled greater separation of the higher molecular weight restriction fragments while the short run allowed the smaller molecular weight restriction fragments to be retained on the gel.

10. Probe preparation

Approximately 12.5-27 ng of each probe template were prepared by PCR amplification and radiolabeled with ^{32}P-dCTP (6000 Ci/mmol) using the random priming method (except probe 10 which was labeled by PCR).

11. Southern blot analyses

Digested genomic DNA isolated from test and control materials was evaluated using Southern blot analyses (Southern, 1975) with modifications.

12. PCR and sequence analyses

Overlapping PCR products were generated that span the insert in MON 89034. These products were sequenced to determine the nucleotide sequence of the insert in MON 89034 as well as the nucleotide sequence of the genomic DNA flanking the 5' and 3' ends of the insert.

The PCR analyses were conducted using 50 ng of genomic DNA template in a 50 µl reaction volume containing a final concentration of 2 mM $MgSO_4$, 0.2 µM of each primer, 0.2 mM each dNTP, and 1 unit of DNA polymerase mix. The specific DNA polymerase mix used to amplify the products was Accuprime *Taq* (Invitrogen). The amplification of Product A was performed under the following cycling conditions: 94°C for 3 minutes; 30 cycles at 94°C for 15 seconds, 57°C for 30 seconds, 68°C for 3 minutes and 30 seconds; 1 cycle at 68°C for 5 minutes. The amplification of Product B was performed under the following cycling conditions: 94°C for 3 minutes; 35 cycles at 94°C for 15 seconds, 66°C for 30 seconds, 68°C for 3 minutes and 30 seconds; 1 cycle at 68°C for 5 minutes. The amplification of Products C, D, and E was performed under the following cycling conditions: 94°C for 3 minutes; 30 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 3 minutes; 1 cycle at 68°C for 5 minutes. The amplification of Products F and G was performed under the following cycling conditions: 94°C for 3 minutes; 30 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute and 30 seconds; 1 cycle at 68°C for 5 minutes.

Aliquots of each PCR product were separated on 1.0 % (w/v) agarose gels and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. The PCR products were sequenced with multiple primers used for PCR amplification in addition to those designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry.

MON 89034 DNA generated the expected size PCR products of approximately 2.5 kb for Product A (lane 4); approximately 3.3 kb for Product B (lane 7); approximately 2.6 kb for Product C (lane 10); approximately 2.6 kb for Product D (lane 14); approximately 3.2 kb for Product E (lane 18), approximately 1.1 kb for Product F (lane 22) and approximately 0.8 kb for Product G (lane 25). The generation of the predicted size PCR products from MON 89034 establishes that the arrangement or linkage of elements in the insert are the same as those in plasmid PV-ZMIR245 and that the elements within each gene cassette are arranged as depicted in the schematic of the insert in Figure A.1.

13. References

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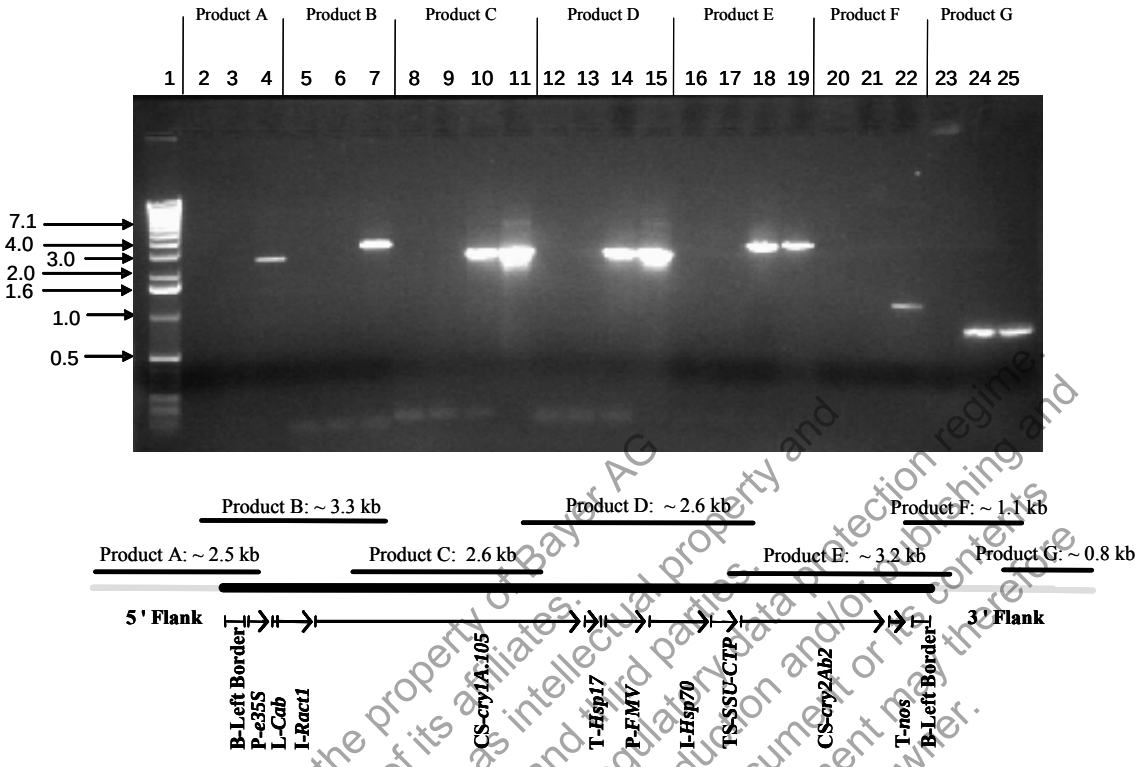

Figure A.1. Overlapping PCR analysis across the insert in MON 89034

PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 89034 were performed on MON 89034 genomic DNA extracted from grain (Lanes 4, 7, 10, 14, 18, 22, and 25). Lanes 3, 6, 9, 13, 17, 21, and 24 contain reactions with conventional corn control DNA while lanes 2, 5, 8, 12, 16, 20, and 23 are reactions containing no template DNA. Lanes 11, 15, and 19 contain reactions with PV-ZMIR245 control DNA. Lane 1 contains Invitrogen 1 kb DNA ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The gel side arrow symbols denote the sizes of DNA (kb) based on the reference DNA size markers on the ethidium bromide stained gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 89034 that appears below the image. Analyses of MON 89034 insert sequence and genomic DNA flanking the insert revealed that the *e35S* promoter that regulates expression of the *cry1A.105* gene has been modified and that the Right Border sequence was replaced by a Left Border sequence. This modification is likely the result of a crossover recombination which occurred prior to the DNA being inserted into the genome.

Lane
1: Invitrogen 1 kb DNA ladder
2: No template DNA control
3: Conventional corn control DNA
4: MON 89034 genomic DNA
5: No template DNA control
6: Conventional corn control DNA
7: MON 89034 genomic DNA
8: No template DNA control
9: Conventional corn control DNA
10: MON 89034 genomic DNA
11: PV-ZMIR245 plasmid
12: No template DNA control
13: Conventional corn control DNA
14: MON 89034 genomic DNA
15: PV-ZMIR245 plasmid
16: No template DNA control
17: Conventional corn control DNA
18: MON 89034 genomic DNA
19: PV-ZMIR245 plasmid
20: No template DNA control
21: Conventional corn control DNA
22: MON 89034 genomic DNA
23: No template DNA control
24: Conventional corn control DNA
25: MON 89034 genomic DNA

APPENDIX B: Materials and Methods for the Characterization of the Cry1A.105 and Cry2Ab2 Proteins in MON 89034

1. Materials and Methods used to Characterize the Cry1A.105 Protein

1.1. Plant-Produced Cry1A.105 Protein

The plant-produced Cry1A.105 protein was purified from grain of MON 89034. The identity of the grain containing MON 89034 was confirmed by event-specific PCR. The purified plant-produced Cry1A.105 protein was stored in a 4 ºC refrigerator in a buffer solution containing 50 mM CAPS, 1 mM PMSF, 2 mM benzamidine-HCl, 1 mM EDTA, 0.8 M NaCl, and 30% (v/v) ethylene glycol, pH 10.0.

1.2. *E. coli*-Produced Cry1A.105 Protein

E. coli-produced Cry1A.105 protein was used as a reference standard for determination of protein concentration and immunoblot analysis using N-terminal peptide antibody. This protein was also used as a reference standard to evaluate equivalence between plant- and *E. coli*-produced Cry1A.105 proteins for the molecular weight and functional activity assay, as a reference and a negative control in glycosylation analysis, and as a reference and a positive control in immunoblot analysis using anti-Cry1A.105 antibody. The *E. coli*-produced Cry1A.105 reference standard was previously characterized. The Cry1A.105 protein was stored in a -80 °C freezer in a buffer solution (25 mM CAPS, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH ~10.3) at a total protein concentration of 1.2 mg/ml by amino acid analysis.

1.3. Assay Controls

Protein molecular weight standards (Bio-Rad, Hercules, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF membranes. The *E. coli*-produced Cry1A.105 reference standard was used to create a standard curve for the quantitative western blot. The Cry1A.105 trypsin-resistant core was used as a negative control for the western blot analysis using N-terminal peptide antibody. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. Transferrin (Amersham Biosciences, Piscataway, NJ) and horseradish peroxidase (Sigma, St. Louis, MO) were used as positive controls for glycosylation analysis. CandyCane™ glycoprotein molecular weight standards were used as molecular weight markers and positive and negative controls for glycosylation analysis.

1.4. Protein Purification

The plant-produced Cry1A.105 protein was purified from the corn grain of MON 89034. The purification procedure was not performed under a GLP study or plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed.

The Cry1A.105 protein was purified at 4°C from an extract of ground grain using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

The ground grain (10 kg) was mixed in PBS extraction buffer [1× PBS: 1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4] for 2.5 hours at approximately a 1:10 sample weight to buffer volume ratio. The slurry was filtered using an Ertel Alsop filter press (Kingston, NY) and the PBS washed filter cakes were retained. The filter cakes were resuspended in ~100 L of CAPS extraction buffer [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine- HCl, and 1% PVPP (w/v), pH 10.8] and stirred for 2 hours. To remove lipids from the extract, CelPure P65 Diatomaceous Earth (Advanced Minerals Corp, Goleta, CA) was added to the slurry at 7.5% (w/v) and allowed to mix for ~10 minutes. The extract was clarified by filtration using the Ertel Alsop filter press and concentrated using a Hollow Fiber Cartridge (Amersham Biosciences, Piscataway, NJ). The contaminant DNA was removed by a combination of benzonase treatment and precipitation with polyethyleneimine. Benzonase was added to a final concentration of ~7 U/ml in the presence of 5 mM $MgCl_2$ and allowed to mix overnight. A polyethyleneimine solution [10% (w/v)] was added to the concentrated extract to a final concentration of 0.05% (w/v) and the extract was clarified by centrifugation to remove the remaining contaminant DNA. An ammonium sulfate pellet (0% – 35% saturation) was prepared by the addition of ammonium sulfate salt to the clarified extract and was allowed to dissolve overnight. After centrifugation, the ammonium sulfate pellet was dissolved over the weekend in ~15 L of resuspension buffer [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 2 mM benzamidine-HCl, pH 10.8]. The resuspended sample was clarified by centrifugation, diafiltrated against fresh buffer (same as the resuspension buffer) to remove any residual ammonium sulfate and concentrated to final volume of ~8 L and CHAPS was added to a final concentration of 0.5 mM.

The concentrated sample was loaded onto a 2.1 L (6.7 cm x 20 cm) Q Sepharose Fast Flow anion exchange resin column, which was equilibrated with AEX buffer A [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, and 0.5 mM CHAPS, pH 10.8]. The bound Cry1A.105 protein was eluted with a linear salt gradient that increased from 0 M to 0.20 M sodium chloride over ~12 L and then maintained a 0.20 M sodium chloride concentration for ~4 L. Next, the salt gradient increased to 0.65 M sodium chloride over ~21 L, and finally stepped up to 1.0 M sodium chloride instantly. Fractions containing the Cry1A.105 protein (based on stained SDS-PAGE gel and western blot analysis of all fractions) were pooled to a final volume of ~10 L. Salt was removed from these pooled fractions by diafiltration with fresh buffer [50 mM EPPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 2 mM benzamidine-HCl, pH 7.5].

The buffer exchanged sample was loaded onto a 2.1 L (13.7 cm x 14.0 cm) Q Sepharose XL anion exchange resin column, which was equilibrated with AEX buffer A [50 mM EPPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, and 0.5 mM CHAPS, pH 7.5]. The bound Cry1A.105 protein was eluted with a linear salt gradient that increased from 0 M to 0.25 M sodium chloride over ~ 4 L and then held at 0.25 M

sodium chloride for ~ 10 L. Next, the salt gradient increased to 0.65 M sodium chloride over ~21 L and finally increased to 1.0 M sodium chloride over ~ 4 L and held constant for ~8L. The fractions containing Cry1A.105 protein (based on stained SDS-PAGE gel and western blot analysis of all fractions) were pooled to a final volume of ~10 L. These fractions were diafiltrated into fresh buffer [50 mM EPPS, 1 mM EDTA, 10 mM DTT, 1mM PMSF, and 2 mM benzamidine-HCl, pH 7.5] to remove salt and concentrated to ~2.0 L using a hollow fiber cartridge.

Prior to the affinity purification step, the sample was diafiltrated into fresh buffer [50 mM EPPS, 1mM EDTA, 1 mM PMSF, 2 mM benzamidine-HCl, and 150 mM NaCl, pH 7.6] and concentrated down to ~1L. The solution containing Cry1A.105 protein was re-circulated over the 9.3 ml (1.75 cm x 2.6 cm) protein A agarose column (Sigma) conjugated with monoclonal anti-Cry1Ac antibody (Strategic Biosolutions, Newark DE). Bound Cry1A.105 protein was eluted using 50 mM CAPS, 1 mM EDTA, 1 mM PMSF, 2 mM benzamidine-HCl, 0.8 M NaCl, and 30% (v/v) ethylene glycol, pH 10.0. After analysis of fractions by lateral flow strips, stained SDS-PAGE gel and western blot, fraction 25 was determined to contain the majority of the full-length Cry1A.105.

1.5. Determination of the Total Protein Concentration

The concentration of the full-length (~130 kDa) plant-produced Cry1A.105 protein was estimated using quantitative immunoblot analysis. The *E. coli*-produced Cry1A.105 protein (amounts ranging from 1 to 6 ng purity corrected for the full-length protein, which was 82%) was used to generate a standard curve. Aliquots of the plant-produced Cry1A.105 protein and reference standard were diluted in deionized water and 5x Laemmli buffer (5x LB), heated at 95.5 °C for 5 min, and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 12-well gel. Three different amounts of the plant-produced protein were loaded in duplicate. Electrophoresis was performed at a constant voltage of 125 V for 15 min followed by a constant voltage of 170 V for 75 min. Pre-stained molecular weight markers (Bio-Rad Precision Plus Dual Color, Hercules, CA) were used to verify electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands observed. Electrotransfer to a 0.45 μm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V.

The membrane was blocked for 1 hour with 5% (w/v) NFDM in 1× PBST. The membrane was probed with a 1:1000 dilution of rabbit anti-Cry1A.105 antibody (lot 070705JL) in 1% (w/v) NFDM in PBST for 60 min. Excess antibody was removed using four 5 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) at a dilution of 1:7500 in 1% (w/v) NFDM in PBST for 60 minutes. Excess HRP-conjugate was removed using three 5 min washes with PBST. All incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ) and exposed (5 min, 10 min, 20 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

The immunoreactive band of the plant-produced Cry1A.105 protein in each lane migrating at the same level as the full-length reference standard protein was quantitated relative to the standard curve. Quantitation was performed using the volume tool and the linear regression method in the Quantity One software (version 4.4.0) after scanning on the Bio-Rad GS-800 densitometer. The concentration of the plant-produced Cry1A.105 was determined by dividing the amount of protein in each lane by the volume of protein loaded in that lane [Microsoft Excel 2002 (10.6730.6718) SP3]. Throughout this summary, all load quantities of plant-produced Cry1A.105 refer to the amount of the full-length protein.

1.6. Western Blot Analysis

Western blot analyses were performed to confirm the identity of the Cry1A.105 protein, the intactness of its N-terminus, and compare immunoreactivity of the plant- and *E. coli*-produced proteins.

The western blot described above (section 1.5) was also used to establish the identity of the plant-produced protein and to compare the immunoreactivity of the plant-produced and *E. coli*-produced Cry1A.105 proteins. Equivalence was demonstrated for bands representing full-length (~130 kDa) proteins that were identified by the anti-Cry1A.105 antibody and showed similar electrophoretic mobility.

1.7. Analysis of the N-terminal Sequence using western blot

A western blot analysis using the N-terminal peptide antibody was performed to confirm the intactness of the N-terminus of the plant-produced Cry1A.105 protein. The N-terminal peptide antibodies were produced against a synthetic peptide consisting of the first 14 amino acids of the Cry1A.105 protein N-terminus. The trypsin resistant core was used as a negative control because its N-terminus was removed by trypsin. The plant-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 reference standard (corrected for the purity of the full-length protein), and the Cry1A.105 trypsin-resistant core (~56 kDa) were each loaded on gels at 20 ng and 40 ng per lane. Each protein was mixed with 5× LB, heated at 96.0 °C for 5 min, and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well gel. Electrophoresis was performed at a constant voltage of 150 V for 5 min followed by a constant voltage of 200 V for 60 min. Pre-stained molecular weight markers (Bio-Rad Precision Plus Dual Color,) were used to verify electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands. Electrotransfer to a 0.45 μm PVDF membrane (Invitrogen) was performed for 90 min at a constant voltage of 25 V.

The membrane was blocked overnight with 5% (w/v) NFDM in 1× PBST. The membrane was probed with a 1:33.33 dilution of rabbit N-terminal peptide antibody (lot 42005MG) in 1% (w/v) NFDM in PBST for 60 min. Excess antibody was removed using three 5 min washes with PBST. Finally, the membrane was probed with HRP-conjugated anti-rabbit IgG (Sigma) at a dilution of 1:7500 in 1% (w/v) NFDM in PBST for

60 minutes. Excess HRP-conjugate was removed using three 5 min washes with PBST. The blocking step was performed at 4 °C. All other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences) and exposed (1 min, 2 min, 5 min, 10 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX-101A automated film processor.

1.8. MALDI-TOF MS Tryptic Mass Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the plant-produced Cry1A.105 protein.

1.8.1. Concentration of Protein

The plant-produced protein (4.5 ml) was diluted to 9.0 ml with Cry1A.105 concentration buffer [25 mM CAPS, 0.02% (w/v) sodium dodecyl sulfate] followed by reduction of the volume to 64 μl using Amicon Ultrafree CL concentrators (Millipore Corporation, Bedford, MA). Sixty microliters of 5× LB was used to wash the concentrator walls and membranes. The membranes were heated with 5× LB for 5 minutes at 95.1°C. Sixteen microliters of this 5× LB was then used as the loading buffer for the Cry1A.105 sample for SDS-PAGE separation.

1.8.2. SDS-PAGE Separation of Proteins

The concentrated plant–produced Cry1A.105 protein was subjected to electrophoresis on an SDS-polyacrylamide gel. The protein sample was loaded in two lanes. Broad Range molecular weight markers (Bio-Rad) were used to estimate molecular weights. Plant-produced Cry1A.105 protein and molecular weight markers were heated at 95.3 °C for 5 min and then applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel. Electrophoresis was performed at constant voltage (125 V for 10 min followed by 170 V for 70 min). Proteins were stained with Bio-Rad Coomassie 1 × stain for 2 hours, and destained by washing with Coomassie R-250 1× destain solution (Bio-Rad) for 2 hours with one change of the destain solution.

1.8.3. In-gel Trypsin Digestion and Sample Preparation

In-gel protein digestion

The bands representing full-length plant-produced Cry1A.105 protein (~130 kDa) were excised from two gel lanes, destained, reduced, alkylated, and subjected to an in-gel trypsin digestion (Williams et al., 1997). Briefly, each gel band was individually destained for 30 min by incubation in 100 μl of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in a microfuge tube. This was repeated two additional times. Following destaining, the gel bands were incubated in 100 μl of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. The protein was reduced in 100 μl of 10 mM dithiothreitol solution for 2 hours at 37 °C. The protein was alkylated by the addition of 100 μl of 200 mM iodoacetic acid. The alkylation reaction was allowed to

proceed at room temperature for 20 min in the dark. The gel bands were incubated in 100 µL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature at which time 100 µl of acetonitrile was added and the incubation was continued for an additional 30 minutes. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove the reducing and alkylating reagents and other salts from the gel. The gel bands were dried in a SpeedVac concentrator, rehydrated with 40 µl 25 mM ammonium bicarbonate containing 33 µg/ml trypsin, and the protein contained in the gel band was digested for 16 hours at 37.1 °C. Digested peptides were extracted for 60-80 min at room temperature with 50 µl 70% (v/v) acetonitrile containing 0.1% (v/v) TFA per gel band. Both extraction supernatants were combined into a single tube and dried in a SpeedVac concentrator. This process of extracting the peptides was repeated two more times. The final dried materials were reconstituted in 5 µl of 0.1% (v/v) TFA.

Sample preparation

An aliquot (4 µl) of the digested sample was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) ZipTip C18 pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. The sample was applied to a ZipTip and eluted with 4 µl of Wash 1 [0.1% (v/v) TFA], followed by 4 µL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], followed by 4 µl of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 4 µl of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

1.8.4. MALDI-TOF Instrumentation and Mass Analysis

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme Peptide Mass Standards kit (Applied Biosystems). Samples (0.5 µl) from each of the desalting steps, as well as a sample of solution taken prior to desalting, were co-crystallized with 0.8 µl α-cyano-4-hydroxy cinnamic acid on the analysis plate. The sample was analyzed in the 500 to 5000 Dalton range using 100 shots at a laser intensity setting of 2781 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected Cry1A.105 (plant) protein sequence based upon the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 5000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 Daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data. The tryptic mass map coverage was considered acceptable if ≥ 40 % of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments.

1.9. Determination of Molecular Weight using SDS-PAGE

Aliquots of the *E. coli*- and plant-produced Cry1A.105 proteins were mixed with 5× LB to a final protein concentration of 10 ng/µl and 2.4 ng/µl, respectively. The plant-produced protein was analyzed in duplicate at 48, 72, and 96 ng of total protein per lane. The *E. coli*-produced Cry1A.105 reference standard was analyzed at 96 ng of purity corrected full-length protein. All samples were heated in a thermo-block at 99 °C for 3 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel (Invitrogen). Electrophoresis was performed at a constant voltage of 125 V for 10 min followed by a constant voltage of 170 V for 70 min.

The gel was stained using the SilverXpress® Silver Staining Kit Protocol (Invitrogen, Carlsbad, CA). The gel was fixed for 10 minutes in 200 ml of fixing solution (90 ml ultra pure water, 100 ml methanol, and 20 ml acetic acid). This was followed by 10 minutes in 100 ml sensitizing solution (105 ml ultra pure water, 100 ml methanol, and 5 ml sensitizer) and repeated once. The excess of the sensitizing solution was removed using two 5 minutes washes in 200 ml ultra pure water. The gel was stained for 15 minutes in 100 ml staining solution (5 ml Stainer A, 5 ml Stainer B, and 90 ml ultra pure water). The stain was removed using two 5 minutes washes of 200 ml ultra pure water. Developing occurred in 100 ml of solution (5 ml developer and 95 ml ultra pure water) for 3 - 15 minutes, and was stopped by addition of 5 ml stopping solution for 10 minutes. The gel was washed three times for 10 minutes each with 200 ml of ultra pure water. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). Molecular weight markers (Bio-Rad Broad-Range) were used to estimate the apparent molecular weight of the plant-produced Cry1A.105 protein. For the purity evaluation, all visible bands within each lane were quantified. Stained bands corresponding to immunoreactive bands identified by anti-Cry1A.105 antibody and migrating from ~56 to ~130 kDa were included in the purity calculation for the protein of interest as they represent various lengths of the insecticidal protein from the ~56 kDa tryptic core to the ~130 kDa full-length protein (Bietlot et al., 1989). The purity and estimated full-length molecular weight of the plant-produced Cry1A.105 protein were reported as the average of the six values obtained by densitometric analysis.

1.10. Determination of Functional Activity by Insect Bioassay

In order to assess the functional activity of the plant-produced Cry1A.105 protein and to compare its activity to the *E. coli*-produced Cry1A.105 reference standard, aliquots of the plant-produced Cry1A.105 protein and *E. coli*-produced Cry1A.105 reference standard protein were used to estimate the effective protein concentration necessary to inhibit the growth of the target insect by 50% (EC_{50} value) and the rate of weight loss as test protein concentration in the diet increased (slope parameter). The functional activity of the plant-produced Cry1A.105 was considered equivalent to that of the *E. coli*-produced protein activity if there was less than a 4-fold difference in mean EC_{50} values. Logistic regression was used to model concentration-response curves for growth inhibition and for EC_{50} determinations.

1.10.1. Insects

Helicoverpa zea (CEW) eggs were obtained from Benzon Research Inc. Insect eggs were incubated at a temperatures ranging from 10^{o} C to 27^{o} C, to achieve the desired hatch time.

1.10.2. Bioassays

CEW were used to measure activity of the plant- and *E. coli*-produced Cry1A.105 protein samples in diet-incorporation bioassay. The bioassay was replicated three times on separate days with separate batches of insects. The plant- and *E. coli*-produced proteins were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and plant-produced Cry1A.105 proteins consisted of a series of five protein levels yielding a dose series ranging from 0.00048 – 0.039 µg Cry1A.105 protein/mL diet with a 3-fold separation factor between dose levels. This dose series was chosen to adequately characterize the dose-effect relationship for the proteins from both sources. Each dose level, including the control, had an equivalent volume of buffer added to the dosing solution. The Cry1A.105 protein dosing solutions were prepared by diluting the protein with purified water and incorporating the solution into an agar-based insect diet. Diet mixture was then dispensed in 1 mL aliquots into a 128 well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD International, Pitman, NJ) and the insects were allowed to feed for a period of 6-7 days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14h:10h, light:dark. The combined weight of the surviving insects at each dose level for each source of protein was recorded at the end of the 6-7 day incubation period.

1.10.3. Statistical analysis

The following three-parameter logistic model, with an extra parameter for the change in variation with the expected weight (equation below), was used to model the dose-response curves for each protein source and each replicate under the PROC NLMIXED procedure in SAS:

Equation:

$$Wt = \frac{W_0}{1 + \left(\frac{DietDose}{EC50}\right)^B} + e$$

$$Var(e) = \left\{ \frac{s \cdot W_0}{1 + \left(\frac{DietDose}{EC50}\right)^B} \right\}^2$$

where Wt is the average CEW larvae weight and *DietDose* is the Cry1A.105 protein diet dose level. The residual variation was assumed to be proportional to the expected mean weight. The parameters that are included in the model are W_0 which represents the expected weight at *DietDose* = 0.0, EC_{50} which represents the concentration needed to inhibit the growth of the target insect by 50%, *B* which reflects the rate of the weight loss as *DietDose* increases, *s* which represents the proportion of the standard deviation to the expected weight, and *e* which denotes the residual (error).

1.11. Glycosylation Analysis

Glycosylation analysis was used to determine whether the plant-produced Cry1A.105 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the plant-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 reference standard, and the positive controls, transferrin (Amersham Biosciences) and horseradish peroxidase (Sigma), were each mixed with 5× LB. These samples were heated at 95 °C for 4 min, cooled, and loaded on a tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel. Each sample was loaded at 48 and 96 ng (purity corrected for the full length protein) per lane. Precision Plus Dual Color pre-stained protein molecular weight markers (Bio-Rad) were loaded to verify electrotransfer of the proteins to the membrane, and the CandyCane™ Glycoprotein Molecular Weight Standards (Molecular Probes, Eugene, OR) were loaded as positive/negative controls and markers for molecular weight. Electrophoresis was performed at a constant voltage of 125 V for 10 min followed by a constant voltage of 170 V for 70 min. Electrotransfer to a 0.2 μm PVDF membrane was performed for 90 min at a constant voltage of 25 V.

Carbohydrate detection was performed directly on the PVDF membrane using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes). The manufacturer's protocol was followed. All steps were performed at room temperature. The PVDF membrane was fixed in 25 ml of a solution containing 50% methanol and 5% glacial acetic acid for 1 hour, and then the solution was changed and the membrane was incubated overnight. Two 15 minute washes (50 ml each) of 3% (v/v) glacial acetic acid (wash solution), were followed by a 20 minute oxidation in 25 ml of the kit supplied oxidizing solution. After oxidation, three 15 minute washes (50 ml each) prepared the

membrane for staining. The blot was incubated in 25 ml of Pro-Q Emerald Staining Solution that was prepared using the kit reagents. After 1 hour of staining in the dark, two 30 minute, 50 ml wash cycles were followed by two 45 minute, 50 ml wash cycles. The final wash cycles included two 25 ml, 1 minute deionized water washes followed by three 25 ml, 5 minute methanol washes (B&J Brand, Muskegon, MI). The blot was then scanned using the BioRad Molecular Imager FX using the Alexa 488 illumination setting (Qunatity One software; version 4.6, build 036) in order to visualize the fluorescing glycosylated proteins.

2.0. Materials and Methods used to Characterize Cry2Ab2 Protein

2.1. Plant-Produced Cry2Ab2 Protein

The plant-produced Cry2Ab2 protein was isolated from ground corn grain of MON 89034. The identity of the grain sample containing MON 89034 was confirmed by event specific PCR. The isolated plant-produced Cry2Ab2 protein was stored in a –80 °C freezer in a buffer solution containing 50 mM CAPS, 2 mM DTT, pH 11, at a total protein concentration of 0.25 mg/ml.

2.2. *E. coli*-Produced Cry2Ab2 Protein

The *E. coli*-produced Cry2Ab2 protein was used as a reference standard in select analyses. These analyses included apparent molecular weight determination by SDS-PAGE, western blot analysis, glycosylation analysis, BioRad protein assay, and the functional activity assay. The *E. coli*-produced Cry2Ab2 protein reference standard has previously been characterized and is referred to as "Cry2Ab2.820" on the Certificate of Analysis.

2.3. Assay Controls

Protein molecular weight standards were used to calibrate SDS polyacrylamide gels and verify protein transfer to PVDF membranes. The *E. coli*-produced Cry2Ab2 reference standard protein was used to estimate the total protein concentration in the BioRad protein assays and also used as the positive control in western blot analysis. Beta-lactoglobulin protein and PTH-amino acid standards were used to verify the performance of the amino acid sequencer. A peptide mixture and an analytical BSA standard were used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis and molecular weight determination, respectively. Transferrin and *E. coli*-produced Cry2Ab2 protein were used as the positive and negative control, respectively, in glycosylation analysis.

2.4. Protein Purification

The plant-produced Cry2Ab2 protein was purified from the grain of MON 89034 prior to initiation of this characterization. Although the purification procedure was not performed under a GLP study or plan, procedures were documented on worksheets and, where

applicable, SOPs were followed. The Cry2Ab2 protein was purified from an extract of ground grain of MON 89034 using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

The isolation of Cry2Ab2 protein from ground corn grain was performed in two 10 kg batches. Prior to extraction of Cry2Ab2 protein, each batch was extracted with 1X PBS buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) to remove contaminant proteins. The PBS extraction procedure consisted of soaking ground grain in PBS for 2 hrs in a 4 °C cold room at approximately 1:10 sample weight to buffer volume ratio. The slurry was clarified by filtration using an Ertel Alsop filter press (Kingston, NY), and the PBS washed solid particle (cake) was retained. Subsequently, the Cry2Ab2 protein in the cake was extracted with extraction buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, 0.5 mM CHAPS, 1% (w/v) PVPP, pH 10.8 at approximately 1:10 sample weight to buffer volume ratio for 2-3 hrs. During extraction, lipids were removed from the extract by adding CelPure P65 diatomaceous earth (Advanced Minerals Corp, Goleta, CA) to the homogenate at ~7.5% (w/v) and allowed to mix for ~10 minutes. The slurry was clarified by filtration using the filter press and the resultant extract from both batches was pooled for a total volume of ~230 L. The pooled extract was concentrated using a 30,000 NMWC Hollow Fiber Cartridge (Amersham Biosciences, Piscataway, NJ) to a final volume of ~35 L. To remove plant genomic DNA, polyethyleneimine, 10% (w/v), was added to the concentrated extract to a final concentration of 0.05% (w/v), the extract was clarified by centrifugation to remove precipitated DNA, and the supernatant was retained. The Cry2Ab2 protein in the supernatant was precipitated by 0% – 35% ammonium sulfate saturation. The 35% ammonium sulfate pellet was recovered by centrifugation and the pellet was dissolved in 20 L of resuspension buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 by mixing in 4 °C cold room. The suspension was clarified by centrifugation and 21 L supernatant was retained, concentrated, and buffer exchanged by diafiltration against Buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 to remove any residual ammonium sulfate salt. The concentrated sample of 13 L was loaded onto an anion exchange column in two batches, Run 1 (6L) and Run 2 (7L). The elution parameters were identical for both runs and thus only the Run1 column parameters are described below.

A portion of concentrated sample was loaded onto a 4.5 L (20 cm x 14.4 cm column) Q Sepharose Fast Flow anion exchange resin column, which was equilibrated with Buffer. The bound Cry2Ab2 protein was eluted with step gradients as follows: 0-20% buffer B (Buffer containing 1M NaCl) in 10 column volumes (CV), and then the gradient was held at 20% buffer B for 4 CV, and then the gradient was increased to 65% buffer B over 10 CV and finally the gradient was stepped up to 100% buffer B and held at 100% buffer B in 2 CV. Fractions, each ~ 4 L, containing Cry2Ab2 protein were identified by Cry2A QuickStix™ (Portland, ME) for further analysis. Based on western blot analysis and SDS-PAGE analysis, fraction 7 from Run 2 was selected for affinity purification.

Subsequently, fraction 7 was concentrated to 400 mL using a 30,000 NMWC Hollow Fiber Cartridge. Approximately one half of the aforementioned sample was buffer exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2) using centrifuge concentrators (30 kDa MWCO), resulting in a final volume of 200 mL. This sample, in two separate batches, was applied to an affinity column (1.0 cm × 2.7 cm) containing Protein G agarose conjugated with Cry2Aa-specific mAb and equilibrated with EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2). The sample was re-circulated through the column for 2 hrs at 100 mL/hr. The column was then washed with 17-20 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2), followed by 5-7 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM Triethylamine, pH 11.4, in five 1.5 mL fractions. Based on SDS-PAGE analysis, fractions containing Cry2Ab2 protein from batch one and two were concentrated and buffer exchanged into a storage buffer containing 50 mM CAPS, 2 mM DTT, pH 11.0, using centrifuge concentrators (30 kDa MWCO). Concentrated samples were then pooled, resulting in a final volume of 2 mL.

The affinity chromatography procedure was repeated beginning with 100 mL of fraction 7 from Run2. As previously described, the sample was concentrated and buffer exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05) to a final volume of 50 mL using centrifuge concentrators (30 kDa MWCO). This sample was re-circulated for 2 hr (60 mL/hr) through an affinity column (1.0 cm × 2.75 cm) prepared with Cry2Aa-specific mAb equilibrated with EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05). The column was then washed with 15 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05) followed by 5 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM Triethylamine, pH 11.4, in five 1.5 mL fractions.

Based on SDS-PAGE analysis, Cry2Ab2 enriched fractions were individually concentrated and buffer exchanged into a storage buffer containing 50 mM CAPS, 2 mM DTT, pH 11, using a centrifuge concentrator (30 kDa MWCO). The concentrated samples were pooled into a final volume of 2.4 mL. The aforementioned sample and the sample from the first run (2 ml) were pooled resulting in a final volume of 4.4 ml. This sample was a clear colorless solution and was assigned lot # Cry2Ab.820_040705.

2.5. Determination of the Total Protein Concentration

The total protein concentration of the purified plant-produced Cry2Ab2 protein was estimated using a BioRad protein assay. The *E. coli*-produced Cry2Ab2 reference standard protein (concentrations ranging from 0.05 to 0.5 mg/mL) was used to prepare a standard curve. The plant-produced Cry2Ab2 total protein concentration was estimated by comparison of absorbance values obtained for the sample to the values of the standard curve. Data were collected using a Bio-Tek Instruments, Inc. Powerwave Xi microplate scanning spectrophotometer (Winooski, VT) employing KC4 software version 3.3 revision 10. Readings were taken at a wavelength of 595 nm.

2.6. Western Blot Analysis

Aliquots of the stock solutions of the plant-produced Cry2Ab2 and reference standard were diluted to a final purity-corrected protein concentration of 2 ng/µl in dilution buffer (50 mM CAPS, 2 mM DTT, pH 11) and 5× sample loading buffer [5x concentrated Laemmli buffer (312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8)]. Samples were then heated to ~100 °C for 5 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well gel. The plant-produced Cry2Ab2 protein was loaded in duplicate at three different loadings of 20, 30, and 40 ng per lane. The *E. coli*-produced Cry2Ab2 reference standard was loaded at 20 ng per lane. Electrophoresis was performed at a constant voltage of 140 V for 20 min followed by a constant voltage of 200 V for 43 min. Pre-stained molecular weight markers included during electrophoresis (BioRad Precision Plus Dual Color, Hercules, CA) were used to verify electrotransfer of protein to the membrane and to estimate the molecular weight of the immunoreactive bands. Samples were electrotransferred to a 0.45 micron PVDF membrane (Invitrogen, Carlsbad, CA) for 60 min at a constant current of 300 mA.

The membrane was blocked for one hour with 5% (w/v) NFDM in PBST. The membrane was probed with a 1:3000 dilution of goat anti-Cry2Ab2 antibody (lot 7227632) in 2% (w/v) NFDM in PBST for one hour. Excess antibody was removed using three 10 min washes with PBST. The membrane was probed with peroxidase-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO) at a dilution of 1:10,000 in 2% (w/v) NFDM in PBST for one hour. Excess peroxidase-conjugate was removed using three 10 min washes with PBST. All procedures, including blocking, and all other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ) and exposed (30 sec, 1 min, 2 min, 3 min, and 7 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

2.7. Analysis of the N-terminal Sequence

An aliquot of the plant-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final purity corrected protein concentration of 80 ng/µl. Molecular weight markers (BioRad Precision Plus Dual Color, Hercules, CA) were used to confirm the transfer of protein to the PVDF membrane. The plant-produced Cry2Ab2 protein was electrophoresed in eight lanes at 2 µg per lane. The Cry2Ab2 containing samples were heated to ~99 °C for 5 min prior to electrophoresis on a pre-cast tris-glycine 4→20% SDS polyacrylamide gel at 140V for 20 min followed by 200 V for 43 min. The gel was then electroblotted to a 0.2 micron PVDF membrane for 60 min at a constant current of 300 mA in a solution containing 10 mM CAPS, 10% (v/v) methanol, pH 11. Protein bands were stained by briefly soaking the membrane with Coomassie Blue R-250 stain (BioRad) and visualized by destaining with a Coomassie R-250 destain solution (BioRad).

The protein bands with molecular weights of approximately 61.3, 49.6 and 27 kDa, (also referred to as band-1, band-2, and band-3, respectively) were excised from the membrane. N-terminal sequence analysis was performed using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise™ Control Software (version 2.1) was used. Chromatographic data were collected using Atlas[99] software (version 2003R1.1, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the three protein bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

2.8. MALDI-TOF MS Tryptic Mass Map Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the plant-produced Cry2Ab2 protein. A protein can be typically identified when 40% of the mass fragments are identified from the analyzed protein (Jiménez et al., 1998).

2.8.1. SDS-PAGE Separation of Proteins

An aliquot of the plant-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final purity corrected protein concentration of 80 ng/µl and 2 µg was electrophoresed in each of five lanes. Broad Range molecular weight markers (BioRad, Hercules, CA) were used to estimate molecular weights. Samples were heated to ~99 °C for 5 min prior to electrophoresis on a pre-cast tris-glycine 4→20% SDS polyacrylamide gel at 140 V for 20 min followed by a constant voltage of 200 V for 46 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained 2 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO), destained 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and followed by 25% (v/v) methanol for 1 hr. Two protein bands, band-1 and band-2, migrating at 61 kDa and 50 kDa, respectively, were identified for tryptic mass map analysis.

2.8.2. In-gel Protein Digestion and Sample Preparation

In-gel protein digestion

The plant-produced full length Cry2Ab2 protein, band-1 migrating at ~61 kDa, and band-2, a proteolytic fragment of the full length Cry2Ab2 protein migrating at ~50 kDa, were excised, destained, reduced, alkylated, and subjected to an in-gel trypsin (Promega, Madison, WI) digestion (Williams et al., 1997). Each gel band was individually destained for 30 min by incubation in 100 µL of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in its own microfuge tube. Following destaining, the gel bands were incubated in 100 µL of 100 mM ammonium bicarbonate buffer for 30 min at room

temperature. Proteins were reduced in 100 µl of 10 mM dithiothreitol solution for 2 hrs at 37 °C. Proteins were then alkylated by the addition of 100 µl of buffer containing 200 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. The gel bands were incubated in 100 µl of 100 mM ammonium bicarbonate for 30 min at room temperature at which time 100 µl of acetonitrile was added and the incubation was continued for an additional 30 minutes. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove the reducing and alkylating reagents, and salts from the gel. The gel bands were dried in a SpeedVac concentrator (Savant, Holbrook, NY), rehydrated with 40 µl 25 mM ammonium bicarbonate containing 33 µg/ml trypsin, and the protein contained in the gel band was digested for 16 hours at 37 °C. Digested peptides were extracted for one hour at room temperature with 50 µl 70% (v/v) acetonitrile containing 0.1% (v/v) TFA per gel band. Supernatants for each extraction were combined into a single tube and dried in a SpeedVac concentrator. This process of extracting the peptides was repeated two more times. The final dried materials were reconstituted in 10 µl of 0.1% (v/v) TFA.

Sample Preparation

A portion (5 µl) of the digested samples was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) ZipTip®C18 pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. Each sample was applied to a ZipTip®C18 and eluted with 5 µL of Wash 1 [0.1% (v/v) TFA], followed by 5 µl of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], followed by 5 µl of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 5 µl of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

2.8.3. MALDI-TOF Instrumentation and Mass Analysis

Mass spectral analyses were performed as follows: mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme™ Peptide Mass Standards kit (Applied Biosystems). Samples (0.3 µl) from each of the desalting steps, as well as a sample of the solution taken prior to desalting, were co-crystallized with 0.75 µl α-cyano-4-hydroxy cinnamic acid (Waters, Milford, MA) on the analysis plate. All samples were analyzed in the 500 to 5000 dalton range using 100 shots at a laser intensity setting of 2318-2460 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected Cry2Ab2 protein sequence deduced from the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 5000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal of ±2 Daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data. The identity of the Cry2Ab2 protein is confirmed if ≥ 40 % of the protein sequence was

identified by matching experimental masses for the tryptic peptide fragments to the expected masses for the fragments.

2.9. Determination of Purity and Molecular Weight

2.9.1. SDS-PAGE

Aliquots of the *E. coli*- and plant-produced Cry2Ab2 proteins were each diluted with sample dilution buffer and 5× sample loading buffer to a final protein concentration of 0.2 µg/µl. Molecular weight markers (BioRad Broad-Range, Hercules, CA) that were used to estimate the apparent molecular weight of the test substance, were diluted to a final total protein concentration of 0.9 µg/µl. The plant-produced Cry2Ab2 protein was analyzed in duplicate at 1, 2, and 3 µg total protein loads per lane. The *E. coli*-produced Cry2Ab2 reference standard was analyzed at 1 µg total protein. All samples were heated at ~102 °C for 5 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 150 V for 82 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained 16 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO), destained 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 6 hr.

Analysis of the gel was performed using a BioRad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). Values for the markers supplied by the manufacturer were used to estimate the apparent molecular weight of each observed band. All visible bands within each lane were quantified using Quantity One software. For the plant-produced Cry2Ab2 protein, purity was estimated as the percent optical density of the ~61 kDa band relative to all bands detected in the lane. Apparent molecular weight and purity were reported as an average of all six loadings containing the plant-produced Cry2Ab2 protein.

2.9.2. MALDI-TOF MS Tryptic Mass Analysis

Determination of the intact mass was attempted for the plant-produced Cry2Ab2 protein using MALDI-TOF mass spectrometry analysis. Prior to analysis, the plant-produced Cry2Ab2 protein and BSA reference protein (NIST, Gaithersberg, MD) were desalted using drop dialysis (Görisch, 1988). A portion of each protein sample (0.3 µl) was spotted on an analysis plate, mixed with 0.75 µl sinapinic acid solution and air-dried. Each sample was analyzed in triplicate. Mass spectral analysis was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF instrument with the supplied Data Explorer software (version 4.0.0.0, Foster City, CA). Mass calibration of the instrument was performed using desalted BSA reference protein.

2.10. Determination of Functional Activity by Insect Bioassay

The purpose of this analysis was to compare the biological activity of the plant- the *E. coli*-produced Cry2Ab2 proteins by determining EC_{50} values in a CEW (*Helicoverpa zea)* diet-incorporation insect bioassay. The EC_{50} value is defined as the concentration of Cry2Ab2 protein in the diet that results in 50% larval growth inhibition. In order to assess the functional activity of the plant-produced Cry2Ab2 protein and to compare its activity to the *E. coli*-produced Cry2Ab2 reference standard, aliquots of the plant- and *E. coli*-produced Cry2Ab2 proteins were used to estimate the effective protein concentration necessary to inhibit the growth of the target insect by 50%. The plant- and *E. coli*-produced Cry2Ab2 proteins were be determined to have comparable functional activity if the difference in mean EC_{50} values between plant- and *E. coli*-produced proteins is less than or equal to three fold.

2.10.1. Insects

Helicoverpa zea (CEW) eggs were obtained from Benzon Research Inc. Insect eggs were incubated at temperatures ranging from 10^{o} C to 27^{o} C, to achieve the desired hatch time.

2.10.2. Bioassays

CEW larvae were used to measure activity of the plant- and *E. coli*-produced Cry2Ab2 protein samples in a laboratory diet-incorporation bioassay. The bioassay was replicated three times on separate days with separate batches of insects. The plant- and *E. coli*-produced proteins were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and plant-produced Cry2Ab2 proteins consisted of a series of seven dilutions and a buffer control yielding a dose series with a 2-fold separation factor ranging from 0.016 – 1.0 µg Cry2Ab2 protein/mL diet. The dose-response curves for each protein included a buffer control. Each buffer control contained an amount of their respective buffer equivalent to the amount of protein in the highest dose level. The Cry2Ab2 protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into an agar-based insect diet. This dose series in diet was chosen to adequately characterize the dose-effect relationship on CEW weight gain for the proteins from both sources. The diet mixture was then dispensed in 1 mL aliquots into a 128 well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD International, Pitman, NJ) and the insects were allowed to feed for a period of seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 h light:10 h dark. The combined weight of the surviving insects at each dose level for each source of protein was recorded at the end of the 7-day incubation period.

2.10.3. Statistical analysis

Data were entered into an Excel spreadsheet and transferred to the Statistics Technology Center for analysis. Dose response modeling and EC_{50} determinations were performed using a 3-parameter logistic regression model (equation 1) under the PROC NLIN procedure in SAS.

Equation 1:
$$Wt = \frac{W_0}{1 + \left(\frac{DietDose}{EC50} \right)^B} + e$$

where *Wt* is the average CEW larvae weight and *DietDose* is the Cry1Ab protein diet dose level. Three parameters that are included in the model; W_0 represents the weight at *DietDose* = 0.0, EC_{50} represents effective concentration to reduce the growth of the target insect by 50%, and *B* reflects the rate of the weight loss as *DietDose* increases, and *e* denotes the residual (error).

2.11. Glycosylation Analysis

Glycosylation analysis was used to determine whether the plant-produced Cry2Ab2 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the plant-produced Cry2Ab2 protein, the *E. coli*-produced Cry2Ab2 reference standard (in this instance, a negative control), and the positive control transferrin (Amersham Biosciences, Piscataway, NJ) were each diluted in dilution buffer and in 5× sample loading buffer to a final purity corrected (total protein for transferrin) concentration of 50 ng/µl. These samples were heated to ~100 °C for 5 min, and loaded along with Precision Plus Dual Color pre-stained protein molecular weight markers (BioRad , Hercules, CA) on a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel. All three samples were loaded at a single loading of 0.5 and 1 µg protein per lane. Electrophoresis was performed at a constant voltage of 140 V for 20 min followed by a constant voltage of 200 V for 47 min. After electrophoresis, proteins were electrotransfered to a 0.45 micron PVDF membrane for one hour at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane containing the 0.5 and 1.0 µg sample loads using the ECL detection system (Amersham Biosciences, Piscataway, NJ). After the electrotransfer of the proteins, the PVDF membrane was incubated in PBS for 10 min, and transferred to a solution of 100 mM acetate buffer, pH 5.5, containing the oxidation reagent, 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 minutes. The oxidation solution was removed from the membrane by two brief rinses followed by three sequential 10 min washes in PBS. The membrane was transferred to a solution of 100 mM acetate buffer, pH 5.5, containing 25 nM biotin hydrazide and incubated for 60 minutes. Biotin hydrazide solution was removed by washing in PBS as previously described. The membrane was blocked with 5% blocking agent in PBS for 60 minutes. The blocking solution was removed by washing in PBS as previously described. The membrane was incubated with streptavidin-HRP conjugate (diluted 1:6000) in acetate buffer for 30 min to detect carbohydrate moieties bound to biotin. Excess streptavidin-HRP was removed by washing in PBS as previously described. Bands were visualized using the ECL detection

system (Amersham Biosciences, Piscataway, NJ). Films were exposed (1 min, 3 min, and 6 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

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APPENDIX C: Materials and Methods for the Safety Assessment of the Cry1A.105 and Cry2Ab2 Proteins

1. Assessment of Digestibility of the Cry1A.105 Protein in Simulated Gastric Fluid

1.1. Cry1A.105 Protein

The test substance was the Cry1A.105 protein. The Cry1A.105 protein was isolated from a fermentation batch of *E. coli* containing pMON96851 expression plasmid. This protein has been characterized and has a total protein concentration of 1.2 mg/ml and a purity of 92 %. Functional activity was confirmed using an insect bioassay with the larvae of a susceptible pest, corn earworm (CEW). The EC_{50} value was 5.8 ng/ml of diet. Prior to its application to the test system, the test substance was stored in a –80 ºC freezer in a test substance storage buffer containing 25 mM CAPS, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH ~10.3.

1.2. Test System

The test system for this study was simulated gastric fluid (SGF) that contains the proteolytic enzyme pepsin. The SGF was prepared using a highly purified form of pepsin (Catalog number P-6887, Sigma Company, St. Louis, MO). The SGF formulation, time course, and experimental parameters used in this study followed the conditions used in the ILSI multi-laboratory evaluation test (Thomas et al., 2004). The SGF was formulated so that ten units of pepsin activity per microgram of total protein from the test substance would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity reported on the product label. One unit of activity is defined as a change in $A_{280\ nm}$ of 0.001 per minute at 37 °C, measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The stock SGF solution was prepared by adding pepsin powder (26.6 mg) to 33.2 ml of an acidic sodium chloride solution (2 mg/ml NaCl, 10 mM HCl, pH 1.3). After the activity of pepsin in SGF was confirmed, the stock SGF solution was diluted to provide approximately 1500 units pepsin activity/ml of solution.

1.3. Sample Preparation

Digestion of the Cry1A.105protein in SGF was evaluated over time by analyzing specimens from all incubation time points. The target digestion temperature was 37 ± 2 ºC.

The Cry1A.105 protein was diluted to 0.6 mg total protein /ml by mixing 200 µl of the protein at 1.2 mg/ml and 200 µl of storage buffer (25 mM CAPS, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH 10.3) and then heated at 44.5 ºC for 10 minutes. Digestion samples were prepared by adding 200 µl of the diluted test substance to a tube containing 800 µl of SGF. The tube contents were vortex mixed and immediately placed in a 37.2 ºC water bath and subsequently vortex mixed every 30 - 60

sec throughout the digestion experiment. Samples (100 µl) were removed at targeted times of 0.5, 2, 5, 10, 20, 30, and 60 min (specimens T1 to T7, respectively) and placed in a tube containing quenching mixture. Quenching mixture contained 35 µl of carbonate buffer [700 mM Na_2CO_3, pH 11.0], and 35 µl of 5× Laemmli buffer (LB) [312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) Bromophenol Blue, and 50% (v/v) glycerol, pH 6.8].

The zero incubation time point (T0) was prepared in a separate tube. SGF (80 µl) was quenched by adding 35 µl of carbonate buffer and 35 µl of 5× LB prior to the addition of 20 µl of the diluted test substance.

All quenched samples were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a -80 ºC freezer until analyzed.

1.4. Experimental Controls

Experimental controls were prepared to determine the stability of the test substance in the test system buffer lacking pepsin [10 mM HCl, 2 mg/ml NaCl, pH 1.3]. These experimental controls were identified with the letter "P". The zero incubation time point (P0) was prepared in a separate tube. Test system buffer (80 µl) was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB prior to the addition of 20 µl of the diluted test substance. The 60 min incubation time point (P7) was prepared by adding 20 µl of the diluted test substance to test system buffer lacking pepsin (80 µl). The tube was vortex mixed and immediately placed in a 37.1 ºC water bath. After 60 min of incubation, the sample was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB.

Additional experimental controls were prepared to evaluate the stability of the pepsin in the test system (SGF) lacking the test substance and to determine if non-specific interaction occurs between the test system components and the antibodies during western blot analysis of the specimens. These experimental controls contained an aliquot of the test system incubated with test substance storage buffer instead of the test substance and were identified with the letter "N". The zero incubation time point (N0) was in a separate tube. Test system (80 µl) was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB prior to addition of 20 µl of the storage buffer (25 mM CAPS, pH 10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT). The 60 min incubation time point (N7) was prepared by adding 20 µl storage buffer to 80 µl of test system. The tube was vortex mixed and placed in a 37.1 ºC water bath. After 60 min of incubation, the sample was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× SB.

All experimental controls were heated at 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a -80 ºC freezer until analyzed.

1.5. SGF Activity Assay

Activity of the SGF was assessed using a pepsin activity assay. The digestibility of the Cry1A.105 protein in SGF was assessed using stained polyacrylamide gels and western blot analysis. The limit of detection (LOD) of the Cry1A.105 protein for these methods was determined concurrently.

The SGF activity assay was used to confirm the suitability of the test system before its use with the test substance. Acceptable activity was defined as a pepsin activity per mg of pepsin powder (0.03 mg of powder per ml of diluted SGF) equal to the activity of pepsin per mg of pepsin powder as determined by the manufacturer (±1000 units/mg). One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a change in the absorbance at 280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 ºC. The assay is used to estimate the amount of soluble peptides present in a TCA solution after pepsin digestion of denatured hemoglobin. Undigested hemoglobin was precipitated with TCA, and the amount of soluble peptides was estimated by measuring the absorbance at 280 nm. The amount of soluble peptide is directly proportional to the amount of protease activity.

Briefly, the SGF was diluted to 0.03 mg of solid material (pepsin) per ml of SGF [the dilution factor (DF) was 26.7]. Acidified hemoglobin [2% (w/v), 5 ml] was added to each of three replicates of the test sample and blank samples and pre-warmed at 37 ± 2 °C for 5-10 minutes prior to starting the reactions. Diluted SGF (1 ml) was added to each replicate of test samples and both test and blank samples were incubated at 37.0 °C for an additional 10 min. The reaction was stopped by addition of 10 ml of chilled 5% (v/v) TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated another 5-10 min at 37.4 °C. Precipitated protein was removed by filtering the test and the blank samples using 0.8 µm syringe filters. Samples of the clarified test and blank samples were read at 280 nm in a Beckman DU-650 Spectrophotometer. The activity of pepsin was calculated using the following equation:

$$\frac{MeanTest_{A280nm} - MeanBlank_{A280nm}}{0.001 \times 10\min \times 1ml} \times DF \,,$$

where 0.001 is the change in the absorbance at 280 nm per min at pH 1.2-2.0 and 37 ± 2 ºC produced by one unit of pepsin activity; 10 min is the reaction time, 1 ml is the amount of SGF added to the reaction; and DF is the dilution factor for the SGF.

1.6. SDS-PAGE

Samples containing 1× LB from the SGF *in vitro* digestion of the Cry1A.105 protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels and tricine running buffer (Invitrogen, Carlsbad, CA). The protein loaded per lane was based on the pre-digestion total protein concentration of the Cry1A.105 protein. All experimental controls were loaded at the same volume as those containing Cry1A.105

protein so that all other components would be comparable. All samples were heated at 100.3 °C for 5 min prior to loading on the gels. Protein markers were used to estimate the relative molecular weight. Electrophoresis was performed at a constant voltage of 125 V for 85 minutes. After electrophoresis, proteins were either visualized by staining the gel with colloidal Brilliant Blue G, or the gel was subjected to electrotransfer of proteins to nitrocellulose membrane for western blot analysis.

1.7. Colloidal Brilliant Blue G Staining

The colloidal Brilliant Blue G staining method was selected because it is an effective method for detecting nanogram quantities of protein on a gel (Neuhoff et al., 1988). Mark12 molecular weight markers (Invitrogen, Carlsbad, CA) were used to estimate the relative molecular weight of visualized proteins and peptides. Based on pre-digestion concentrations, approximately 0.7 µg of total protein was loaded per lane. After separation of proteins, the gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and stained for approximately 20 h in 1× Brilliant Blue G-colloidal stain solution containing 20% (v/v) methanol. The gels were briefly destained for 30 s in a 10% (v/v) acetic acid, 25% (v/v) methanol solution and completely destained for ~5 h in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer. The results of the *in vitro* digestibility of Cry1A.105 protein were determined by visual examination of the stained gels.

The approximate molecular weights of the full-size protein and proteolytic fragment observed on the colloidal Brilliant Blue G stained gels were visually determined relative to the positions of the molecular weight markers.

The LOD of the Cry1A.105 protein using the colloidal Brilliant Blue G staining procedure was determined. Various dilutions of the zero time point (T0) digestion specimen were loaded onto a separate gel that was run concurrently with the gel used to assess digestibility. Aliquots of the T0 digestion sample representing approximately 700, 350, 100, 50, 20, 10, 5, and 2.5 ng total protein were used for the stained LOD gel.

1.8. Western Blot Analysis

Specimens from the SGF *in vitro* digestions were also analyzed using western blotting. Based on pre-digestion concentrations, approximately 20 ng of total protein were loaded per lane. Following electrophoresis, pre-stained molecular weight markers (Precision Plus Protein Standards, Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. Proteins were electrotransferred to nitrocellulose membranes (0.45 µm pore size, Invitrogen) for 90 min at a constant voltage of 25 V.

Membranes were blocked overnight in a 4 ºC refrigerator with 5% (w/v) non-fat dry milk (NFDM) in phosphate buffered saline containing Tween-20 (PBST) buffer. All subsequent incubations (described below) were performed at room temperature. Membranes were incubated with rabbit anti–Cry1A.105 antibody diluted 1:2,000 in PBST containing 1% (w/v) NFDM for 1 h. Excess serum was removed by three 10 min

washes with PBST. The membrane was incubated with HRP–conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1:10,000 in PBST containing 1% (w/v) NFDM for 1 h and again washed (three 10 min washes) with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) and exposed (2, 5, and 10 minutes) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

The approximate molecular weights of the full-size protein observed on the western blots were visually determined relative to the positions of the molecular weight markers.

The LOD for the Cry1A.105 protein using the western blot analysis procedure was determined. Various dilutions of the zero time point (T0) digestion specimen were loaded onto a separate gel that was run concurrently with the digestion western blot gel and subjected to the same western blot procedure as described above. Aliquots of the T0 digestion sample representing approximately 7, 3.5, 2, 1, 0.5, 0.2, 0.1, and 0.05 ng total protein were used for the western blot LOD analysis.

2.0. Digestibility of the Cry2Ab2 Protein in Simulated Gastric Fluid

2.1. Cry2Ab2 Protein

The test substance is the *E. coli*-produced Cry2Ab2 protein. The *E. coli*-produced Cry2Ab2 protein is referred to as Cry2Ab2.820 in the Certificate of Analysis. The protein was isolated from a fermentation batch of *E. coli* containing the pMON70520 expression plasmid. This protein has been characterized and has a total protein concentration of 0.5 mg/ml, and a purity of 87%. Activity was confirmed using an insect bioassay with the larvae of a susceptible pest, corn earworm (*Helicoverpa zea*, CEW). The EC_{50} value was 0.25 µg/ml of diet. The test substance was stored prior to use in a –80 ºC freezer in buffer containing 50 mM CAPS, 2 mM DTT, pH 11.

2.2. Test System

The test system for this study was simulated gastric fluid (SGF) that contains the proteolytic enzyme pepsin. The SGF was prepared using a highly purified form of pepsin (Catalog number P-6887, Sigma Company, St. Louis, MO). The SGF was formulated so that ten units of pepsin activity per microgram of total protein from the test substance would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity reported on the product label. One unit of activity is defined as a change in $A_{280\ nm}$ of 0.001 per minute at 37 °C, measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The stock SGF solution was prepared by adding pepsin powder (26.6 mg) to 33.2 ml of an acidic sodium chloride solution (2 mg/ml NaCl, 10 mM HCl, pH ~1.3). After the activity of pepsin in SGF was confirmed, the stock SGF solution was diluted to provide approximately 1944 units pepsin activity/ml of solution.

2.3. Sample Preparation

Digestion of the test substance in SGF was evaluated over time by analyzing specimens from all incubation time points. The target digestion temperature was 37 ± 2 ºC.

Digestion samples were prepared by adding 252 µl of the test substance to a tube containing 648 µl of SGF. The tube contents were vortex mixed and immediately placed in a 37.2 ºC water bath. The tube with the digestion mixture was regularly vortexed (every 30-60 sec) throughout the digestion experiment to prevent test substance precipitation. Samples (100 µl) were removed at targeted times of 0.5, 2, 5, 10, 20, 30, and 60 min (specimens T1 to T7, respectively) and placed in a tube containing quenching mixture. Quenching mixture contained 35 µl of carbonate buffer [700 mM Na_2CO_3, pH 11], and 35 µl of 5× Laemmli buffer (LB) [312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) Bromophenol Blue, and 50% (v/v) glycerol, pH 6.8].

The zero incubation time point (T0) was prepared in a separate tube. SGF (72 µl) was quenched by adding 35 µl of carbonate buffer and 35 µl of 5× LB prior to adding 28 µl of test substance.

All quenched samples were heated at 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a -80 ºC freezer until analyzed.

2.4. Experimental Controls

Experimental controls were prepared to determine the stability of the test substance in the test system buffer lacking pepsin [10 mM HCl, 2 mg/ml NaCl, pH 1.3]. These experimental controls were identified with the letter "P". The zero incubation time point (P0) was prepared in a separate tube. Test system buffer (72 µl) was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB prior to addition of 28 µl of the test substance. The 60 min incubation time point (P7) was prepared by adding 28 µl test substance to test system buffer lacking pepsin (72 µl). The tube was vortex mixed and immediately placed in a 37.4 ºC water bath. After 60 min of incubation, the sample was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB.

Additional experimental controls were prepared to evaluate the stability of the pepsin in the test system (SGF) lacking the test substance and to determine if non-specific interaction occurs between the test system components and the antibodies during western blot analysis of the specimens. These experimental controls contained an aliquot of the test system incubated with test substance storage buffer instead of the test substance and were identified with the letter "N". The zero incubation time point (N0) was in a separate tube. Test system (72 µl) was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB prior to addition of 28 µl of storage buffer (50 mM CAPS, 2 mM DTT, pH 11). The 60 min incubation time point (N7) was prepared by adding 28 µl storage buffer

to 72 µl of test system. The tube was vortex mixed and placed in a 37.4 ºC water bath. After 60 min of incubation, the sample was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB.

All experimental controls were heated at 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a -80 ºC freezer until analyzed.

2.5. SGF Activity Assay

Activity of the SGF was assessed using a pepsin activity assay. The digestibility of the Cry2Ab2 protein in SGF was assessed using stained polyacrylamide gels and western blot analysis. The limit of detection (LOD) of the Cry2Ab2 protein for these methods was determined concurrently.

The SGF activity assay was used to confirm the suitability of the test system before its use with the test substance. Acceptable activity was defined as a pepsin activity per mg of pepsin powder (0.03 mg of powder per ml of SGF) equal to the activity of pepsin per mg of pepsin powder as determined by the manufacturer (±1000 units/mg). One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a change in the absorbance at 280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 ºC.

The assay is used to estimate the amount of soluble peptides present in a TCA solution after pepsin digestion of denatured hemoglobin. Undigested hemoglobin was precipitated with TCA, and the amount of soluble peptides was estimated by measuring the absorbance at 280 nm. The amount of soluble peptide is directly proportional to the amount of protease activity.

The SGF was diluted to 0.03 mg of solid material (pepsin) per ml of SGF [the dilution factor (DF) was 26.7]. Acidified hemoglobin [2% (w/v), 5 ml] was added to each of three replicates of the test sample and blank samples and pre-warmed at 37 ± 2 °C for 5-10 minutes prior to starting the reactions. Diluted SGF (1 ml) was added to each replicate of test samples and both test and blank samples were incubated at 37.0 °C for an additional 10 min. The reaction was stopped by addition of 10 ml of 5% (v/v) chilled TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated another 5-10 min at 37.4 °C. Precipitated protein was removed by filtering the test and the blank samples using 0.8 µm syringe filters. Samples of the clarified test and blank samples were read at 280 nm in a Beckman DU-650 Spectrophotometer. The activity of pepsin was calculated using the following equation:

$$\frac{MeanTest_{A280nm} - Mean\,Blank_{A280nm}}{0.001 \times 10\,\min \times 1\,ml} \times DF \,,$$

where 0.001 is the change in the absorbance at 280 nm per min at pH 1.2-2.0 and 37 ± 2 ºC produced by one unit of pepsin activity; 10 min is the reaction time; 1 ml is the amount of SGF added to the reaction; and DF is the dilution factor for the SGF.

2.6. SDS-PAGE

Samples containing 1× LB from the SGF *in vitro* digestion of the Cry2Ab2 protein were separated by SDS-PAGE using pre-cast tricine10-20% polyacryamide gradient mini-gels and tricine running buffer (Invitrogen, Carlsbad, CA). The protein loading per lane was based on pre-digestion total protein concentration of the Cry2Ab2 protein. All experimental controls were loaded at the same volume as those containing Cry2Ab2 protein so that all other components would be comparable. All samples were heated at 95.3 °C for 5 min prior to loading on the gels. Mark12 molecular weight markers (Invitrogen, Carlsbad, CA) were loaded to estimate the relative molecular weight of proteins and peptides visualized by staining. Electrophoresis was performed at a constant voltage of 125 V for 90 min. After electrophoresis, proteins were either visualized by staining the gel with colloidal Brilliant Blue G, or the gel was subjected to electrotransfer of proteins to polyvinylidene difluoride (PVDF) membrane for western blot analysis.

2.7. Colloidal Brilliant Blue G Staining

The colloidal Brilliant Blue G staining method was selected because it is an effective method for detecting nanogram quantities of protein on a gel (Neuhoff et al., 1988). Based on pre-digestion concentrations, approximately 0.8 µg of total protein was loaded per lane. After separation of proteins, the gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and stained for 15 h in 1× Brilliant Blue G-colloidal stain solution containing 20% (v/v) methanol. The gels were briefly destained for 30 s in 10% (v/v) acetic acid, 25% (v/v) methanol and completely destained for 23 h in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer. The results of the *in vitro* digestibility of Cry2Ab2 protein were determined by visual examination of the stained gels.

The LOD of the Cry2Ab2 protein using the colloidal Brilliant Blue G staining procedure was determined. Various dilutions of the zero time point (T0) digestion specimen were loaded onto a separate gel that was run concurrently with the gel used to assess digestibility. Aliquots of the T0 digestion sample representing approximately 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, and 0.0025 µg total protein were used for the stained LOD gel.

2.8. Western Blot Analysis

Specimens from the SGF *in vitro* digestions were also analyzed using western blotting. Based on pre-digestion concentrations, approximately 20 ng of total protein were loaded per lane. Following electrophoresis, pre-stained molecular weight markers (Precision Plus Protein Standards, Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane and estimate size of proteins and peptides. Proteins were electrotransferred to PVDF membranes (0.45 µm pore size, Invitrogen) for 90 min at a constant current of 25 V.

Membranes were blocked overnight in a 4 ºC refrigerator with 5% (w/v) non-fat dry milk (NFDM) in phosphate buffered saline containing Tween-20 (PBST) buffer. All subsequent incubations (described below) were performed at room temperature. Membranes were incubated with goat anti-Cry2Ab2 antibody diluted 1:3,000 in PBST containing 1% (w/v) NFDM for 70 min. Excess serum was removed by three 5 min washes with PBST. The membrane was incubated with HRP–conjugated anti-goat IgG (Sigma) at a dilution of 1:10,000 in PBST containing 1% (w/v) NFDM for 1 h and again washed (three 5 min washes) with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ) and exposed (1, 2, 5, and 10 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

The LOD for the Cry2Ab2 protein using the western blot analysis procedure was determined. Various dilutions of the zero time point (T0) digestion specimen were loaded onto a separate gel that was run concurrently with the digestion western blot gel and subjected to the same western blot procedure as described above. Aliquots of the T0 digestion sample representing approximately 10, 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 ng total protein were used for the western blot LOD analysis.

3. Materials and Methods Used to Establish Equivalence of the Cry1A.105 Proteins Produced in *E. coli* and in MON 89034

3.1. Plant-Produced Cry1A.105 Protein

The plant-produced Cry1A.105 protein was purified from grain of MON 89034. The identity of the grain containing MON 89034 was confirmed by event-specific PCR. The purified plant-produced Cry1A.105 protein was stored in a 4 ºC refrigerator in a buffer solution containing 50 mM CAPS, 1 mM PMSF, 2 mM benzamidine-HCl, 1 mM EDTA, 0.8 M NaCl, and 30% (v/v) ethylene glycol, pH 10.0.

3.2. *E. coli*-produced Cry1A.105 Reference Standard

E. coli-produced Cry1A.105 protein was used as a reference standard for determination of protein concentration and immunoblot analysis using N-terminal peptide antibody. This protein was also used as a reference standard to evaluate equivalence between plant- and *E. coli*-produced Cry1A.105 proteins for the molecular weight and functional activity assay, as a reference and a negative control in glycosylation analysis, and as a reference and a positive control in immunoblot analysis using anti-Cry1A.105 antibody. The *E. coli*-produced Cry1A.105 reference standard was previously characterized. The Cry1A.105 protein was stored in a -80 °C freezer in a buffer solution (25 mM CAPS, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH ~10.3) at a total protein concentration of 1.2 mg/ml by amino acid analysis.

3.3. Assay Controls

Protein molecular weight standards (Bio-Rad, Hercules, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF membranes. The *E. coli*-produced Cry1A.105 reference standard was used to create a standard curve for the quantitative western blot. The Cry1A.105 trypsin-resistant core was used as a negative control for the western blot analysis using N-terminal peptide antibody. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. Transferrin (Amersham Biosciences, Piscataway, NJ) and horseradish peroxidase (Sigma, St. Louis, MO) were used as positive controls for glycosylation analysis. CandyCane™ glycoprotein molecular weight standards were used as molecular weight markers and positive and negative controls for glycosylation analysis.

3.4. Protein Purification

The Cry1A.105 protein was purified at 4°C from an extract of ground grain using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

The ground grain (10 kg) was mixed in PBS extraction buffer [1× PBS: 1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4] for 2.5 hours at approximately a 1:10 sample weight to buffer volume ratio. The slurry was filtered using an Ertel Alsop filter press (Kingston, NY) and the PBS washed filter cakes were retained. The filter cakes were resuspended in ~100 L of CAPS extraction buffer [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine- HCl, and 1% PVPP (w/v), pH 10.8] and stirred for 2 hours. To remove lipids from the extract, CelPure P65 Diatomaceous Earth (Advanced Minerals Corp, Goleta, CA) was added to the slurry at 7.5% (w/v) and allowed to mix for ~10 minutes. The extract was clarified by filtration using the Ertel Alsop filter press and concentrated using a Hollow Fiber Cartridge (Amersham Biosciences, Piscataway, NJ). The contaminant DNA was removed by a combination of benzonase treatment and precipitation with polyethyleneimine. Benzonase was added to a final concentration of ~7 U/ml in the presence of 5 mM $MgCl_2$ and allowed to mix overnight. A polyethyleneimine solution [10% (w/v)] was added to the concentrated extract to a final concentration of 0.05% (w/v) and the extract was clarified by centrifugation to remove the remaining contaminant DNA. An ammonium sulfate pellet (0% – 35% saturation) was prepared by the addition of ammonium sulfate salt to the clarified extract and was allowed to dissolve overnight. After centrifugation, the ammonium sulfate pellet was dissolved over the weekend in ~15 L of resuspension buffer [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 2 mM benzamidine-HCl, pH 10.8]. The resuspended sample was clarified by centrifugation, diafiltrated against fresh buffer (same as the resuspension buffer) to remove any residual ammonium sulfate and concentrated to final volume of ~8 L and CHAPS was added to a final concentration of 0.5 mM.

The concentrated sample was loaded onto a 2.1 L (6.7 cm x 20 cm) Q Sepharose Fast Flow anion exchange resin column, which was equilibrated with AEX buffer A [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, and 0.5 mM CHAPS, pH 10.8]. The bound Cry1A.105 protein was eluted with a linear salt gradient that increased from 0 M to 0.20 M sodium chloride over ~12 L and then maintained a 0.20 M sodium chloride concentration for ~4 L. Next, the salt gradient increased to 0.65 M sodium chloride over ~21 L, and finally stepped up to 1.0 M sodium chloride instantly. Fractions containing the Cry1A.105 protein (based on stained SDS-PAGE gel and western blot analysis of all fractions) were pooled to a final volume of ~10 L. Salt was removed from these pooled fractions by diafiltration with fresh buffer [50 mM EPPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, and 2 mM benzamidine-HCl, pH 7.5].

The buffer exchanged sample was loaded onto a 2.1 L (13.7 cm x 14.0 cm) Q Sepharose XL anion exchange resin column, which was equilibrated with AEX buffer A [50 mM EPPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, and 0.5 mM CHAPS, pH 7.5]. The bound Cry1A.105 protein was eluted with a linear salt gradient that increased from 0 M to 0.25 M sodium chloride over ~ 4 L and then held at 0.25 M sodium chloride for ~ 10 L. Next, the salt gradient increased to 0.65 M sodium chloride over ~21 L and finally increased to 1.0 M sodium chloride over ~ 4 L and held constant for ~8L. The fractions containing Cry1A.105 protein (based on stained SDS-PAGE gel and western blot analysis of all fractions) were pooled to a final volume of ~10 L. These fractions were diafiltrated into fresh buffer [50 mM EPPS, 1 mM EDTA, 10 mM DTT, 1mM PMSF, and 2 mM benzamidine-HCl, pH 7.5] to remove salt and concentrated to ~2.0 L using a hollow fiber cartridge.

Prior to the affinity purification step, the sample was diafiltrated into fresh buffer [50 mM EPPS, 1mM EDTA, 1 mM PMSF, 2 mM benzamidine-HCl, and 150 mM NaCl, pH 7.6] and concentrated down to ~1L. The solution containing Cry1A.105 protein was re-circulated over the 9.3 ml (1.75 cm x 2.6 cm) protein A agarose column (Sigma) conjugated with monoclonal anti-Cry1Ac antibody (Strategic Biosolutions, Newark DE). Bound Cry1A.105 protein was eluted using 50 mM CAPS, 1 mM EDTA, 1 mM PMSF, 2 mM benzamidine-HCl, 0.8 M NaCl, and 30% (v/v) ethylene glycol, pH 10.0. After analysis of fractions by lateral flow strips, stained SDS-PAGE gel and western blot, fraction 25 was determined to contain the majority of the full-length Cry1A.105.

3.5. Protein Concentration

The concentration of the full-length (~130 kDa) plant-produced Cry1A.105 protein was estimated using quantitative immunoblot analysis. The *E. coli*-produced Cry1A.105 protein (amounts ranging from 1 to 6 ng purity corrected for the full-length protein, which was 82%) was used to create a standard curve. Aliquots of the plant-produced Cry1A.105 protein and reference standard were diluted in deionized water and 5x Laemmli buffer (5x LB), heated at 95.5 °C for 5 min, and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 12-well gel. Three different amounts of the plant-produced protein were loaded in duplicate. Electrophoresis was performed at a constant voltage of 125 V for 15 min followed by a constant voltage of 170 V for 75 min.

Pre-stained molecular weight markers (Bio-Rad Precision Plus Dual Color, Hercules, CA) were used to verify electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands observed. Electrotransfer to a 0.45 μm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V.

The membrane was blocked for 1 hour with 5% (w/v) NFDM in 1× PBST. The membrane was probed with a 1:1000 dilution of rabbit anti-Cry1A.105 antibody in 1% (w/v) NFDM in PBST for 60 min. Excess antibody was removed using four 5 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) at a dilution of 1:7500 in 1% (w/v) NFDM in PBST for 60 minutes. Excess HRP-conjugate was removed using three 5 min washes with PBST. All incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ) and exposed (5 min, 10 min, 20 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

The immunoreactive band of the plant-produced Cry1A.105 protein in each lane migrating at the same level as the full-length reference standard protein was quantitated relative to the standard curve. Quantitation was performed using the volume tool and the linear regression method in the Quantity One software (version 4.4.0) after scanning on the Bio-Rad GS-800 densitometer. The concentration of the plant-produced Cry1A.105 was determined by dividing the amount of protein in each lane by the volume of protein loaded in that lane [Microsoft Excel 2002 (10.6730.6718) SP3]. All load quantities of plant-produced Cry1A.105 refer to the amount of the full-length protein.

3.6. Western Blot Analysis

Immunoblot analysis was performed to confirm the identity of the Cry1A.105 protein, the intactness of its N-terminus, and compare immunoreactivity of the plant- and *E. coli*-produced proteins.

3.6.1. Western Blot Analysis Using anti-Cry1A.105 Antibody

The western blot described in section 5.2 was also used to establish the identity of the plant-produced protein and to compare the immunoreactivity of the plant-produced and *E. coli*-produced Cry1A.105 proteins. Equivalence was demonstrated for bands representing full-length (~130 kDa) proteins that were identified by the anti-Cry1A.105 antibody and showed similar electrophoretic mobility.

3.6.2. Western Blot Analysis Using the N-terminal Peptide Antibody

A western blot analysis using the N-terminal peptide antibody was performed to confirm the intactness of the N-terminus of the plant-produced Cry1A.105 protein. The N-terminal peptide antibodies were produced against a synthetic peptide consisting of the first 14 amino acids of the Cry1A.105 protein N-terminus. The trypsin resistant core

was used as a negative control because its N-terminus was removed by trypsin. The plant-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 reference standard (corrected for the purity of the full-length protein), and the Cry1A.105 trypsin-resistant core (~56 kDa) were each loaded on gels at 20 ng and 40 ng per lane. Each protein was mixed with 5× LB, heated at 96.0 °C for 5 min, and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well gel. Electrophoresis was performed at a constant voltage of 150 V for 5 min followed by a constant voltage of 200 V for 60 min. Pre-stained molecular weight markers (Bio-Rad Precision Plus Dual Color,) were used to verify electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands. Electrotransfer to a 0.45 μm PVDF membrane (Invitrogen) was performed for 90 min at a constant voltage of 25 V.

The membrane was blocked overnight with 5% (w/v) NFDM in 1× PBST. The membrane was probed with a 1:33.33 dilution of rabbit N-terminal peptide antibody (lot 42005MG) in 1% (w/v) NFDM in PBST for 60 min. Excess antibody was removed using three 5 min washes with PBST. Finally, the membrane was probed with HRP-conjugated anti-rabbit IgG (Sigma) at a dilution of 1:7500 in 1% (w/v) NFDM in PBST for 60 minutes. Excess HRP-conjugate was removed using three 5 min washes with PBST. The blocking step was performed at 4 °C. All other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences) and exposed (1 min, 2 min, 5 min, 10 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX-101A automated film processor.

3.7. MALDI-TOF Tryptic Mass Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the plant-produced Cry1A.105 protein.

3.7.1. Concentration of Protein

The plant-produced protein (4.5 ml) was diluted to 9.0 ml with Cry1A.105 concentration buffer [25 mM CAPS, 0.02% (w/v) sodium dodecyl sulfate] followed by concentration down to 64 μl using Amicon Ultrafree CL concentrators (Millipore Corporation, Bedford, MA). Sixty microliters of 5× LB was used to wash the concentrator walls and membranes. The membranes were heated with 5× LB for 5 minutes at 95.1°C. Sixteen microliters of this 5× LB was then used as the loading buffer for the Cry1A.105 sample for SDS-PAGE separation.

3.7.2. SDS-PAGE Separation

The concentrated plant–produced Cry1A.105 protein was subjected to electrophoresis on an SDS-polyacrylamide gel. The protein sample was loaded in two lanes. Broad Range molecular weight markers (Bio-Rad) were used to estimate molecular weights. Plant-produced Cry1A.105 protein and molecular weight markers were heated at 95.3 °C for 5 min and then applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well

mini-gel. Electrophoresis was performed at constant voltage (125 V for 10 min followed by 170 V for 70 min). Proteins were stained with Bio-Rad Coomassie 1 × stain for 2 hours, and destained by washing with Coomassie R-250 1× destain solution (Bio-Rad) for 2 hours with one change of the destain solution.

3.7.3. In-gel Protein Digestion

The bands representing full-length plant-produced Cry1A.105 protein (~130 kDa) were excised from two gel lanes, destained, reduced, alkylated, and subjected to an in-gel trypsin digestion (Williams et al., 1997). Briefly, each gel band was individually destained for 30 min by incubation in 100 μL of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in a microfuge tube. This was repeated two additional times. Following destaining, the gel bands were incubated in 100 μL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. The protein was reduced in 100 μL of 10 mM dithiothreitol solution for 2 hours at 37 °C. The protein was alkylated by the addition of 100 μL of 200 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. The gel bands were incubated in 100 μL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature at which time 100 μL of acetonitrile was added and the incubation was continued for an additional 30 minutes. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove the reducing and alkylating reagents and other salts from the gel. The gel bands were dried in a SpeedVac concentrator, rehydrated with 40 μL 25 mM ammonium bicarbonate containing 33 μg/ml trypsin, and the protein contained in the gel band was digested for 16 hours at 37.1 °C. Digested peptides were extracted for 60-80 min at room temperature with 50 μL 70% (v/v) acetonitrile containing 0.1% (v/v) TFA per gel band. Both extraction supernatants were combined into a single tube and dried in a SpeedVac concentrator. This process of extracting the peptides was repeated two more times. The final dried materials were reconstituted in 5 μL of 0.1% (v/v) TFA.

3.7.4. Sample Preparation

An aliquot (4 μl) of the digested sample was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) ZipTip C18 pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. The sample was applied to a ZipTip and eluted with 4 μL of Wash 1 [0.1% (v/v) TFA], followed by 4 μL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], followed by 4 μL of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 4 μL of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

3.7.5. MALDI-TOF Instrumentation and Mass Analysis

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme Peptide Mass Standards kit (Applied Biosystems). Samples (0.5 μL) from each of the desalting steps, as well as a sample of solution taken prior to desalting, were co-crystallized with 0.8 μL α-cyano-4-hydroxy cinnamic acid on the analysis plate. The sample was analyzed in the

500 to 5000 Dalton range using 100 shots at a laser intensity setting of 2781 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected Cry1A.105 (plant) protein sequence based upon the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 5000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 Daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data. The tryptic mass map coverage was considered acceptable if ≥ 40 % of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments.

3.8. Molecular Weight and Purity Estimation by SDS-PAGE

Aliquots of the *E. coli*-produced reference standard and plant-produced Cry1A.105 proteins were mixed with 5× LB to a final protein concentration of 10 ng/µl and 2.4 ng/µl, respectively. The plant-produced protein was analyzed in duplicate at 48, 72, and 96 ng of total protein per lane. The *E. coli*-produced Cry1A.105 reference standard was analyzed at 96 ng of purity corrected full-length protein. All samples were heated in a thermo-block at 99 °C for 3 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel (Invitrogen). Electrophoresis was performed at a constant voltage of 125 V for 10 min followed by a constant voltage of 170 V for 70 min.

The gel was stained using the SilverXpress® Silver Staining Kit Protocol (Invitrogen, Carlsbad, CA). The gel was fixed for 10 minutes in 200 ml of fixing solution (90 ml ultra pure water, 100 ml methanol, and 20 ml acetic acid). This was followed by 10 minutes in 100 ml sensitizing solution (105 ml ultra pure water, 100 ml methanol, and 5 ml sensitizer) and repeated once. The excess of the sensitizing solution was removed using two 5 minutes washes in 200 ml ultra pure water. The gel was stained for 15 minutes in 100 ml staining solution (5 ml Stainer A, 5 ml Stainer B, and 90 ml ultra pure water). The stain was removed using two 5 minutes washes of 200 ml ultra pure water. Developing occurred in 100 ml of solution (5 ml developer and 95 ml ultra pure water) for 3 - 15 minutes, and was stopped by addition of 5 ml stopping solution for 10 minutes. The gel was washed three times for 10 minutes each with 200 ml of ultra pure water. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). Broad-Range molecular weight markers (Bio-Rad) were used to estimate the apparent molecular weight of the plant-produced Cry1A.105 protein. For the purity evaluation, all visible bands within each lane were quantified. Stained bands corresponding to immunoreactive bands identified by anti-Cry1A.105 antibody and migrating from ~56 to ~130 kDa were

included in the purity calculation for the protein of interest as they represent various lengths of the insecticidal protein from the ~56 kDa tryptic core to the ~130 kDa full-length protein (Bietlot et al., 1989). The purity and estimated full-length molecular weight of the plant-produced Cry1A.105 protein were reported as the average of the six values obtained by densitometric analysis.

3.9. Glycosylation Analysis

Glycosylation analysis was used to determine whether the plant-produced Cry1A.105 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the plant-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 reference standard, and the positive controls, transferrin (Amersham Biosciences) and horseradish peroxidase (Sigma), were each mixed with 5× LB. These samples were heated at 95 °C for 4 min, cooled, and loaded on a tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel. Each sample was loaded at 48 and 96 ng (purity corrected for the full length protein) per lane. Precision Plus Dual Color pre-stained protein molecular weight markers (Bio-Rad) were loaded to verify electrotransfer of the proteins to the membrane, and the CandyCane™ Glycoprotein Molecular Weight Standards (Molecular Probes, Eugene, OR) were loaded as positive/negative controls and markers for molecular weight. Electrophoresis was performed at a constant voltage of 125 V for 10 min followed by a constant voltage of 170 V for 70 min. Electrotransfer to a 0.2 μm PVDF membrane was performed for 90 min at a constant voltage of 25 V.

Carbohydrate detection was performed directly on the PVDF membrane using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes). The manufacturer's protocol was followed. All steps were performed at room temperature. The PVDF membrane was fixed in 25 ml of a solution containing 50% methanol and 5% glacial acetic acid for 1 hour, and then the solution was changed and the membrane was incubated overnight. Two 15 minute washes (50 ml each) of 3% (v/v) glacial acetic acid (wash solution), were followed by a 20 minute oxidation in 25 ml of the kit supplied oxidizing solution. After oxidation, three 15 minute washes (50 ml each) prepared the membrane for staining. The blot was incubated in 25 ml of Pro-Q Emerald Staining Solution that was prepared using the kit reagents. After 1 hour of staining in the dark, two 30 minute, 50 ml wash cycles were followed by two 45 minute, 50 ml wash cycles. The final wash cycles included two 25 ml, 1 minute deionized water washes followed by three 25 ml, 5 minute methanol washes (B&J Brand, Muskegon, MI). The blot was then scanned using the BioRad Molecular Imager FX using the Alexa 488 illumination setting (Qunatity One software; version 4.6, build 036) in order to visualize the fluorescing glycosylated proteins.

3.10. Functional Activity Assay

In order to assess the functional activity of the plant-produced Cry1A.105 protein and to compare its activity to the *E. coli*-produced Cry1A.105 reference standard, aliquots of the plant-produced Cry1A.105 protein and *E. coli*-produced Cry1A.105 reference standard protein were analyzed in diet-incorporation insect bioassay.

The total protein concentration of the *E. coli*-produced Cry1A.105 protein aliquots was 1.2 mg/mL, with a purity of 92%, and a purity corrected concentration of 1.1 mg Cry1A.105/mL. The Cry1A.105 protein concentration of the plant-produced Cry1A.105 protein aliquots was 3 μg/mL. The control substances used in the bioassays were buffers of the same composition used for storing the *E. coli*-produced and plant-produced proteins. The *E. coli*-produced reference standard was suspended in 25 mM CAPS, pH ~10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, 0.2 mM DTT buffer solution. The composition of the plant storage buffer was 50 mM CAPS, 1.0 mM PMSF, 2.0 mM benzamidine-HCl, 1 mM EDTA, 0.8 M NaCl, 30% (v/v) ethylene glycol, pH 10.0. The plant-produced and *E. coli*-produced Cry1A.105 proteins were stored at 4° C and -80° C, respectively, and the buffers for both proteins were stored at 4° C.

3.10.1. Insects

CEW were obtained from Benzon Research Inc. Insect eggs were incubated at a temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

3.10.2. Bioassays

CEW were used to measure activity of the plant- and *E. coli*-produced Cry1A.105 protein samples. The bioassay was replicated three times on separate days with separate batches of insects. The plant- and *E. coli*-produced proteins were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and plant-produced Cry1A.105 proteins consisted of a series of five protein levels yielding a dose series ranging from 0.00048 – 0.039 μg Cry1A.105 protein/mL diet with a 3-fold separation factor between dose levels. This dose series was chosen to adequately characterize the dose-effect relationship for the proteins from both sources. Each dose level, including the control, had an equivalent volume of buffer added to the dosing solution. The Cry1A.105 protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into an agar-based insect diet. Diet mixture was then dispensed in 1 mL aliquots into a 128 well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD International, Pitman, NJ) and the insects were allowed to feed for a period of 6-7 days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14h:10h, light:dark. The combined weight of the surviving insects at each dose level for each source of protein was recorded at the end of the 6-7 day incubation period.

3.10.3. Dose-response Modeling and Results

The following three-parameter logistic model, with an extra parameter for the change in variation with the expected weight (equation below), was used to model the dose-response curves for each protein source and each replicate under the PROC NLMIXED procedure in SAS:

Equation:

$$Wt = \frac{W_0}{1 + \left(\frac{DietDose}{EC50} \right)^B} + e$$

$$Var(e) = \left\{ \frac{s \cdot W_0}{1 + \left(\frac{DietDose}{EC50} \right)^B} \right\}^2$$

where *Wt* is the average CEW larvae weight and *DietDose* is the Cry1A.105 protein diet dose level. The residual variation was assumed to be proportional to the expected mean weight. The parameters that are included in the model are W_0 which represents the expected weight at *DietDose* = 0.0, EC_{50} which represents the concentration needed to inhibit the growth of the target insect by 50%, *B* which reflects the rate of the weight loss as *DietDose* increases, *s* which represents the proportion of the standard deviation to the expected weight, and *e* which denotes the residual (error).

4. Materials and Methods Used to Establish Equivalence of the Cry2Ab2 Proteins Produced in *E. coli* and in MON 89034

4.1. Cry2Ab2 Protein

The plant-produced Cry2Ab2 protein was isolated from ground grain of MON 89034. The identity of the grain sample containing MON 89034 was confirmed by event specific PCR. The isolated plant-produced Cry2Ab2 protein was stored in a –80 °C freezer in a buffer solution containing 50 mM CAPS, 2 mM DTT, pH 11 at a total protein concentration of 0.25 mg/mL.

4.2. Reference Standard

The *E. coli*-produced Cry2Ab2 protein was used as a reference standard in select analyses. These analyses included apparent molecular weight determination by SDS-PAGE, western blot analysis, glycosylation analysis, BioRad protein assay, and the functional activity assay.

4.3. Assay Controls

Protein molecular weight standards were used to calibrate SDS polyacrylamide gels and verify protein transfer to PVDF membranes. The *E. coli*-produced Cry2Ab2 reference standard protein was used to estimate the total protein concentration in the BioRad

protein assays and also used as the positive control in western blot analysis. Beta-lactoglobulin protein and PTH-amino acid standards were used to verify the performance of the amino acid sequencer. A peptide mixture and an analytical BSA standard were used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis and molecular weight determination, respectively. Transferrin and E. coli-produced Cry2Ab2 protein were used as the positive and negative control, respectively, in glycosylation analysis.

4.4. Protein Purification

The Cry2Ab2 protein was purified from an extract of ground grain of MON 89034 using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

The isolation of Cry2Ab2 protein from ground corn grain was performed in two 10 kg batches. Prior to extraction of Cry2Ab2 protein, each batch was extracted with 1X PBS buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) to remove contaminant proteins. The PBS extraction procedure consisted of soaking ground grain in PBS for 2 hrs in a 4 °C cold room at approximately 1:10 sample weight to buffer volume ratio. The slurry was clarified by filtration using an Ertel Alsop filter press (Kingston, NY), and the PBS washed solid particle (cake) was retained. Subsequently, the Cry2Ab2 protein in the cake was extracted with extraction buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, 0.5 mM CHAPS, 1% (w/v) PVPP, pH 10.8 at approximately 1:10 sample weight to buffer volume ratio for 2-3 hrs. During extraction, lipids were removed from the extract by adding CelPure P65 diatomaceous earth (Advanced Minerals Corp, Goleta, CA) to the homogenate at ~7.5% (w/v) and allowed to mix for ~10 minutes. The slurry was clarified by filtration using the filter press and the resultant extract from both batches was pooled for a total volume of ~230 L. The pooled extract was concentrated using a 30,000 NMWC Hollow Fiber Cartridge (Amersham Biosciences, Piscataway, NJ) to a final volume of ~35 L. To remove plant genomic DNA, polyethyleneimine, 10% (w/v), was added to the concentrated extract to a final concentration of 0.05% (w/v), the extract was clarified by centrifugation to remove precipitated DNA, and the supernatant was retained. The Cry2Ab2 protein in the supernatant was precipitated by 0% – 35% ammonium sulfate saturation. The 35% ammonium sulfate pellet was recovered by centrifugation and the pellet was dissolved in 20 L of resuspension buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 by mixing in 4 °C cold room. The suspension was clarified by centrifugation and 21 L supernatant was retained, concentrated, and buffer exchanged by diafiltration against Buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 to remove any residual ammonium sulfate salt. The concentrated sample of 13 L was loaded onto an anion exchange column in two batches, Run 1 (6L) and Run 2 (7L). The elution parameters were identical for both runs and thus only the Run1 column parameters are described below.

A portion of concentrated sample was loaded onto a 4.5 L (20 cm x 14.4 cm column) Q Sepharose Fast Flow anion exchange resin column, which was equilibrated with Buffer. The bound Cry2Ab2 protein was eluted with step gradients as follows: 0-20% buffer B (Buffer containing 1M NaCl) in 10 column volumes (CV), and then the gradient was held at 20% buffer B for 4 CV, and then the gradient was increased to 65% buffer B over 10 CV and finally the gradient was stepped up to 100% buffer B and held at 100% buffer B in 2 CV. Fractions, each ~ 4 L, containing Cry2Ab2 protein were identified by Cry2A QuickStix™ (Portland, ME) for further analysis. Based on western blot analysis and SDS-PAGE analysis, fraction 7 from Run 2 was selected for affinity purification.

Subsequently, fraction 7 was concentrated to 400 mL using a 30,000 NMWC Hollow Fiber Cartridge. Approximately one half of the aforementioned sample was buffer exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2) using centrifuge concentrators (30 kDa MWCO), resulting in a final volume of 200 mL. This sample, in two separate batches, was applied to an affinity column (1.0 cm × 2.7 cm) containing Protein G agarose conjugated with Cry2Aa-specific mAb and equilibrated with EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2). The sample was re-circulated through the column for 2 hrs at 100 mL/hr. The column was then washed with 17-20 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2), followed by 5-7 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM Triethylamine, pH 11.4, in five 1.5 mL fractions. Based on SDS-PAGE analysis, fractions containing Cry2Ab2 protein from batch one and two were concentrated and buffer exchanged into a storage buffer containing 50 mM CAPS, 2 mM DTT, pH 11.0, using centrifuge concentrators (30 kDa MWCO). Concentrated samples were then pooled, resulting in a final volume of 2 mL.

The affinity chromatography procedure was repeated beginning with 100 mL of fraction 7 from Run2. As previously described, the sample was concentrated and buffer exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05) to a final volume of 50 mL using centrifuge concentrators (30 kDa MWCO). This sample was re-circulated for 2 hr (60 mL/hr) through an affinity column (1.0 cm × 2.75 cm) prepared with Cry2Aa-specific mAb equilibrated with EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05). The column was then washed with 15 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05) followed by 5 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM Triethylamine, pH 11.4, in five 1.5 mL fractions.

Based on SDS-PAGE analysis, Cry2Ab2 enriched fractions were individually concentrated and buffer exchanged into a storage buffer containing 50 mM CAPS, 2 mM DTT, pH 11, using a centrifuge concentrator (30 kDa MWCO). The concentrated samples were pooled into a final volume of 2.4 mL. The aforementioned sample and the sample from the first run (2 mL) were pooled resulting in a final volume of 4.4 mL.

4.5. Total Protein Concentration by BioRad Assay

The total protein concentration of the purified plant-produced Cry2Ab2 protein was estimated using a BioRad protein assay. The *E. coli*-produced Cry2Ab2 reference standard protein (concentrations ranging from 0.05 to 0.5 mg/mL) was used to prepare a standard curve. The plant-produced Cry2Ab2 total protein concentration was estimated by comparison of absorbance values obtained for the sample to the values of the standard curve. Data were collected using a Bio-Tek Instruments, Inc. Powerwave Xi microplate scanning spectrophotometer (Winooski, VT) employing KC4 software version 3.3 revision 10. Readings were taken at a wavelength of 595 nm.

4.6. Western Blot Analysis

Equivalence would be demonstrated if the full-length plant-produced Cry2Ab2 protein was identified by the anti-Cry2Ab2 antibody and exhibited similar electrophoretic mobility compared to the *E. coli*-produced Cry2Ab2 reference standard. Aliquots of the stock solutions of the plant-produced Cry2Ab2 and reference standard were diluted to a final purity-corrected protein concentration of 2 ng/µL in dilution buffer (50 mM CAPS, 2 mM DTT, pH 11) and 5× sample loading buffer [5x concentrated Laemmli buffer (312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8)]. Samples were then heated to ~100 °C for 5 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well gel. The plant-produced Cry2Ab2 protein was loaded in duplicate at three different loadings of 20, 30, and 40 ng per lane. The *E. coli*-produced Cry2Ab2 reference standard was loaded at 20 ng per lane. Electrophoresis was performed at a constant voltage of 140 V for 20 min followed by a constant voltage of 200 V for 43 min. Pre-stained molecular weight markers included during electrophoresis (BioRad Precision Plus Dual Color, Hercules, CA) were used to verify electrotransfer of protein to the membrane and to estimate the molecular weight of the immunoreactive bands. Samples were electrotransferred to a 0.45 micron PVDF membrane (Invitrogen, Carlsbad, CA) for 60 min at a constant current of 300 mA.

The membrane was blocked for one hour with 5% (w/v) NFDM in PBST. The membrane was probed with a 1:3000 dilution of goat anti-Cry2Ab2 antibody (lot 7227632) in 2% (w/v) NFDM in PBST for one hour. Excess antibody was removed using three 10 min washes with PBST. The membrane was probed with peroxidase-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO) at a dilution of 1:10,000 in 2% (w/v) NFDM in PBST for one hour. Excess peroxidase-conjugate was removed using three 10 min washes with PBST. All procedures, including blocking, and all other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ) and exposed (30 sec, 1 min, 2 min, 3 min, and 7 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

4.7. MALDI-TOF Tryptic Mass Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the plant-produced Cry2Ab2 protein. A protein can be typically identified when 40% of the mass fragments are identified from the analyzed protein (Jiménez et al., 1998).

4.7.1. SDS-PAGE Separation of Proteins

An aliquot of the plant-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final purity corrected protein concentration of 80 ng/μL and 2 μg was electrophoresed in each of five lanes. Broad Range molecular weight markers (BioRad, Hercules, CA) were used to estimate molecular weights. Samples were heated to ~99 °C for 5 min prior to electrophoresis on a pre-cast tris-glycine 4→20% SDS polyacrylamide gel at 140 V for 20 min followed by a constant voltage of 200 V for 46 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained 2 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO), destained 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and followed by 25% (v/v) methanol for 1 hr. Two protein bands, band-1 and band-2, migrating at 61 kDa and 50 kDa, respectively, were identified for tryptic mass map analysis.

4.7.2. In-gel Protein Digestion

The plant-produced full length Cry2Ab2 protein, band-1 migrating at ~61 kDa, and band-2, a proteolytic fragment of the full length Cry2Ab2 protein migrating at ~50 kDa, were excised, destained, reduced, alkylated, and subjected to an in-gel trypsin (Promega, Madison, WI) digestion (Williams et al., 1997). Each gel band was individually destained for 30 min by incubation in 100 μL of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in its own microfuge tube. Following destaining, the gel bands were incubated in 100 μL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. Proteins were reduced in 100 μL of 10 mM dithiothreitol solution for 2 hrs at 37 °C. Proteins were then alkylated by the addition of 100 μL of buffer containing 200 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. The gel bands were incubated in 100 μL of 100 mM ammonium bicarbonate for 30 min at room temperature at which time 100 μL of acetonitrile was added and the incubation was continued for an additional 30 minutes. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove the reducing and alkylating reagents, and salts from the gel. The gel bands were dried in a SpeedVac concentrator (Savant, Holbrook, NY), rehydrated with 40 μL 25 mM ammonium bicarbonate containing 33 μg/mL trypsin, and the protein contained in the gel band was digested for 16 hours at 37 °C. Digested peptides were extracted for one hour at room temperature with 50 μL 70% (v/v) acetonitrile containing 0.1% (v/v) TFA per gel band. Supernatants for each extraction were combined into a single tube and dried in a SpeedVac concentrator. This process of extracting the peptides was repeated two more times. The final dried materials were reconstituted in 10 μL of 0.1% (v/v) TFA.

4.7.3. Sample Preparation

A portion (5 µl) of the digested samples was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) ZipTip®C18 pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. Each sample was applied to a ZipTip®C18 and eluted with 5 µL of Wash 1 [0.1% (v/v) TFA], followed by 5 µL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], followed by 5 µL of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 5 µL of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

4.7.4. MALDI-TOF Instrumentation and Mass Analysis

Mass spectral analyses were performed as follows: mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme™ Peptide Mass Standards kit (Applied Biosystems). Samples (0.3 µL) from each of the desalting steps, as well as a sample of the solution taken prior to desalting, were co-crystallized with 0.75 µL α-cyano-4-hydroxy cinnamic acid (Waters, Milford, MA) on the analysis plate. All samples were analyzed in the 500 to 5000 dalton range using 100 shots at a laser intensity setting of 2318-2460 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected Cry2Ab2 protein sequence deduced from the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 5000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal of ±2 daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data. The identity of the Cry2Ab2 protein is confirmed if ≥ 40 % of the protein sequence was identified by matching experimental masses for the tryptic peptide fragments to the expected masses for the fragments.

4.8. Analysis of the N-terminal Sequence

An aliquot of the plant-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final purity corrected protein concentration of 80 ng/µL. Molecular weight markers (BioRad Precision Plus Dual Color, Hercules, CA) were used to confirm the transfer of protein to the PVDF membrane. The plant-produced Cry2Ab2 protein was electrophoresed in eight lanes at 2 µg per lane. The Cry2Ab2 containing samples were heated to ~99 °C for 5 min prior to electrophoresis on a pre-cast tris-glycine 4→20% SDS polyacrylamide gel at 140V for 20 min followed by 200 V for 43 min. The gel was then electroblotted to a 0.2 micron PVDF membrane for 60 min at a constant current of 300 mA in a solution containing 10 mM CAPS, 10% (v/v) methanol, pH 11. Protein

bands were stained by briefly soaking the membrane with Coomassie Blue R-250 stain (BioRad) and visualized by destaining with a Coomassie R-250 destaining solution (BioRad).

The protein bands with molecular weights of approximately 61.3, 49.6 and 27 kDa, (also referred to as band-1, band-2, and band-3, respectively) were excised from the membrane. N-terminal sequence analysis was performed using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise™ Control Software (version 2.1) was used. Chromatographic data were collected using Atlas[99] software (version 2003R1.1, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the three protein bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

4.9. Molecular Weight Estimation by SDS-PAGE

Aliquots of the test substance and reference standard protein were each diluted with sample dilution buffer and 5× sample loading buffer to a final protein concentration of 0.2 µg/µL. Molecular weight markers (BioRad Broad-Range, Hercules, CA) that were used to estimate the apparent molecular weight of the test substance, were diluted to a final total protein concentration of 0.9 µg/µL. The plant-produced Cry2Ab2 protein was analyzed in duplicate at 1, 2, and 3 µg total protein loads per lane. The E. coli-produced Cry2Ab2 reference standard was analyzed at 1 µg total protein. All samples were heated at ~102 °C for 5 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 150 V for 82 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained 16 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO), destained 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 6 hr.

Analysis of the gel was performed using a BioRad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). Values for the markers supplied by the manufacturer were used to estimate the apparent molecular weight of each observed band. All visible bands within each lane were quantified using Quantity One software. For the plant-produced Cry2Ab2 protein, purity was estimated as the percent optical density of the ~61 kDa band relative to all bands detected in the lane. Apparent molecular weight and purity were reported as an average of all six loadings containing the plant-produced Cry2Ab2 protein.

4.10. Molecular Weight Determination using MALDI-TOF MS

Determination of the intact mass was attempted for the plant-produced Cry2Ab2 protein using MALDI-TOF mass spectrometry analysis. This analysis was not specified in the characterization plan and is therefore a plan deviation. However, there is no impact on the protein characterization.

Prior to analysis, the plant-produced Cry2Ab2 protein and BSA reference protein (NIST, Gaithersberg, MD) were desalted using drop dialysis (Görisch, 1988). A portion of each protein sample (0.3 µL) was spotted on an analysis plate, mixed with 0.75 µL sinapinic acid solution and air-dried. Each sample was analyzed in triplicate. Mass spectral analysis was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF instrument with the supplied Data Explorer software (version 4.0.0.0, Foster City, CA). Mass calibration of the instrument was performed using desalted BSA reference protein.

4.11. Functional Activity Assay

The purpose of this analysis was to compare the biological activity between plant-produced Cry2Ab2 protein and the *E. coli*-produced Cry2Ab2 protein by determining EC_{50} values in a CEW diet-incorporation insect bioassay. The EC_{50} value is defined as the concentration of Cry2Ab2 protein in the diet that results in 50% growth inhibition. In order to assess the functional activity of the plant-produced Cry2Ab2 protein and to compare its activity to the *E. coli*-produced Cry2Ab2 reference standard, aliquots of the plant-produced Cry2Ab2 protein and *E. coli*-produced Cry2Ab2 reference standard protein were used to estimate the effective protein concentration necessary to inhibit the growth of the target insect by 50%.

The total protein concentration of the *E. coli*-produced Cry2Ab2 protein aliquots was 0.50 mg/mL, with a purity of 87%, and a purity corrected concentration of 0.4 mg Cry2Ab2/mL. The total protein concentration of the plant-produced Cry2Ab2 protein aliquots was 0.25 mg/mL with a purity of 33%, and a purity corrected concentration of 0.1 mg Cry2Ab2/mL. Both proteins were suspended in 50 mM CAPS, 2 mM DTT, pH 11.0, buffer. The control substance used in the bioassays was buffer of the same composition used to store the *E. coli*-produced and plant-produced proteins, 50 mM CAPS, 2 mM DTT, pH 11, buffer. The plant-produced and *E. coli*-produced Cry2Ab2 proteins were stored in a -80° C freezer and the buffer was stored in a 4° C refrigerator.

4.11.1. Insects

CEW *(Helicoverpa zea*) eggs were obtained from Benzon Research Inc. Insect eggs were incubated at temperatures ranging from 10^{o} C to 27^{o} C, to achieve the desired hatch time.

4.11.2. Bioassays

CEW were used to measure activity of the plant- and *E. coli*-produced Cry2Ab2 protein samples in accordance with the Monsanto SOP BR-ME-0044-03. The bioassay was replicated three times on separate days with separate batches of insects. The plant- and *E. coli*-produced proteins were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and plant-produced Cry2Ab2 proteins consisted of a series of seven dilutions and a buffer control yielding a dose series with a 2-fold separation factor ranging from 0.016 – 1.0 µg Cry2Ab2 protein/mL diet. The dose-response curves for each protein included a buffer control. Each buffer control contained an amount of their respective buffer equivalent to the amount of protein in the highest dose level. The Cry2Ab2 protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into an agar-based insect diet. This dose series in diet was chosen to adequately characterize the dose-effect relationship on CEW weight gain for the proteins from both sources. The diet mixture was then dispensed in 1 mL aliquots into a 128 well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD International, Pitman, NJ) and the insects were allowed to feed for a period of seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 h light:10 h dark. The combined weight of the surviving insects at each dose level for each source of protein was recorded at the end of the 7-day incubation period.

4.11.3. Dose Response Modeling

Data were entered into an Excel spreadsheet and transferred to the Statistics Technology Center for analysis. Dose response modeling and EC_{50} determinations were performed using a 3-parameter logistic regression model (equation 1) under the PROC NLIN procedure in SAS.

Equation 1:
$$Wt = \frac{W_0}{1+\left(\frac{DietDose}{EC50}\right)^B} + e$$

where Wt is the average CEW larvae weight and $DietDose$ is the Cry1Ab protein diet dose level. Three parameters that are included in the model; W_0 represents the weight at $DietDose = 0.0$, EC_{50} represents effective concentration to reduce the growth of the target insect by 50%, and B reflects the rate of the weight loss as $DietDose$ increases, and e denotes the residual (error).

4.12. Glycosylation Analysis

Glycosylation analysis was used to determine whether the plant-produced Cry2Ab2 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the plant-produced Cry2Ab2 protein, the *E. coli*-produced Cry2Ab2 reference standard (in this instance, a negative control), and the positive control transferrin (Amersham Biosciences, Piscataway, NJ) were each diluted in dilution buffer and in 5× sample loading buffer to a final purity corrected (total protein for transferrin) concentration of 50 ng/µL. These samples were heated to ~100 °C for 5 min, and loaded along with Precision Plus Dual Color pre-stained protein molecular weight markers (BioRad , Hercules, CA) on a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel. All three samples were loaded at a single loading of 0.5 and 1 µg protein per lane. Electrophoresis was performed at a constant voltage of 140 V for 20 min followed by a constant voltage of 200 V for 47 min. After electrophoresis, proteins were electrotransfered to a 0.45 micron PVDF membrane for one hour at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane containing the 0.5 and 1.0 µg sample loads using the ECL detection system (Amersham Biosciences, Piscataway, NJ). After the electrotransfer of the proteins, the PVDF membrane was incubated in PBS for 10 min, and transferred to a solution of 100 mM acetate buffer, pH 5.5, containing the oxidation reagent, 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 minutes. The oxidation solution was removed from the membrane by two brief rinses followed by three sequential 10 min washes in PBS. The membrane was transferred to a solution of 100 mM acetate buffer, pH 5.5, containing 25 nM biotin hydrazide and incubated for 60 minutes. Biotin hydrazide solution was removed by washing in PBS as previously described. The membrane was blocked with 5% blocking agent in PBS for 60 minutes. The blocking solution was removed by washing in PBS as previously described. The membrane was incubated with streptavidin-HRP conjugate (diluted 1:6000) in acetate buffer for 30 min to detect carbohydrate moieties bound to biotin. Excess streptavidin-HRP was removed by washing in PBS as previously described. Bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ). Films were exposed (1 min, 3 min, and 6 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

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APPENDIX D: Summary of The Tryptic Masses of the Cry1A.105 And Cry2Ab2 Proteins Identified Using MALDI-TOF MS

Table D.1. Summary of the Tryptic Masses Identified for the Full-length Plant-produced Cry1A.105 Protein Using MALDI-TOF Mass Spectrometry

Only experimental masses that matched expected masses are listed in the table.

Observed Mass (Da)					Expected Mass (Da)	Δ[1]	AA Position[2]	Fragment Sequence(s)
No Desalting[3]	Desalting[4]							
	Wash 1	Wash 2	Wash 3	Wash 4				
515.15	515.22	515.25	515.27	515.27	515.34	0.19	525-528	ARIR
	529.16				529.27	0.11	695-698	QPER
579.13	579.21	579.25	579.28		579.33	0.20	749-752	YQLR
589.12	589.18	589.23	589.26	589.25	589.31[5] 589.28[5]	0.19 0.16	225-228 1023-1027	DWIR VCPGR
				606.01	605.31	0.70	666-670	ELSEK
	611.25	611.29	611.32	611.29	611.36	0.11	936-940	VHSIR
621.17		621.29	621.31	621.32	621.37	0.20	1028-1032	GYILR
649.16	649.94	649.29	649.32	649.99	649.37	0.21	254-258	TYPIR
	688.26	688.30			688.37	0.11	94-99	NQAISR
727.14	727.25	727.28	727.31		727.35	0.21	229-233	YNQFR
731.15	731.24	731.29	731.33		731.36	0.21	424-429	QGFSHR
764.18	764.27	764.33	764.35	764.35	764.39	0.21	88-93	IEEFAR
	781.28	781.32		781.34	781.38	0.10	193-198	YNDLTR

[1] A difference of less than one dalton between the observed (first column where this mass is documented) and expected masses was necessary for consideration as a match.

[2] AA position refers to amino acid position within the predicted Cry1A.105 sequence.

[3] Sample, 0.5 µL, was analyzed prior to Zip Tip desalting

[4] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations of acetonitrile: Wash 1 – 0% (v/v), Wash 2 – 20% (v/v), Wash 3 – 50% (v/v), and Wash 4 - 90% (v/v) acetonitrile.

Table D.1 (cont.). Summary of the Tryptic Masses Identified for the Full-length Plant-produced Cry1A.105 Protein Using MALDI-TOF Mass Spectrometry

Observed Mass (Da)					Expected Mass (Da)	Δ[1]	AA[2] Position	Fragment Sequence(s)
	Desalting[3]							
No Desalting[4]	Wash 1	Wash 2	Wash 3	Wash 4				
	784.26				784.37	0.11	853-859	TQDGHAR
804.22	804.33	804.39	804.43	804.41	804.46	0.24	259-265	TVSQLTR
816.17	816.29	816.33	816.36		816.40	0.23	218-224	VWGPDSR
	854.94			855.00	854.41	0.53	1114-1120	SYTDGRR
907.21		907.40	907.43	907.43	907.46	0.25	174-181	DVSVFGQR
925.24	925.37	925.42	925.46	925.45	925.47	0.23	529-536	YASTTNLR
940.26		940.45	940.47	940.48	940.51	0.25	361-368	TLSSTLYR
976.26		976.44	976.48	976.47	976.50	0.24	430-437	LSHVSMFR
1007.31	1007.44	1007.49		1007.52	1007.55	0.24	537-545	IYVTVAGER
1066.17	1065.97	1066.38		1066.03	1066.43	0.26	1121-1128	ENPCEFNR
1074.28		1074.50		1074.52	1074.55	0.27	282-292	GSAQGIEGSIR
				1078.51	1078.55	0.04	682-690	NLLQDSNFK
1089.30		1089.52	1089.54	1089.55	1089.57	0.27	491-501	GPGFTGGDILR

[1] A difference of less than one dalton between the observed (first column where this mass is documented) and expected masses was necessary for consideration as a match.

[2] AA position refers to amino acid position within the predicted Cry1A.105 sequence.

[3] Sample, 0.5 µL, was analyzed prior to Zip Tip desalting

[4] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations of acetonitrile: Wash 1 – 0% (v/v), Wash 2 – 20% (v/v), Wash 3 – 50% (v/v), and Wash 4 - 90% (v/v) acetonitrile.

Table D.1 (cont.) Summary of the Tryptic Masses Identified for the Full-length Plant-produced Cry1A.105 Protein Using MALDI-TOF Mass Spectrometry

Observed Mass (Da)					Expected Mass (Da)	Δ^1	AA[2] Position	Fragment Sequence(s)
No Desalting[3]	Desalting[4] Wash 1	Wash 2	Wash 3	Wash 4				
1144.29		1144.53	1144.55	1144.54	1144.57	0.28	450-458	APMFSWIHR
1203.40		1203.64	1203.67	1203.66	1203.68	0.28	350-360	IVAQLGQGVYR
1237.30		1237.55	1237.58	1237.57	1237.60	0.30	182-192	WGFDAATINSR
1253.36		1253.61	1253.64	1253.63	1253.65	0.29	438-449	SGFSNSSVSIIR
1258.36		1258.61	1258.63	1258.64	1258.65	0.29	199-209	LIGNYTDHAVR
1269.40		1269.65			1269.69	0.29	479-490	AHTLQSGTTVVR
1398.34		1398.63	1398.65	1398.65	1398.67	0.33	116-127	EWEADPTNPALR
1424.32	1424.53	1424.62			1424.65	0.33	994-1005	GHVDVEEQNNQR
				1576.81	1576.81[5] 1576.87[5]	0.00 0.06	682-694 623-637	NLLQDSNFKDINR AVNALFTSTNQLGLK
				1598.81	1598.71	0.10	1120-1131	RENPCEFNRGYR
1800.47		1800.84	1800.84	1800.84	1800.87	0.40	753-767	GYIEDSQDLEIYSIR
1900.48		1900.88	1900.89	1900.89	1900.91	0.43	266-281	EIYTNPVLENFDGSFR
1902.52			1902.92	1902.90	1902.96	0.44	100-115	LEGLSNLYQIYAESFR

[1] A difference of less than one dalton between the observed (first column where this mass is documented) and expected masses was necessary for consideration as a match.

[2] AA position refers to amino acid position within the predicted Cry1A.105 sequence.

[3] Sample, 0.5 µL, was analyzed prior to Zip Tip desalting

[4] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations of acetonitrile: Wash 1 – 0% (v/v), Wash 2 – 20% (v/v), Wash 3 – 50% (v/v), and Wash 4 - 90% (v/v) acetonitrile.

[5] Two expected fragments having nearly identical masses were matched to one observed mass.

Table D.1 (cont.) Summary of the Tryptic Masses Identified for the Full-length Plant-produced Cry1A.105 Protein Using MALDI-TOF Mass Spectrometry

Observed Mass (Da)					Expected Mass (Da)	Δ[1]	AA[2] Position	Fragment Sequence(s)
No Desalting[3]	Desalting[4] Wash 1	Wash 2	Wash 3	Wash 4				
1955.58			1955.98	1955.97	1956.01	0.43	1006-1022	SVLVVPEWEAEVSQEVR
2097.69			2098.14	2098.12	2098.15	0.46	860-878	LGNLEFLEEKPLVGEALAR
2107.63			2108.07	2108.05	2108.09	0.46	602-619	FELIPVTATLEAEYNLER
2133.62			2134.08		2133.11	0.51	503-522	TSGGPFAYTIVNINGQLPQR
2148.59		2149.03	2149.06	2149.02	2149.05	0.46	404-423	SGTVDSLDEIPPQNNNVPPR
2196.64			2197.11	2197.06	2197.11	0.47	293-311	SPHLMDILNSITIYTDAHR
			2211.11	2211.05	2211.13	0.02	430-449	LSHVSMFRSGFSNSSVSIIR
		2277.12			2277.10[5] 2277.15[5]	0.02 0.03	753-771 403-423	GYIEDSQDLEIYSIRYNAK KSGTVDSLDEIPPQNNNVPPR
2615.78			2616.33	2616.37	2616.36	0.58	941-964	EAYLPELSVIPGVNAAIFEELEGR
3728.03			3729.00		3728.87	0.84	369-402	RPFNIGINNQQLSVLDGTEFAYGTSS NLPSAVYR

[1] A difference of less than one dalton between the observed (first column where this mass is documented) and expected masses was necessary for consideration as a match.

[2] AA position refers to amino acid position within the predicted Cry1A.105 sequence.

[3] Sample, 0.5 µL, was analyzed prior to Zip Tip desalting

[4] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations of acetonitrile: Wash 1 – 0% (v/v), Wash 2 – 20% (v/v), Wash 3 – 50% (v/v), and Wash 4 - 90% (v/v) acetonitrile.

[5] Two expected fragments having nearly identical masses were matched to one observed mass.

Table D.2. Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the 61 kDa Plant-Produced Cry2Ab2 Protein

Observed Mass[1] (Da)					Expected Mass (Da)	Δ[2]	AA[3] Position	Fragment Sequence(s)
No Desalting[4]	Desalting[5]							
	Wash 1	Wash 2	Wash 3	Wash 4				
				506.58	506.25	0.33	100-103	ETEK
		552.44	552.45	552.45	552.31	0.13	538-541	YTLR
553.35	553.40				553.27	0.08	218-221	NYTR
560.38	560.44	560.45			560.32	0.06	237-241	GLNTR
	646.39	646.48	646.47		646.32	0.07	439-443	NEDLR
	659.44		659.44		659.38[6]	0.06	563-568	VTINGR
					659.41[6]	0.03	69-75	VGSLVGK
677.47	677.53	677.54	677.54	677.54	677.37	0.10	104-108	FLNQR
709.46	709.52	709.54	709.54	709.54	709.36	0.10	410-416	SGAFTAR
		724.57			724.39	0.18	514-519	TFISEK
730.54		730.63	730.63	730.63	730.45	0.09	77-82	ILSELR
903.61		903.72	903.72	903.72	903.49	0.12	109-116	LNTDTLAR
993.62	993.70	993.73	993.73	993.73	993.48	0.14	520-528	FGNQGDSLR
		1022.73	1022.72		1022.45	0.28	380-388	SWLDSGSDR

[1] Only experimental masses that matched to an expected mass are listed in the table.

[2] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match (Except for mass fragment 144-164).

[3] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 9.VI.

[4] Sample, 0.3 µL, was analyzed prioto desalting.

[5] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile].

[6] Two expected fragments having nearly identical masses were matched to one observed mass.

Table D.2 (cont.) Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the 61 kDa Plant-Produced Cry2Ab2 Protein

Observed Mass[1] (Da)					Expected Mass (Da)	Δ^2	AA[3] Position	Fragment Sequence(s)
No Desalting[4]	Desalting[5] Wash 1	Wash 2	Wash 3	Wash 4				
1033.71		1033.83	1033.82	1033.82	1033.56	0.15	553-562	VISSIGNSTIR
1053.79		1053.91	1053.91	1053.91	1053.64	0.15	429-438	NISGVPLVVR
1060.21		1060.80	1060.79	1060.79	1060.52	0.31	242-249	LHDMLEFR
1076.69		1076.80	1076.80	1076.80	1076.53	0.16	466-474	AYMVSVHNR
1080.67	1080.76	1080.79		1080.79	1080.51	0.16	529-537	FEQNNTTAR
	1163.92				1164.60	0.68	100-108	ETEKFLNQR
1184.78		1184.92	1184.91	1184.90	1184.60	0.18	453-465	NIASPSGTPGGAR
1197.82	1197.94	1197.96	1197.95	1197.95	1197.65	0.17	444-452	RPLHYNEIR
1216.79		1216.92	1216.92	1216.91	1216.61	0.18	134-143	QVDNFLNPNR
1492.91		1493.13	1493.06	1493.06	1492.69[6]	0.22	417-428	GNSNYFPDYFIR
					1492.75[6]	0.16	211-221	TYRDYLKNYTR
1680.15		1680.31			1680.94	0.79	429-443	NISGVPLVVRNEDLR
1904.24	1904.45	1904.46	1904.43	1904.41	1903.95	0.29	117-133	VNAELTGLQANVEEFNR

[1] Only experimental masses that matched to an expected mass are listed in the table.

[2] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match (Except for mass fragment 144-164).

[3] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 9.VI.

[4] Sample, 0.3 µL, was analyzed prior to desalting.

[5] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile].

[6] Two expected fragments having nearly identical masses were matched to one observed mass.

Table D.2 (cont.) Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the 61 kDa Plant-Produced Cry2Ab2 Protein

Observed Mass[1] (Da)					Expected Mass (Da)	Δ^2	AA[3] Position	Fragment Sequence(s)
No Desalting[4]	Desalting[5] Wash 1	Wash 2	Wash 3	Wash 4				
1919.28			1919.47	1919.47	1919.01	0.27	83-99	NLIFPSGSTNLMQDILR
2311.25		2311.68			2311.06	0.19	569-590	VYTATNVNTTTNNDGVNDNGAR
			2334.77	2334.78	2333.23	1.54	144-164	NAVPLSITSSVNTMQQLFLNR
2339.51			2339.75	2339.69	2339.15	0.36	389-409	EGVATVTNWQTESFETTLGLR
2451.70			2451.98	2451.97	2451.34	0.36	320-343	LSNTFPNIVGLPGSTTHALLAAR

[1] Only experimental masses that matched to an expected mass are listed in the table.

[2] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match (Except for mass fragment 144-164).

[3] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 9.VI.

[4] Sample, 0.3 µL, was analyzed prior to desalting.

[5] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile], as described in the methods section (Section 5.4.3).

Table D.3. Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the 50 kDa Fragment of the Plant-Produced Cry2Ab2 Protein

Observed Mass[1] (Da)					Expected Mass (Da)	Δ^2	AA[3] Position	Fragment Sequence(s)
No Desalting[4]	Desalting[5] Wash 1	Wash 2	Wash 3	Wash 4				
		552.44	552.45		552.31	0.13	394-397	YTLR
553.37	553.41				553.27	0.10	74-77	NYTR
560.40	560.45	560.45	560.46		560.32	0.08	93-97	GLNTR
		646.47	646.48		646.32	0.15	295-299	NEDLR
659.40	659.45			660.20	659.38	0.02	419-424	VTINGR
709.49	709.54	709.54	709.55	709.57	709.36	0.13	266-272	SGAFTAR
993.66		993.73	993.74	993.77	993.48	0.18	376-384	FGNQGDSLR
			1022.73		1022.45	0.28	236-244	SWLDSGSDR
1033.74		1033.84	1033.85		1033.56	0.18	409-418	VSSIGNSTIR
1053.83		1053.92	1053.93	1053.97	1053.64	0.19	285-294	NISGVPLVVR
1060.25		1060.80	1060.81		1060.52	0.27	98-105	LHDMLEFR
1072.30					1072.54	0.24	70-77	DYLKNYTR
1076.73		1076.81	1076.82	1076.84	1076.53	0.20	322-330	AYMVSVHNR
1080.71	1080.79	1080.79	1080.80		1080.51	0.20	385-393	FEQNNTTAR

[1] Only experimental masses that matched to an expected mass are listed in the table.

[2] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match.

[3] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 10.VI.

[4] Sample, 0.3 µL, was analyzed prior to desalting.

[5] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile].

Table D.3 (cont.) Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the 50 kDa Fragment of the Plant-Produced Cry2Ab2 Protein.

Observed Mass[1] (Da)					Expected Mass (Da)	Δ[2]	AA[3] Position	Fragment Sequence(s)
No	Desalting[5]							
Desalting[4]	Wash 1	Wash 2	Wash 3	Wash 4				
1184.81		1184.91	1184.93		1184.60	0.21	309-321	NIASPSGTPGGAR
1197.87		1197.97	1197.98	1198.02	1197.65	0.22	300-308	RPLHYNEIR
1492.96		1493.09	1493.10	1493.12	1492.69[6]	0.27	273-284	GNSNYFPDYFIR
					1492.75[6]	0.21	67-77	TYRDYLKNYTR
1680.22		1680.31	1680.35		1680.94	0.72	285-299	NISGVPLVVRNEDLR
1844.28					1843.96	0.32	50-66	DVILNADEWGISAATLR
		1875.16			1874.79	0.37	78-92	DYSNYCINTYQSAFK
		2311.67			2311.06	0.61	425-446	VYTATNVNTTTNNDGVNDNGAR
			2339.79		2339.15	0.64	245-265	EGVATVTNWQTESFETTLGLR
2451.81			2452.02	2452.03	2451.34	0.47	176-199	LSNTFPNIVGLPGSTTTHALLAAR

[1] Only experimental masses that matched to an expected mass are listed in the table.

[2] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match.

[3] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 10.VI.

[4] Sample, 0.3 µL, was analyzed prior to desalting.

[5] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile]

[6] Two expected fragments having nearly identical masses were matched to one observed mass.

APPENDIX E: Materials and Methods used for the Estimation of Cry1Ab.105 and Cry2Ab2 Protein Levels in Tissues of MON 89034

1. Test, control, and reference substances

1.1. Test substance

The test substance was MON 89034 grown in 2005 U.S. field trials at five sites.

1.2. Control substance

The negative control substance was a conventional corn with a similar genetic background to the test plants grown in 2005 U.S. field trials at five sites.

1.3. Characterization of test and control substances

The identities of the test and control substances were confirmed by verifying the chain-of-custody documentation prior to analysis. To further confirm the identities of the test and control substances, event-specific polymerase chain reaction (PCR) analyses were conducted on seed and grain samples. The identities of grain samples harvested from the field were verified by PCR and the verification of identity was referenced back to the seed used for planting.

1.4. Reference substances

Four *E. coli*-produced protein standards were used and the certificates of analysis were archived with the study data. A Cry1A.105 protein standard (lot 20-100086) was used as the reference substance for the analysis of Cry1A.105 protein levels. The purity-corrected protein concentration of the purified standard was 1.0 mg/ml by amino acid composition analysis. The purity was 80% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis.

A Cry2Ab2 protein standard (lot 20-100071) was used as the reference substance for the analysis of Cry2Ab2 protein levels. The purity-corrected protein concentration of the purified standard was 0.4 mg/ml by amino acid composition analysis. The purity was 87% as determined by SDS-PAGE and densitometric analysis.

2.0. Generation of plant samples

2.1. Summary of field design

Plants were grown at five field sites in the U.S. during the 2005 field season: Jefferson County, IA; Warren County, IL; Clinton County, IL; York County, NE; and Fayette

County, OH. These field sites were located within the major corn-growing regions of the U.S. and provided a variety of environmental conditions. At each site, three replicated plots of MON 89034, as well as the conventional control, were planted using a randomized complete block field design. Overseason leaf (OSL 1-4), overseason root (OSR 1-4), over season whole plant (OSWP 1-4), pollen, silk, forage, stover, forage-root, senescent root, and grain tissues were collected from each replicated plot at all field sites. The over season samples (leaf, root, and whole plant) were collected four times at different growth stages: (1) V2 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, and (4) pre-VT stage. The identification of corn growth and development stages was based on the descriptions in "How a Corn Plant Develops" (Ritchie et al., 1997). Throughout the field production, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. All tissue samples, except grain, were stored and shipped on dry ice to the Monsanto processing facility in Creve Coeur, Missouri. Grain samples were stored and shipped at ambient temperature.

2.2. Description of the collected tissues

Overseason Leaf
The youngest immature whorl leaf (2 - 4 inches) samples were collected from 15 plants from each of the test and control plots. Overseason leaf (OSL) samples were collected at the four growth stages defined in Section 2.1. The leaves corresponding to each growth stage were pooled from each plot during collection.

Overseason Whole Plant
Two whole plants were collected from each of the test and control plots. A whole plant sample consists of shoot tissue (above-ground portion of the plant including leaves, tassels, ears, etc.), minus the roots. The two whole plant samples were pooled from each plot during collection. OSWP samples were collected at the four growth stages defined in Section 2.1.

Overseason Root
The OSR samples were the below ground root mass that had been cut from the corresponding OSWP sample. The two root samples were pooled from each plot during collection. OSR tissue samples were collected were collected at the four growth stages defined in Section 2.1.

Pollen
Approximately 5 g of pollen was collected non-systematically from each test and control replicated plot at each site at the R1 plant growth stage. Any debris in the pollen sample was removed using a fine mesh sieve.

Silk
Silks were collected non-systematically from five primary ears of plants from each test and control plot at each site. The samples were collected at the time of hand pollination

(the R1 plant growth stage). The silk tissue samples were pooled from each plot during collection.

Forage Root
Forage root is defined as the root ball removed from the plant sampled for forage. Forage root tissue samples were collected at the early dent growth stage (R4 - R6) from each test and control plot. Collection of the forage root tissue was similar to that used for the overseason root tissue. The forage root tissue samples were pooled from two plants from each plot during collection.

Senescent Root
Senescent root is the below ground root ball removed from plants sampled for stover. Two senescent root samples were collected from each test and control plot and corresponded to the plants that had been collected for stover. Collection of the senescent root tissue was similar to that used for the overseason root tissue. The senescent root tissue samples were pooled from each plot during collection.

Grain
All hand-pollinated ears in each test and control plot were collected at the R6 growth stage (physiological maturity). The ears were shucked and dried to moisture content of 10-15%. All cobs were shelled prior to shipping.

2.3. Tissue processing and protein extraction methods

2.3.1. Processing

All tissue samples produced at the field sites were shipped to Monsanto for processing. During the processing step, dry ice was combined with the samples (except pollen) and then vertical cutters or mixers were used to thoroughly grind and mix the tissues. All processed tissue samples were stored in a −80°C freezer during the study.

2.3.2. Extraction

The Cry1A.105 and Cry2Ab2 proteins were extracted from corn tissues as using standard operating procedures. All processed tissues were kept on dry ice during extract preparation. All tissues were extracted using a Harbil mixer. Insoluble material was removed from the extracts by using a Serum Filter System (Fisher Scientific, Pittsburgh, PA), or by centrifugation. The extracts were aliquoted and stored in a -80°C freezer until ELISA analyses.

2.4. ELISA reagents and methods

2.4.1. Cry1A.105 antibodies

Goat polyclonal antibodies (lot 7509175) specific for the Cry1A.105 protein were purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 0.93 mg/ml by spectrophotometric methods. The purified antibody was stored in a phosphate buffered saline (1X PBS) buffer (pH 7.4) containing 0.001 M KH_2PO_4, 0.01 M $Na_2HPO_4 \cdot 7H_2O$, 0.137 M NaCl, and 0.0027 M KCl.

The purified Cry1A.105 antibodies were coupled with biotin (Sigma, St. Louis, MO) according to the manufacturer's instructions and assigned lot 7509180. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP).

2.4.2. Cry2Ab2 antibodies

Mouse monoclonal antibody (lot G-800601) specific for the Cry2Ab2 protein was purified using Protein-A Agarose affinity chromatography. The concentration of the purified IgG was determined to be 1.0 mg/ml by spectrophotometric methods. Production of the Cry2Ab2 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 0.02 M $Na_2HPO_4 \cdot 7H_2O$ and 0.15 M NaCl with 0.05% NaN_3 added as a preservative.

Purified Cry2Ab2 antibodies (lot 7381862) were coupled with biotin (Sigma) according to the manufacturer's instructions and assigned lot 7381898. The detection reagent was NeutrAvidin-HRP.

2.4.3. Cry1A.105 ELISA method

Goat anti-Cry1A.105 capture antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) and immobilized onto 96-well microtiter plates at 5.0 µg/ml followed by incubation in a 4°C refrigerator for $\geq$8 h. Prior to each step in the assay, plates were washed with 1X PBS containing 0.05% (v/v) Tween-20 (1X PBST). For grain tissue only, plates were blocked with the addition of 100 to 150 µl per well of 1X PBST with 9% non-fat dried milk (NFDM) for 30 to 90 minutes at 37°C. Cry1A.105 protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured Cry1A.105 protein was detected by the addition of 100 µl per well of biotinylated goat anti-Cry1A.105 antibodies and NeutrAvidin-HRP (Pierce). Plates were developed by adding 100 µl per well of HRP substrate, 3,3',5,5'-tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantitation of the Cry1A.105 protein was accomplished by interpolation from a Cry1A.105 protein standard curve that ranged from 0.438 – 14 ng/ml.

2.4.4. Cry2Ab2 ELISA method

Mouse anti-Cry2Ab2 capture antibody was diluted in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and immobilized onto 96-well microtiter plates at 5.0 µg/ml followed by incubation in a 4°C refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1X PBST. Cry2Ab2 protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured Cry2Ab2 protein was detected by the addition of 100 µl per well of biotinylated goat anti-Cry2Ab2 antibodies and NeutrAvidin-HRP. Plates were developed by adding 100 µl per well of TMB. The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantitation of the Cry2Ab2 protein was accomplished by interpolation from a Cry2Ab2 protein standard curve that ranged from 0.219 – 7 ng/ml.

2.4.5. Sensitivity of the ELISA methods

The limits of detection (LOD) and limits of quantitation (LOQ) for the Cry1A.105 and Cry2Ab2 protein ELISA methods are summarized in the table below.

	Cry1A.105		Cry2Ab2	
Tissue Type	**LOD[1] (µg/g fwt)**	**LOQ[2] (µg/g fwt)**	**LOD[1] (µg/g fwt)**	**LOQ[2] (µg/g fwt)**
Forage	0.372	0.44	0.191	0.44
Leaf	0.568	0.66	0.081	0.44
Pollen	0.412	1.1	0.055	0.11
Root	0.254	0.33	0.056	0.22
Silk	0.275	0.44	0.040	0.22
Grain	0.262	1.1	0.123	0.22

[1] The limit of detection (LOD) was calculated as the mean value plus three SD using the data generated with conventional sample extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

[2] The limit of quantitation (LOQ) was calculated based on the lowest standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

Reference

Ritchie, S.W., J.J. Hanway, and G.O. Benson. 1997. How a Corn Plant Develops: Special Report #48. Iowa State University of Science and Technology Cooperative Extension Service, Ames, IA.

APPENDIX F: Materials and Methods used for Compositional Analysis of MON 89034

1.0. Test, control and reference substances

1.1. Test substance

The test substance was MON 89034. Forage and grain tissues of corn MON 89034 were evaluated in this study.

1.2. Control substance

The control substance was conventional corn hybrid with genetic background representative of MON 89034. The forage and grain tissues of the control substance were evaluated in this study.

1.3. Reference substances

The reference substances were 15 conventional commercial corn hybrids. A single replicate of the forage and grain tissues from each reference substance was evaluated in this study. The following conventional corn hybrids were analysed:

Vendor/Hybrid	Starting Seed Lot No.	Field Site
Golden Harvest/ H8751	REF-0404-14931-S	IA
Golden Harvest/ H9231	REF-0404-14932-S	IA
Northrup King/ N60-N2	REF-0404-14933-S	IA
Burrus/ 590	REF-0404-14934-S	IL-1
Mycogen/ 2784	REF-0404-14935-S	IL-1
Dekalb/ DKC62-15	REF-0404-14936-S	IL-1
Pfister/ 2730	REF-0404-14937-S	IL-2
Mycogen/ 2E685	REF-0404-14938-S	IL-2
Dekalb/ DKC61-42	REF-0404-14939-S	IL-2
Dekalb/ DKC60-15	REF-0404-14940-S	NE
Mycogen/ 2P682	REF-0404-14941-S	NE
Mycogen/ 2A791	REF-0404-14942-S	NE
Seed Consultants / SC1124A	REF-0404-14943-S	OH
Crow's/ 4908	REF-0404-14944-S	OH
Asgrow/ RX708	REF-0404-14945-S	OH

2.0. Test, control and reference substance characterization

The identity of the forage and grain samples from each test, control, and reference substance was verified by confirming the chain-of-custody documentation supplied with the forage and grain collected from the plots. The grain from the test, control, and reference substances was further characterized by event-specific PCR analysis of DNA extracted from grain to confirm the presence or absence of each event. The presence or absence of MON 89034 in respective samples of the grain from the test and control substances was confirmed. All forage samples were characterized by the confirmation of chain-of-custody records.

3.0. Field trial description

Forage and grain of the test, control, and reference substances were collected at five replicated field sites in the U.S. Seed was planted in a randomized complete block design with three replicates per block of each test, control, and reference substance. All the samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. The five U.S. sites were: Site 1-Jefferson County, IA; Site 2-Jersey County, IL; Site 3-Warren County, IL; Site 4-York County, NE; and Site 5-Fayette County, OH. Forage and grain samples were harvested from all plots and shipped on dry ice (forage) or ambient temperature (grain) to Monsanto Company, St. Louis, MO, USA. A sub-sample for use in compositional analysis was obtained from each bulk forage and grain sample generated in the field. Each sub-sample was ground, stored in a -20°C freezer located at Monsanto Company (St. Louis, MO), and then shipped, overnight, on dry ice to Covance Laboratories, Inc. (Madison, WI) for analyses. The labels on the samples shipped to Covance Laboratories, Inc. listed the composition protocol number, a unique sample number, line/event number, tissue type, and storage conditions.

4.0. Analytical methods

A total of 90 ground forage and grain samples were analyzed by Covance Laboratories, Inc. Compositional analyses of the forage samples included proximates (protein, fat, ash, and moisture), ADF, NDF, minerals (calcium and phosphorus), and carbohydrates by calculation. Compositional analyses of the grain samples included proximates (protein, fat, ash, and moisture), ADF, NDF, TDF, amino acids, fatty acids, vitamins (B_1, B_2, B_6, E, niacin, and folic acid), anti-nutrients (phytic acid and raffinose), secondary metabolites (furfural, ferulic acid, and p-coumaric acid), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and carbohydrates by calculation. The methods used for compositional analyses are summarized below.

4.1. Acid detergent fiber

The method used was based on an USDA Agriculture Handbook No. 379 (1970) method. The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash was used to remove the fats and pigments. The lignocellulose fraction was collected on the frit and determined gravimetrically. The limit of quantitation of this method was 0.1% fw.

4.2. Amino acid composition

The method used was based on AOAC International (2000) method 982.30 that estimates the levels of 18 amino acids in the sample: alanine, arginine, aspartic acid (including asparagine), cystine (including cysteine), glutamic acid (including glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis using sodium hydroxide. Sulfur-containing amino acids required an oxidation using performic acid prior to hydrolysis with hydrochloric acid. Analysis of the remaining amino acids was accomplished through direct hydrolysis with hydrochloric acid. The individual amino acids were quantitated using an automated amino acid analyzer. The limit of quantitation of this method was 0.1 mg/g fw. The reference standards were Beckman K18, 2.5 µmol/mL per constituent except cystine (1.25 µmol/mL), lot number S407158; Sigma L-Tryptophan, 100%, lot number 063K0382; Fluka L-Cysteic Acid Monohydrate, 100%, lot number 1157629; Sigma L-Methionine Sulfone, 100%, lot number 12H3349.

4.3. Ash

The method used was based on AOAC International (2000) method 923.03. The sample was placed in an electric furnace at 550 °C and ignited to drive off volatile organic compounds. The nonvolatile matter remaining was quantitated gravimetrically and the percent ash was determined by calculation. The limit of quantitation of this method was 0.1% fw.

4.4. Carbohydrates

The method used was based on an USDA Agriculture Handbook No. 74 (1973) method. The limit of quantitation of this method was 0.1% fw. Carbohydrate values were calculated by difference using the fresh weight-derived data and the following equation:

% carbohydrates = 100% – (% protein + % fat + % ash + % moisture)

4.5. Fat by acid hydrolysis

The method used was based on AOAC International (2000) methods 922.06 and 954.02. The forage sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted using diethyl ether followed by hexane. The extract was

evaporated under nitrogen, re-dissolved in hexane and filtered through a sodium sulfate column. The hexane extract was then evaporated again under nitrogen, dried, and weighed. The limit of quantitation of this method was 0.1% fw.

4.6. Fat by soxhlet extraction

The method used was based on AOAC International (2000) method 960.39. The grain sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was evaporated, dried, and weighed. The limit of quantitation of this method was 0.1% fw.

4.7. Fatty acids

The method used was based on AOCS (1997) method Ce 1-62 that estimates the levels of 22 fatty acids in the sample: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:0 palmitic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic, 18:3 gamma linolenic acid, 20:0 arachidic acid, 20:1 eicosenoic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, and 22:0 behenic acid. Lipid in grain samples was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% (weight/volume) boron trifluoride:methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The limit of quantitation of this method was 0.003% fw. The reference standards were Nu Chek Prep GLC reference standard Hazelton no. 1, used as 100%, lot number D13-0; Nu Chek Prep GLC reference standard Hazelton no. 2, used as 100%, lot number M13-0; Nu Chek Prep GLC reference standard Hazelton no. 3, used as 100%, lot number MA13-0; Nu Chek Prep GLC reference standard Hazelton no. 4, used as 100%, lot number D13-0; Nu Chek Prep methyl gamma linolenate, used as 100%, lot number U-63M-MA19-0; and Sigma methyl tridecanoate, used as 100%, lot number 035K1392.

4.8. Folic acid

The method used was based on AOAC International (2000) methods 960.46 and 992.05 and Methods of Analysis for Infant Formulas (1973), Section C-2. The grain sample was hydrolyzed in potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis, the sample was treated with a chicken-pancreas enzyme and incubated approximately 18 hours to liberate the bound folic acid. The amount of folic acid was turbidimetrically determined by comparing the growth response of the bacteria *Lactobacillus casei* in the sample versus the growth response in folic acid standard. The limit of quantitation of this method was 0.06 µg/g fw. The reference standard was USP folic acid 98%, lot number P.

4.9. Furaldehyde

The method used was based on a literature method (Albala-Hurtado et al., 1997). The grain sample was extracted with 4% trichloroacetic acid. The level of 2-furaldehyde (furfural) in the extract was determined by HPLC with UV quantitation. The reference standard was ACROS 2-furaldehyde, 99%, lot number A018806701. The quantitation limit of this method was calculated to be 0.5 ppm.

4.10. Minerals/ICP Emission Spectrometry.

The method used was based on AOAC International (2000) methods 984.27 and 985.01 and a literature method (Dahlquist and Knoll, 1978). Samples were dried, precharred, and ashed overnight at 500°. Ashed samples were treated with hydrochloric acid, dried, and dissolved in 5% (v/v) hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, using inductively coupled plasma, with the emission of the standard solutions. The limits of quantitation of this method and Spex CertiPrep reference standards are listed in the table below.

Mineral	Lot Numbers	Concentration (µg/ml)	Limit of Quantitation (ppm)
Calcium	SC5179247, SC5179249	201.0, 1001	20.0
Copper	SC5179247, SC5179248	2.01, 10.04	0.50
Iron	SC5179247, SC5179250	9.99, 50.2	2.00
Magnesium	SC5179247, SC5179248	49.93, 250.0	20.0
Manganese	SC5179247, SC5179248	2.01, 10.06	0.30
Phosphorus	SC5179247, SC5179249	200.7, 1005	20.0
Potassium	SC5179247, SC5179249	199.9, 1007	100
Sodium	SC5179247, SC5179249	201.7, 1007	100
Zinc	SC5179247, SC5179248	9.92, 49.82	0.40

4.11. Moisture

The method used was based on AOAC International (2000) methods 926.08 and 925.09. Samples were dried in a vacuum oven at 100°C to a constant weight. The moisture loss was determined and converted to percent moisture. The limit of quantitation of this method was 0.1% fw.

4.12. Neutral detergent fiber, enzyme method

The method used was based on AACC (1998) method 32.20 and an USDA Agriculture Handbook No. 379 (1970) method. Samples were placed in a fritted vessel and washed with a neutral boiling detergent solution to dissolve the protein, carbohydrate, enzyme,

and ash. Fats and pigments were removed using an acetone wash. The hemicellulose, cellulose, and lignin fractions were collected on a frit and determined gravimetrically. The limit of quantitation of this method was 0.1% fw.

4.13. Niacin

The method used was based on AOAC International (2000) method 944.13. The grain sample was hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was turbidimetrically determined by comparing the growth response of the bacteria *Lactobacillus plantarum* in the samples versus the growth response in niacin standard. The limit of quantitation of this method was 0.3 µg/g fw. The reference standard was USP, niacin, 100%, lot number H2C121.

4.14. p-Coumaric and ferulic acids

The method was based on a literature method (Hagerman and Nicholson, 1982). The grain samples were extracted with methanol using ultrasonication, and the extracts were then hydrolyzed using 4N sodium hydroxide, buffered using acetic acid/sodium hydroxide, acidified with 3N hydrochloric acid, and filtered. The levels of p-coumaric and ferulic acids in the extracts were determined by RP-HPLC with UV quantitation. The reference standards were ACROS p-Hydroxycinnamic acid (p-coumaric acid), 97.9%, lot number A018661301 and ACROS 4-Hydroxy-3-methoxycinnamic acid (ferulic acid), 100%, lot number A014010401. The limit of quantitation for both analytes was calculated to be approximately 50.0 ppm using the following equation:

$$(\text{conc. of lowest standard}) \times (\text{vol}) \times (\text{dil}) / (\text{sample weight}) = \text{quantitation limit (ppm)}$$

4.15. Phytic acid

The method used was based on two literature methods (Lehrfeld 1989; 1994). Grain samples were extracted using 0.5M HCl with ultrasonication. Purification and concentration was performed using a silica-based anion exchange (SAX) column. Sample analysis was conducted using a macroporous polymer HPLC column [PRP-1, 5µm (150 x 4.1 mm)] connected to a refractive index detector. The limit of quantitation of this method was approximately 0.1% fw. The reference standard was Aldrich phytic acid, dodecasodium salt hydrate, 95%, lot number 01913EC.

4.16. Protein

The method used was based on AOAC International (2000) methods 955.04 and 979.09 and two literature methods (Bradstreet, 1965; Kalthoff and Sandell, 1948). Protein and other nitrogenous compounds in the sample were reduced to ammonia by digestion of the sample with sulfuric acid containing a mercury catalyst mixture. The acid digest was made alkaline, and the ammonia was distilled and titrated with a standard acid. The percent nitrogen was determined and converted to percent protein by multiplication with 6.25. The limit of quantitation of this method was 0.1% fw.

4.17. Pyridoxine/vitamin B_6

The method used was based on AOAC International (2000) method 961.15. The grain sample was hydrolyzed with dilute sulfuric acid. The amount of pyridoxine was turbidimetrically determined by comparing the growth response of the yeast *Saccharomyces carlsbergensis* in the sample with the growth response in a pyridoxine standard. The limit of quantitation of this method was 0.07 µg/g fw. The reference standard was USP pyridoxine, 100%, lot number P.

4.18. Raffinose

This method was based on two literature methods (Mason and Slover, 1971; Brobst, 1972). The grain samples were extracted with deionized water and the extracts treated with an hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The reference standard was Sigma, D(+)-Raffinose Pentahydrate Sigma Ultra, 99%, lot number 073K0938. The limit of quantitation of this method: The acceptable range for an 8/2.5 dilution was 0.05-0.9%.

4.19. Riboflavin/vitamin B_2

The method used was based on AOAC International (2000) method 940.33. The grain sample was hydrolyzed with dilute HCl and pH adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the bacteria, *Lactobacillus casei*, in the sample hydrolysate with the bacterial growth response in varying amounts of riboflavin standard. The bacterial growth response was measured turbidimetrically. The limit of quantitation of this method was 0.2 µg/g fw. The reference standard was USP riboflavin, 100%, lot number N0C021.

4.20. Thiamin/vitamin B_1

The method used was based on AOAC International (2000) methods 942.23, 953.17, and 957.17. The grain sample was autoclaved under weak acid conditions to extract the thiamin. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamin. The solution was purified on an ion-exchange column. An aliquot was taken and reacted with potassium ferricyanide to convert thiamin to thiochrome. The thiochrome was extracted into isobutyl alcohol and read on a fluorometer against a known standard. The limit of quantitation of this method was 0.01 mg/100g fw. The reference standard was USP, thiamin, 100%, lot number O.

4.21. Total dietary fiber

The method used was based on AOAC International (2000) method 985.29. Duplicate grain samples were gelatinized with alpha-amylase and digested with enzymes to break

down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The samples were filtered and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using the protein and ash values. The limit of quantitation of this method was approximately 1.0% fw.

4.22. Vitamin E

The method used was based on three literature methods (Cort et al., 1983; McMurray et al., 1980; Speek et al., 1985). Grain samples were saponified to break down fat and release vitamin E. The saponified mixture was extracted with ethyl ether and quantitated directly by HPLC on a silica column. The limit of quantitation of this method was approximately 0.005 mg/g fw. The reference standard was USP alpha tocopherol, 100%, lot number M.

5.0. Control of bias

The test, control, and reference substances from each respective plot within the field sites were produced under similar agronomic conditions. To control and/or minimize bias, the samples were analyzed in the order specified by a computer-generated randomized sample list. The Study Director generated the randomized sample list and forwarded it to Covance Laboratories, Inc. prior to analysis.

6.0. Statistical analysis

The data processing and statistical analysis methodology is described in the sections below.

6.1. Data processing

After compositional analyses were performed at Covance Laboratories, Inc., data spreadsheets were sent to Monsanto Company. The data were reviewed, formatted, and sent to Certus International, Inc. for statistical analysis. A statistical sub-report was generated by Certus and sent to Monsanto Company. The following formulas were used for re-expression of the data for statistical analysis:

Component	From (X)	To	Formula
Proximates (excluding moisture), Fiber, Raffinose, Phytic Acid	% FW	% DW	X/d
Furfural, p-Coumaric Acid, Ferulic Acid	ppm FW	ug/g DW	X/d
Calcium, Phosphorus, Magnesium, Potassium, Sodium	ppm FW	% DW	$(X/d) \times 10^{-4}$
Copper, Iron, Manganese, Zinc	ppm FW	mg/kg DW	X/d
Vitamin B1	mg/100g FW	mg/kg DW	10 (X/d)
Vitamin E	mg/g FW	mg/kg DW	10^3 (X/d)
Niacin, Folic Acid, Vitamin B2, Vitamin B6	ug/g FW	mg/kg DW	X/d
Amino Acids (AA)	mg/g FW	% DW	X/(10*d)
Fatty Acids (FA)	% FW	% Total FA	$(100)X_j/\Sigma X_j$, for each FA j
'd' is the fraction of the sample that is dry matter.			

The following 16 compositional analytes with >50% of observations below the LOQ of their respective assay were excluded from statistical analysis: sodium, furfural, raffinose, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecenoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, and 20:4 arachidonic acid.

The following additional seven observations for forage and grain tissue samples were below the LOQ: 16:1 palmitoleic acid (five values in grain); and vitamin E (two values in grain). To include a complete data set for these analytes in the statistical analysis, a value equal to half the quantitation limit was assigned for these seven data points.

The data was assessed for potential outliers using a studentized PRESS residuals calculation. Two outliers were identified in the data set: copper (Site 4, Test MON 89034, Rep 1) and iron (Site 1, Reference H8751, Rep 2). The identified copper and iron values were considered outliers and were removed from further analysis. The outlier test procedure was reapplied to all remaining copper and iron data to detect potential outliers that were masked in the first analysis. Only one iron value (Site 1, Test MON 89034, Rep 1) identified in the second analysis was considered an outlier and removed from further analysis.

6.2. Statistical methodology

At the field sites, the test, control, and reference substances were grown in single plots randomly assigned within each of three replication blocks. The compositional components for the test and control substances were statistically analyzed using a mixed

model analysis of variance. The data from the five replicated sites were analyzed separately and as a combined data set.

Individual replicated site analyses used the model:

$$Y_{ij} = U + T_i + B_j + e_{ij} ,$$

where Y_{ij} = unique individual observation, U = overall mean, T_i = hybrid effect, B_j = random block effect, and e_{ij} = residual error.

Combined site analyses used the model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk} ,$$

where Y_{ijk} = unique individual observation, U = overall mean, T_i = hybrid effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by hybrid interaction effect, and e_{ijk} = residual error. For each compositional component, the forage and grain from the test substance was compared to the conventional control.

A range of observed values from the reference substances was determined for each analytical component. Additionally, the reference substances data were used to develop population tolerance intervals. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. For each compositional component, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of commercial references (George et al., 2004; Ridley et al., 2002). Each tolerance interval estimate was based upon one observation per unique reference substance. Individual substances with multiple observations were summarized within sites to obtain a single estimate for inclusion in tolerance interval calculations. Because negative quantities are not possible, calculated negative lower tolerance bounds were set to zero. SAS® software was used to generate all summary statistics and perform all analyses (SAS Software Release 9.1, 2002-2003). Report tables present p-values from SAS® as either <0.001 or the actual value truncated to three decimal places.

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APPENDIX G: Compositional Analyses Data for Individual Sites

This appendix contains the compositional analysis tables for the five individual sites as follows:

Site No. (State)	Table
1 (IA)	G.1-G.7
2 (IL)	G.8-G.14
3 (IL)	G.15-G.22
4 (NE)	G.23-G.30
5 (OH)	G.31-G.38

Table G.1. Comparison of the proximates, fiber, and mineral content in forage collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	33.94 ± 2.44 (32.27 - 35.85)	32.16 ± 2.44 (30.00 - 35.59)	1.78 ± 3.06 (0.27 - 2.79)	-5.28,8.83	0.577	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fiber (% DW)	41.26 ± 0.80 (39.51 - 42.46)	42.26 ± 0.80 (40.23 - 43.96)	-1.00 ± 1.13 (-2.16 - -0.12)	-3.59,1.59	0.400	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.24 ± 0.0065 (0.24 - 0.24)	0.26 ± 0.0065 (0.25 - 0.28)	-0.023 ± 0.0090 (-0.036 - -0.014)	-0.044,-0.0024	0.033	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.24 ± 0.0048 (0.24 - 0.25)	0.24 ± 0.0048 (0.23 - 0.25)	0.0018 ± 0.0039 (-0.0019 - 0.0041)	-0.0072,0.011	0.654	(0.14 - 0.25) [0.071,0.32]
Proximate						
Ash (% DW)	4.21 ± 0.26 (3.32 - 4.67)	4.46 ± 0.26 (4.22 - 4.65)	-0.25 ± 0.36 (-1.19 - 0.42)	-1.08,0.59	0.515	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	85.50 ± 0.47 (85.20 - 85.68)	85.51 ± 0.47 (84.51 - 86.46)	-0.013 ± 0.60 (-0.85 - 1.18)	-1.40,1.38	0.983	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	74.87 ± 0.44 (74.40 - 75.40)	73.83 ± 0.44 (72.70 - 74.40)	1.03 ± 0.51 (0.40 - 1.70)	-0.13,2.20	0.075	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	8.90 ± 0.16 (8.85 - 8.98)	8.49 ± 0.16 (8.24 - 8.87)	0.41 ± 0.21 (0.12 - 0.63)	-0.085,0.91	0.092	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	1.39 ± 0.60 (0.89 - 2.13)	1.54 ± 0.60 (0.92 - 2.75)	-0.15 ± 0.66 (-0.61 - 0.23)	-1.66,1.36	0.823	(0.58 - 3.11) [0,4.54]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.2. Comparison of the of amino acid content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Alanine (% DW)	0.88 ± 0.017 (0.87 - 0.88)	0.81 ± 0.017 (0.79 - 0.84)	0.064 ± 0.024 (0.037 - 0.089)	0.0078,0.12	0.030	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.51 ± 0.0095 (0.50 - 0.52)	0.46 ± 0.0095 (0.46 - 0.47)	0.050 ± 0.013 (0.036 - 0.062)	0.020,0.081	0.005	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.77 ± 0.011 (0.77 - 0.78)	0.71 ± 0.011 (0.70 - 0.73)	0.061 ± 0.015 (0.038 - 0.078)	0.026,0.097	0.003	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.25 ± 0.0039 (0.24 - 0.26)	0.23 ± 0.0039 (0.23 - 0.23)	0.017 ± 0.0056 (0.011 - 0.023)	0.0045,0.030	0.014	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	2.27 ± 0.039 (2.26 - 2.28)	2.09 ± 0.039 (2.03 - 2.16)	0.18 ± 0.055 (0.12 - 0.24)	0.054,0.31	0.011	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.41 ± 0.0065 (0.40 - 0.41)	0.38 ± 0.0065 (0.37 - 0.39)	0.026 ± 0.0091 (0.012 - 0.035)	0.0052,0.047	0.020	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.34 ± 0.0057 (0.34 - 0.34)	0.32 ± 0.0057 (0.31 - 0.32)	0.023 ± 0.0081 (0.015 - 0.030)	0.0041,0.041	0.022	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.39 ± 0.0099 (0.39 - 0.40)	0.37 ± 0.0099 (0.36 - 0.38)	0.025 ± 0.014 (0.016 - 0.041)	-0.0075,0.057	0.114	(0.32 - 0.45) [0.23,0.51]
Leucine (% DW)	1.49 ± 0.034 (1.48 - 1.51)	1.37 ± 0.034 (1.33 - 1.41)	0.12 ± 0.047 (0.098 - 0.16)	0.013,0.23	0.032	(1.14 - 1.68) [0.77,1.92]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.2 (cont.). Comparison of the of amino acid content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Lysine (% DW)	0.35 ± 0.0062 (0.33 - 0.36)	0.32 ± 0.0062 (0.32 - 0.33)	0.022 ± 0.0081 (0.0042 - 0.033)	0.0029,0.040	0.028	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.25 ± 0.0043 (0.25 - 0.27)	0.23 ± 0.0043 (0.22 - 0.24)	0.026 ± 0.0061 (0.024 - 0.028)	0.012,0.040	0.003	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.58 ± 0.013 (0.57 - 0.59)	0.53 ± 0.013 (0.52 - 0.54)	0.050 ± 0.019 (0.041 - 0.067)	0.0066,0.094	0.028	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	1.05 ± 0.019 (1.04 - 1.05)	0.98 ± 0.019 (0.95 - 1.01)	0.071 ± 0.027 (0.041 - 0.10)	0.0097,0.13	0.028	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.60 ± 0.0085 (0.60 - 0.61)	0.56 ± 0.0085 (0.55 - 0.57)	0.046 ± 0.012 (0.034 - 0.058)	0.019,0.074	0.004	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.37 ± 0.0051 (0.37 - 0.37)	0.34 ± 0.0051 (0.33 - 0.36)	0.029 ± 0.0072 (0.016 - 0.039)	0.012,0.046	0.004	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.062 ± 0.0011 (0.061 - 0.063)	0.061 ± 0.0011 (0.058 - 0.063)	0.0016 ± 0.0015 (-0.0015 - 0.0050)	-0.0018,0.0051	0.311	(0.043 - 0.059) [0.032,0.072]
Tyrosine (% DW)	0.43 ± 0.012 (0.42 - 0.43)	0.36 ± 0.012 (0.35 - 0.37)	0.063 ± 0.018 (0.052 - 0.072)	0.023,0.10	0.006	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.53 ± 0.012 (0.53 - 0.54)	0.50 ± 0.012 (0.48 - 0.51)	0.035 ± 0.016 (0.020 - 0.055)	-0.0026,0.073	0.063	(0.42 - 0.55) [0.35,0.62]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.3. Comparison of the fatty acid content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.21 ± 0.043 (9.12 - 9.31)	9.23 ± 0.043 (9.15 - 9.34)	-0.023 ± 0.060 (-0.14 - 0.16)	-0.16,0.11	0.706	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.11 ± 0.0081 (0.11 - 0.11)	0.11 ± 0.0081 (0.11 - 0.12)	-0.0017 ± 0.011 (-0.0034 - 0.00039)	-0.028,0.025	0.889	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.80 ± 0.016 (1.79 - 1.83)	1.81 ± 0.016 (1.77 - 1.85)	-0.0070 ± 0.023 (-0.055 - 0.063)	-0.061,0.047	0.772	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	25.08 ± 0.12 (24.87 - 25.36)	24.75 ± 0.12 (24.55 - 24.92)	0.34 ± 0.17 (0.099 - 0.81)	-0.057,0.73	0.083	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	61.79 ± 0.12 (61.56 - 62.00)	61.98 ± 0.12 (61.74 - 62.18)	-0.19 ± 0.17 (-0.45 - 0.25)	-0.58,0.20	0.298	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.21 ± 0.026 (1.20 - 1.23)	1.34 ± 0.026 (1.25 - 1.43)	-0.13 ± 0.037 (-0.23 - -0.022)	-0.21,-0.040	0.009	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.37 ± 0.0057 (0.36 - 0.39)	0.37 ± 0.0057 (0.36 - 0.38)	0.0036 ± 0.0081 (-0.019 - 0.032)	-0.015,0.022	0.670	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.27 ± 0.0050 (0.26 - 0.28)	0.27 ± 0.0050 (0.25 - 0.28)	0.0018 ± 0.0064 (-0.014 - 0.011)	-0.013,0.016	0.784	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.15 ± 0.0035 (0.14 - 0.16)	0.14 ± 0.0035 (0.14 - 0.15)	0.0030 ± 0.0049 (-0.0071 - 0.017)	-0.0083,0.014	0.562	(0.12 - 0.19) [0.081,0.23]

[1] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.4. Comparison of the fiber and proximate content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	5.39 ± 0.30 (4.76 - 5.80)	5.19 ± 0.30 (4.76 - 5.68)	0.20 ± 0.42 (-0.92 - 1.04)	-0.76,1.17	0.641	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fiber (% DW)	11.31 ± 0.28 (10.78 - 12.08)	10.68 ± 0.28 (9.93 - 11.22)	0.63 ± 0.38 (-0.44 - 1.20)	-0.25,1.52	0.136	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fiber (% DW)	15.37 ± 0.46 (14.70 - 16.28)	14.22 ± 0.46 (13.62 - 15.25)	1.15 ± 0.65 (-0.55 - 2.50)	-0.34,2.65	0.112	(12.99 - 18.03) [9.20,20.27]
Proximate						
Ash (% DW)	1.48 ± 0.056 (1.38 - 1.56)	1.44 ± 0.056 (1.35 - 1.49)	0.043 ± 0.063 (0.021 - 0.076)	-0.10,0.19	0.513	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	83.38 ± 0.23 (83.29 - 83.55)	84.52 ± 0.23 (84.28 - 84.74)	-1.13 ± 0.32 (-1.42 - -0.98)	-1.88,-0.39	0.008	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	8.06 ± 0.096 (7.89 - 8.16)	8.09 ± 0.096 (7.86 - 8.21)	-0.030 ± 0.14 (-0.080 - 0.030)	-0.34,0.28	0.830	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	11.89 ± 0.19 (11.73 - 11.98)	10.85 ± 0.19 (10.70 - 11.00)	1.04 ± 0.27 (0.87 - 1.28)	0.41,1.67	0.005	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.24 ± 0.047 (3.16 - 3.33)	3.19 ± 0.047 (3.13 - 3.24)	0.050 ± 0.067 (-0.0014 - 0.12)	-0.10,0.20	0.479	(2.66 - 3.71) [2.20,4.55]

[1] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.5. Comparison of the mineral content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Mineral						
Calcium (% DW)	0.0064 ± 0.00014 (0.0062 - 0.0066)	0.0058 ± 0.00014 (0.0056 - 0.0059)	0.00063 ± 0.00020 (0.00043 - 0.00090)	0.00018,0.0011	0.012	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	1.89 ± 0.69 (1.86 - 1.95)	2.82 ± 0.69 (1.68 - 4.54)	-0.92 ± 0.98 (-2.59 - 0.18)	-3.17,1.33	0.372	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	23.54 ± 1.22 (23.02 - 24.06)	25.49 ± 1.00 (24.07 - 27.02)	-1.96 ± 1.58 (-4.01 - -1.33)	-5.69,1.77	0.254	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.13 ± 0.0021 (0.13 - 0.13)	0.12 ± 0.0021 (0.12 - 0.13)	0.0036 ± 0.0030 (-0.0012 - 0.0087)	-0.0034,0.011	0.269	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	8.34 ± 0.34 (7.62 - 9.32)	6.99 ± 0.34 (6.84 - 7.17)	1.35 ± 0.45 (0.78 - 2.36)	0.30,2.40	0.017	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.34 ± 0.0049 (0.34 - 0.35)	0.34 ± 0.0049 (0.33 - 0.35)	0.0050 ± 0.0070 (-0.0068 - 0.015)	-0.011,0.021	0.496	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.37 ± 0.0060 (0.36 - 0.38)	0.37 ± 0.0060 (0.36 - 0.38)	-0.00086 ± 0.0084 (-0.010 - 0.0055)	-0.020,0.019	0.921	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	26.50 ± 0.58 (25.91 - 26.89)	25.46 ± 0.58 (24.53 - 26.04)	1.04 ± 0.82 (0.098 - 2.18)	-0.85,2.93	0.239	(16.72 - 34.04) [7.16,38.55]

[1] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.6. Comparison of the vitamin content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Vitamin						
Folic Acid (mg/kg DW)	0.41 ± 0.048 (0.39 - 0.43)	0.51 ± 0.048 (0.49 - 0.53)	-0.10 ± 0.059 (-0.11 - -0.098)	-0.24,0.034	0.121	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	28.14 ± 0.74 (26.27 - 30.05)	29.01 ± 0.74 (27.34 - 29.85)	-0.88 ± 1.04 (-3.57 - 2.71)	-3.28,1.52	0.424	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	3.05 ± 0.16 (2.39 - 3.38)	3.23 ± 0.16 (3.05 - 3.36)	-0.18 ± 0.21 (-0.66 - 0.11)	-0.67,0.30	0.411	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.45 ± 0.11 (1.37 - 1.52)	1.45 ± 0.11 (1.36 - 1.51)	-0.0076 ± 0.15 (-0.12 - 0.16)	-0.36,0.34	0.961	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	6.74 ± 0.11 (6.49 - 6.99)	6.73 ± 0.11 (6.67 - 6.80)	0.016 ± 0.16 (-0.31 - 0.32)	-0.35,0.38	0.922	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	6.33 ± 0.71 (5.73 - 6.70)	4.87 ± 0.71 (2.72 - 6.00)	1.46 ± 1.01 (-0.27 - 3.83)	-0.86,3.78	0.185	(2.71 - 13.94) [0,20.49]

[1] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.7. Comparison of the antinutrient and secondary metabolites content in grain collected at Site 1 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Antinutrient						
Phytic Acid (% DW)	0.78 ± 0.039 (0.77 - 0.80)	0.75 ± 0.039 (0.70 - 0.83)	0.028 ± 0.055 (-0.037 - 0.073)	-0.099,0.15	0.629	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	2458.21 ± 53.43 (2366.74 - 2525.31)	2571.23 ± 53.43 (2472.77 - 2669.85)	-113.03 ± 75.57 (-303.11 - 9.81)	-287.29,61.23	0.173	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	172.95 ± 6.96 (166.11-177.48)	172.63 ± 6.96 (167.76-176.90)	0.32 ± 9.63 (-10.80 – 9.73)	-21.89,22.53	0.974	(99.30 – 285.75) [0,378.57]

[1] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.8. Comparison of the fiber, mineral, and proximates content in forage collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	25.53 ± 1.39 (24.18 - 26.38)	27.40 ± 1.39 (24.53 - 32.26)	-1.87 ± 1.97 (-6.22 - 1.85)	-6.42,2.68	0.371	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fiber (% DW)	36.81 ± 1.89 (33.99 - 39.94)	36.96 ± 1.89 (35.78 - 37.65)	-0.15 ± 2.67 (-3.47 - 4.16)	-6.32,6.02	0.956	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.17 ± 0.0093 (0.16 - 0.18)	0.17 ± 0.0093 (0.15 - 0.19)	0.0044 ± 0.012 (-0.017 - 0.017)	-0.023,0.031	0.716	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.24 ± 0.014 (0.23 - 0.24)	0.20 ± 0.014 (0.18 - 0.24)	0.041 ± 0.019 (0.0048 - 0.064)	-0.0036,0.085	0.066	(0.14 - 0.25) [0.071,0.32]
Proximate						
Ash (% DW)	3.55 ± 0.30 (3.30 - 3.93)	3.57 ± 0.30 (2.96 - 4.24)	-0.015 ± 0.43 (-0.94 - 0.97)	-1.00,0.97	0.972	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	86.92 ± 0.87 (84.98 - 88.60)	88.22 ± 0.87 (85.87 - 89.57)	-1.30 ± 1.23 (-4.23 - 2.73)	-4.13,1.53	0.320	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	69.03 ± 0.68 (68.50 - 69.40)	66.53 ± 0.68 (65.90 - 67.70)	2.50 ± 0.96 (1.70 - 3.30)	0.29,4.71	0.031	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	7.53 ± 0.39 (6.95 - 8.41)	6.63 ± 0.39 (6.06 - 7.52)	0.90 ± 0.56 (-0.30 - 2.35)	-0.38,2.18	0.144	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	2.00 ± 0.45 (0.88 - 3.17)	1.58 ± 0.45 (1.16 - 2.37)	0.42 ± 0.64 (-1.49 - 1.95)	-1.05,1.88	0.528	(0.58 - 3.11) [0,4.54]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.9. Comparison of the amino acid content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Alanine (% DW)	0.70 ± 0.027 (0.66 - 0.76)	0.71 ± 0.027 (0.67 - 0.78)	-0.0092 ± 0.033 (-0.012 - -0.0057)	-0.084,0.066	0.785	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.45 ± 0.011 (0.43 - 0.47)	0.45 ± 0.011 (0.44 - 0.47)	0.0049 ± 0.015 (-0.0068 - 0.015)	-0.031,0.040	0.760	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.62 ± 0.019 (0.60 - 0.66)	0.62 ± 0.019 (0.60 - 0.67)	0.0013 ± 0.023 (-0.0033 - 0.0070)	-0.052,0.054	0.956	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.22 ± 0.0058 (0.22 - 0.23)	0.22 ± 0.0058 (0.21 - 0.23)	0.0044 ± 0.0069 (-0.0022 - 0.014)	-0.011,0.020	0.538	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	1.78 ± 0.073 (1.69 - 1.95)	1.81 ± 0.073 (1.70 - 2.00)	-0.025 ± 0.085 (-0.051 - -0.0056)	-0.22,0.17	0.779	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.37 ± 0.0084 (0.36 - 0.39)	0.37 ± 0.0084 (0.36 - 0.38)	0.0048 ± 0.011 (0.0023 - 0.0062)	-0.020,0.030	0.665	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.29 ± 0.0082 (0.27 - 0.31)	0.29 ± 0.0082 (0.28 - 0.30)	0.0011 ± 0.0087 (-0.012 - 0.011)	-0.019,0.021	0.898	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.32 ± 0.014 (0.30 - 0.36)	0.32 ± 0.014 (0.30 - 0.36)	0.00013 ± 0.017 (-0.0070 - 0.010)	-0.038,0.038	0.993	(0.32 - 0.45) [0.23,0.51]
Leucine (% DW)	1.15 ± 0.055 (1.09 - 1.27)	1.17 ± 0.055 (1.08 - 1.33)	-0.017 ± 0.064 (-0.052 - 0.0032)	-0.16,0.13	0.803	(1.14 - 1.68) [0.77,1.92]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.9 (cont.). Comparison of the amino acid content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Lysine (% DW)	0.32 ± 0.0090 (0.29 - 0.33)	0.30 ± 0.0090 (0.29 - 0.31)	0.017 ± 0.012 (0.0028 - 0.031)	-0.0093,0.044	0.170	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.22 ± 0.0056 (0.21 - 0.23)	0.22 ± 0.0056 (0.20 - 0.23)	0.0033 ± 0.0079 (-0.0066 - 0.014)	-0.015,0.021	0.682	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.46 ± 0.019 (0.44 - 0.50)	0.46 ± 0.019 (0.43 - 0.52)	-0.0020 ± 0.022 (-0.018 - 0.0087)	-0.052,0.048	0.929	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	0.89 ± 0.031 (0.84 - 0.96)	0.88 ± 0.031 (0.84 - 0.95)	0.0040 ± 0.037 (-0.0064 - 0.011)	-0.082,0.090	0.917	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.48 ± 0.014 (0.46 - 0.50)	0.49 ± 0.014 (0.46 - 0.52)	-0.0096 ± 0.018 (-0.021 - 0.0088)	-0.051,0.032	0.607	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.31 ± 0.0081 (0.30 - 0.33)	0.31 ± 0.0081 (0.29 - 0.32)	0.0067 ± 0.0091 (-0.00043 - 0.011)	-0.014,0.028	0.484	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.054 ± 0.0024 (0.053 - 0.056)	0.055 ± 0.0024 (0.052 - 0.061)	-0.00092 ± 0.0026 (-0.0055 - 0.0014)	-0.0070,0.0051	0.735	(0.043 - 0.059) [0.032,0.072]
Tyrosine (% DW)	0.34 ± 0.012 (0.33 - 0.36)	0.35 ± 0.012 (0.32 - 0.38)	-0.0023 ± 0.013 (-0.024 - 0.015)	-0.033,0.028	0.863	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.45 ± 0.016 (0.42 - 0.50)	0.45 ± 0.016 (0.43 - 0.49)	0.0016 ± 0.020 (-0.011 - 0.0084)	-0.045,0.048	0.938	(0.42 - 0.55) [0.35,0.62]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.10. Comparison of the fatty acid content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.21 ± 0.065 (9.17 - 9.24)	9.08 ± 0.065 (8.91 - 9.23)	0.12 ± 0.089 (-0.013 - 0.33)	-0.080,0.33	0.199	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.13 ± 0.0022 (0.12 - 0.13)	0.14 ± 0.0022 (0.13 - 0.14)	-0.0093 ± 0.0029 (-0.011 - -0.0071)	-0.016,-0.0027	0.012	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.96 ± 0.027 (1.89 - 2.02)	1.82 ± 0.027 (1.76 - 1.85)	0.14 ± 0.024 (0.12 - 0.18)	0.088,0.20	<0.001	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	25.30 ± 0.29 (25.03 - 25.68)	25.78 ± 0.29 (25.34 - 26.66)	-0.48 ± 0.32 (-0.98 - -0.15)	-1.21,0.25	0.168	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	61.34 ± 0.22 (61.02 - 61.54)	61.14 ± 0.22 (60.51 - 61.53)	0.20 ± 0.27 (-0.051 - 0.51)	-0.42,0.82	0.471	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.22 ± 0.011 (1.21 - 1.23)	1.21 ± 0.011 (1.19 - 1.23)	0.014 ± 0.016 (-0.014 - 0.036)	-0.022,0.051	0.390	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.41 ± 0.0055 (0.40 - 0.42)	0.39 ± 0.0055 (0.38 - 0.40)	0.020 ± 0.0057 (0.017 - 0.023)	0.0073,0.034	0.007	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.29 ± 0.0052 (0.28 - 0.29)	0.29 ± 0.0052 (0.29 - 0.29)	-0.0035 ± 0.0073 (-0.0040 - -0.0029)	-0.020,0.013	0.644	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.15 ± 0.0042 (0.14 - 0.15)	0.16 ± 0.0042 (0.15 - 0.16)	-0.013 ± 0.0057 (-0.019 - -0.0013)	-0.026,0	0.050	(0.12 - 0.19) [0.081,0.23]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.11. Comparison of the fiber and proximate content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	4.96 ± 0.51 (3.82 - 6.05)	5.55 ± 0.51 (4.37 - 7.00)	-0.59 ± 0.71 (-3.18 - 1.69)	-2.24,1.06	0.435	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fiber (% DW)	10.00 ± 0.53 (9.83 - 10.11)	10.50 ± 0.53 (9.48 - 11.22)	-0.50 ± 0.75 (-1.16 - 0.34)	-2.24,1.24	0.524	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fiber (% DW)	14.49 ± 0.70 (13.39 - 15.06)	14.93 ± 0.70 (13.17 - 15.84)	-0.43 ± 0.99 (-2.45 - 1.88)	-2.71,1.84	0.671	(12.99 - 18.03) [9.20,20.27]
Proximate						
Ash (% DW)	1.40 ± 0.038 (1.35 - 1.47)	1.43 ± 0.038 (1.34 - 1.48)	-0.025 ± 0.053 (-0.11 - 0.046)	-0.15,0.097	0.648	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	85.63 ± 0.27 (84.90 - 86.11)	85.67 ± 0.27 (84.94 - 86.22)	-0.044 ± 0.27 (-0.11 - 0.012)	-0.68,0.59	0.875	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	8.46 ± 0.22 (8.15 - 9.02)	8.43 ± 0.22 (8.06 - 9.08)	0.030 ± 0.31 (-0.93 - 0.87)	-0.69,0.75	0.925	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	9.67 ± 0.29 (9.14 - 10.35)	9.67 ± 0.29 (9.22 - 10.50)	0.0034 ± 0.23 (-0.15 - 0.30)	-0.53,0.53	0.988	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.30 ± 0.093 (3.26 - 3.36)	3.23 ± 0.093 (3.09 - 3.45)	0.066 ± 0.11 (-0.20 - 0.21)	-0.18,0.31	0.553	(2.66 - 3.71) [2.20,4.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.12. Comparison of the mineral content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Mineral						
Calcium (% DW)	0.0048 ± 0.00018 (0.0046 - 0.0049)	0.0048 ± 0.00018 (0.0046 - 0.0050)	-0.00004 ± 0.00021 (-0.00006 - -0.00003)	-0.00054,0.00045	0.845	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	1.76 ± 0.11 (1.51 - 2.21)	1.36 ± 0.11 (1.26 - 1.43)	0.40 ± 0.14 (0.16 - 0.78)	0.071,0.73	0.023	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	20.86 ± 0.42 (19.23 - 21.79)	19.48 ± 0.42 (19.03 - 19.71)	1.38 ± 0.60 (-0.47 - 2.75)	0.012,2.76	0.048	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.12 ± 0.0022 (0.11 - 0.12)	0.11 ± 0.0022 (0.11 - 0.12)	0.0044 ± 0.0029 (-0.0066 - 0.011)	-0.0023,0.011	0.170	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	6.54 ± 0.17 (6.11 - 6.78)	6.19 ± 0.17 (6.03 - 6.47)	0.35 ± 0.24 (-0.36 - 0.71)	-0.20,0.89	0.180	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.33 ± 0.0062 (0.31 - 0.35)	0.33 ± 0.0062 (0.32 - 0.35)	0.0070 ± 0.0088 (-0.031 - 0.026)	-0.013,0.027	0.449	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.38 ± 0.0084 (0.37 - 0.40)	0.37 ± 0.0084 (0.36 - 0.40)	0.011 ± 0.011 (-0.030 - 0.035)	-0.014,0.035	0.345	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	20.50 ± 0.52 (18.91 - 22.12)	19.26 ± 0.52 (18.81 - 20.03)	1.24 ± 0.74 (-1.13 - 3.19)	-0.46,2.94	0.131	(16.72 - 34.04) [7.16,38.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.13. Comparison of the vitamin content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Vitamin						
Folic Acid (mg/kg DW)	0.30 ± 0.017 (0.27 - 0.33)	0.33 ± 0.017 (0.30 - 0.36)	-0.030 ± 0.024 (-0.058 - 0.0038)	-0.086,0.026	0.249	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	32.34 ± 1.44 (30.61 - 34.84)	32.70 ± 1.44 (31.03 - 35.75)	-0.36 ± 2.04 (-4.17 - 3.81)	-5.06,4.34	0.865	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	3.17 ± 0.18 (3.05 - 3.27)	2.84 ± 0.18 (2.39 - 3.16)	0.33 ± 0.26 (0.030 - 0.66)	-0.27,0.93	0.242	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.46 ± 0.069 (1.35 - 1.65)	1.53 ± 0.069 (1.45 - 1.61)	-0.072 ± 0.098 (-0.22 - 0.20)	-0.30,0.15	0.484	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	6.49 ± 0.14 (6.27 - 6.64)	6.53 ± 0.14 (6.45 - 6.63)	-0.042 ± 0.16 (-0.37 - 0.20)	-0.41,0.33	0.800	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	7.16 ± 0.26 (6.47 - 7.91)	6.96 ± 0.26 (6.65 - 7.40)	0.20 ± 0.35 (-0.93 - 1.08)	-0.62,1.01	0.591	(2.71 - 13.94) [0,20.49]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.14. Comparison of the antinutrient and secondary metabolites content in grain collected at Site 2 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Antinutrient						
Phytic Acid (% DW)	0.86 ± 0.041 (0.83 - 0.87)	0.78 ± 0.041 (0.69 - 0.85)	0.081 ± 0.059 (-0.013 - 0.18)	-0.055,0.22	0.206	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	2057.02 ± 106.60 (1923.50 - 2298.73)	2184.45 ± 106.60 (2033.94 - 2265.73)	-127.43 ± 150.76 (-330.17 - 264.79)	-475.07,220.22	0.422	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	196.97 ± 7.65 (185.76 – 214.62)	195.82 ± 7.65 (188.08- 210.13)	1.15 ± 10.81 (-24.37 – 25.37)	-23.78,26.08	0.917	(99.30 – 285.75) [0,378.57]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.15. Comparison of the fiber and mineral content in forage collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	29.76 ± 1.78 (26.56 - 33.83)	28.84 ± 1.78 (25.00 - 31.08)	0.92 ± 2.27 (-3.89 - 3.89)	-4.32,6.15	0.696	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fiber (% DW)	39.23 ± 2.36 (34.41 - 42.19)	37.97 ± 2.36 (35.41 - 42.21)	1.26 ± 2.13 (-1.11 - 5.89)	-3.66,6.19	0.570	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.22 ± 0.016 (0.22 - 0.22)	0.21 ± 0.016 (0.19 - 0.23)	0.011 ± 0.022 (-0.014 - 0.034)	-0.038,0.061	0.615	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.22 ± 0.0097 (0.22 - 0.23)	0.20 ± 0.0097 (0.19 - 0.21)	0.026 ± 0.014 (0.016 - 0.033)	-0.0052,0.058	0.090	(0.14 - 0.25) [0.071,0.32]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.16. Comparison of the proximates content in forage collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Proximate						
Ash (% DW)	4.36 ± 0.24 (3.99 - 4.57)	4.13 ± 0.24 (3.78 - 4.47)	0.23 ± 0.34 (-0.48 - 0.74)	-0.55,1.01	0.522	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	86.08 ± 0.54 (84.93 - 86.69)	86.94 ± 0.54 (86.69 - 87.13)	-0.85 ± 0.76 (-2.05 - -0.060)	-2.60,0.89	0.292	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	74.03 ± 0.82 (73.00 - 74.70)	75.13 ± 0.82 (73.00 - 76.80)	-1.10 ± 0.99 (-2.40 - 0)	-3.38,1.18	0.297	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	8.15 ± 0.36 (7.59 - 8.75)	7.85 ± 0.36 (7.54 - 8.07)	0.30 ± 0.51 (-0.33 - 1.21)	-0.88,1.48	0.574	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	1.41 ± 0.19 (1.20 - 1.75)	1.08 ± 0.19 (0.77 - 1.31)	0.32 ± 0.27 (0.037 - 0.51)	-0.30,0.95	0.262	(0.58 - 3.11) [0,4.54]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.17. Comparison of the amino acid content in grain collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Alanine (% DW)	0.69 ± 0.020 (0.67 - 0.71)	0.71 ± 0.020 (0.67 - 0.74)	-0.028 ± 0.025 (-0.070 - 0.0025)	-0.085,0.028	0.282	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.47 ± 0.012 (0.44 - 0.49)	0.45 ± 0.012 (0.41 - 0.47)	0.017 ± 0.017 (-0.023 - 0.056)	-0.022,0.057	0.342	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.63 ± 0.014 (0.60 - 0.66)	0.64 ± 0.014 (0.61 - 0.66)	-0.0031 ± 0.019 (-0.040 - 0.028)	-0.047,0.041	0.875	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.21 ± 0.0055 (0.20 - 0.21)	0.22 ± 0.0055 (0.21 - 0.23)	-0.0091 ± 0.0078 (-0.022 - 0.0052)	-0.027,0.0088	0.274	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	1.77 ± 0.053 (1.71 - 1.82)	1.84 ± 0.053 (1.73 - 1.90)	-0.077 ± 0.068 (-0.19 - 0.040)	-0.23,0.079	0.286	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.38 ± 0.0066 (0.36 - 0.39)	0.37 ± 0.0066 (0.36 - 0.38)	0.0089 ± 0.0094 (-0.0046 - 0.017)	-0.013,0.031	0.372	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.29 ± 0.0061 (0.28 - 0.30)	0.29 ± 0.0061 (0.28 - 0.30)	0.0017 ± 0.0083 (-0.0026 - 0.0066)	-0.017,0.021	0.841	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.34 ± 0.0097 (0.32 - 0.34)	0.34 ± 0.0097 (0.33 - 0.36)	-0.0078 ± 0.014 (-0.018 - 0.012)	-0.039,0.024	0.587	(0.32 - 0.45) [0.23,0.51]
Leucine (% DW)	1.15 ± 0.041 (1.12 - 1.18)	1.21 ± 0.041 (1.13 - 1.26)	-0.064 ± 0.051 (-0.13 - 0.015)	-0.18,0.053	0.243	(1.14 - 1.68) [0.77,1.92]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.17 (cont.). Comparison of the amino acid content in grain collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.]
Amino Acid (% DW)						
Lysine (% DW)	0.33 ± 0.0098 (0.31 - 0.35)	0.31 ± 0.0098 (0.29 - 0.32)	0.021 ± 0.014 (0.017 - 0.023)	-0.011,0.053	0.163	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.20 ± 0.0056 (0.20 - 0.20)	0.21 ± 0.0056 (0.20 - 0.22)	-0.012 ± 0.0071 (-0.017 - -0.0048)	-0.029,0.0039	0.117	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.46 ± 0.015 (0.45 - 0.48)	0.48 ± 0.015 (0.45 - 0.50)	-0.016 ± 0.019 (-0.039 - 0.016)	-0.060,0.027	0.414	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	0.88 ± 0.020 (0.87 - 0.91)	0.88 ± 0.020 (0.83 - 0.91)	0.00035 ± 0.025 (-0.035 - 0.035)	-0.057,0.057	0.989	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.46 ± 0.011 (0.45 - 0.49)	0.48 ± 0.011 (0.46 - 0.50)	-0.019 ± 0.014 (-0.053 - 0)	-0.051,0.014	0.227	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.31 ± 0.0085 (0.30 - 0.32)	0.31 ± 0.0085 (0.30 - 0.32)	0.00064 ± 0.010 (-0.015 - 0.013)	-0.023,0.024	0.951	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.050 ± 0.0025 (0.048 - 0.052)	0.050 ± 0.0025 (0.045 - 0.054)	-0.00022 ± 0.0032 (-0.0051 - 0.0068)	-0.0075,0.0071	0.946	(0.043 - 0.059) [0.032,0.072]
Tyrosine (% DW)	0.36 ± 0.024 (0.35 - 0.36)	0.32 ± 0.024 (0.24 - 0.38)	0.034 ± 0.021 (-0.014 - 0.11)	-0.015,0.082	0.150	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.46 ± 0.011 (0.45 - 0.48)	0.46 ± 0.011 (0.45 - 0.48)	-0.00051 ± 0.016 (-0.019 - 0.021)	-0.036,0.035	0.974	(0.42 - 0.55) [0.35,0.62]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.18. Comparison of the fatty acid content in grain collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.29 ± 0.069 (9.12 - 9.46)	9.10 ± 0.069 (9.06 - 9.16)	0.19 ± 0.092 (0.050 - 0.30)	-0.019,0.41	0.068	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.12 ± 0.0018 (0.12 - 0.13)	0.13 ± 0.0018 (0.12 - 0.13)	-0.0055 ± 0.0026 (-0.012 - -0.0013)	-0.011,0.00046	0.066	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.98 ± 0.024 (1.93 - 2.03)	1.82 ± 0.024 (1.79 - 1.85)	0.16 ± 0.032 (0.13 - 0.18)	0.090,0.24	<0.001	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	24.75 ± 0.18 (24.14 - 25.25)	23.82 ± 0.18 (23.62 - 24.11)	0.94 ± 0.23 (0.52 - 1.15)	0.40,1.47	0.003	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	61.87 ± 0.23 (61.19 - 62.42)	63.17 ± 0.23 (62.80 - 63.41)	-1.30 ± 0.28 (-1.62 - -1.00)	-1.95,-0.66	0.001	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.17 ± 0.014 (1.12 - 1.22)	1.18 ± 0.014 (1.15 - 1.21)	-0.013 ± 0.018 (-0.033 - 0.024)	-0.054,0.029	0.505	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.39 ± 0.0063 (0.38 - 0.40)	0.37 ± 0.0063 (0.36 - 0.37)	0.025 ± 0.0088 (0.015 - 0.032)	0.0047,0.045	0.021	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.28 ± 0.0034 (0.27 - 0.28)	0.27 ± 0.0034 (0.27 - 0.28)	0.0022 ± 0.0046 (-0.0050 - 0.0073)	-0.0083,0.013	0.644	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.14 ± 0.0053 (0.13 - 0.15)	0.14 ± 0.0053 (0.14 - 0.15)	0.0017 ± 0.0075 (-0.012 - 0.011)	-0.016,0.019	0.830	(0.12 - 0.19) [0.081,0.23]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.19. Comparison of the fiber and proximates content in grain collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	5.53 ± 0.37 (4.53 - 6.10)	4.95 ± 0.37 (4.54 - 5.46)	0.58 ± 0.50 (-0.32 - 1.42)	-0.57,1.73	0.277	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fiber (% DW)	8.98 ± 0.84 (8.59 - 9.49)	9.63 ± 0.84 (8.48 - 11.75)	-0.65 ± 1.19 (-2.26 - 0.20)	-3.40,2.10	0.601	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fiber (% DW)	15.11 ± 0.90 (14.02 - 17.02)	14.75 ± 0.90 (12.82 - 17.62)	0.36 ± 1.25 (-3.61 - 4.20)	-2.53,3.24	0.782	(12.99 - 18.03) [9.20,20.27]
Proximate						
Ash (% DW)	1.38 ± 0.092 (1.35 - 1.44)	1.31 ± 0.092 (1.28 - 1.35)	0.069 ± 0.13 (0.0050 - 0.13)	-0.23,0.37	0.607	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	85.86 ± 0.31 (85.08 - 86.52)	85.68 ± 0.31 (85.53 - 85.84)	0.18 ± 0.44 (-0.44 - 0.84)	-0.85,1.20	0.699	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	9.79 ± 0.16 (9.51 - 10.10)	9.60 ± 0.16 (9.51 - 9.77)	0.19 ± 0.23 (0 - 0.33)	-0.34,0.72	0.441	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	9.11 ± 0.27 (8.54 - 9.67)	9.58 ± 0.27 (9.38 - 9.80)	-0.47 ± 0.38 (-1.26 - 0.29)	-1.34,0.41	0.252	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.65 ± 0.099 (3.50 - 3.89)	3.43 ± 0.099 (3.22 - 3.75)	0.22 ± 0.13 (0.15 - 0.29)	-0.079,0.52	0.127	(2.66 - 3.71) [2.20,4.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.20. Comparison of the mineral content in grain collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Mineral						
Calcium (% DW)	0.0040 ± 0.00009 (0.0038 - 0.0042)	0.0041 ± 0.00009 (0.0040 - 0.0044)	-0.00014 ± 0.00011 (-0.00027 - -0.00001)	-0.00039,0.00010	0.216	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	1.61 ± 0.57 (1.50 - 1.72)	1.81 ± 0.57 (1.61 - 1.93)	-0.19 ± 0.81 (-0.43 - 0.10)	-2.06,1.68	0.818	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	19.62 ± 0.27 (19.23 - 19.91)	20.28 ± 0.27 (19.34 - 20.89)	-0.66 ± 0.39 (-1.66 - 0.38)	-1.55,0.23	0.126	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.11 ± 0.0026 (0.11 - 0.12)	0.11 ± 0.0026 (0.11 - 0.12)	-0.00043 ± 0.0027 (-0.0055 - 0.0051)	-0.0067,0.0058	0.879	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	5.65 ± 0.14 (5.43 - 5.80)	5.74 ± 0.14 (5.57 - 5.85)	-0.084 ± 0.13 (-0.36 - 0.15)	-0.39,0.22	0.540	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.32 ± 0.0077 (0.31 - 0.32)	0.32 ± 0.0077 (0.30 - 0.34)	0.0021 ± 0.0097 (-0.015 - 0.013)	-0.020,0.025	0.831	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.37 ± 0.0075 (0.37 - 0.38)	0.36 ± 0.0075 (0.35 - 0.38)	0.011 ± 0.011 (-0.0066 - 0.022)	-0.014,0.035	0.342	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	19.73 ± 0.33 (19.35 - 20.00)	20.13 ± 0.33 (19.39 - 20.67)	-0.40 ± 0.44 (-0.66 - -0.040)	-1.42,0.61	0.387	(16.72 - 34.04) [7.16,38.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.21. Comparison of the vitamin content in grain collected at Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Vitamin						
Folic Acid (mg/kg DW)	0.37 ± 0.0059 (0.35 - 0.38)	0.32 ± 0.0059 (0.32 - 0.33)	0.045 ± 0.0084 (0.028 - 0.057)	0.025,0.064	<0.001	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	31.66 ± 1.26 (27.14 - 34.70)	30.16 ± 1.26 (29.06 - 31.59)	1.49 ± 1.78 (-4.44 - 5.64)	-2.62,5.61	0.426	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	2.81 ± 0.23 (2.66 - 2.98)	2.65 ± 0.23 (2.55 - 2.76)	0.15 ± 0.33 (-0.10 - 0.33)	-0.63,0.94	0.657	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.43 ± 0.060 (1.38 - 1.51)	1.28 ± 0.060 (1.20 - 1.39)	0.15 ± 0.085 (0.0051 - 0.27)	-0.046,0.35	0.115	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	5.82 ± 0.17 (5.65 - 6.13)	5.89 ± 0.17 (5.67 - 6.07)	-0.070 ± 0.25 (-0.38 - 0.46)	-0.64,0.50	0.784	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	6.66 ± 0.18 (6.43 - 6.98)	6.38 ± 0.18 (6.05 - 6.82)	0.28 ± 0.19 (-0.39 - 0.71)	-0.16,0.72	0.182	(2.71 - 13.94) [0,20.49]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.22. Comparison of the antinutrient and secondary metabolite content in grain collected at the Site 3 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Antinutrient						
Phytic Acid (% DW)	0.66 ± 0.046 (0.56 - 0.79)	0.67 ± 0.046 (0.65 - 0.70)	-0.0089 ± 0.066 (-0.14 - 0.13)	-0.16,0.14	0.896	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	1995.62 ± 93.94 (1790.25 - 2124.58)	1961.58 ± 93.94 (1878.66 - 2122.02)	34.04 ± 103.91 (-88.41 - 240.51)	-205.59,273.66	0.751	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	186.61 ± 8.59 (172.39 – 195.01)	188.43 ± 8.59 (171.29 – 198.38)	-1.82 ± 9.99 (-5.95 – 1.11)	-24.86,21.22	0.860	(99.30 – 285.75) [0,378.57]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.23. Comparison of the fiber and mineral content in forage collected at Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	24.19 ± 1.74 (22.60 - 27.08)	24.29 ± 1.74 (19.93 - 26.90)	-0.11 ± 2.47 (-4.03 - 7.15)	-5.80,5.58	0.965	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fiber (% DW)	37.93 ± 2.41 (35.64 - 39.24)	32.96 ± 2.41 (31.44 - 34.62)	4.97 ± 3.41 (2.83 - 7.47)	-2.89,12.84	0.182	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.17 ± 0.0084 (0.16 - 0.18)	0.16 ± 0.0084 (0.13 - 0.17)	0.010 ± 0.012 (-0.017 - 0.049)	-0.017,0.038	0.415	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.25 ± 0.023 (0.23 - 0.28)	0.17 ± 0.023 (0.15 - 0.21)	0.080 ± 0.032 (0.024 - 0.13)	0.0064,0.15	0.036	(0.14 - 0.25) [0.071,0.32]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.24. Comparison of the proximates content in forage collected at Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Proximate						
Ash (% DW)	3.20 ± 0.29 (2.93 - 3.38)	4.39 ± 0.29 (3.30 - 5.10)	-1.19 ± 0.42 (-1.72 - -0.37)	-2.15,-0.23	0.021	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	88.16 ± 0.65 (86.86 - 88.84)	84.98 ± 0.65 (84.36 - 85.29)	3.18 ± 0.82 (1.57 - 4.41)	1.29,5.07	0.004	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	71.73 ± 1.01 (69.70 - 74.30)	72.23 ± 1.01 (70.10 - 74.70)	-0.50 ± 1.31 (-3.50 - 4.20)	-3.51,2.51	0.711	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	7.03 ± 0.38 (6.34 - 7.52)	8.02 ± 0.38 (7.63 - 8.66)	-0.99 ± 0.54 (-2.32 - -0.23)	-2.23,0.25	0.104	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	1.61 ± 0.43 (0.63 - 2.33)	2.62 ± 0.43 (2.18 - 2.91)	-1.00 ± 0.46 (-2.28 - -0.15)	-2.05,0.049	0.059	(0.58 - 3.11) [0,4.54]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.25. Comparison of the amino acid content in grain collected at the Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Alanine (% DW)	0.73 ± 0.023 (0.64 - 0.78)	0.77 ± 0.023 (0.76 - 0.78)	-0.043 ± 0.033 (-0.13 - 0.0071)	-0.12,0.032	0.223	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.44 ± 0.018 (0.38 - 0.48)	0.47 ± 0.018 (0.46 - 0.48)	-0.028 ± 0.025 (-0.090 - 0.010)	-0.085,0.029	0.289	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.63 ± 0.020 (0.56 - 0.67)	0.67 ± 0.020 (0.66 - 0.68)	-0.041 ± 0.028 (-0.11 - -0.0031)	-0.11,0.024	0.182	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.24 ± 0.0042 (0.23 - 0.25)	0.24 ± 0.0042 (0.23 - 0.25)	0.00021 ± 0.0060 (-0.012 - 0.012)	-0.014,0.014	0.973	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	1.86 ± 0.062 (1.63 - 2.01)	1.99 ± 0.062 (1.96 - 2.00)	-0.12 ± 0.088 (-0.33 - 0.011)	-0.33,0.080	0.200	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.36 ± 0.012 (0.32 - 0.39)	0.38 ± 0.012 (0.38 - 0.39)	-0.023 ± 0.017 (-0.067 - 0.00013)	-0.061,0.016	0.212	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.29 ± 0.0094 (0.25 - 0.32)	0.31 ± 0.0094 (0.30 - 0.31)	-0.016 ± 0.013 (-0.050 - 0.0033)	-0.046,0.015	0.266	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.34 ± 0.012 (0.30 - 0.37)	0.36 ± 0.012 (0.36 - 0.36)	-0.019 ± 0.017 (-0.056 - 0.0027)	-0.057,0.020	0.294	(0.32 - 0.45) [0.23,0.51]
Leucine (% DW)	1.25 ± 0.040 (1.09 - 1.33)	1.33 ± 0.040 (1.30 - 1.35)	-0.080 ± 0.057 (-0.21 - -0.015)	-0.21,0.051	0.195	(1.14 - 1.68) [0.77,1.92]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.25 (cont.). Comparison of the amino acid content in grain collected at the Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Lysine (% DW)	0.30 ± 0.013 (0.26 - 0.32)	0.31 ± 0.013 (0.31 - 0.32)	-0.018 ± 0.018 (-0.056 - 0.0089)	-0.060,0.024	0.349	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.23 ± 0.0038 (0.22 - 0.24)	0.23 ± 0.0038 (0.22 - 0.24)	-0.0036 ± 0.0054 (-0.014 - 0.0072)	-0.016,0.0090	0.528	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.49 ± 0.016 (0.43 - 0.52)	0.52 ± 0.016 (0.51 - 0.53)	-0.030 ± 0.023 (-0.080 - -0.0037)	-0.083,0.023	0.231	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	0.90 ± 0.028 (0.79 - 0.97)	0.94 ± 0.028 (0.93 - 0.96)	-0.043 ± 0.040 (-0.15 - 0.012)	-0.14,0.049	0.314	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.49 ± 0.014 (0.44 - 0.54)	0.52 ± 0.014 (0.52 - 0.53)	-0.031 ± 0.020 (-0.087 - 0.0053)	-0.077,0.015	0.160	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.31 ± 0.011 (0.27 - 0.34)	0.34 ± 0.011 (0.33 - 0.35)	-0.024 ± 0.015 (-0.052 - -0.00037)	-0.058,0.011	0.155	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.054 ± 0.0020 (0.051 - 0.056)	0.055 ± 0.0020 (0.052 - 0.057)	-0.00057 ± 0.0029 (-0.0032 - 0.0035)	-0.0072,0.0060	0.846	(0.043 - 0.059) [0.032,0.072]
Tyrosine (% DW)	0.36 ± 0.011 (0.32 - 0.38)	0.39 ± 0.011 (0.38 - 0.40)	-0.029 ± 0.016 (-0.066 - -0.0014)	-0.065,0.0073	0.103	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.46 ± 0.016 (0.40 - 0.49)	0.48 ± 0.016 (0.48 - 0.49)	-0.026 ± 0.022 (-0.084 - 0.0093)	-0.078,0.026	0.277	(0.42 - 0.55) [0.35,0.62]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.26. Comparison of the fatty acid content in grain collected at the Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.26 ± 0.050 (9.14 - 9.35)	9.23 ± 0.050 (9.19 - 9.29)	0.037 ± 0.071 (-0.051 - 0.14)	-0.13,0.20	0.619	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.14 ± 0.010 (0.13 - 0.14)	0.14 ± 0.010 (0.13 - 0.14)	0.00086 ± 0.015 (-0.00036 - 0.0019)	-0.033,0.035	0.954	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.88 ± 0.019 (1.86 - 1.89)	1.85 ± 0.019 (1.82 - 1.87)	0.029 ± 0.027 (0.021 - 0.034)	-0.033,0.090	0.318	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	25.60 ± 0.23 (25.42 - 25.75)	25.59 ± 0.23 (24.96 - 25.98)	0.0060 ± 0.28 (-0.41 - 0.66)	-0.63,0.64	0.983	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	61.12 ± 0.24 (60.85 - 61.27)	61.19 ± 0.24 (60.77 - 61.91)	-0.071 ± 0.32 (-0.64 - 0.35)	-0.82,0.67	0.830	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.18 ± 0.020 (1.17 - 1.18)	1.19 ± 0.020 (1.16 - 1.20)	-0.0096 ± 0.022 (-0.031 - 0.019)	-0.059,0.040	0.669	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.38 ± 0.0020 (0.38 - 0.39)	0.38 ± 0.0020 (0.38 - 0.39)	0.0039 ± 0.0023 (0.00019 - 0.0078)	-0.0014,0.0091	0.130	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.28 ± 0.0012 (0.28 - 0.28)	0.29 ± 0.0012 (0.28 - 0.29)	-0.0043 ± 0.0016 (-0.0071 - -0.0018)	-0.0081,-0.00052	0.030	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.17 ± 0.016 (0.15 - 0.18)	0.16 ± 0.016 (0.13 - 0.18)	0.0092 ± 0.018 (-0.0098 - 0.022)	-0.031,0.050	0.612	(0.12 - 0.19) [0.081,0.23]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.27. Comparison of the fiber and proximates content in grain collected at the Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	5.55 ± 0.41 (5.06 - 5.94)	5.41 ± 0.41 (5.28 - 5.49)	0.14 ± 0.58 (-0.43 - 0.49)	-1.19,1.47	0.817	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fiber (% DW)	10.52 ± 0.42 (10.43 - 10.69)	9.05 ± 0.42 (8.64 - 9.38)	1.47 ± 0.55 (1.07 - 2.05)	0.20,2.75	0.028	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fiber (% DW)	16.51 ± 0.66 (16.27 - 16.76)	15.63 ± 0.66 (15.07 - 16.69)	0.88 ± 0.93 (-0.17 - 1.63)	-1.26,3.03	0.368	(12.99 - 18.03) [9.20,20.27]
Proximate						
Ash (% DW)	1.34 ± 0.042 (1.25 - 1.38)	1.35 ± 0.042 (1.30 - 1.40)	-0.013 ± 0.059 (-0.043 - 0.030)	-0.15,0.12	0.826	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	85.11 ± 0.22 (84.99 - 85.29)	85.11 ± 0.22 (84.75 - 85.31)	-0.0055 ± 0.31 (-0.28 - 0.28)	-0.73,0.72	0.986	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	12.40 ± 0.27 (12.10 - 12.80)	12.77 ± 0.27 (12.10 - 13.10)	-0.37 ± 0.38 (-1.00 - 0.20)	-1.23,0.50	0.357	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	10.31 ± 0.15 (10.26 - 10.35)	10.39 ± 0.15 (10.33 - 10.49)	-0.078 ± 0.21 (-0.19 - 0.019)	-0.57,0.42	0.725	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.25 ± 0.12 (3.19 - 3.28)	3.15 ± 0.12 (3.05 - 3.35)	0.097 ± 0.16 (-0.069 - 0.23)	-0.27,0.47	0.562	(2.66 - 3.71) [2.20,4.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.28. Comparison of the mineral content in grain collected at the Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Mineral						
Calcium (% DW)	0.0050 ± 0.00016 (0.0048 - 0.0054)	0.0050 ± 0.00016 (0.0047 - 0.0051)	0.00008 ± 0.00023 (-0.00024 - 0.00064)	-0.00044,0.00061	0.722	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	2.15 ± 0.13 (1.92 - 2.38)	1.67 ± 0.11 (1.54 - 1.75)	0.48 ± 0.17 (0.38 - 0.63)	0.086,0.87	**0.023**	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	21.47 ± 0.93 (19.45 - 25.23)	20.02 ± 0.93 (19.33 - 20.71)	1.45 ± 1.32 (-0.98 - 5.90)	-1.60,4.49	0.304	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.10 ± 0.0038 (0.10 - 0.11)	0.11 ± 0.0038 (0.11 - 0.12)	-0.0075 ± 0.0047 (-0.018 - 0.0036)	-0.018,0.0032	0.145	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	6.85 ± 0.33 (6.45 - 7.45)	7.11 ± 0.33 (6.66 - 8.00)	-0.26 ± 0.47 (-1.54 - 0.78)	-1.35,0.82	0.590	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.29 ± 0.010 (0.27 - 0.31)	0.30 ± 0.010 (0.29 - 0.31)	-0.011 ± 0.012 (-0.038 - 0.016)	-0.038,0.016	0.357	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.34 ± 0.0082 (0.32 - 0.37)	0.35 ± 0.0082 (0.34 - 0.35)	-0.0086 ± 0.0087 (-0.029 - 0.014)	-0.029,0.012	0.352	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	21.39 ± 0.80 (20.07 - 23.74)	22.46 ± 0.80 (21.75 - 23.44)	-1.07 ± 1.14 (-3.37 - 1.53)	-3.69,1.54	0.371	(16.72 - 34.04) [7.16,38.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.29. Comparison of the vitamin content in grain collected at the Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Vitamin						
Folic Acid (mg/kg DW)	0.27 ± 0.017 (0.26 - 0.28)	0.26 ± 0.017 (0.23 - 0.29)	0.0069 ± 0.020 (-0.012 - 0.045)	-0.040,0.053	0.740	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	31.47 ± 0.86 (30.39 - 33.52)	30.38 ± 0.86 (30.26 - 30.49)	1.09 ± 1.21 (-0.10 - 3.15)	-1.70,3.88	0.394	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	3.20 ± 0.13 (3.07 - 3.44)	2.98 ± 0.13 (2.76 - 3.22)	0.22 ± 0.18 (-0.15 - 0.68)	-0.21,0.64	0.274	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.25 ± 0.065 (1.24 - 1.26)	1.45 ± 0.065 (1.30 - 1.55)	-0.20 ± 0.092 (-0.30 - -0.049)	-0.41,0.017	0.066	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	6.36 ± 0.29 (6.15 - 6.47)	6.02 ± 0.29 (5.37 - 6.44)	0.34 ± 0.39 (-0.088 - 1.10)	-0.57,1.25	0.412	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	6.88 ± 0.55 (6.19 - 7.28)	6.95 ± 0.55 (6.73 - 7.23)	-0.074 ± 0.74 (-1.04 - 0.55)	-1.78,1.63	0.923	(2.71 - 13.94) [0,20.49]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.30. Comparison of the antinutrient and secondary metabolites content in grain collected at Site 4 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Antinutrient						
Phytic Acid (% DW)	0.60 ± 0.058 (0.53 - 0.73)	0.61 ± 0.058 (0.56 - 0.68)	-0.0070 ± 0.077 (-0.15 - 0.14)	-0.18,0.17	0.929	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	2119.34 ± 94.97 (2041.28 - 2200.68)	2090.08 ± 94.97 (2071.35 - 2116.04)	29.26 ± 134.31 (-30.06 - 84.64)	-280.47,338.99	0.833	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	196.33 ± 6.94 (184.63 - 212.09)	177.32 ± 6.94 (172.92 - 180.67)	19.01 ± 9.81 (6.27 - 39.16)	-3.62,41.64	0.088	(99.30 - 285.75) [0,378.57]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.31. Comparison of the fiber and mineral content in forage collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	31.31 ± 1.70 (26.92 - 34.93)	23.58 ± 1.70 (23.06 - 24.48)	7.73 ± 2.40 (3.72 - 10.45)	2.20,13.26	0.012	(26.72 - 38.94) [16.76,43.76]
Neutral Detergent Fiber (% DW)	43.21 ± 2.11 (40.07 - 46.82)	37.87 ± 2.11 (35.06 - 41.38)	5.34 ± 1.98 (5.00 - 5.58)	0.78,9.90	0.027	(33.70 - 46.74) [25.94,55.67]
Mineral						
Calcium (% DW)	0.18 ± 0.014 (0.17 - 0.21)	0.15 ± 0.014 (0.14 - 0.17)	0.030 ± 0.018 (-0.0023 - 0.063)	-0.011,0.071	0.131	(0.11 - 0.29) [0.016,0.38]
Phosphorus (% DW)	0.27 ± 0.019 (0.24 - 0.32)	0.22 ± 0.019 (0.22 - 0.23)	0.049 ± 0.027 (0.0094 - 0.10)	-0.012,0.11	0.102	(0.14 - 0.25) [0.071,0.32]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.32. Comparison of the proximates content in forage collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Proximate						
Ash (% DW)	3.19 ± 0.29 (2.51 - 3.61)	2.97 ± 0.29 (2.59 - 3.38)	0.22 ± 0.39 (-0.87 - 0.86)	-0.67,1.12	0.582	(3.40 - 5.45) [1.93,6.31]
Carbohydrates (% DW)	87.86 ± 0.91 (85.66 - 89.13)	87.82 ± 0.91 (86.94 - 88.77)	0.037 ± 0.85 (-1.28 - 1.03)	-1.93,2.00	0.966	(84.88 - 88.39) [83.05,90.74]
Moisture (% FW)	71.33 ± 1.21 (70.10 - 73.10)	69.93 ± 1.21 (69.20 - 71.00)	1.40 ± 1.40 (-0.90 - 3.50)	-1.84,4.64	0.348	(64.90 - 77.40) [57.62,86.45]
Protein (% DW)	7.50 ± 0.49 (6.65 - 8.49)	7.50 ± 0.49 (7.30 - 7.79)	0.0046 ± 0.67 (-0.65 - 0.70)	-1.53,1.54	0.994	(6.58 - 8.82) [4.78,10.38]
Total Fat (% DW)	1.45 ± 0.42 (0.77 - 2.23)	1.71 ± 0.42 (1.34 - 2.32)	-0.26 ± 0.38 (-0.57 - -0.093)	-1.14,0.61	0.507	(0.58 - 3.11) [0,4.54]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.33. Comparison of the amino acid content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Alanine (% DW)	0.86 ± 0.023 (0.82 - 0.89)	0.87 ± 0.023 (0.86 - 0.89)	-0.018 ± 0.027 (-0.069 - 0.023)	-0.081,0.045	0.534	(0.67 - 0.96) [0.48,1.08]
Arginine (% DW)	0.51 ± 0.010 (0.50 - 0.52)	0.50 ± 0.010 (0.49 - 0.51)	0.011 ± 0.014 (0.0046 - 0.016)	-0.021,0.044	0.443	(0.37 - 0.49) [0.33,0.56]
Aspartic acid (% DW)	0.74 ± 0.019 (0.73 - 0.76)	0.74 ± 0.019 (0.72 - 0.76)	0.00048 ± 0.026 (-0.038 - 0.039)	-0.060,0.061	0.985	(0.57 - 0.77) [0.43,0.90]
Cystine (% DW)	0.24 ± 0.0060 (0.23 - 0.25)	0.24 ± 0.0060 (0.24 - 0.25)	-0.0014 ± 0.0059 (-0.014 - 0.0076)	-0.015,0.012	0.817	(0.20 - 0.24) [0.18,0.27]
Glutamic acid (% DW)	2.20 ± 0.063 (2.10 - 2.29)	2.21 ± 0.063 (2.18 - 2.26)	-0.015 ± 0.078 (-0.16 - 0.10)	-0.19,0.16	0.852	(1.71 - 2.41) [1.25,2.75]
Glycine (% DW)	0.41 ± 0.0072 (0.40 - 0.41)	0.40 ± 0.0072 (0.40 - 0.41)	0.0035 ± 0.010 (-0.0074 - 0.015)	-0.020,0.027	0.741	(0.32 - 0.40) [0.28,0.46]
Histidine (% DW)	0.34 ± 0.0072 (0.33 - 0.35)	0.33 ± 0.0072 (0.33 - 0.34)	0.0036 ± 0.0080 (-0.0083 - 0.014)	-0.015,0.022	0.664	(0.26 - 0.33) [0.22,0.38]
Isoleucine (% DW)	0.42 ± 0.011 (0.41 - 0.43)	0.42 ± 0.011 (0.41 - 0.42)	0.0013 ± 0.014 (-0.0052 - 0.0063)	-0.032,0.035	0.930	(0.32 - 0.45) [0.23,0.51]
Leucine (% DW)	1.50 ± 0.050 (1.41 - 1.57)	1.53 ± 0.050 (1.51 - 1.55)	-0.032 ± 0.055 (-0.13 - 0.044)	-0.16,0.096	0.582	(1.14 - 1.68) [0.77,1.92]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.33 (cont.). Comparison of the amino acid content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Amino Acid (% DW)						
Lysine (% DW)	0.35 ± 0.0073 (0.35 - 0.35)	0.35 ± 0.0073 (0.34 - 0.36)	0.0018 ± 0.010 (-0.012 - 0.011)	-0.022,0.026	0.864	(0.24 - 0.34) [0.20,0.40]
Methionine (% DW)	0.24 ± 0.0066 (0.23 - 0.24)	0.23 ± 0.0066 (0.23 - 0.24)	0.0062 ± 0.0054 (-0.0037 - 0.017)	-0.0063,0.019	0.284	(0.17 - 0.22) [0.14,0.25]
Phenylalanine (% DW)	0.58 ± 0.018 (0.55 - 0.61)	0.59 ± 0.018 (0.58 - 0.60)	-0.0084 ± 0.020 (-0.054 - 0.032)	-0.055,0.039	0.692	(0.45 - 0.65) [0.32,0.73]
Proline (% DW)	0.95 ± 0.026 (0.90 - 1.00)	0.97 ± 0.026 (0.96 - 0.98)	-0.016 ± 0.028 (-0.064 - 0.026)	-0.081,0.050	0.598	(0.83 - 1.11) [0.68,1.21]
Serine (% DW)	0.56 ± 0.016 (0.54 - 0.59)	0.57 ± 0.016 (0.55 - 0.60)	-0.010 ± 0.023 (-0.052 - 0.034)	-0.063,0.043	0.664	(0.45 - 0.62) [0.34,0.71]
Threonine (% DW)	0.33 ± 0.012 (0.30 - 0.35)	0.34 ± 0.012 (0.33 - 0.35)	-0.0095 ± 0.014 (-0.032 - 0.012)	-0.042,0.023	0.524	(0.29 - 0.37) [0.24,0.41]
Tryptophan (% DW)	0.060 ± 0.0015 (0.055 - 0.064)	0.058 ± 0.0015 (0.057 - 0.059)	0.0016 ± 0.0021 (-0.0038 - 0.0072)	-0.0031,0.0064	0.449	(0.043 - 0.059) [0.032,0.072]
Tyrosine (% DW)	0.35 ± 0.051 (0.22 - 0.42)	0.37 ± 0.051 (0.25 - 0.42)	-0.022 ± 0.071 (-0.21 - 0.14)	-0.19,0.14	0.764	(0.25 - 0.40) [0.17,0.52]
Valine (% DW)	0.54 ± 0.013 (0.53 - 0.55)	0.54 ± 0.013 (0.53 - 0.55)	0.0068 ± 0.016 (-0.015 - 0.021)	-0.030,0.044	0.682	(0.42 - 0.55) [0.35,0.62]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.34. Comparison of the fatty acid content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fatty Acid (% Total FA)						
16:0 Palmitic (% Total FA)	9.00 ± 0.026 (8.98 - 9.03)	8.97 ± 0.026 (8.94 - 9.01)	0.022 ± 0.037 (-0.032 - 0.064)	-0.065,0.11	0.575	(9.10 - 12.55) [6.12,15.67]
16:1 Palmitoleic (% Total FA)	0.13 ± 0.014 (0.13 - 0.14)	0.11 ± 0.014 (0.048 - 0.14)	0.026 ± 0.018 (-0.0012 - 0.079)	-0.015,0.067	0.177	(0.050 - 0.19) [0,0.28]
18:0 Stearic (% Total FA)	1.84 ± 0.012 (1.82 - 1.86)	1.81 ± 0.012 (1.80 - 1.82)	0.031 ± 0.016 (0.017 - 0.059)	-0.0058,0.068	0.087	(1.57 - 2.45) [0.86,2.98]
18:1 Oleic (% Total FA)	24.07 ± 0.29 (23.38 - 24.53)	24.28 ± 0.29 (23.98 - 24.85)	-0.22 ± 0.38 (-1.48 - 0.51)	-1.10,0.67	0.588	(21.17 - 35.33) [7.51,46.46]
18:2 Linoleic (% Total FA)	62.98 ± 0.30 (62.56 - 63.61)	62.86 ± 0.30 (62.37 - 63.16)	0.12 ± 0.37 (-0.60 - 1.24)	-0.74,0.98	0.762	(50.33 - 63.59) [39.41,76.74]
18:3 Linolenic (% Total FA)	1.15 ± 0.012 (1.13 - 1.17)	1.16 ± 0.012 (1.15 - 1.18)	-0.0082 ± 0.015 (-0.049 - 0.016)	-0.042,0.026	0.596	(0.93 - 1.52) [0.63,1.77]
20:0 Arachidic (% Total FA)	0.38 ± 0.0033 (0.38 - 0.39)	0.37 ± 0.0033 (0.37 - 0.38)	0.012 ± 0.0046 (0.0058 - 0.022)	0.00099,0.022	0.035	(0.32 - 0.47) [0.23,0.54]
20:1 Eicosenoic (% Total FA)	0.28 ± 0.0021 (0.28 - 0.28)	0.28 ± 0.0021 (0.27 - 0.28)	0.0038 ± 0.0029 (0.0022 - 0.0049)	-0.0027,0.010	0.215	(0.23 - 0.32) [0.15,0.39]
22:0 Behenic (% Total FA)	0.17 ± 0.012 (0.15 - 0.20)	0.16 ± 0.012 (0.14 - 0.17)	0.013 ± 0.012 (-0.0020 - 0.029)	-0.015,0.040	0.311	(0.12 - 0.19) [0.081,0.23]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.35. Comparison of the fiber and proximates content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Fiber						
Acid Detergent Fiber (% DW)	5.99 ± 0.52 (5.33 - 7.24)	5.27 ± 0.52 (4.17 - 6.22)	0.72 ± 0.74 (-0.84 - 3.07)	-0.98,2.42	0.358	(4.11 - 6.33) [2.77,7.56]
Neutral Detergent Fiber (% DW)	9.48 ± 0.42 (8.87 - 9.79)	8.87 ± 0.42 (8.57 - 9.44)	0.61 ± 0.59 (0.26 - 1.22)	-0.75,1.96	0.334	(8.20 - 11.30) [5.93,13.63]
Total Dietary Fiber (% DW)	14.34 ± 0.34 (13.80 - 14.94)	13.82 ± 0.34 (13.50 - 14.32)	0.52 ± 0.24 (0.30 - 0.65)	-0.035,1.07	0.062	(12.99 - 18.03) [9.20,20.27]
Proximate						
Ash (% DW)	1.43 ± 0.048 (1.37 - 1.53)	1.44 ± 0.048 (1.31 - 1.51)	-0.0025 ± 0.055 (-0.099 - 0.063)	-0.13,0.12	0.964	(1.12 - 1.62) [0.74,1.96]
Carbohydrates (% DW)	84.26 ± 0.19 (83.99 - 84.59)	83.80 ± 0.19 (83.58 - 84.03)	0.46 ± 0.14 (0.41 - 0.56)	0.15,0.78	0.009	(82.91 - 86.78) [81.08,88.80]
Moisture (% FW)	8.89 ± 0.10 (8.71 - 9.01)	8.60 ± 0.10 (8.36 - 8.89)	0.29 ± 0.14 (-0.18 - 0.65)	-0.039,0.61	0.076	(7.60 - 15.30) [0.45,19.52]
Protein (% DW)	11.15 ± 0.20 (10.83 - 11.43)	11.31 ± 0.20 (11.15 - 11.52)	-0.15 ± 0.25 (-0.69 - 0.19)	-0.72,0.42	0.558	(9.33 - 11.82) [7.54,13.13]
Total Fat (% DW)	3.15 ± 0.098 (3.05 - 3.21)	3.46 ± 0.098 (3.14 - 3.68)	-0.31 ± 0.14 (-0.50 - 0.070)	-0.63,0.0082	0.054	(2.66 - 3.71) [2.20,4.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.36. Comparison of the mineral content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Mineral						
Calcium (% DW)	0.0049 ± 0.00016 (0.0048 - 0.0049)	0.0046 ± 0.00016 (0.0045 - 0.0047)	0.00026 ± 0.00023 (0.00013 - 0.00037)	-0.00027,0.00079	0.288	(0.0031 - 0.0049) [0.0016,0.0059]
Copper (mg/kg DW)	1.35 ± 0.65 (1.33 - 1.39)	2.68 ± 0.65 (1.84 - 4.29)	-1.32 ± 0.86 (-2.96 - -0.51)	-3.31,0.66	0.163	(1.15 - 3.56) [0,4.20]
Iron (mg/kg DW)	21.37 ± 0.84 (20.59 - 21.76)	25.74 ± 0.84 (22.83 - 28.26)	-4.38 ± 1.19 (-6.50 - -2.24)	-7.12,-1.63	0.006	(18.04 - 29.22) [8.88,34.51]
Magnesium (% DW)	0.13 ± 0.0024 (0.13 - 0.14)	0.13 ± 0.0024 (0.13 - 0.14)	-0.0014 ± 0.0028 (-0.0046 - 0.00096)	-0.0080,0.0052	0.636	(0.099 - 0.14) [0.075,0.17]
Manganese (mg/kg DW)	6.56 ± 0.17 (6.09 - 6.85)	6.52 ± 0.17 (6.38 - 6.66)	0.039 ± 0.17 (-0.29 - 0.32)	-0.34,0.42	0.817	(5.56 - 8.64) [3.17,9.99]
Phosphorus (% DW)	0.35 ± 0.0055 (0.34 - 0.36)	0.35 ± 0.0055 (0.35 - 0.36)	-0.00071 ± 0.0067 (-0.011 - 0.0058)	-0.016,0.015	0.917	(0.25 - 0.37) [0.18,0.45]
Potassium (% DW)	0.36 ± 0.0041 (0.35 - 0.36)	0.35 ± 0.0041 (0.35 - 0.35)	0.0040 ± 0.0058 (-0.0029 - 0.011)	-0.0092,0.017	0.503	(0.32 - 0.40) [0.26,0.46]
Zinc (mg/kg DW)	22.13 ± 0.55 (21.25 - 22.95)	22.25 ± 0.55 (21.76 - 22.92)	-0.11 ± 0.78 (-0.81 - 1.19)	-1.90,1.68	0.889	(16.72 - 34.04) [7.16,38.55]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.37. Comparison of the vitamin content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Vitamin						
Folic Acid (mg/kg DW)	0.39 ± 0.025 (0.32 - 0.48)	0.35 ± 0.025 (0.32 - 0.37)	0.041 ± 0.035 (-0.043 - 0.11)	-0.040,0.12	0.276	(0.13 - 0.45) [0.012,0.69]
Niacin (mg/kg DW)	26.78 ± 0.70 (25.72 - 28.00)	25.71 ± 0.70 (24.93 - 26.19)	1.07 ± 0.90 (-0.47 - 3.07)	-1.00,3.14	0.268	(16.17 - 29.19) [6.97,37.83]
Vitamin B1 (mg/kg DW)	3.11 ± 0.49 (2.96 - 3.40)	2.99 ± 0.49 (2.85 - 3.06)	0.12 ± 0.69 (-0.088 - 0.34)	-1.47,1.71	0.866	(2.19 - 5.60) [0.37,6.35]
Vitamin B2 (mg/kg DW)	1.52 ± 0.089 (1.44 - 1.61)	1.39 ± 0.089 (1.16 - 1.52)	0.13 ± 0.13 (-0.038 - 0.45)	-0.16,0.42	0.322	(1.34 - 1.91) [0.91,2.30]
Vitamin B6 (mg/kg DW)	5.68 ± 0.30 (5.28 - 6.00)	6.11 ± 0.30 (5.95 - 6.38)	-0.43 ± 0.43 (-0.72 - -0.19)	-1.42,0.56	0.348	(5.08 - 7.47) [3.12,9.30]
Vitamin E (mg/kg DW)	6.83 ± 0.81 (5.55 - 8.62)	8.01 ± 0.81 (6.35 - 9.02)	-1.18 ± 1.15 (-2.35 - -0.40)	-3.82,1.46	0.331	(2.71 - 13.94) [0,20.49]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table G.38. Comparison of the antinutrients and secondary metabolites content in grain collected at the Site 5 from MON 89034 and conventional corn

Analytical Component	Test Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference(Test minus Control) Mean ± S.E. (Range)	95% CI (Lower,Upper)	p-Value	Commercial (Range) [99% Tolerance Int.[1]]
Antinutrient						
Phytic Acid (% DW)	0.85 ± 0.018 (0.85 - 0.86)	0.86 ± 0.018 (0.83 - 0.88)	-0.010 ± 0.023 (-0.031 - 0.025)	-0.064,0.044	0.676	(0.50 - 0.94) [0.21,1.22]
Secondary Metabolite						
Ferulic Acid (µg/g DW)	2026.73 ± 69.44 (1954.76 - 2092.23)	1932.90 ± 69.44 (1898.73 - 1979.22)	93.82 ± 91.31 (-24.47 - 171.48)	-116.73,304.37	0.334	(1412.68 - 2297.36) [1136.69,2806.24]
p-Coumaric Acid (µg/g DW)	218.38 ± 10.54 (187.79 - 253.04)	185.63 ± 10.54 (182.20 - 189.17)	32.75 ± 12.71 (-1.39 - 70.84)	3.44,62.07	0.032	(99.30 - 285.75) [0,378.57]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

APPENDIX H: Supplementary Compositional Analysis Data

The purpose of this Appendix is to provide statistical summaries of re-expressed amino acid and fatty acid corn composition data for the combined sites.

The grain fatty acid, total fat, amino acid, protein and moisture data from MON 89034, and the conventional control, LH198 x LH172, and 15 unique commercial reference corn hybrids were selected from the data set. The corn test, control and references were grown in U.S. field trials during 2004. The test and control substances were grown in single plots randomly assigned within each of three replication blocks at each of five sites (1-5).

Analytes with greater than fifty percent of observations below the assay's limit of quantitation (LOQ) were excluded from summaries and analysis. Otherwise, results below the quantitation limit were assigned a value equal to half the quantitation limit. The following analytes of interest were assigned values:

		Obs. Below LOQ				
Analyte	**Units**	**N**	**(%)**	**Total N**	**LOQ**	**Value Assigned**
Cottonseed Fatty Acid						
16:1 Palmitoleic	% FW	6	6.7	90	0.0030	0.0015
22:0 Behenic	% FW	1	1.1	90	0.0030	0.0015

The following formulas were used for re-expression of amino acid and fatty acid data:

Component	**From (X)**	**To**	**Formula**[1]
Amino Acids (AA)	mg/g FW	% Total Protein	X/(10*fp)
		% Total AA	$(100)X_j/\Sigma X_j$, for each AA j
Fatty Acid (FA)	% FW	% Total Fat	X/(ff)
		% DW	X/(d)
[1]fp is the protein fraction of fresh weight obtained by proximate analysis = (% protein /100); ff is the total fat fraction of fresh weight obtained by proximate analysis = (% total fat / 100); d is the fraction of the sample that is dry matter.			

2. Statistical Approach

Summary statistics for the analytes of interest were generated using SAS®[1] software.

3. Results Discussion

Statistical results of re-expression of amino acid and fatty acid components are summarized in Tables H.1-H.4. For each amino acid and fatty acid component re-expression, the overall mean, standard error (S.E.), and the range of observed values are

[1] SAS Software Release 9.1 (TS1M3). Copyright (c) 2002-2003 by SAS Institute Inc., Cary, NC, USA.

presented. In addition, the overall range of observed values for commercial references is presented in the desired unit.

Table H.1. Combined-Site Statistical Summary for Amino Acid Content as Percent Total Amino Acid in the Grain of MON 89034, Control and Commercial Corn

Component	MON 89034 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	Commercial [Range]
Alanine (% Total AA)	7.48 (0.041) [7.18 - 7.75]	7.56 (0.037) [7.34 - 7.84]	[7.31 - 8.04]
Arginine (% Total AA)	4.66 (0.061) [4.28 - 5.01]	4.55 (0.045) [4.25 - 4.87]	[3.85 - 4.83]
Aspartic acid (% Total AA)	6.62 (0.033) [6.43 - 6.82]	6.59 (0.024) [6.45 - 6.76]	[6.17 - 6.82]
Cystine (% Total AA)	2.27 (0.052) [2.07 - 2.89]	2.25 (0.027) [2.09 - 2.45]	[1.97 - 2.48]
Glutamic acid (% Total AA)	19.21 (0.11) [18.57 - 19.77]	19.38 (0.073) [18.86 - 19.82]	[18.68 - 20.26]
Glycine (% Total AA)	3.76 (0.050) [3.45 - 4.03]	3.72 (0.039) [3.53 - 3.98]	[3.39 - 3.95]
Histidine (% Total AA)	3.03 (0.018) [2.93 - 3.16]	3.01 (0.019) [2.91 - 3.16]	[2.64 - 3.24]
Isoleucine (% Total AA)	3.53 (0.034) [3.36 - 3.87]	3.54 (0.029) [3.35 - 3.79]	[3.40 - 3.80]
Leucine (% Total AA)	12.71 (0.13) [11.95 - 13.54]	12.87 (0.12) [11.98 - 13.81]	[12.38 - 14.14]

Table H.1 (contd.). Combined-Site Statistical Summary for Amino Acid Content as Percent Total Amino Acid in the Grain of MON 89034, Control and Commercial Corn

Component[a]	MON 89034 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	Commercial [Range]
Lysine (% Total AA)	3.20 (0.056) [2.90 - 3.57]	3.12 (0.038) [2.92 - 3.41]	[2.66 - 3.21]
Methionine (% Total AA)	2.23 (0.044) [2.08 - 2.75]	2.19 (0.026) [2.01 - 2.42]	[1.71 - 2.21]
Phenylalanine (% Total AA)	5.01 (0.036) [4.82 - 5.28]	5.03 (0.038) [4.74 - 5.27]	[4.97 - 5.48]
Proline (% Total AA)	9.12 (0.086) [8.43 - 9.51]	9.10 (0.072) [8.36 - 9.38]	[8.73 - 9.65]
Serine (% Total AA)	5.06 (0.038) [4.78 - 5.29]	5.12 (0.032) [4.90 - 5.32]	[4.74 - 5.44]
Threonine (% Total AA)	3.20 (0.036) [2.83 - 3.35]	3.20 (0.028) [2.90 - 3.32]	[2.91 - 3.34]
Tryptophan (% Total AA)	0.55 (0.0094) [0.49 - 0.64]	0.54 (0.0086) [0.50 - 0.59]	[0.40 - 0.60]
Tyrosine (% Total AA)	3.58 (0.11) [2.04 - 3.84]	3.49 (0.11) [2.29 - 3.85]	[2.36 - 3.80]
Valine (% Total AA)	4.78 (0.034) [4.58 - 4.99]	4.75 (0.019) [4.65 - 4.94]	[4.40 - 4.96]

[a] AA = amino acid.

Table H.2. Combined-Site Statistical Summary for Amino Acid Content as Percent Total Protein in the Grain of MON 89034, Control and Commercial Corn

Component	MON 89034 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	Commercial [Range]
Alanine (% Total Protein)	7.37 (0.12) [6.17 - 8.33]	7.48 (0.052) [7.19 - 7.77]	[7.18 - 8.08]
Arginine (% Total Protein)	4.60 (0.11) [3.66 - 5.72]	4.50 (0.052) [4.21 - 4.85]	[3.87 - 4.82]
Aspartic acid (% Total Protein)	6.52 (0.12) [5.38 - 7.72]	6.52 (0.030) [6.34 - 6.72]	[6.01 - 6.88]
Cystine (% Total Protein)	2.23 (0.032) [2.00 - 2.42]	2.22 (0.026) [2.10 - 2.40]	[1.91 - 2.45]
Glutamic acid (% Total Protein)	18.93 (0.31) [15.80 - 21.35]	19.16 (0.12) [18.41 - 19.91]	[18.26 - 20.37]
Glycine (% Total Protein)	3.71 (0.088) [3.07 - 4.59]	3.68 (0.037) [3.44 - 3.90]	[3.32 - 3.99]
Histidine (% Total Protein)	2.99 (0.055) [2.47 - 3.53]	2.97 (0.016) [2.87 - 3.08]	[2.56 - 3.16]
Isoleucine (% Total Protein)	3.48 (0.068) [2.91 - 4.01]	3.50 (0.036) [3.28 - 3.73]	[3.37 - 3.77]
Leucine (% Total Protein)	12.53 (0.22) [10.61 - 13.84]	12.73 (0.15) [11.75 - 13.59]	[12.17 - 14.22]

Table H.2 (contd.). Combined-Site Statistical Summary for Amino Acid Content as Percent Total Protein in the Grain of MON 89034, Control and Commercial Corn

Component	MON 89034 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	Commercial [Range]
Lysine (% Total Protein)	3.16 (0.090) [2.48 - 4.06]	3.08 (0.035) [2.86 - 3.32]	[2.57 - 3.27]
Methionine (% Total Protein)	2.19 (0.025) [2.03 - 2.37]	2.16 (0.025) [2.01 - 2.37]	[1.75 - 2.15]
Phenylalanine (% Total Protein)	4.93 (0.085) [4.15 - 5.56]	4.97 (0.051) [4.65 - 5.30]	[4.82 - 5.49]
Proline (% Total Protein)	8.99 (0.17) [7.63 - 10.69]	8.99 (0.073) [8.34 - 9.46]	[8.44 - 9.50]
Serine (% Total Protein)	4.99 (0.081) [4.23 - 5.71]	5.06 (0.029) [4.92 - 5.25]	[4.62 - 5.41]
Threonine (% Total Protein)	3.15 (0.064) [2.66 - 3.79]	3.16 (0.030) [2.90 - 3.35]	[2.82 - 3.41]
Tryptophan (% Total Protein)	0.54 (0.0053) [0.50 - 0.58]	0.54 (0.0078) [0.48 - 0.58]	[0.39 - 0.59]
Tyrosine (% Total Protein)	3.53 (0.13) [2.00 - 4.24]	3.45 (0.12) [2.25 - 3.89]	[2.38 - 3.95]
Valine (% Total Protein)	4.71 (0.092) [3.87 - 5.56]	4.70 (0.026) [4.52 - 4.88]	[4.37 - 4.90]

Table H.3. Combined-Site Statistical Summary for Fatty Acid Content as Percent Dry Weight in the Grain of MON 89034, Control and Commercial Corn

Component	MON 89034 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	Commercial [Range]
16:0 Palmitic (% DW)	0.29 (0.0050) [0.26 - 0.34]	0.29 (0.0052) [0.26 - 0.33]	[0.28 - 0.42]
16:1 Palmitoleic (% DW)	0.0040 (0.00009) [0.0033 - 0.0044]	0.0039 (0.00019) [0.0016 - 0.0047]	[0.0016 - 0.0064]
18:0 Stearic (% DW)	0.060 (0.0014) [0.053 - 0.073]	0.057 (0.0011) [0.052 - 0.067]	[0.044 - 0.084]
18:1 Oleic (% DW)	0.79 (0.013) [0.68 - 0.91]	0.78 (0.014) [0.72 - 0.87]	[0.58 - 1.18]
18:2 Linoleic (% DW)	1.95 (0.026) [1.85 - 2.20]	1.95 (0.041) [1.77 - 2.27]	[1.57 - 2.14]
18:3 Linolenic (% DW)	0.037 (0.00051) [0.034 - 0.041]	0.038 (0.00069) [0.034 - 0.042]	[0.031 - 0.045]
20:0 Arachidic (% DW)	0.012 (0.00025) [0.011 - 0.014]	0.012 (0.00023) [0.011 - 0.014]	[0.0096 - 0.017]
20:1 Eicosenoic (% DW)	0.0088 (0.00015) [0.0078 - 0.010]	0.0087 (0.00018) [0.0074 - 0.010]	[0.0066 - 0.011]
22:0 Behenic (% DW)	0.0049 (0.00015) [0.0042 - 0.0061]	0.0048 (0.00018) [0.0037 - 0.0061]	[0.0040 - 0.0060]

Table H.4. Combined-Site Statistical Summary for Fatty Acid Content as Percent Dry Weight in the Grain of MON 89034, Control and Commercial Corn

Component	MON 89034 Mean (S.E.) [Range]	Control Mean (S.E.) [Range]	Commercial [Range]
16:0 Palmitic (% Total Fat)	8.74 (0.051) [8.51 - 9.29]	8.69 (0.030) [8.55 - 8.90]	[8.75 - 12.18]
16:1 Palmitoleic (% Total Fat)	0.12 (0.0027) [0.11 - 0.14]	0.12 (0.0057) [0.046 - 0.13]	[0.046 - 0.18]
18:0 Stearic (% Total Fat)	1.80 (0.020) [1.68 - 1.92]	1.73 (0.0087) [1.69 - 1.81]	[1.44 - 2.35]
18:1 Oleic (% Total Fat)	23.73 (0.21) [22.34 - 25.36]	23.68 (0.23) [22.52 - 25.60]	[20.18 - 33.87]
18:2 Linoleic (% Total Fat)	58.78 (0.34) [56.57 - 61.07]	59.17 (0.33) [56.90 - 61.17]	[48.24 - 61.46]
18:3 Linolenic (% Total Fat)	1.13 (0.0090) [1.04 - 1.18]	1.16 (0.016) [1.10 - 1.34]	[0.85 - 1.42]
20:0 Arachidic (% Total Fat)	0.37 (0.0042) [0.34 - 0.40]	0.36 (0.0027) [0.34 - 0.37]	[0.29 - 0.46]
20:1 Eicosenoic (% Total Fat)	0.26 (0.0024) [0.25 - 0.28]	0.27 (0.0026) [0.24 - 0.28]	[0.22 - 0.31]
22:0 Behenic (% Total Fat)	0.15 (0.0048) [0.12 - 0.19]	0.15 (0.0038) [0.12 - 0.17]	[0.12 - 0.18]