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October 24, 2006

Dr. Neil E. Hoffman
Director, Environmental Risk Analysis Programs
USDA-APHIS-BRS
4700 River Road, Unit 98
Riverdale, MD 20737

Re: Request for the Determination of Non-regulated Status for MON 89034

Dear Dr. Hoffman:

Monsanto Company requests that the USDA-APHIS review the enclosed petition for the determination of non-regulated status for corn MON 89034.

Monsanto has developed, by the use of recombinant DNA techniques, MON 89034 as a second generation insect protection corn product to provide enhanced benefits for the control of lepidopteran insect pests. MON 89034 produces the Cry1A.105 and Cry2Ab2 proteins which belong to a family of insecticidal proteins derived from *Bacillus thuringiensis* (Bt). Compared to YieldGard® Corn Borer corn (MON 810), MON 89034 will better serve corn growers' needs by controlling a wider spectrum of lepidopteran pests. In addition, the combination of both Cry1A.105 and Cry2Ab2 proteins in a single plant provides a more effective insect resistance management tool.

The data submitted in this petition support the conclusion that MON 89034 is not likely to pose an increased plant pest potential or to have an adverse environmental impact compared to conventional corn. The introduction of MON 89034 is expected to provide economic, environmental and health benefits due to the protection of corn yield, reduction of chemical insecticide usage, reduction of mycotoxin levels in corn grain, and the increase of Bt corn product durability. The introduction of MON 89034 is not expected to change the current U.S. corn cultivation practices and the management of weeds, diseases and insects except for the control of lepidopteran insect pests.

® YieldGard is a registered trademark of Monsanto Technology LLC.

Monsanto Company, therefore, requests a determination from APHIS that the new biotechnology-derived corn MON 89034, any progeny derived from crosses between MON 89034 and conventional corn, and any progeny derived from crosses of MON 89034 with other deregulated biotechnology-derived corn be granted non-regulated status under 7 CFR Part 340.

The enclosed "Petition for the Determination of Non-regulated Status for MON 89034" contains relevant information upon which to make a determination. We are claiming the attachment (copies of study reports submitted to the U.S. EPA) as confidential business information (CBI). Therefore, we are also submitting a CBI deleted version of this petition which can be released to the public.

Contained within this package are:

- six copies (2 original and 4 copies) of the CBI deleted version of the petition
- one copy (original) of the CBI version of the petition including CBI attachment

Should you have any questions or require further information regarding this request or the enclosed petition, please feel free to contact Dr. Russell Schneider, Monsanto Regulatory Affairs Director in Washington DC at (202) 383-2866, or myself at (314) 694-2943.

Yours sincerely,

Yong Gao, PhD
Regulatory Affairs Manager

cc: Dr. Russell Schneider
Ms. Carolyn Carrera

MONSANTO

Petition for the Determination of Non-regulated Status for MON 89034

The undersigned submits this petition under 7 CFR Part 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR Part 340

October 24, 2006

OECD Unique Identifier: MON-89034-3
Monsanto Petition Number: 06-CR-166U

Prepared and Submitted by

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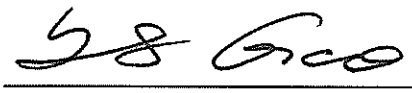
Release of Information

Monsanto is submitting the information in this petition for review by the USDA as part of the regulatory process. By submitting this information, Monsanto does not authorize its release to any third party. In the event the USDA receives a Freedom of Information Act request, pursuant to 5 U.S.C., § 552 and 7 CFR Part 1, covering all or some of this information, Monsanto expects that, in advance of the release of the document(s), USDA will provide Monsanto with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns. Monsanto expects that no information that has been identified as CBI (confidential business information), will be provided to any third party. Monsanto understands that a CBI-deleted copy of this information may be made available to the public in a reading room and by individual request, as part of a public comment period. Except in accordance with the foregoing, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

Certification

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



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Petition for the Determination of Non-regulated Status for MON 89034

Summary

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167) to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulation 7 CFR Part 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

Monsanto Company is submitting this request to APHIS for a determination of non-regulated status for the new biotechnology-derived corn product, MON 89034, any progeny derived from crosses between MON 89034 and conventional corn, and any progeny derived from crosses of MON 89034 with other biotechnology-derived corn which has been granted non-regulated status under 7 CFR Part 340.

Corn (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. Insect pests in the corn fields, if not properly controlled, significantly reduce corn yields and grain quality. In 1997, Monsanto commercialized a biotechnology-derived corn product, YieldGard® Corn Borer corn (i.e., MON 810) which contains the *Bacillus thuringiensis* (Bt) *cry1Ab* gene. The expression of Cry1Ab protein in corn plants provides effective protection against damage caused by lepidopteran insect pests, such as European corn borer. Since the launch of MON 810, several other Bt corn products have also been commercialized in the U.S., including YieldGard® Rootworm corn which expresses Cry3Bb1 protein that confers protection against coleopteran pests. In 2005, approximately 35% of the total U.S. corn acreage was planted with corn seed possessing insect resistance trait.

Monsanto has recently developed MON 89034 as a second generation insect protection corn product to provide enhanced benefits for the control of lepidopteran insect pests. MON 89034 produces the Cry1A.105 and Cry2Ab2 proteins derived from *Bacillus thuringiensis*, which are active against lepidopteran insect pests. Compared to MON 810, MON 89034 will better serve corn growers' need for controlling a wider spectrum of lepidopteran pests and help assure the durability of Bt corn. MON 89034 provides outstanding control of *Ostrinia* species such as European corn borer (ECB) and Asian corn borer, and *Diatraea* species such as southwestern corn borer (SWCB) and sugarcane borer. Control of these insects provided by MON 89034 is comparable to MON 810. MON 89034 also provides a high level control of fall armyworm (FAW) throughout the season, whereas MON 810 principally controls fall armyworm larvae during vegetative growth stage. Furthermore, MON 89034 provides significantly improved protection from

damage caused by corn earworm (CEW) than MON 810. In addition to the wider spectrum of insect control, the combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant, MON 89034, provides a much more effective insect resistance management (IRM) tool. The results of mathematical modeling indicate that biotechnology-derived plants expressing two Cry proteins will have significantly greater durability than plants producing either of the single proteins if the cross-resistance between the Cry proteins is low and the mortality of susceptible insects caused by each of the individual proteins is at least 90%. Comparative biophysical studies indicate that the Cry1A.105 and Cry2Ab2 proteins have important differences in their mode of action, specifically in the way in which they bind to the lepidopteran midgut. Therefore, the probability of cross-resistance between these two proteins is low. Furthermore, *in vitro* and *in planta* studies with Cry1A.105 and Cry2Ab2 demonstrate that both proteins are highly active against the primary lepidopteran pests of corn (ECB, SWCB, CEW, and FAW), particularly ECB, achieving close to or greater than the critical 95% level of control in all cases. With these properties, MON 89034 should be durable with a significantly smaller structured refuge than is necessary for Bt corn products producing a single insecticidal protein.

The data and information presented in this application demonstrate that MON 89034 is not likely to pose an increased plant pest potential or to have an adverse environmental impact compared to conventional corn. This conclusion is based on multiple lines of evidence. The first is the detailed molecular characterization of the inserted DNA. Results confirm the insertion of a single functional copy of *cry1A.105* and *cry2Ab2* expression cassettes at a single locus within the corn genome. The second is a detailed biochemical characterization of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034. In addition, the data demonstrate that the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 are equivalent to the respective Cry1A.105 and Cry2Ab2 proteins produced by recombinant strains of *Escherichia coli*, which were used in the various safety assessment studies. The third line of evidence is an assessment of the toxicity and allergenicity potential of the Cry1A.105 and Cry2Ab2 proteins based on extensive information collected and studies performed on the two proteins. The results demonstrate with reasonable certainty that the Cry1A.105 and Cry2Ab2 proteins are unlikely to be allergens or toxins. The fourth line of evidence is the compositional and nutritional assessment which confirms that MON 89034 grain and forage are compositionally equivalent to and as safe as those of conventional corn. The fifth line of evidence is the extensive evaluation of the MON 89034 phenotypic and agronomic characteristics and ecological interactions, which demonstrates that MON 89034 is not likely to have an increased plant pest potential compared to the conventional corn. Finally, an assessment on the potential impact on non-target organisms (NTO) and endangered species concludes that MON 89034 is unlikely to have adverse effects on these organisms under the conditions of use.

MON 89034 was produced by *Agrobacterium*-mediated transformation of corn with PV-ZMIR245, which is a binary vector containing 2T-DNAs. 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* (neomycin phosphotransferase II)

expression cassette. During transformation, both T-DNAs were inserted into the genome. The *nptII* gene was used as the selectable marker which was needed for selection of the transformed cells. Once the transgenic cells were identified, the selectable marker gene was no longer needed. Therefore, traditional breeding was used to isolate plants that only contain the *cry1A.105* and *cry2Ab2* expression cassettes (T-DNA I) and do not contain the *nptII* expression cassette (T-DNA II), thereby, producing marker-free corn MON 89034. Molecular characterization of MON 89034 by Southern blot analyses 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 are present in the inserted DNA as expected with the exception that the *e35S* promoter, which 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. 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 was demonstrated by the fact that the Southern blot fingerprint of MON 89034 was maintained for seven generations tested in the breeding history. Additionally, T-DNA II analysis of multiple generations of MON 89034 indicated that there were no T-DNA II elements present other than those which are common to T-DNA I, including *35S* promoter, *nos* 3´ end sequence, and Left Border sequence. Furthermore, these generations have been shown not to contain any backbone sequence from plasmid PV-ZMIR245. The stability was further confirmed by the fact that the inheritance of the lepidopteran protection trait in MON 89034 follows Mendelian segregation principle.

The expression levels of Cry1A.105 and Cry2Ab2 proteins were determined in MON 89034 tissues produced from multiple field sites in the major U.S. corn production regions. The results demonstrated that both Cry1A.105 and Cry2Ab2 proteins were expressed in all tissues collected, including leaf, root, forage, silk, pollen, grain and stover. The mean Cry1A.105 protein levels (µg/g dwt) across all test sites were 5.9 in grain, 42 in forage, 12 in pollen, 520 in leaves of plants at V2-V4 stage, 120 in leaves of plants at pre-VT stage, 12 in forage root, and 50 in stover. In tissues harvested throughout the growing season, mean Cry1A.105 protein levels (µg/g dwt) across all test sites varied from 72-520 in leaf, 11-79 in root, and 42-380 in whole plant. In general, levels of the Cry1A.105 protein declined over the growing season. The mean Cry2Ab2 protein levels (µg/g dwt) across all test sites were 1.3 in grain, 38 in forage, 0.64 in pollen, 180 in leaves of plants at V2-V4 stage, 160 in leaves of plants at pre-VT stage, 21 in forage root, and 62 in stover. In tissues harvested throughout the growing season, mean Cry2Ab2 protein levels (µg/g dwt) across all test sites varied from 130-180 in leaf, 21-58 in root, and 38-130 in whole plant. In general, levels of the Cry2Ab2 protein declined over the growing season.

Safety assessment studies require large amount of the Cry1A.105 and Cry2Ab2 proteins. The expression levels of the two proteins in MON 89034 were too low to allow for purification of sufficient quantities of the two proteins directly from MON 89034 for use

in the safety assessment studies. Therefore, it was necessary to produce the Cry1A.105 and Cry2Ab2 proteins in a high-expressing recombinant host organism, *E. coli*. The proteins produced by *E. coli* were engineered to match the amino acid sequences of their counterparts expressed in MON 89034. Thus, the physicochemical and functional equivalence of MON 89034-produced and *E. coli*-produced proteins was examined to ensure that the proteins from the two host sources were equivalent so that the *E. coli*-produced proteins could be used as surrogates in the studies. Small quantities of the Cry1A.105 and Cry2Ab2 proteins were purified from the grain of MON 89034. Large quantities of Cry1A.105 and Cry2Ab2 proteins were produced and purified from *E. coli*. The proteins from the two sources were characterized and the equivalence was evaluated based a panel of analytical tests and assays, including Western blot analysis; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); N-terminal sequence analysis; glycosylation analysis; and insect activity bioassay. The results provide 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 history of safe use and data from multiple studies support the safety of MON 89034 and the Cry1A.105 and Cry2Ab2 proteins. The two proteins belong to a family of Cry proteins from *Bacillus thuringiensis*, an organism which has been used commercially in the U.S. for over four decades to produce microbial pesticides. Bt corn expressing several Cry1 proteins and Bt cotton expressing both Cry1 and Cry2Ab2 proteins have been cultivated in large areas in the U.S. and other countries for up to a decade. The extremely low mammalian toxicity of Bt-based microbial insecticides and Cry proteins has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins. Cry1A.105 and Cry2Ab2 proteins do not share any amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins which have adverse effects to mammals. This has been shown by extensive assessments with bioinformatic tools, such as FASTA sequence alignment search and an eight-amino acid sliding window search. Cry1A.105 and Cry2Ab2 proteins are rapidly digestible in simulated gastric fluids (SGF). Greater than 95% to 99% of these proteins were digested in SGF in less than 30 seconds. Proteins that are rapidly digestible in mammalian gastrointestinal systems are unlikely to be allergens 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 the highest does levels tested, which are 2072 and 2198 mg/kg body weight for Cry1A.105 and Cry2Ab2 proteins, respectively. The dietary safety assessment based on the acute toxicity data and corn product dietary pattern establishes that the margins of exposure (MOE) for the overall U.S. population are ≥199,000 and 981,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively. And the MOEs are ≥79,400 and 390,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively, for children aged 3-5 years old, an age group with the highest corn consumption per unit body weight. For poultry and livestock, the MOEs range between 1,930–13,500 and 2,160–47,600 for the Cry1A.105 and Cry2Ab2 proteins, respectively. Taken together, these data indicate that food and feed drived from MON 89034 which contains the Cry1A.105 and Cry2Ab2 proteins are safe for consumption.

Compositional assessment of the grain and forage from multiple field sites in the major U.S. corn production regions demonstrate that MON 89034 is nutritionally and compositionally equivalent to, and as safe and nutritious as its conventional counterpart. The compositional analyses were conducted on a total of 77 components, nine in forage and 68 in grain, including protein, fat, carbohydrate, fiber, ash, moisture, amino acids, fatty acids, vitamins, anti-nutrients, secondary metabolites and minerals. The results were within the range of data expected for these types of analyses. Of the 77 components analyzed, the results of 16 components were below the limit of quantitation for over half of the analyses conducted. As such, these 16 components were not included in the statistical analyses. Among the remaining 61 components, analyses of the data across all test sites (combined sites) indicate that there were no statistical differences for 58 components. The three analytes with statistical differences were phosphorus in forage, and stearic acid (C18:0) and arachidic acid (C20:0) in grain. The differences observed are generally small (3.4 – 19.2%) considering the range of natural variability, and the mean levels and ranges of these three analytes of MON 89034 are well within the ranges of values observed for the 15 reference commercial corn hybrids grown along the side of MON 89034, and within the ranges in the International Life Sciences Institute crop composition database, as well as within the published literature ranges. Therefore, it is concluded that the slight difference observed for these 3 components is not biologically meaningful, and MON 89034 and the control corn are compositionally equivalent.

The phenotypic, agronomic, and ecological interaction assessment indicates that MON 89034 is comparable to conventional corn and is unlikely to have any increased plant pest risk. An important element in assessing plant pest potential and environmental impact of MON 89034 is to compare MON 89034 to conventional corn. The assessment is based initially on the concept of familiarity, which USDA recognizes plays an important role in assessments. Familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant variety whose biological properties and plant pest potential are known to experts. Familiarity considers the biology of the crop, the introduced trait, the receiving environment and the interaction among these factors, and provides a basis for comparative risk assessment between a biotechnology-derived plant and its conventional counterpart. Results from the phenotypic and agronomic characteristics assessments indicate that MON 89034 does not possess characteristics that would confer an increased plant pest risk compared to conventional corn. The assessments are based on a combination of laboratory experiments and field studies conducted by scientists who are familiar with the production and evaluation of corn. In each of these studies, MON 89034 was compared to an appropriate conventional corn hybrid which has a genetic background similar to MON 89034 but does not possess the lepidopteran-protection trait. In addition, multiple commercial corn hybrids were also employed to provide a range of values that are common to the commercial corn hybrids for each measured characteristic. These assessments included five seed germination parameters, two pollen characteristics, 14 plant growth and development characteristics, and more than 70 observations for each of the plant-insect, plant-disease and plant responses to abiotic stressor interactions.

Seed dormancy and germination characterization observed no viable hard seed in any of the temperature regimes tested. No statistically significant differences were detected between MON 89034 and the control corn for pollen diameter or viability. The phenotypic and agronomic characteristics data collected from 18 field test sites in 2004 and 2005 demonstrate that no significant differences were detected between MON 89034 and the control corn for seedling vigor, early stand count, final stand count, days to 50% pollen shed, days to 50% silking, stay green, ear height, dropped ears, root lodged plants, grain moisture, test weight and yield. Differences in plant height and number of stalk lodged plants were detected in 2004 trials but not in 2005 trials. In 2004 trials, plant height was slightly lower for MON 89034 compared to control (84.1 vs. 85.4 inches), and stalk lodged plants were less for MON 89034 than for the control (0.8 vs. 2.4 per plot). The magnitude of these differences are generally small, similar differences are not detected in 2005 trials, and the mean values of MON 89034 fall well within the ranges of values observed for the 23 reference commercial corn hybrids grown along the side of MON 89034. Therefore, these two differences observed in 2004 field trials are not considered biologically meaningful and are unlikely to contribute to increased plant pest potential. In addition to the phenotypic and agronomic characteristics, observational data on the presence of and differential response to biotic (insects, diseases) and abiotic (drought, wind, nutrient deficiency) stressors were collected in the two years of field trials to examine the ecological interactions of MON 89034 compared with those of the conventional control corn. Based on 255 comparative observations recorded over two years, no repeatable differences were observed across sites between MON 89034 and the control in their susceptibility or tolerance to the ecological stressors assessed. The result supports the conclusion that compared to conventional corn, the ecological interactions between MON 89034 and insects, diseases, and abiotic stressors were not altered except for the introduced lepidopteran-protection trait.

The environmental assessment of MON 89034 and Cry1A.105 and Cry2Ab2 proteins indicates that these two proteins pose no adverse effect on non-target organisms (NTOs) and endangered species under the conditions of use. The assessment took into consideration several components, including the familiarity with the mode of action of Cry proteins, the activity spectra of the Cry1A.105 and Cry2Ab2 proteins, the expression levels of the two proteins in MON 89034, the environmental fate of the proteins, the lack of interaction between the two proteins, and feeding tests of the two proteins or MON 89034 corn materials to representative NTOs. The tested NTOs include one mammalian species (mice), two avian species (broiler chicken and bobwhite quail), one aquatic species (Daphnia), two species of soil decomposers (Collembola and earthworm), and four beneficial insect species (honeybee, minute pirate bugs, ladybird beetle, and parasitic wasp). The estimates of MOEs for the non-target insects exposed to Cry1A.105 and Cry2Ab2 proteins are ≥14. Risk assessments of potential effects on the federally listed threatened or endangered species indicated that only Karner blue butterfly (*Lycaeides melissa samuelis*) has the potential to occur in proximity to corn fields. There are only two Wisconsin counties in the Midwestern corn belt where potential temporal overlap between Karner blue butterfly larvae and corn pollen shed is likely. Based on a conservative risk analysis model, it is shown that the margin of safety for Karner blue is ≥12 fold using the highest possible pollen concentration from MON 89034. Taken

together, these data support the conclusion that MON 89034 is unlikely to have adverse effects on NTOs and endangered species under the conditions of use.

The potential for MON 89034 outcrossing to sexually compatible species is unlikely in the U.S. Corn and annual teosinte (*Zea mays* subsp. *mexicana*) are genetically compatible, wind-pollinated and, in areas of Mexico and Guatemala, freely hybridize when in close proximity to each other. However, teosinte is not present in the U.S. other than as an occasional botanical garden specimen. Differences in factors such as flowering time, geographical separation and development factors make natural crosses in the U.S. highly unlikely. In contrast with corn and teosinte, special techniques are required to hybridize corn and *Tripsacum*. With the exception of *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with corn, and the open literature indicates that the offspring of the cross show varying levels of sterility. *Tripsacum*-corn hybrids have not been observed in the field. Therefore, the environmental consequence of pollen transfer from MON 89034 to other wild plant species is considered negligible.

Finally, an assessment of current corn agronomic practices determined that the introduction of MON 89034 will not impact cultivation practices and the management of weeds, diseases and insects except for the control of lepidopteran insect pests. As with other lepidopteran-protected Bt corn, MON 89034 provides a simple and highly effective means for controlling lepidopteran pests. The approach is environmentally benign and helps to preserve beneficial insects and decrease cultivation input, and requires fewer chemical insecticide applications. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides not only a wider spectrum of pest control but also a better insect resistance management tool.

Based on the data and information presented in this submission, it is concluded that MON 89034 is not likely to pose an increased plant pest potential or to have an adverse environmental impact compared to conventional corn. The successful adoption of MON 89034 is expected to increase economic, environmental and health benefits due to the protection of corn yields, decrease of chemical insecticide usage, reduction of mycotoxin levels in corn grain, and increase of Bt corn product durability. Therefore, Monsanto Company requests a determination from APHIS that MON 89034 and any progeny derived from crosses between MON 89034 and other commercial corn varieties be granted non-regulated status under 7 CFR Part 340.

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Abbreviations, Acronyms and Definitions

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

2T-DNA	Plasmid vector containing two separate T-DNA regions each surrounded by left and right borders of the Ti plasmid
35S	The promoter and leader from the cauliflower mosaic virus (CaMV) 35S RNA
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*
ADF	Acid detergent fiber
ANOVA	Analysis of Variance
AOCS	American Oil Chemists Society
AOSA	Association of Official Seed Analysts
BCW	Black cutworm, *Agrotis ipsilon*
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
Cab	The 5' untranslated leader of the gene encoding wheat chlorophyll a/b-binding protein
CaMV	cauliflower mosaic virus
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CBI	Confidential business information
CEW	Corn earworm, *Helicoverpa zea*
CFR	Code of Federal Regulations
Cry	Crystal proteins from *Bacillus thuringiensis*
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 Cry1A.105 protein
Cry2Ab2	A Cry2 class crystal protein from *Bacillus thuringiensis* subsp. *kurstaki*.
cry2Ab2	Coding sequence for Cry2Ab protein
CTAB	Hexadecyltrimethylammonium bromide
CTP	Chloroplast transit peptide
CV	Column volume
DAP	Days after planting

dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DT_{50}	Time to 50% dissipation of a protein in soil
DT_{90}	Time to 90% dissipation of a protein in soil
DTT	Dithiothreitol
DW	Dry weight
dwt	Dry weight of tissue
E. coli	*Escherichia coli*
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*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	United States Environmental Protection Agency
EPPS	4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
FA	Fatty acid
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FAW	Fall armyworm, *Spodoptera frugiperda*
FDA	United States Food and Drug Adminstration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMV	Figwort mosaic virus 35S promoter
FW	Fresh weight
fwt	Fresh weight of tissue
GDU	Growing degree units
HEPES	N-[2-(Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]
HRP	Horseradish peroxidase
Hsp17	The 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
IgG	Immunoglobulin G
ILSI-CCD	International Life Sciences Institute crop composition database
IRM	Insect resistance management

LB buffer	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]
LC_{50}	LC stands for lethal concentration. LC_{50} is the concentration of a substance which causes the death of 50% (one half) of a group of test organisms
Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
LOD	Limit of detection
LOQ	Limit of quantitation
LSD	Least significant difference
MALDI-TOF MS	Matix-assisted laser desorption/ionization time-of-flight mass spectrometry
MEEC	Maximum expected environmental concentration
MH^+	Protonated mass ion
MMT	Million metric tones
MOE	Margin of exposure
MON 810	A Monsanto corn product, producing the insecticidal Bt Cry1Ab protein
MON 863	A Monsanto corn product, producing the insecticidal Bt Cry3Bb1 protein
MON 89034	A Monsanto corn product, and the subject of this application, which produces Bt Cry1A.105 and Cry2Ab2 proteins
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut-off
MWM	Molecular weight marker
N/A	Not applicable
NCGA	National Corn Growers Association
NDF	Neutral detergent fiber
NFDM	Non-fat dried milk
NK603	A Monsanto corn product producing the glyphosate-tolerant CP4 EPSPS protein
NMWC	Nominal molecular weight cut-off
NOEC	No Observable Effect Concentration
NOEL	No Observable Effect Level
nos	The nopaline synthase gene from *Agrobacterium tumefaciens*
nptII	Coding sequence of neomycin phosphotransferase II gene that confers resistance to neomycin and kanamycin
NPTII	Neomycin phosphotransferase II

NTO	Non-target organism
OECD	Organization for Economic Co-operation and Development
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
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% (v/v) Tween-20
PCR	Polymerase chain reaction
PIP	Plant-incorporated protectant
PMSF	Phenylmethanesulfonyl fluoride
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*
SAP	Scientific Advisory Panel organized by US EPA
SAS	Statistical Analysis System
SCB	Sugarcane borer, *Diatraea saccharalis*
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SGF	simulated gastric fluid
SSU-CTP	The DNA encoding the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit including the first intron
SWCB	Southwestern corn borer, *Diatraea grandiosella*
TDF	Total dietary fiber
T-DNA I	Transfer DNA containing the *cry1A.105* and *cry2Ab2* expression cassettes in plasmid vector PV-ZMIR245

T-DNA II	Transfer DNA containing the *nptII* gene cassette in plasmid vector PV-ZMIR245
T-DNA	Transfer DNA
TFA	Trifluoroacetic acid
TMB	3,3',5,5'-tetramethylbenzidene
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylenesorbitan monolaurate
USDA-APHIS	United States Department of Agriculture – Animal and Plant Health Inspection Service
USDA-ERS	United States Department of Agriculture – Economic Research Service
USDA-NASS	United States Department of Agriculture – National Agricultural Statistics Service
USDA-NRCS	United States Department of Agriculture – National Resources Conservation Service
USFWS	United States Fish and Wildlife Service
WBCW	Western bean cutworm, *Striacosta albicosta*

I. Rationale for the Development of MON 89034

I.A. Basis for the Request for a Determination of Nonregulated Status under 7 CFR Part 340.6

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772) and the Plant Quarantine Act (7 U.S.C. § 151-167), to prevent the introduction and dissemination of plant pests into the United States. The APHIS regulation 7 CFR Part 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article.

I.B. MON 89034

Monsanto has developed a biotechnology-derived corn, MON 89034, which produces the Cry1A.105 and Cry2Ab2 insecticidal proteins and is protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry1A.105 is a modified *Bacillus thuringiensis* (Bt) Cry1A protein. Cry2Ab2 is a protein derived from Bt (subsp. *kurstaki*). The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides effective insect control against a broad range of lepidopteran insect pests and offers an effective and durable insect-resistance management tool.

I.C. Rationale for the Development of MON 89034 and Benefits

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, approximately 42.5 million acres of corn planting area in the U.S. (or 52% of the total U.S. corn acreage) was planted with biotechnology-derived corn seed, and 28.6 million acres (or 35% of the total corn acreage) were planted with corn seed possessing insect resistance (Bt) trait (USDA-NASS, 2006).

In the U.S., both corn yields and grain quality are significantly negatively impacted by insect pests (James, 2003). In 1997, Monsanto commercialized the biotechnology-derived corn product, YieldGard® Corn Borer corn (i.e., MON 810) which contains Bt *cry1Ab* gene that encodes the Cry1Ab protein, providing 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

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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 pests such as ECB and CEW. In addition to the benefits as an effective insect pest management tool, MON 810 also resulted in lower levels of harmful mycotoxins in corn grain, which has resulted in improved food and feed safety of the corn products. The mycotoxin reduction effect was due to the decrease of insect damage to the Bt corn ears and thereby reducing the entering routes by mycotoxin-producing fungi. The mycotoxin reduction 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; Brookes and Barfoot, 2005). Within ten years since the first Bt corn product introduction, the safe and effective use of Bt corn has been adopted globally on over 44 million acres in 2005 to control several primary insect pests in 12 countries (James, 2005).

Recently, Monsanto developed MON 89034 as a second generation insect protection corn to provide enhanced benefits for the control of lepidopteran pests. Compared to MON 810, MON 89034 will even better serve corn growers' need for controlling a wider spectrum of lepidopteran pests, reducing the levels of mycotoxins in grain, enabling more efficient plant breeding of this multi-genic trait into superior hybrids, and reducing the refuge acreage needed for insect resistance management. By producing efficacious 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 frugiperda*) and black cutworm (BCW, *Agrotis ipsilon*) compared to Cry1Ab. The Cry2Ab2 protein provides improved control over Cry1Ab 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 will enhance the economic benefits to growers and improve the quality of grain and the safety of food and feed products derived from the grain. In addition, MON 89034 was developed to allow the efficient introgression of two insect protection genes into improved corn germplasm, which will reduce the time and costs for the introduction of new improved varieties 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:

1) *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 (FAW) 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. 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.

2) *Improved Insect Resistant Management (IRM)*: MON 89034 produces two different Bt proteins, Cry1A.105 and Cry2Ab2, both are highly efficacious against a variety of lepidopteran pests. The mechanism of insecticidal activity or mode of action of Cry proteins consists of a number of steps e.g., solubilization, activation, receptor binding, oligomerization, 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 mode of action, which influences the interactions of these proteins with susceptible insects without qualitatively influencing their host range. Several lines of evidence establish that Cry1A.105 and Cry2Ab2 have important differences in their mode of actions, particularly in the way in which they bind to the lepidopteran midgut. These proteins have different primary 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 in MON 89034, the likelihood of resistance evolution is significantly reduced compared to single protein products. 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.

3) *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, as such it will provide greater yield protection and result in continuous reduction for potential mycotoxin

contamination of the corn products. Corn ears that are protected from feeding damage caused by ECB, CEW, and FAW would have fewer ports of entry for invasion by mycotoxin-producing fungi, which will make the grain even safer to consume. 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 insect control spectrum of MON 89034 compared to MON 810, it is expected that there will be a greater benefit to food and feed consumed globally.

4) *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 proteins 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 Bt 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 costly because of duplicated work to introgress two transformation events into new germplasms. MON 89034 can reduce the time and cost factors in breeding programs to introgress only a single transformation event.

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

I.D. Adoption of MON 89034

Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2006, approximately 40% of the total corn acreage in the U.S. was planted with corn seed possessing insect resistance (Bt) trait (USDA-NASS, 2006). Growers are expected to adopt MON 89034 in those regions of the U.S where lepidopteran pest pressure is high. The major area of adoption is likely to be the U.S. corn belt including Illinois, Indiana, Iowa, Kansas, Kentucky, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin.

I.E. Submissions to Other Regulatory Agencies

I.E.1. Submissions to the EPA

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act [7 U.S.C. §136(u)], are subject to regulation by the Environmental Protection Agency (EPA). Pesticides produced *in planta*, referred to as plant-incorporated protectants (PIP's), are also subject to regulation by the EPA.

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 Cry1A.105 and Cry2Ab2 proteins in 2005. In July 2006, EPA established a temporary exemption from the requirement of a tolerance for the plant-incorporated protectant Bt Cry1A.105 protein and the genetic material necessary for its production in all corn commodities including field corn, sweet corn, and popcorn (40 CFR §174.453). Likewise, temporary exemption from the requirement of a tolerance for Bt Cry2Ab2 protein and the genetic material necessary for its production in corn was also granted by EPA in July 2006 (40 CFR §174.454).

Monsanto has filed an application, dated September 28, 2006, to the EPA for the registration of the plant-incorporated protectant *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 proteins, and the genetic material (vector PV-ZMIR245) necessary for their production including field corn, sweet corn, and popcorn.

I.E.2. Submission to the FDA

MON 89034 falls within the scope of the Food and Drug Administration's (FDA) policy statement concerning regulation of products derived from new plant varieties, including those produced through genetic engineering. Monsanto has voluntarily initiated and will complete a consultation process with FDA prior to commercial distribution of this product. A safety and nutritional assessment of food and feed derived from MON 89034 was submitted to the FDA dated October 13, 2006.

I.E.3. Submissions to Foreign Government Agencies

Regulatory submissions for import and production approvals will be made to countries that import U.S. corn grain and have regulatory approval processes in place. These will include submissions to a number of foreign government regulatory agencies, including Japan's Ministry of Agriculture, Forestry and Fisheries (MAFF) and the Ministry of Health, Labor and Welfare (MHLW); the Canadian Food Inspection Agency (CFIA) and Health Canada; Argentina's National Advisory Committee on Agricultural Biotechnology (CONABIA) and the National Service of Agricultural and Food Health and Quality (SENASA); the European Food Safety Authority (EFSA), and the regulatory authorities in many other countries. As appropriate, notifications of import will be made to importing countries that do not have a formal approval process.

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II. The Biology of Corn

This section summarizes the biology of corn based on: 1) the consensus document on the biology of corn (maize) developed as part of the Organization for Economic Co-operation and Development (OECD, 2003); 2) a summary prepared by the Biotechnology Regulatory Services of the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS, 2006); 3) information provided in the USDA petition for MON 88017 (Monsanto Company petition No. 04-CR-108U); and 4) other published literature.

II.A. Corn as a Crop

Corn (*Zea mays* L.), or maize, is one of the few major crop species indigenous to the Western Hemisphere. It is a member of the *Maydeae* tribe of the grass family, *Poaceae*. It is a robust monoecious annual plant, which requires the help of man to disperse its seeds for propagation and survival. Corn is an efficient plant for capturing the energy of the sun and converting it into food, and adapts readily to different conditions of humidity, sunlight, altitude, and temperature.

Corn is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) in total global production. In 2004, corn was planted globally on 146.7 million ha with a total production of 723.9 million metric tones (MMT) (FAOSTAT, 2006). The top three production countries in 2004 were: USA (299.9 MMT), China (130.4 MMT) and Brazil (41.8 MMT). In the U.S., corn is grown in almost all the states, and in 2004, it produced 11.8 billion bushels of corn grain with a market value of $24 billion (USDA-NASS, 2006).

In industrialized countries corn has two major uses: 1) as animal feed in the form of grain, forage or silage; and 2) as a raw material for wet- or dry-milled processed products such as high fructose corn syrup, oil, starch, glucose, and dextrose (Tsaftaris, 1995). These processed products are used as ingredients in many industrial applications and in human food products. In developing countries, corn is used in a variety of ways. In Latin American countries such as Mexico, one of the main uses of corn is for food. In Africa, corn is consumed as a food in the sub-Saharan region, and in Asia it is generally used to feed animals (Morris, 1998).

Corn has been studied extensively, and it seems that the domestication of corn occurred in southern Mexico 7,000 to 10,000 years ago. 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 know it today, cannot survive in the wild because the female inflorescence (the ear) restricts seed dispersal. Although grown extensively throughout the world, corn is not considered a persistent weed or one difficult to control. A summary of the history, taxonomy, genetics, and life cycle of corn is

presented below, followed by a discussion of gene flow between cultivated corn and its wild relatives.

II.B. History of Corn

Corn originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus discovered the Western Hemisphere, corn was being grown by the indigenous people from Chile to southern Canada. Columbus noted the presence of corn on the north coast of Cuba in November 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.

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 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 United States appears 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 led eventually 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 distinct 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).

II.C. Taxonomy

Corn (*Zea mays* L.) is a member of the tribe *Maydae*, which is included in the subfamily *Panicoideae* of the grass family *Gramineae*. **Table II-1** summarizes the taxonomic classification of corn and its close relatives.

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.

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

The other genus included in the *Maydae* tribe is *Tripsacum*. *Tripsacum* includes 16 species with a basic set of 18 chromosomes (n = 18), and the different species of *Tripsacum* include multiples of 18 chromosomes ranging from 2n = 36 to 2n = 108 (**Table II-1**). An additional species, *Tripsacum hermaphrodita* (*Anthephora hermaphrodita*), has been described in the literature but has not been formally included in this taxonomic classification. In addition to their occurrence in other regions of the Americas, five *Tripasacum* species are native to the U.S. (USDA-NRCS, 2006). *Tripsacum dactyloides,* or Eastern gamagrass, is the most widely distributed species and can be found in the Midwestern, Eastern and Southern regions of the U.S. The other species have a more limited geographical distribution and can be found in the following states and territories: *Tripsacum floridanum* in Florida, *Tripsacum lanceolatum* in Arizona and New Mexico, *Tripsacum fasciculatum* in Puerto Rico, and *Tripsacum*

hermaphrodita in Florida, Hawaii, Puerto Rico and the Virgin Islands (USDA-NRCS, 2006). Twelve of 16 *Tripsacum* species are native to Mexico and Guatemala. *Tripsacum australe* and two other species are native to South America. The center of variation for *Tripsacum* is the western slopes of Mexico, the same area where teosinte is frequently found. The habitat preferences of *Tripsacum* are similar to those for teosinte: seasonally dry, summer rains, elevation of 1500 m, and limestone soils (Wilkes, 1972).

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

II.D. Genetics

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

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

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

The corn genome is approximately 5×10^6 kb in size (OECD, 2003). It includes highly repeated sequences that constitute about 20% of the genome; these sequences are present in about ten superabundant sequence types. There are more than 1000 different moderately repetitive sequence families, collectively representing 40% of the genome. The remaining 40% of the genome contains single-copy sequences, or more than 10^6 gene-size regions.

Table II-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*

II.E. Life Cycle

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

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

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

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

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

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

II.F. Hybridization

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

The fundamental concepts for development of hybrid corn were defined by 1920 (Sprague, 1946). Basic studies on the genetic composition of a corn variety were conducted to determine the effects of self pollinating (or inbreeding, which is the opposite of outcrossing) within a corn variety (Shull, 1908). Because corn naturally cross-fertilizes, the genetic composition of each plant is not known. Continuous selfing of individuals for 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. Recent techniques such as marker-assisted breeding have reduced the time and number of generations required to produce pure inbreds (Youseff and Juvik, 2001).

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 the growers (Shull, 1909).

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

II.G. Pollination

II.G.1. Outcrossing with Wild *Zea* Species

Annual teosinte (*Zea mays* subsp. *mexicana*) and corn are wind-pollinated, self-compatible, and are highly variable, interfertile species (Wilkes, 1972 and 1989). Corn and teosinte are genetically compatible, and in areas of Mexico and Guatemala they freely hybridize when they are in proximity to each other and other conditions are favorable. Teosinte exists primarily as a weed around the margins of corn fields, and the frequency of hybrids between teosinte and corn has been studied. A frequency of one F_1 hybrid (corn × teosinte) for every 500-corn plants has been reported for the Chalco region of the Valley of Mexico (Wilkes, 1972). The F_1 hybrid of teosinte by corn is robust and fertile and is capable of backcrossing to corn. Intercrossing and gene exchange between teosinte and corn occurs freely, and, accompanied by selection, teosinte had a significant role in the evolution of corn. Recently, Evans and Kermicle (2001) have shown that although corn can introgress into teosinte, there is incompatibility between some corn populations and certain types of teosinte, resulting in low fitness of some hybrids that prevents a high rate of introgression.

Although corn easily crosses with teosinte, teosinte is not present in the U.S. corn belt. The natural distribution of teosinte is limited to the seasonally dry, subtropical zone with summer rain along the western escarpment of Mexico and Guatemala and the Central Plateau of Mexico (Wilkes, 1972; Gonzalez and Corral, 1997). Dependent upon the human characterization of teosinte with its local environment, it may be considered a weed. However, it has been noted that populations of teosinte have been in decline for several decades because of increased grazing and urbanization in Mexico (Wilkes, 1995). Except for special plantings, there are no reports of teosinte occurring in the United States.

II.G.2. Outcrossing with *Tripsacum* Species

Tripsacum evolved by polyploidy, whereas corn and teosinte have undergone introgressive hybridization at the diploid level (2n = 20). The diploid forms of *Tripsacum* (2n = 36) are morphologically distinct and allopathic in their distribution (Wilkes, 1989). *Tripsacum* species are perennials and seem to be more closely related to the genus *Manisuris* than to either corn or teosinte (Goodman, 1976). *Tripsacum* received greater interest in the evolution of corn after Mangelsdorf and Reeves (1931) successfully crossed corn and *Tripsacum dactyloides* (2n = 36). The cross by Mangelsdorf and Reeves (1931) was made with the diploid *Tripsacum dactyloides* (2n = 36) as the male parent. Silks of the female corn parent were cut to permit successful pollination. The cross had 28 chromosomes and was male sterile. Five other *Tripsacum* species have been crossed with corn, and Galinat (1988) has mapped more than 50 homologous loci on the chromosomes of corn and *Tripsacum*.

In contrast with corn and teosinte, which can be easily hybridized, both in the wild and by controlled pollinations, special techniques are required to hybridize corn and *Tripsacum*.

With the exception of *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with corn, and the offspring of the cross show varying levels of sterility. *Tripsacum*-corn hybrids have not been observed in the field, and *Tripsacum*-teosinte hybrids have not been produced (Wilkes, 1972).

In recent years additional research has been conducted on the hybridization of corn with *Tripsacum* species. Eubanks (1995, 1998) has developed a method for transferring *Tripsacum* genes into corn. In this method two wild relatives of corn, *Tripsacum* and diploid perennial teosinte (*Zea diploperennis*), were crossed to produce a hybrid, which is called tripsacorn and can be used to generate corn-tripsacorn hybrids. The use of tripsacorn is intended to confer resistance to pests and disease, drought tolerance, and improved uniformity. Eubanks (2000) has claimed that traits such as apomixis, totipotency, perennialism, adaptation to adverse soil conditions and to a carbon dioxide enriched atmosphere can be transmitted to corn by such techniques. While these discoveries show promise, additional research needs to be conducted to determine their widespread applicability.

In experiments with *Tripsacum dactyloides* or Eastern gamagrass, DeWald and others at the USDA successfully obtained a true *Tripsacum* cytoplasm with a corn nuclear background (DeWald et al., 1999). In this experiment Eastern gamagrass was used as the female parent and corn as the male or pollen donor. The *Tripsacum*-derived mitochondrial chondrome and chloroplast plastome in these hybrids contribute to the seed qualities of the plants, but the nuclear genome appeared to be totally corn in origin. These results suggest that any possibility of corn contributing genetic material to Eastern gamagrass through random pollen flow in agricultural situations is extremely remote, and if this were to occur, the resultant genome would be lacking in most or all of the corn chromosomal complement.

II.G.3. Outcrossing with Cultivated *Zea* Varieties

Corn is wind-pollinated, and the distances that viable pollen can travel depend on prevailing wind patterns, humidity, and temperature. Occasionally it has been found that corn pollen can travel up to 3.2 km (2 miles) by wind under favorable conditions. All corn will interpollinate, except for certain popcorn varieties and hybrids that have one of the dent-sterile gametophyte factors (Ga^s, Ga, and ga allelic series on chromosome 4). Pollen of a specific hybrid can be carried by wind to pollinate other dent corn, sweet corn, and popcorn if the popcorn does not carry the dent-sterile gametophyte factor. Corn pollen, therefore, moves freely within an area, lands on silks of the same cultivar or different cultivars, germinates almost immediately after pollination, and completes fertilization within 24 hours. Although there may be some minor differences in the rate of pollen germination and pollen tube elongation on some genotypes, corn pollen is promiscuous. It is estimated each corn plant can shed more than 10 million pollen grains.

Certification standards for distances between different corn genotypes have been established to assist in the production of hybrid corn having desired levels of purity. A specific isolation field to produce commercial seed will be located so that the seed parent

is no less than 660 feet (200 m) from other corn of a similar type (i.e., if seed parent is a yellow, dent corn it should be isolated at least 660 ft from other yellow, dent corn). The distance of 660 ft can be modified because of size of field, number of border rows, and different maturity dates of flower, provided no receptive silks are available at the time pollen is being shed from the contaminating field.

II.H. Weediness of Corn

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

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

II.I. Characteristics of the Recipient Corn Material

The corn germplasm that was utilized as the initial recipient of the transgenes in MON 89034 is the proprietary inbred line, LH172 (Eggerling 1994). This inbred line was used because it responds well to transformation with *Agrobacterium* and tissue regeneration.

The inbred line LH172 was developed from the cross of (LH82 x LH122) x LH82. LH172 is characterized as a non-Stiff-Stalk, yellow dent corn. Its parental line LH82 is considered to represent a unique heterotic group that typically is crossed to Stiff Stalk lines to produce commercial F_1 hybrids. The progenitors of LH82 include Pioneer 3558, W153R (Wisconsin Agricultural Experiment Station release), and 'Krug' (open-pollinated variety). Parental line LH122 was derived from Pioneer 3535.

II.J. Corn as a Test System in this Petition

In developing the data in support of this petition, appropriate test and control materials were developed, and where feasible, conventional reference corn materials were used to establish a range of responses expected for commercial corn in the U.S. In general, the background of the test material was matched with that of the control material so that the effect of the genetic insert could be assessed in an unbiased manner. The transgenic

(MON 89034) and non-transgenic corn lines were used as test and control (and/or reference) materials, respectively. Further descriptions of the test, control, and reference materials are provided in the methods and materials sections for each trial or study.

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III. Description of the Transformation System

III.A. Transformation System

MON 89034 was developed through *Agrobacterium*-mediated transformation of the corn LH172 to express the *Bacillus thuringiensis* insecticidal proteins Cry1A.105 and Cry2Ab2 using the plasmid vector PV-ZMIR245 (**Figure III-1**). *Agrobacterium*-mediated transformation is a well-documented process for the transfer and integration of exogenous DNA into a plant's nuclear chromosome. MON 89034 was produced using the 2T-DNA transformation plasmid vector PV-ZMIR245 which contains two separate T-DNA's. 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. During transformation, both T-DNAs were inserted into the genome. Traditional breeding was then used to isolate plants that only contain the T-DNA I (*cry1A.105* and the *cry2Ab2* expression cassettes) and do not contain the T-DNA II (*nptII* expression cassette). This resulted in the production of marker-free, lepidopteran-protected corn – MON 89034.

The use of selectable marker genes, such as *nptII*, is essential to select transformed cells due to the relatively small number of target cells in which integration of the foreign DNA occurs. Once the transgenic cells are identified, the selectable marker gene is no longer needed. For commercial development, it is desirable to produce marker-free plants. Co-transformation of plants with *Agrobacterium tumefaciens* 2T-DNA binary vector system is an effective approach to generate marker-free plants. This approach takes advantage of *Agrobacterium*-mediated transfer of two different T-DNAs bearing the gene of interest and the selectable marker gene, and their subsequent integration at sufficiently unlinked sites in the primary tranformants followed by genetic segregation in the progenies. 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). *Agrobacterium tumefaciens* strain ABI, containing plasmid PV-ZMIR245 was induced to be virulent by the use of acetosyringone. Strain ABI also contains a disarmed Ti plasmid that is incapable of inducing tumor formation because of the deletion of the phytohormone genes originally present in the *Agrobacterium* Ti plasmid. Following inoculation with *Agrobacterium*, the immature 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 plants 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 III-2** displays the process map of the major steps involved in the transformation, selection, and development of MON 89034.

III.B. References

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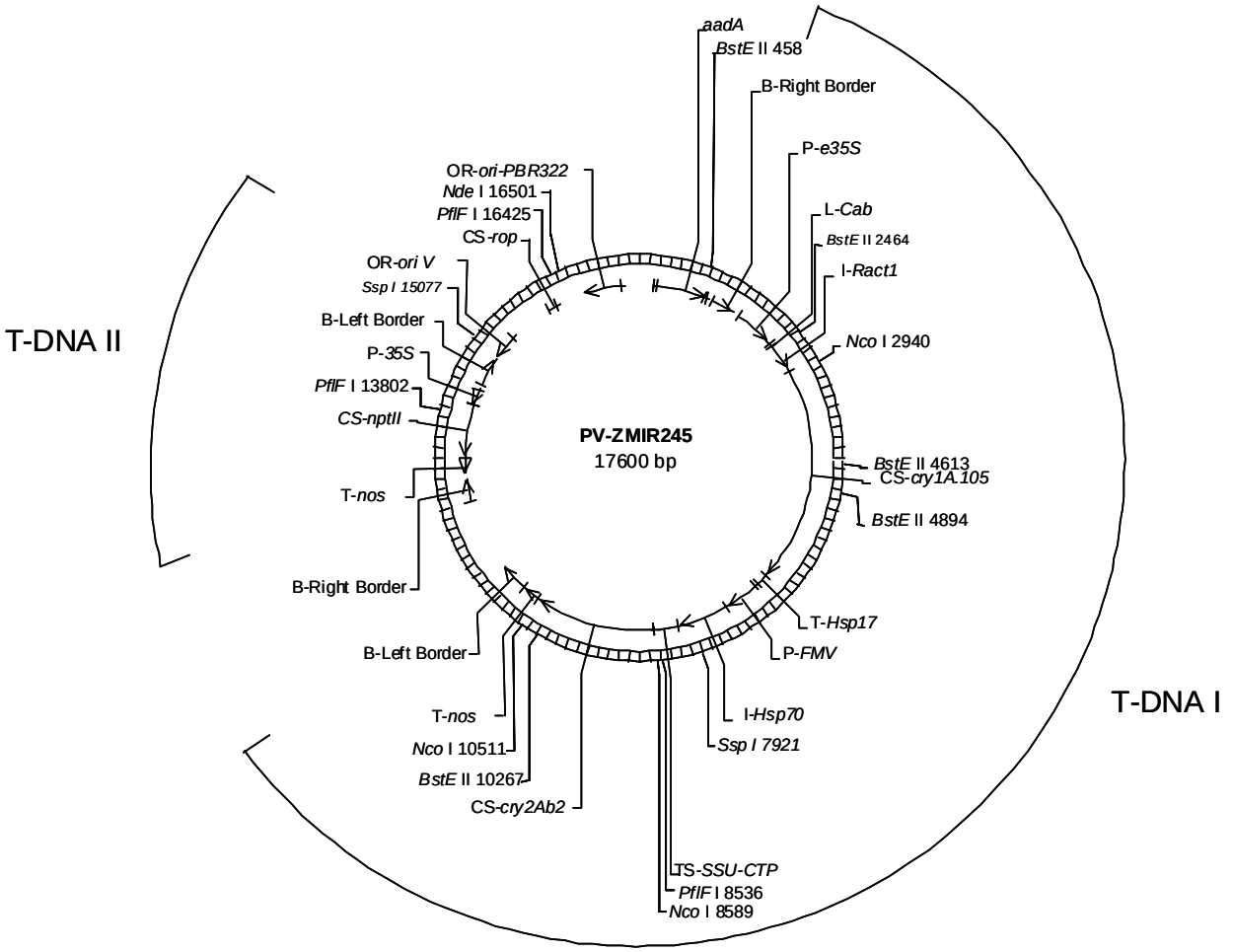

Figure III-1. Plasmid map of vector PV-ZMIR245

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-DNA I and T-DNA 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.

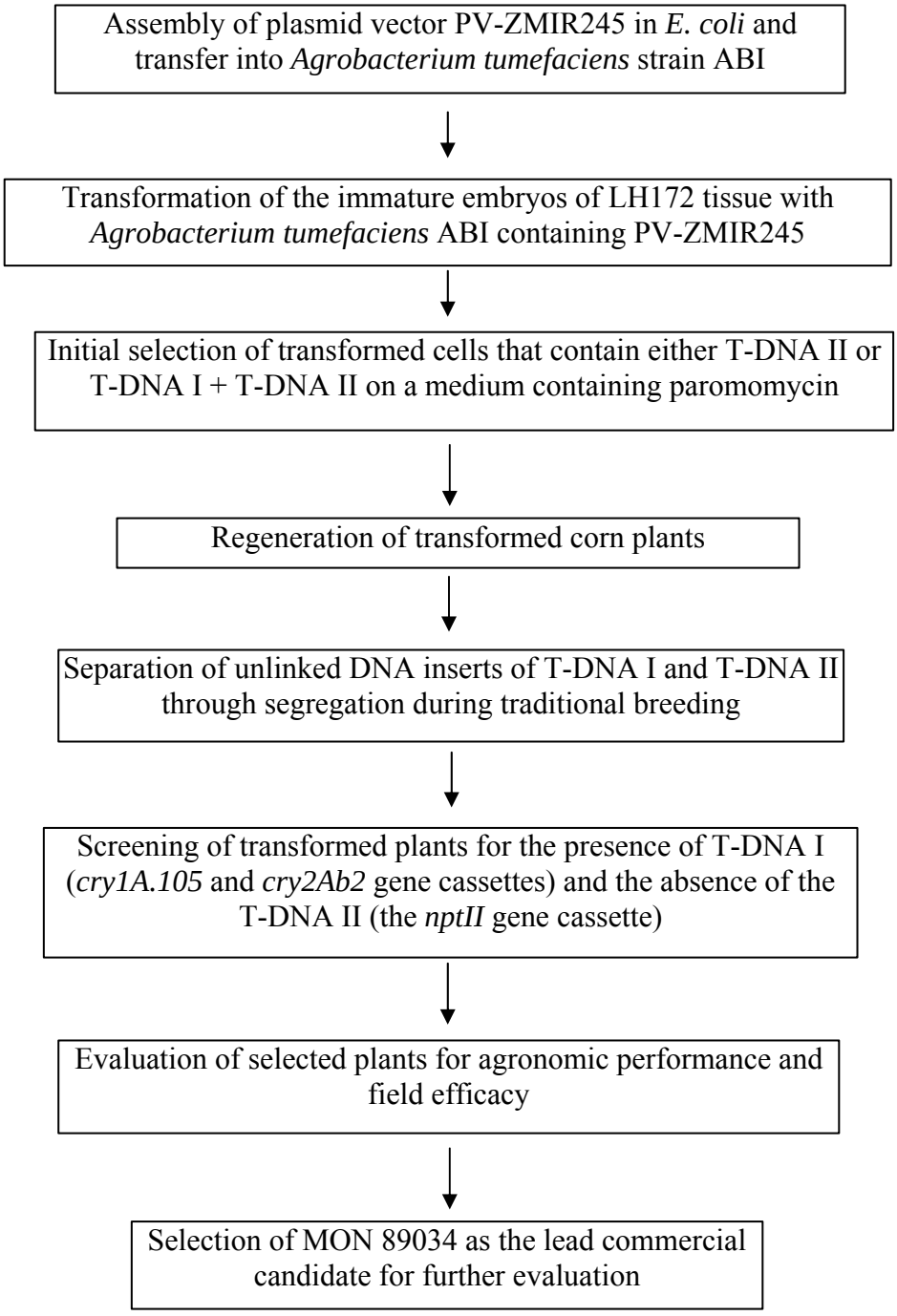

Figure III-2. Process map for transformation, selection, regeneration and evaluation of MON 89034

IV. Donor Genes and Regulatory Sequences

This section describes the donor genes and regulatory sequences used in the development of MON 89034 and the deduced amino acid sequences of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034.

IV.A. Vector PV-ZMIR245

The plasmid vector PV-ZMIR245 (**Figure III-1**) was used for the transformation of corn cells to produce MON 89034. It was constructed at Monsanto's research laboratories in St. Louis, Missouri, using molecular biology techniques. PV-ZMIR245 is a 2T-DNA transformation plasmid vector which contains two separate T-DNAs.

The first T-DNA, designated as T-DNA I, contains two expression cassettes – *cry1A.105* and *cry2Ab2* expression cassettes. An expression cassette, or gene cassette, is defined as a coding sequence and the sequences necessary for proper expression of the coding sequence (e.g., promoter, intron, and 3' end sequence). The first cassette in T-DNA I contains the *cry1A.105* coding sequence under the regulation of the *e35S* promoter, *Ract1* intron, and the *Hsp17* 3' end sequence. The second cassette in T-DNA I contains the coding sequence for a chloroplast transit peptide (*SSU-CTP*) and the *cry2Ab2* coding sequence under the regulation of the *FMV* promoter, the *Hsp70* intron, and the *nos* 3' end sequence.

The second T-DNA, designated as T-DNA II, contains the *nptII* expression cassette. The *nptII* coding sequence is under the regulation of the CaMV *35S* promoter and *nos* 3' end sequence. The *nptII* coding sequence encodes the neomycin phosphotransferase II protein which confers the bacterial cells resistance to kanamycin and was used during the initial selection process. Traditional breeding was used to isolate plants that contain the *cry1A.105* and the *cry2Ab2* expression cassettes (T-DNA I) but do not contain the *nptII* expression cassette (T-DNA II), therefore producing marker-free, lepidopteran-protected MON 89034 (see **Section III**). The specific genetic elements and origins of the various components used to construct plasmid vector PV-ZMIR245 are provided in **Table IV-1**. The following sections provide further descriptions on these genetic elements.

IV.B. T-DNA I

IV.B.1. The *cry1A*.105 Coding Sequence 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. The Cry1A.105 is a modified Bt Cry1A protein with amino acid sequence identity to Cry1Ab, Cry1Ac and Cry1F proteins of 90.0%, 93.6% and 76.7%, respectively (see **Section VI**). The deduced amino acid sequence of the Cry1A.105 protein produced in MON 89034 is presented in **Figure IV-1**.

Table IV-1. Summary of the genetic elements in plasmid 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 for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985) (GenBank accession X03043). The *aadA* 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 24 bp 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*	2942-6475	Coding sequence for *Bacillus thuringiensis* Cry1A.105 protein (Monsanto unpublished data)

Table IV-1 continues on next page.

[1] B – border region
[2] P - promoter
[3] L - leader
[4] I - intron
[5] CS – coding sequence. The sequence length listed in this table for *cry1A*.105 includes one stop codon which accounts for 3 bp.

Table IV-1 (continued). Summary of the genetic elements in plasmid 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	The 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[3]-*cry2Ab2*	8591-10498	Coding sequence for 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	The 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	Sequences used in DNA cloning
B-Left Border	10845-11286	DNA region from *Agrobacterium tumefaciens* containing the 25 pb left border sequence used for transfer of the T-DNA (Barker et al., 1983)

Table IV-1 continues on next page.

[1] T – transcript termination sequence
[2] TS – targeting sequence
[3] CS – coding sequence. The sequence length listed in this table for *cry2Ab2* includes two stop codons which account for 6 bp.

Table IV-1 (continued). Summary of the genetic elements in plasmid 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	The 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	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	Sequences used in DNA cloning
P-*35S*	14084-14407	The promoter and 31 pb 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)

Table IV-1 continues on next page.

[1] OR-origin of replication

Table IV-1 (continued). Summary of the genetic elements in plasmid vector PV-ZMIR245

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	15383-16119	Sequences 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	Sequences used in DNA cloning
OR-*ori-PBR322*	16739-17327	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978)
Intervening Sequence	17328-17600	Sequences used in DNA cloning

IV.B.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) with a duplicated enhancer region (Kay et al., 1987). The cassette also 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).

IV.B.3. The *cry2Ab2* Coding Sequence 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 is a variant of the wild-type Cry2Ab2 protein isolated from *Bacillus thuringiensis* subsp. *kurstaki*. The deduced amino acid sequence of the Cry2Ab2 protein, together with the chloroplast transit peptide (CTP), produced in MON 89034 is shown in **Figure IV-2**.

```
   1  MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51  VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101  EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151  QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201  GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251  DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301  NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351  VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401  YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451  PMFSWIHRSA EFNNIIASDS ITQIPLVKAH TLQSGTTVVR GPGFTGGDIL
 501  RRTSGGPFAY TIVNINGQLP QRYRARIRYA STTNLRIYVT VAGERIFAGQ
 551  FNKTMDTGDP LTFQSFSYAT INTAFTFPMS QSSFTVGADT FSSGNEVYID
 601  RFELIPVTAT LEAEYNLERA QKAVNALFTS TNQLGLKTNV TDYHIDQVSN
 651  LVTYLSDEFC LDEKRELSEK VKHAKRLSDE RNLLQDSNFK DINRQPERGW
 701  GGSTGITIQG GDDVFKENYV TLSGTFDECY PTYLYQKIDE SKLKAFTRYQ
 751  LRGYIEDSQD LEIYSIRYNA KHETVNVPGT GSLWPLSAQS PIGKCGEPNR
 801  CAPHLEWNPD LDCSCRDGEK CAHHSHHFSL DIDVGCTDLN EDLGVWVIFK
 851  IKTQDGHARL GNLEFLEEKP LVGEALARVK RAEKKWRDKR EKLEWETNIV
 901  YKEAKESVDA LFVNSQYDQL QADTNIAMIH AADKRVHSIR EAYLPELSVI
 951  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-1. 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-2. Deduced amino acid sequence of the chloroplast transit peptide and the Cry2Ab2 protein produced in MON 89034

The chloroplast transit peptide (CTP) is underlined. Accumulation of the Cry2Ab2 protein is targeted to the chloroplasts using CTP. The CTP is typically cleaved from the mature protein on uptake into the chloroplasts and is then rapidly degraded (see **Section VI.B.1** for more detail).

IV.B.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). The cassette also 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).

IV.B.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 as 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-DNA I and T-DNA 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).

IV.C. T-DNA II

IV.C.1. The *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 *nptII* are eliminated due to the action of the paromomycin. The T-DNA II, and therefore the *nptII* gene, is segregated out at the F1 generation.

IV.C.2. The *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 cassette also containts the coding sequence for the NPTII protein (Beck et al., 1982), followed by the 3' nontranslated region of the nopaline synthase (T-*nos*) sequence from

Agrobacterium tumefaciens which terminates the transcription and directs polyadenylation (Bevan et al., 1983).

IV.C.3. T-DNA borders

The Right and Left T-DNA borders are described under **Section IV.B.5**.

IV.D. Genetic Elements outside the T-DNA Borders

The backbone region outside of the inserted DNA contains two origins of replication which are necessary for replication and maintenance of the plasmid in bacteria, as well as a bacterial selectable marker gene, *aadA*, which encodes an aminoglycoside-modifying enzyme that provides 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-1**.

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V. Genetic Analysis

Characterization of the DNA insert in MON 89034 was conducted by Southern blot analyses, PCR, and DNA sequencing. The results demonstrated that MON 89034 contains a single functional copy of *cry1A.105* and *cry2Ab2* expression cassettes. The complete insert includes: (1) the *cry1A.105* coding sequence whole transcription is directed by a modified *e35S* promoter, the 5' untranslated leader of the wheat chlorophyll a/b-binding protein (*Cab*), the intron sequence from rice actin gene (*Ract1*), and the transcriptional termination and polyadenylation sequence derived from the 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3 (*Hsp17*); and (2) the *cry2Ab2* coding sequence whole transcription is directed by figwort mosaic virus *35S* promoter (*FMV*), the first intron from the maize heat shock protein 70 gene (*Hsp70*), the DNA region containing the targeting sequence for the chlorophyll transit peptide of maize ribulose 1,5-bisphosphate carboxylase small subunit and the first intron (*SSU-CTP*), and the transcriptional termination and polyadenylation sequence derived from the 3' termination sequence of the nopaline synthase (*nos*) coding sequence from *Agrobacterium tumefaciens*. This T-DNA (T-DNA I) was inserted into the corn genome and results in the synthesis of Cry1A.105 and Cry2Ab2 proteins from the expression of the *cry1A.105* and *cry2Ab2* genes. The chlorophyll transit peptide (CTP) is present to direct the Cry2Ab2 protein to corn plastids.

The PCR and DNA sequencing analysis of the complete DNA insert and adjacent genomic DNA in MON 89034 confirmed the organization of the genetic elements within the insert. PCR primers were designed to amplify seven overlapping regions of DNA that span the entire length of the insert, and the sequencing results of the amplified DNA fragments confirmed that the sequence of the DNA insert in MON 89034 matched the designed, corresponding sequences in PV-ZMIR245 with one exception. This exception is 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 was replaced by a Left Border sequence in MON 890343. 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 V-1**). 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.

Analysis of the stability of the integrated DNA demonstrated that a unique Southern blot fingerprint of MON 89034 was maintained in seven generations during conventional breeding, thereby confirming the stability of the insert. Additionally, T-DNA II analysis of multiple generations in the MON 89034 breeding history indicated that there were no detectable T-DNA II elements other than those which are common to T-DNA I, i.e., *35S* promoter, *nos* 3´ end sequence, Left Border sequence. Furthermore, these generations

were shown not to contain any detectable backbone sequence from plasmid PV-ZMIR245.

The following sections describe the molecular analysis experiments and results. Southern blot, PCR and DNA sequencing analyses were used to assess the following: 1) number of insertions of the integrated expression cassettes; 2) number of copies of the integrated expression cassettes; 3) intactness of the expression cassettes; 4) the presence or absence of plasmid backbone sequences; 5) the stability of the inserted DNA during conventional breeding; and 6) organization of the insert in MON 89034. Detailed materials and methods of Southern blot and PCR analyses are provided in **Appendix A.** Maps of plasmid vector PV-ZMIR245 annotated with the probes used in the Southern analyses are shown in **Figures V-2** and **V-3**. The generations used in the studies are depicted in **Figure-17**. The overall results of the molecular analyses are summarized in a linear map (**Figure V-4**) which illustrates the organization of the insert, the corn genomic DNA flanking the insert, the restriction sites, and expected sizes of the DNA fragments after restriction digestion. The description of the genetic elements of the DNA insert in MON 89034 is provided in **Table V-1**.

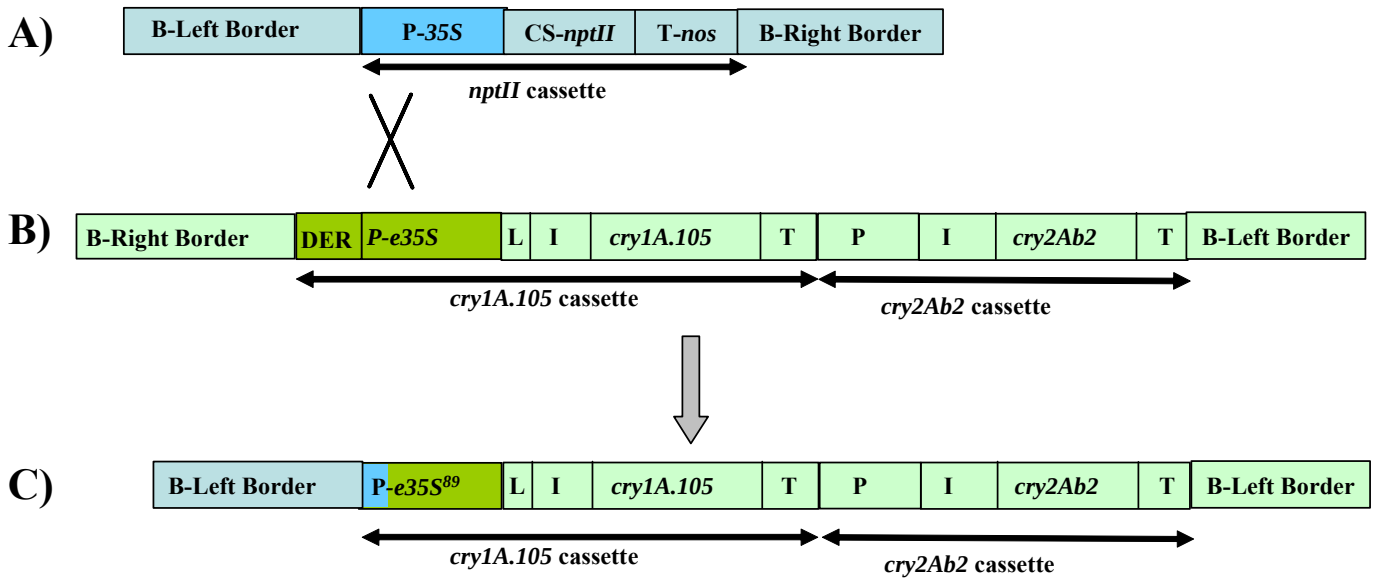


Figure V-1. Description of the recombination process that explains the modified 5'end of the insert

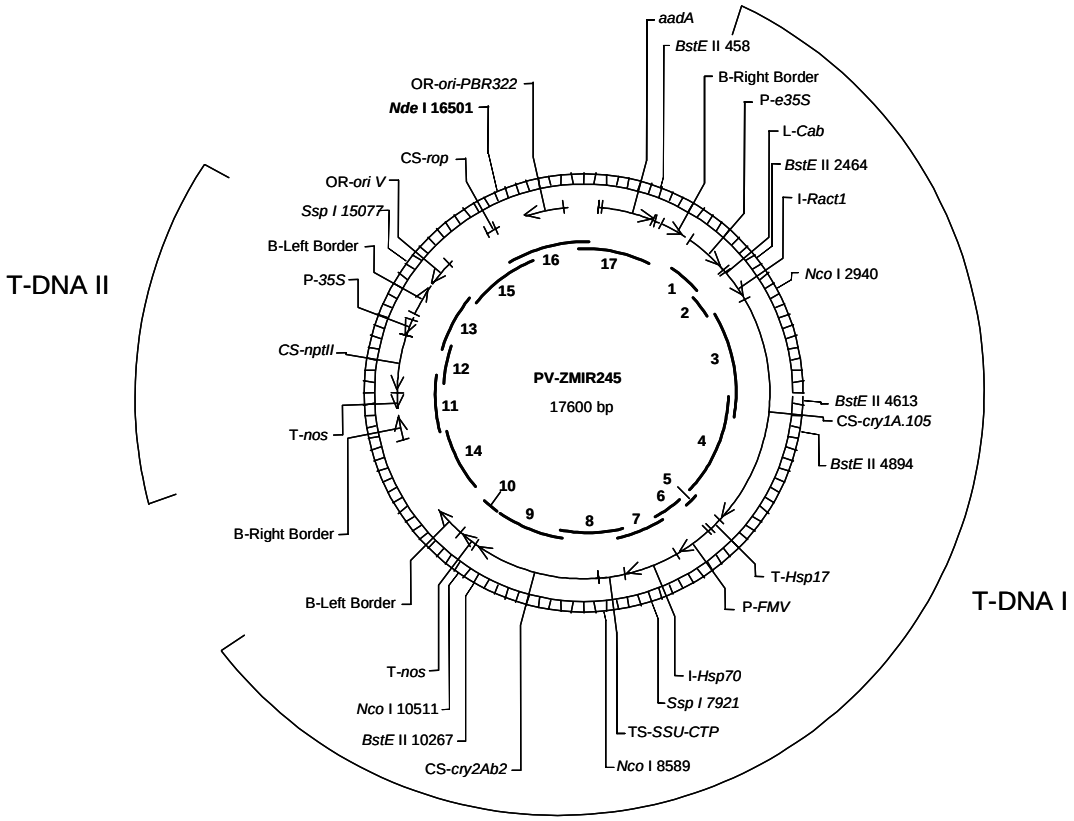
A) Illustration of the T-DNA II of plasmid PV-ZMIR245.

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 **Table V-1** and **Figure V-4**.

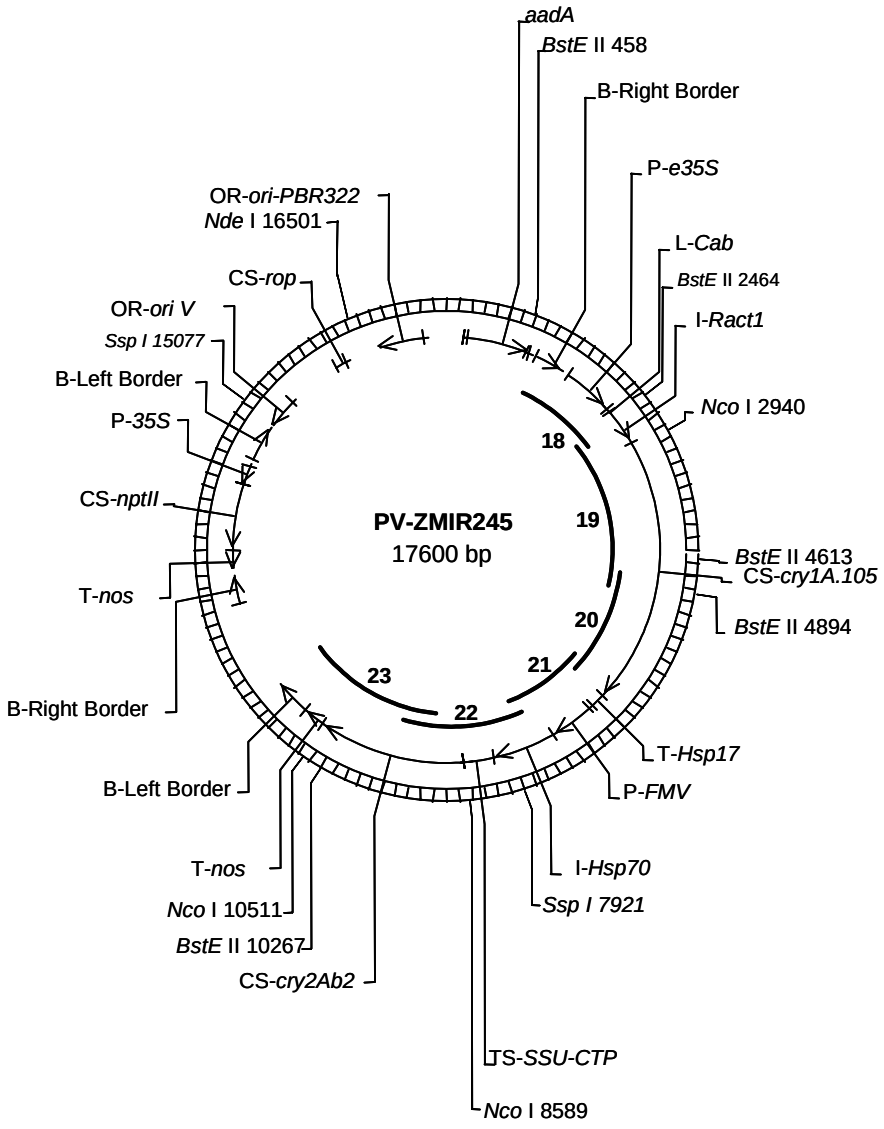
The diagram illustrates a recombination event, which likely occurred prior to or during the process of T-DNA transfer to the plant cells, between the DNA sequences near the *35S* promoters in T-DNA I and T-DNA II. Due to this recombination event, the reconstituted *e35S* promoter in MON 89034 (referred to as modified *e35S* or $e35S^{89}$) no longer has the duplicated enhancer elements (DER) 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 Number	DNA Probe Description	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 V-2. Plasmid map of vector PV-ZMIR245 showing probes 1-17 used in Southern blot analyses

A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown. The 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 I and T-DNA II.



Probe Number	DNA Probe Description	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 V-3. Plasmid map of vector PV-ZMIR245 showing probes 18 – 23 used in Southern blot analyses

A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown. The 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.

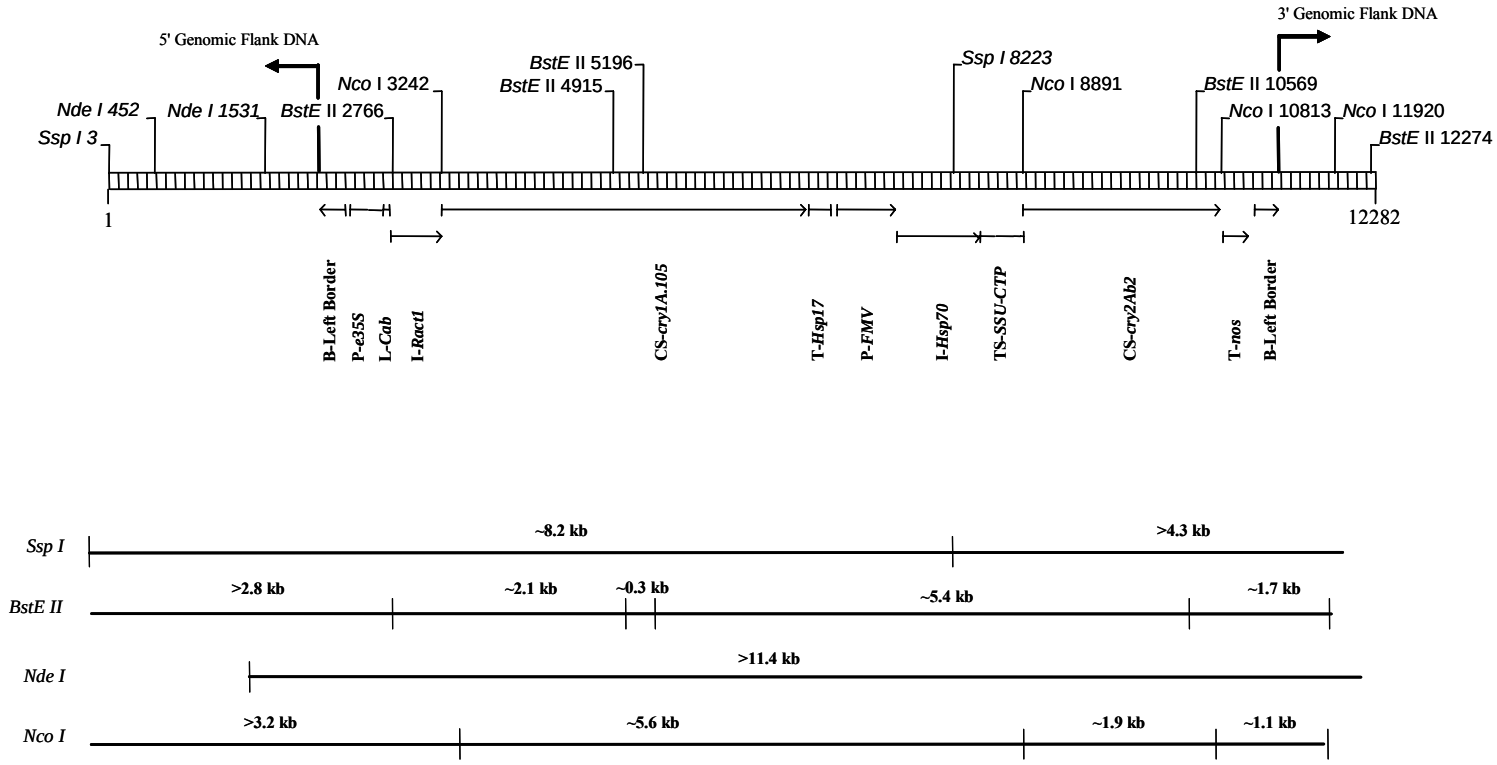


Figure V-4. Schematic representation of the insert and genomic flanking sequences in MON 89034

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 V-1. 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	2061-2299	The 239 bp DNA region from the B-Left Border region remaining after integration
Intervening Sequence	2300-2349	Sequences used in DNA cloning
P-*e35S*[89]	2350-2651	Modified *e35S* promoter and 9 bp leader resulting from a recombination between the *e35S* and *35S* promoters
Intervening Sequence	2652-2677	Sequences used in DNA cloning
L[2]-*Cab*	2678-2738	The 5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al. 1985)
Intervening Sequence	2739-2754	Sequences used in DNA cloning
I[3]-*Ract1*	2755-3234	Intron from the rice actin gene (McElroy et al. 1991)
Intervening Sequence	3235-3243	Sequences used in DNA cloning
CS[4]-*cry1A.105*	3244-6777	Coding sequence for the *Bacillus thuringiensis* Cry1A.105 protein (Monsanto unpublished data)
Intervening Sequence	6778-6808	Sequences used in DNA cloning
T[5]-*Hsp17*	6809-7018	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	7019-7085	Sequences used in DNA cloning

Table V-1 continues on next page.

[1] B – border region
[2] L - leader
[3] I - intron
[4] CS – coding sequence of *cry1A.105*, including one stop codon which accounts for 3 bp.
[5] T – transcript termination sequence

Table V-1 (continued). Summary of genetic elements in MON 89034

Genetic Element	Location in Sequence	Function (Reference)
P[1]-*FMV*	7086-7649	The figwort mosaic virus 35S promoter (Rogers, 2000)
Intervening Sequence	7650-7671	Sequences 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	Sequences 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[3]-*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	Sequences used in DNA cloning
T-*nos*	10827-11079	The 3' nontranslated region of the nopaline synthase (*nos*) coding sequence from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al. 1983)
Intervening Sequence	11080-11146	Sequences used in DNA cloning
B-Left Border	11147-11377	The 230 bp DNA region from the 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] CS – Coding sequence for Cry2Ab2, including two stop codons which account for 6 bp.

V.A. Insert and Copy Number

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. The number of restriction fragments detected indicates the number of inserts present in MON 89034. The number of copies of the T-DNA I integrated at a single locus 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 T-DNA I, probing with T-DNA I should result in two bands, each representing a portion of the T-DNA I along with adjacent, corn plant genomic DNA. The blot was examined with overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18 – 23 in **Figure V-3**). The results of this analysis are shown in **Figure V-5**. 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.

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 is not unexpected because several genetic elements comprising T-DNA I were 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 in all lanes, including those containing the conventional corn control DNA, and therefore they are considered to be endogenous background bands. Plasmid PV-ZMIR245 DNA mixed with conventional corn control DNA and digested with *Ssp I* (lanes 9 and 10) produced the expected bands at approximately 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 approximately 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 an approximately 13.0 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 approximately 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. The approximately 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 greater than 4.3 kb (refer to **Figure V-4**).

These results indicate that MON 89034 contains one copy of T-DNA I that resides at a single locus of integration on an approximately 13.0 kb *Nde* I restriction fragment.

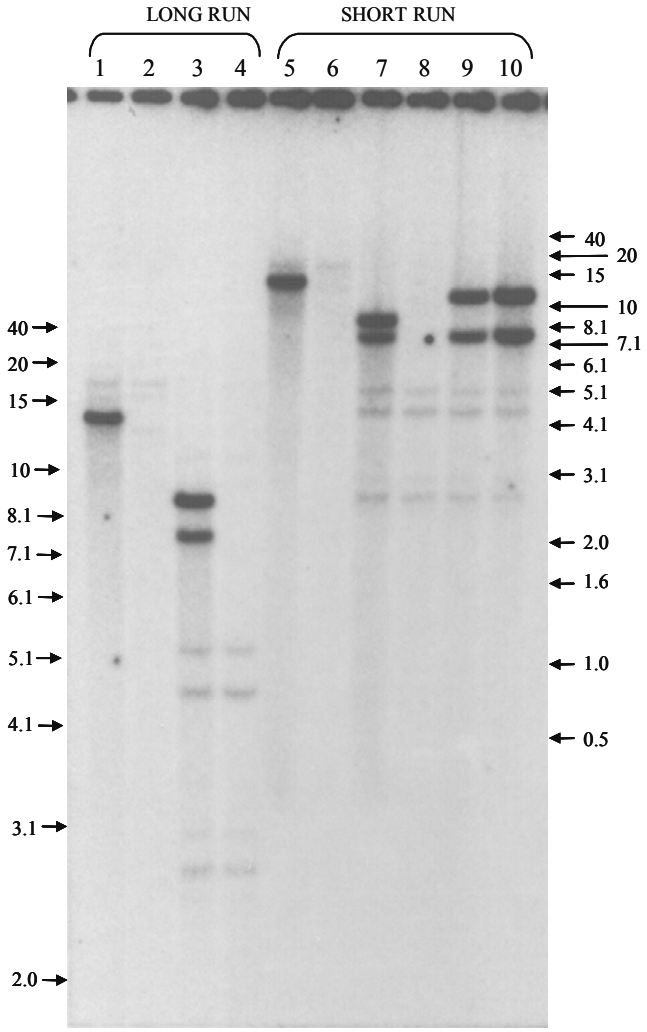


Figure V-5. Southern blot analysis of MON 89034 – insert and copy number analysis

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 – 23 in **Figure V-3**). Each lane contains approximately 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]

V.B. Intactness of c*ry1A.105* and *cry2Ab2* Expression Cassettes

The presence of all the elements of the inserted *cry1A.105* and *cry2Ab2* expression cassettes was assessed by digestion of the test DNA with the restriction enzyme *Ssp* I, *Nco* I, or *BstE* II.

Digestion with *Ssp* I releases two border fragments with the expected size of approximately 8.2 and greater than 4.3 kb (refer to **Figure V-4**). The approximately 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 greater than 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.

Digestion of the test substance with *Nco* I releases two internal restriction fragments and two border fragments (refer to **Figure V-4**). The 5' border fragment is expected to be greater than 3.2 kb and contains genomic DNA flanking the 5' end of the insert, the Left Border sequence, modified *e35S* promoter sequence, the *Cab* leader, and the *Ract1* intron. The approximately 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 approximately 1.9 kb internal fragment contains the *cry2Ab2* coding sequence. The 3' border fragment is expected to be approximately 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 the test substance with *Bst*E II generates two border fragments and three internal fragments (refer to **Figure V-4**). The 5' border fragment is expected to be greater than 2.8 kb and contains genomic DNA flanking the 5' end of the insert, Left Border sequence, modified *e35S* promoter sequence, and the *Cab* leader sequence. The 3' border fragment is expected to be approximately 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 in **Figure V-2**).

V.B.1. *e35S* Promoter/*Cab* Leader

Figure V-6 shows the Southern blot examined with the probe of *e35S* promoter including the *Cab* leader (probe 1 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) 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 at approximately 10.0 kb.

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

V.B.2. *Ract 1* Intron

Figure V-7 shows the Southern blot examined with *Ract 1* intron probe (probe 2 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) 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 at approximately 10 kb. The migration of the approximately 10 kb fragment is slightly higher than indicated by the size marker band. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the reference DNA size markers (Sambrook and Russell, 2001).

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

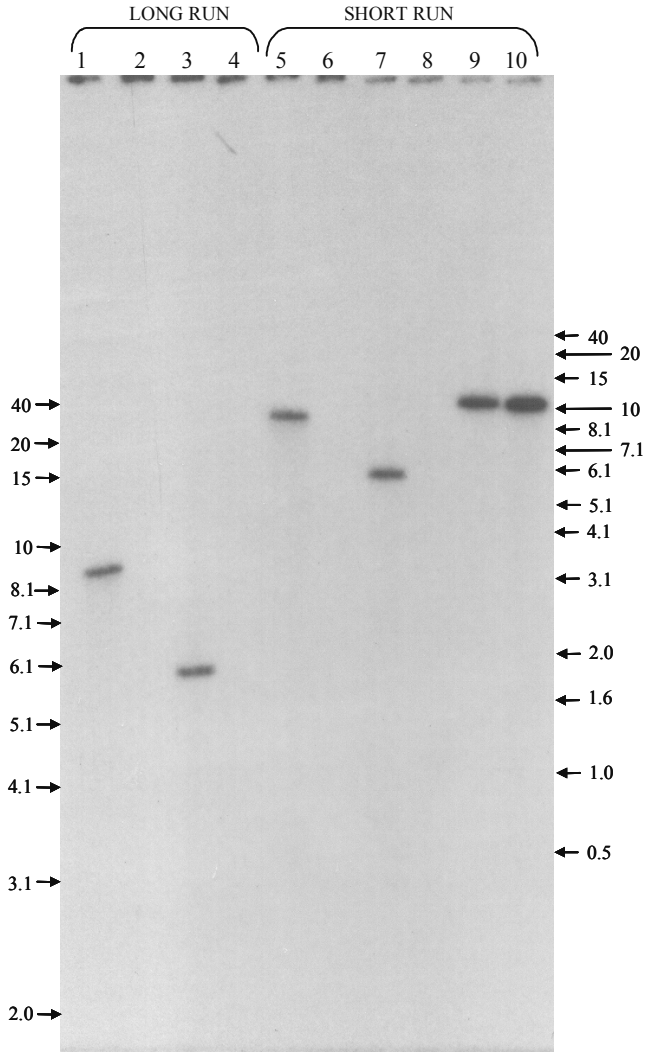


Figure V-6. Southern blot analysis of MON 89034 – *e35S* promoter/*Cab* leader

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *e35S* promoter and *Cab* leader region (probe 1 in **Figure V-2**). Each lane contains approximately 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]

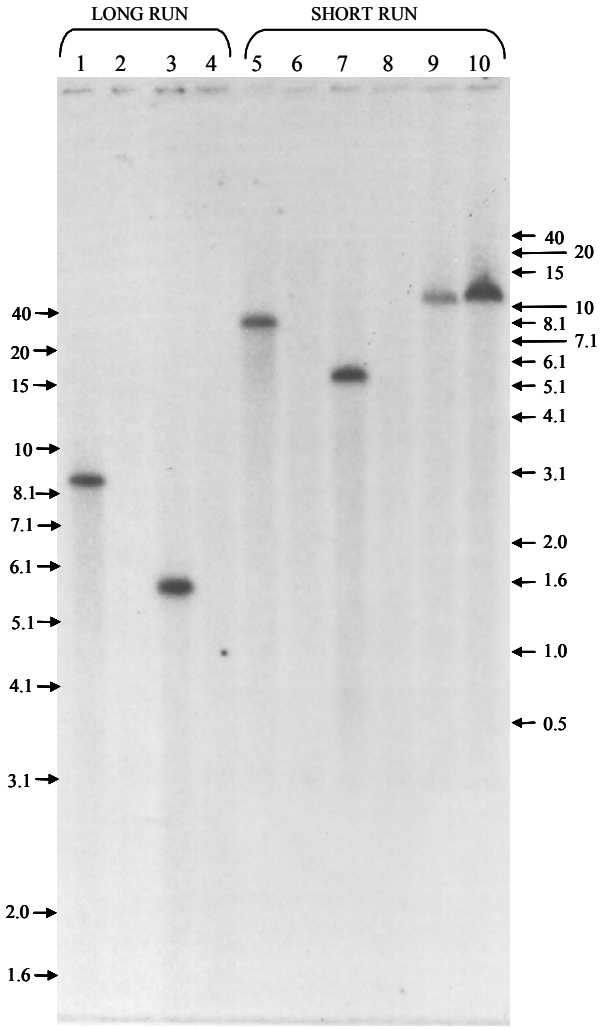


Figure V-7. Southern blot analysis of MON 89034 – *Ract1* intron

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *Ract1* intron (probe 2 in **Figure V-2**). Each lane contains approximately 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]

V.B.3. *cry1A.105* Coding Sequence

Figure V-8 shows the Southern blot examined with overlapping probes spanning the *cry1A.105* coding sequence (probes 3 and 4 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) showed no detectable 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 approximately 5.6 kb. The migration of the approximately 5.6 kb fragment is slightly higher than indicated by the reference DNA size markers. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the reference DNA size markers (Sambrook and Russell, 2001).

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

V.B.4. *Hsp17* 3' End Sequence

Figure V-9 shows the Southern blot examined with *Hsp17* 3' end sequence probe (probe 5 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) showed no detectable hybridization bands, as expected for the negative control. Conventional corn control DNA spiking with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at approximately 5.6 kb.

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

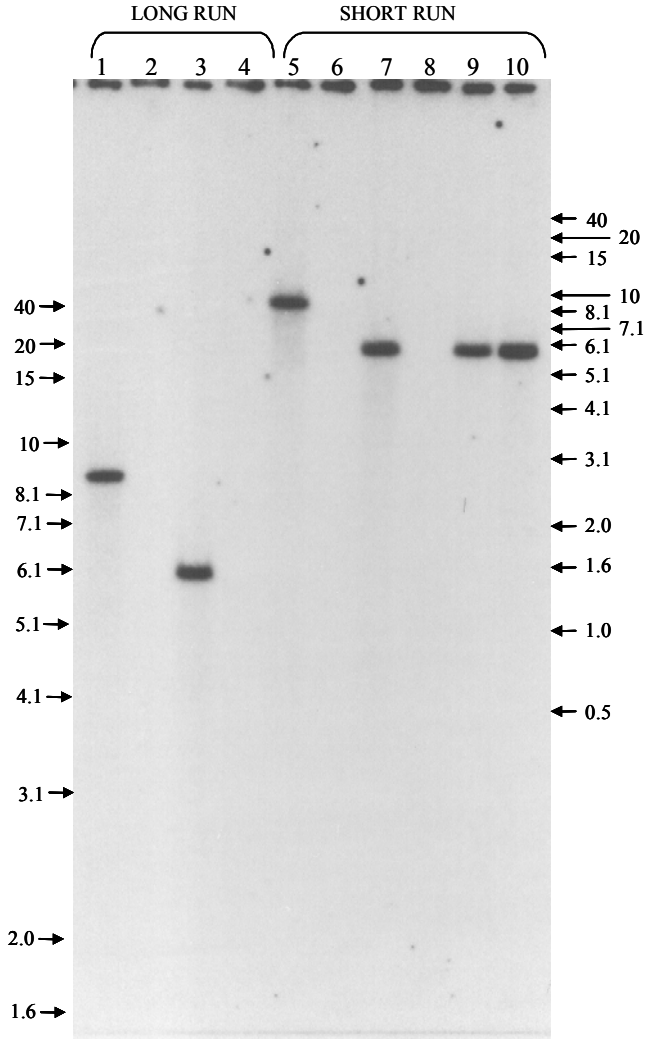


Figure V-8. Southern blot analysis of MON 89034 – *cry1A.105* coding sequence

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with overlapping ^{32}P-labeled probes that spanned the *cry1A.105* coding sequence (probes 3 and 4 in **Figure V-2**). Each lane contains approximately 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]

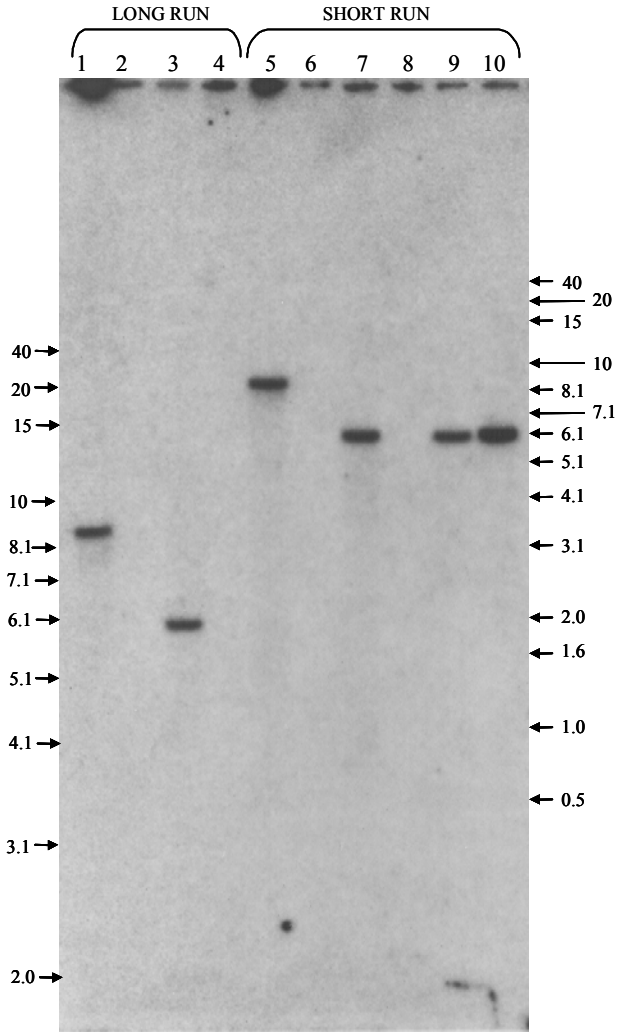


Figure V-9. Southern blot analysis of MON 89034 – *Hsp17* 3' end sequence

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp17* 3' end sequence (probe 5 in **Figure V-2**). Each lane contains approximately 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]

V.B.5. *FMV* Promoter

Figure V-10 shows the Southern blot examined with *FMV* promoter probe (probe 6 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) 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 at approximately 5.6 kb. The migration of the approximately 5.6 kb fragment is slightly higher than indicated by the reference DNA size markers. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the reference DNA size markers (Sambrook and Russell, 2001).

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

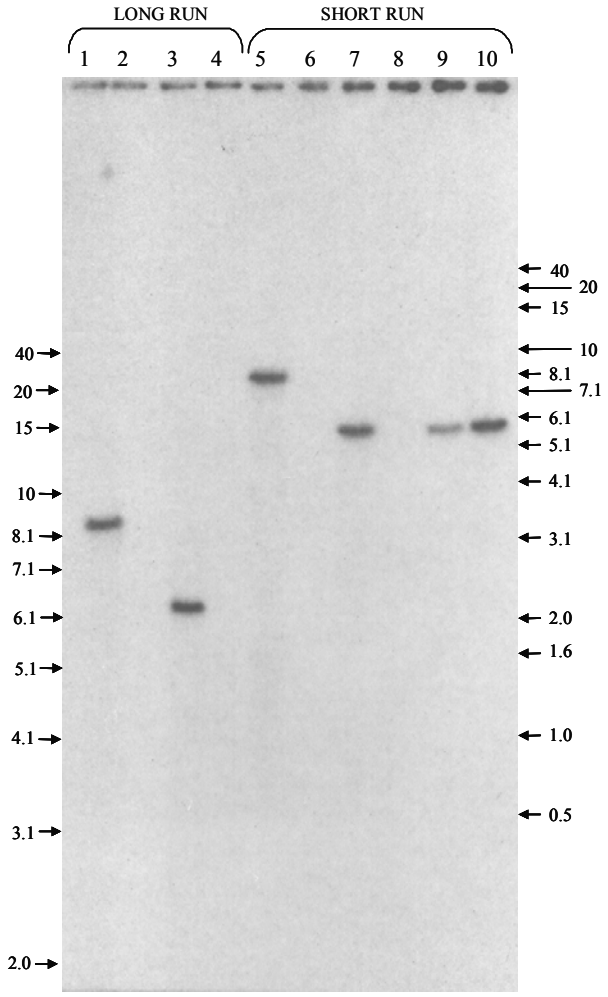


Figure V-10. Southern blot analysis of MON 89034 – *FMV* promoter

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *FMV* promoter (probe 6 in **Figure V-2**). Each lane contains approximately 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]

V.B.6. *Hsp70* Intron

Figure V-11 shows the Southern blot examined with *Hsp70* intron probe (probe 7 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) 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 corn 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 approximately 5.6 kb in addition to the endogenous bands. The migration of the approximately 5.6 kb fragment is slightly higher than indicated by the reference DNA size markers. The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the reference DNA size markers (Sambrook and Russell, 2001).

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced two expected bands of approximately 8.2 and 7.4 kb in addition to the endogenous bands. The approximately 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 (refer to **Figure V-4**). The approximately 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 single unique band of approximately 5.6 kb in addition to the endogenous bands. No unexpected bands were detected, indicating that MON 89034 contains no additional, detectable *Hsp70* intron elements other than those associated with the *cry2Ab2* cassette.

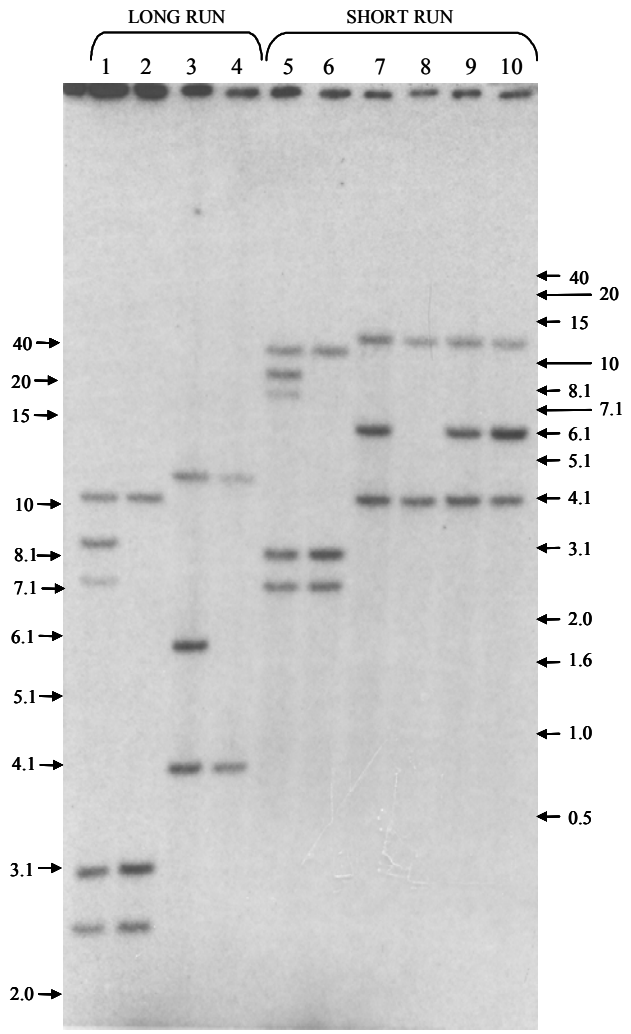


Figure V-11. Southern blot analysis of MON 89034 – *Hsp70* intron

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp70* intron (probe 7 in **Figure V-2**). Each lane contains approximately 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]

V.B.7. *SSU-CTP* Targeting Sequence / *cry2Ab2* Coding Sequence

Figure V-12 shows the Southern blot examined with overlapping probes spanning the *SSU-CTP* targeting sequence / *cry2Ab2* coding sequence (probes 8 and 9 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) produced several hybridization signals. This is not unexpected because the *SSU-CTP* targeting sequence was originally derived from corn. These hybridization signals result from the probe 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, therefore they are considered to be endogenous background. Endogenous bands were not detected in the long runs of the *Nco* I digests (lane 4) because they ran off the gel.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size bands at approximately 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 approximately 7.4 kb in addition to the endogenous bands. The approximately 7.4 kb band is consistent with the expected band of >4.3 kb (refer to **Figure V-4**). 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 approximately 5.6 and 1.9 kb. The migration of the approximately 5.6 and 1.9 kb fragments is slightly higher than indicated by the reference DNA size markers in the long run (lane 3) but run concurrently with the bands produced by PV-ZMIR245 in the short run (lane 7, 9, and 10). The altered migrations may be due to the difference in salt concentrations between the corn DNA sample and the reference DNA size markers (Sambrook and Russell, 2001). No unexpected bands were detected, indicating that MON 89034 contains no additional, detectable *SSU-CTP/cry2Ab2* elements other than those associated with the *cry2Ab2* cassette.

V.B.8. *nos* 3' End Sequence

Figure V-13 shows the Southern blot examined with *nos* 3' end sequence probe (probe 10 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *BstE* II (lanes 4 and 8) showed no detectable hybridization bands, as expected for the negative control. Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *BstE* II (lanes 9 and 10) produced the expected size band at approximately 7.8 kb.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced a single, unique band of approximately 7.4 kb that is consistent with the expected band of >4.3 kb (refer to **Figure V-4**). MON 89034 DNA digested with *BstE* II (lanes 3 and 7) produced the expected single unique band of approximately 1.7 kb. No unexpected bands were detected indicating that MON 89034 contains no additional *nos* 3' elements other than those associated with the *cry2Ab2* cassette.

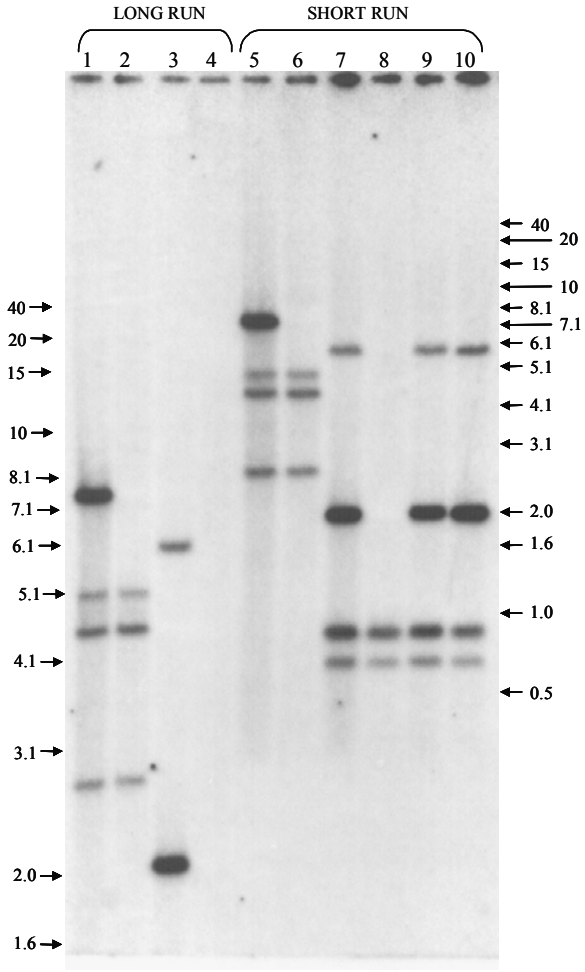


Figure V-12. Southern blot analysis of MON 89034 – *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. 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 in **Figure V-2**). Each lane contains approximately 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]

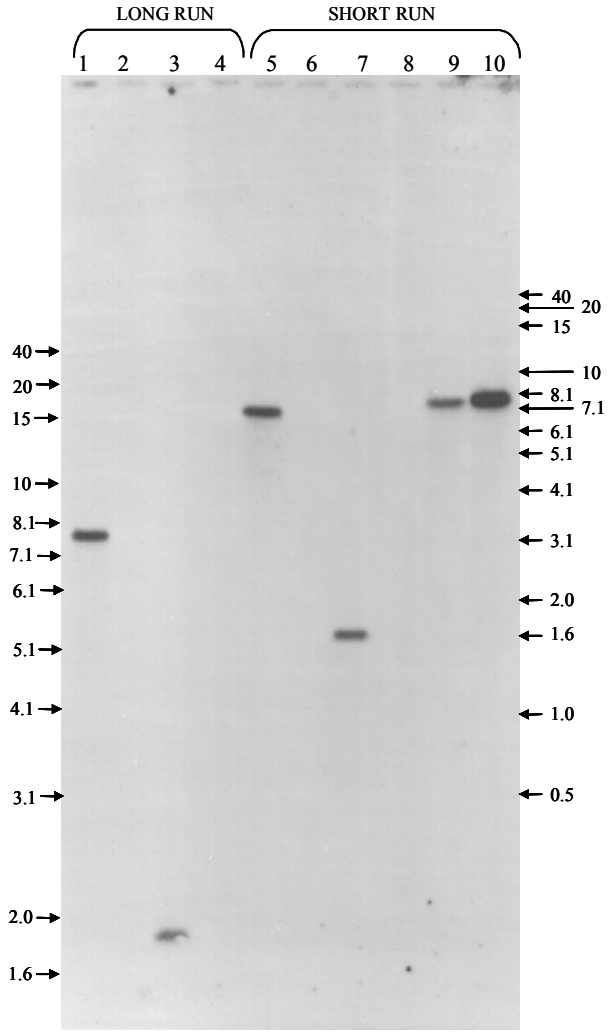


Figure V-13. Southern blot analysis of MON 89034 – *nos* 3' end sequence

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *nos* 3' end sequence (probe 10 in **Figure V-2**). Each lane contains approximately 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]

V.C. Analysis of the Presence or Absence of the Plasmid Backbone

Figure V-14 shows the Southern blot analysis to determine the presence or absence of plasmid PV-ZMIR245 backbone. 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 (detected with probes 14 – 17 of **Figure V-2**) 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 approximately 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.

V.D. Southern Blot Analysis to Confirm the Absence of T-DNA II

V.D.1. Analysis of the Presence or 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 desired proteins are produced in MON 89034. **Figure V-15** shows the Southern blot analysis used to determine the presence or absence of the *nptII* coding sequence. 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 in **Figure V-2**) showed no detectable hybridization bands, as expected for the negative control.

Conventional corn control DNA spike with plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of approximately 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* coding sequence.

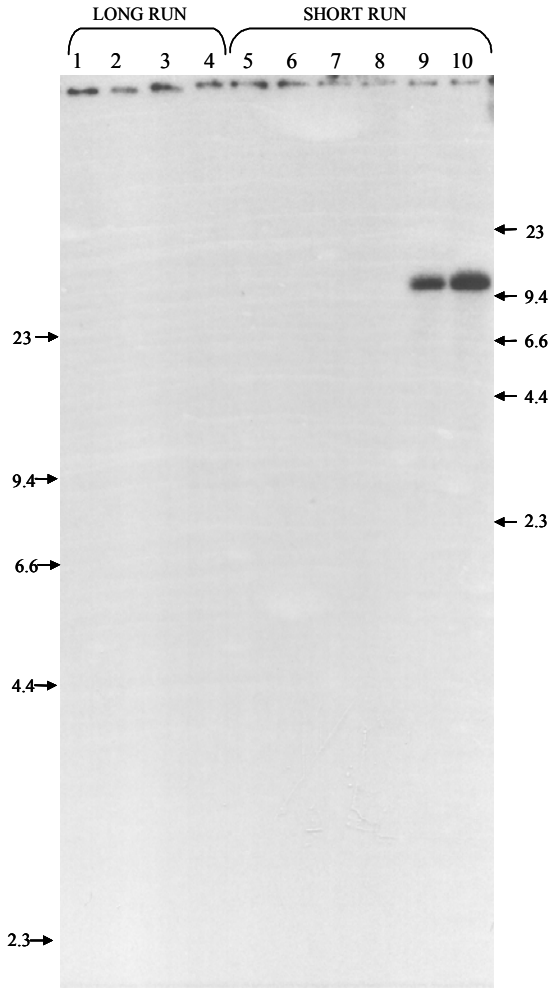


Figure V-14. Southern blot analysis of MON 89034 – PV-ZMIR245 backbone sequence

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17 in **Figure V-2**). Each lane contains approximately 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]

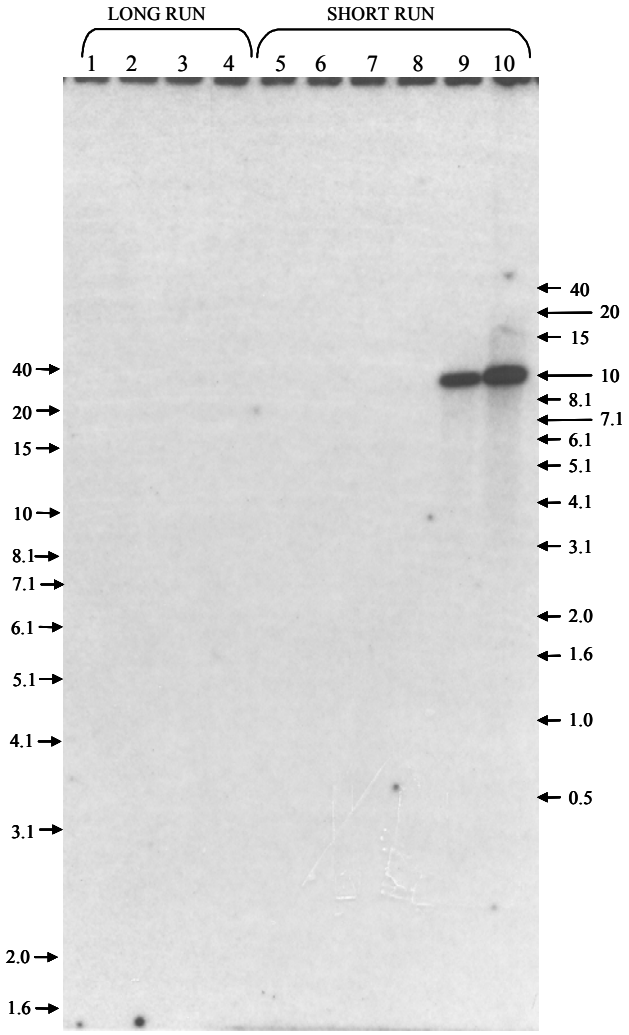


Figure V-15. Southern blot analysis of MON 89034 – *nptII* coding sequence

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. 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 arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with a ^{32}P-labeled probe that spanned the *npt II* coding sequence (probe 12 in **Figure V-2**). Each lane contains approximately 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]

V.D.2. Analysis of the Presence or Absence of the Overall T-DNA II Region

This analysis further 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 shared with the T-DNA I expression cassettes. The analysis confirmed that the F1 plants that contained T-DNA II elements were segregated out and that the plants selected for further development contained only T-DNA I.

Figure V-16 shows the Southern blot analysis to determine the presence or absence of T-DNA II. Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) and *BstE* II (lanes 4 and 8) examined with overlapping probe spanning T-DNA II (probes 11, 12, and 13 in **Figure V-2**) showed no hybridization bands.

Conventional corn control DNA spiked with plasmid PV-ZMIR245 DNA digested with *BstE* II (lanes 9 and 10) produced the two expected size bands at approximately 7.8 and 2.0 kb. It should be noted that the overlapping probe spanning T-DNA II contains the *35S* promoter, *nos* 3' end sequence, and Left Border sequences which are also contained in T DNA I. Therefore, the T-DNA II probe is expected to hybridize to fragments which are common to T-DNA I.

MON 89034 DNA digested with *Ssp* I (lanes 1 and 5) produced two bands of approximately 8.2 and 7.4 kb. The 8.2 kb band is consistent with the 8.2 kb band detected with the *e35S* promoter/*Cab* leader region probe (refer to **Figure V-6**, lanes 1 or 5). The 7.4 kb band is consistent with the 7.4 kb band detected with *nos* 3' end sequence probe (refer to **Figure V-13**, lanes 1 or 5).

MON 89034 DNA digested with *BstE* II (lanes 3 and 7) produced two bands of approximately 4.2 and 1.7 kb. The approximately 4.2 kb band is consistent with the >2.8 kb expected band for T-DNA I digested with *BstE* II (refer to **Figure V-4**), and the approximately 1.7 kb band is consistent with the T-DNA I specific band observed in **Figure V-13 (**lanes 3 and 7). No unexpected bands were detected, indicating that MON 89034 contains no additional, detectable T-DNA II elements other than those that are common to T-DNA I.

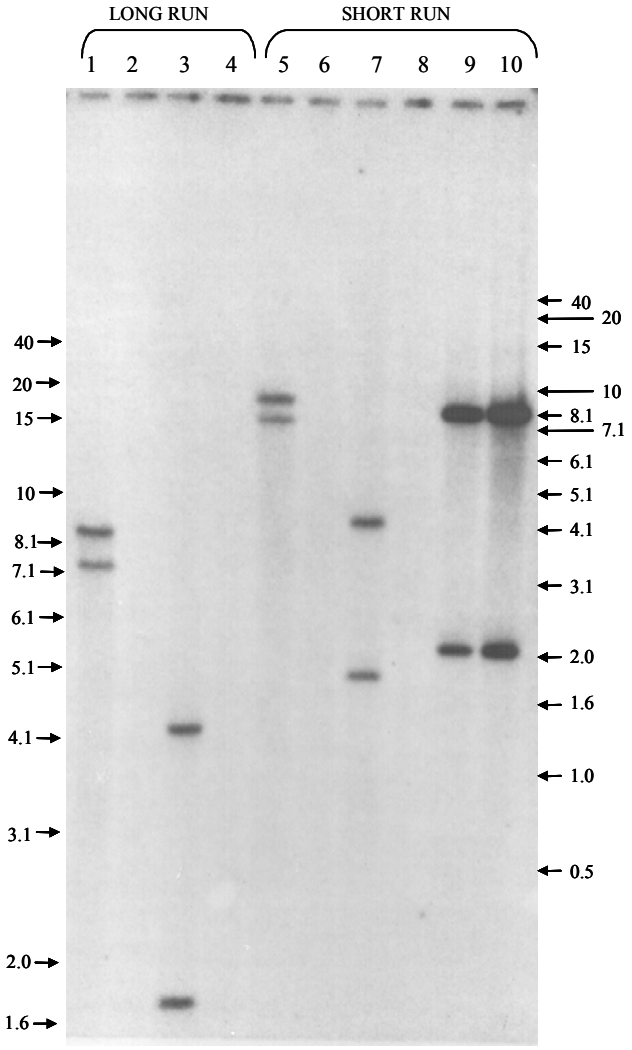


Figure V-16. Southern blot analysis of MON 89034 – T-DNA II

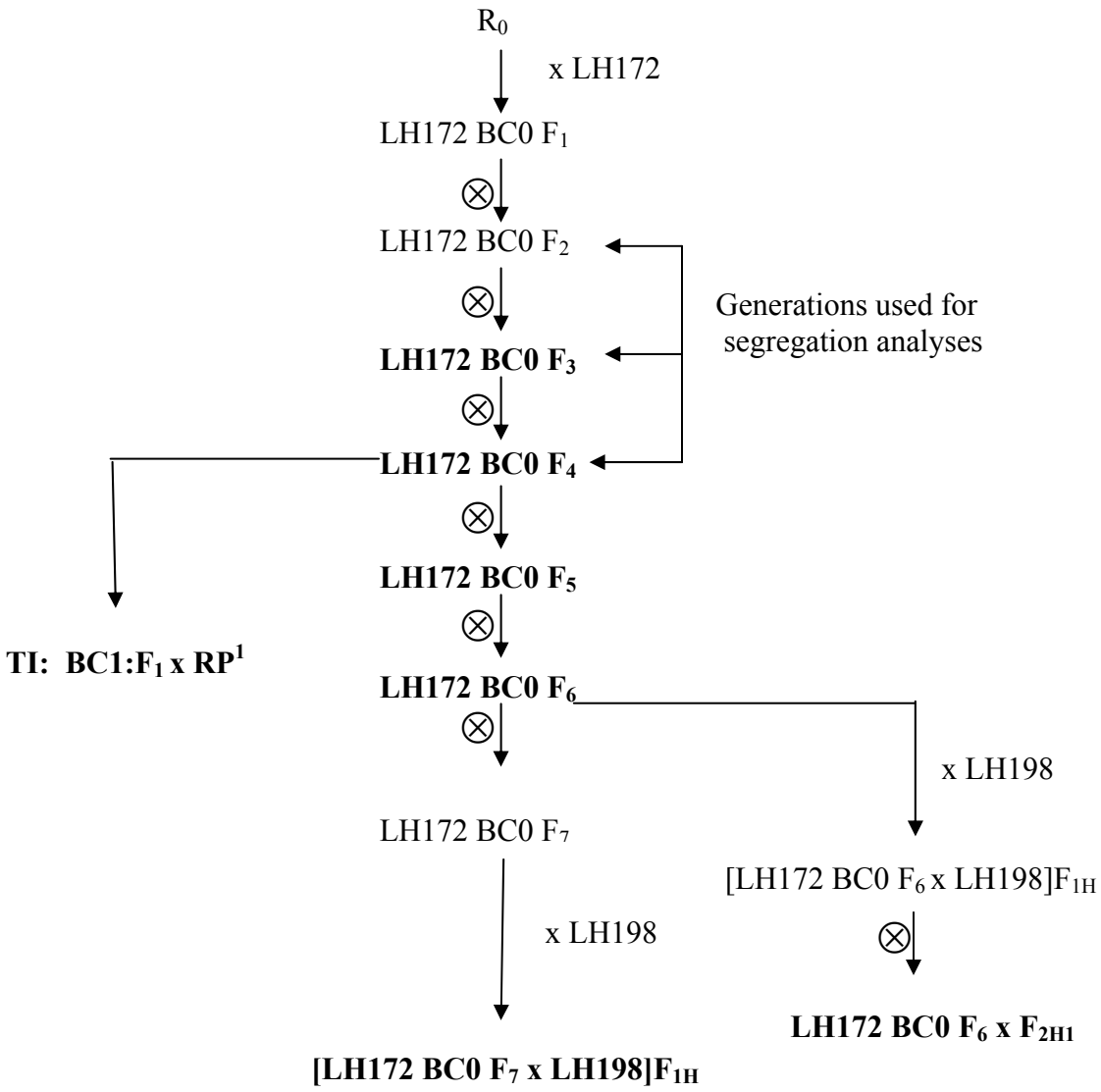
The long run enabled greater separation of the higher molecular weight restriction fragments while the short run allowed the smaller molecular weight restriction fragments to be retained on the gel. The gel side arrow symbols denote the sizes of DNA (kb) based on the reference DNA size markers on the ethidium bromide stained gel. The arrows on the left side of the gel are for the long run lanes, and the arrows on the right side of the gel are for the short run lanes. The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13 in **Figure V-2**). Each lane contains approximately 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]

V.E. Insert Stability across Generations of MON 89034

In order to demonstrate the stability of MON 89034 across generations, additional Southern blot analyses were performed using DNA samples obtained from multiple generations in the MON 89034 breeding history. For reference, the breeding history of MON 89034 is presented in **Figure V-17**. The specific generations tested are indicated in the legends of **Figures V-18**, **V-19**, and **V-20**. In 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 approximately 8.2 and >4.3 kb (refer to **Figure V-4**). The approximately 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 (refer to **Figure V-4**).

Plasmid PV-ZMIR245 DNA was spiked into the conventional corn control DNA which served as a positive hybridization control. All samples were subjected to the same digestion and probing procedure. Individual Southern blots were examined with three probe sets: (1) 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 V-3**); (2) The absence of the *nptII* selectable marker and unique T-DNA II genetic elements was confirmed using overlapping probes spanning T-DNA II (probes 11, 12, and 13 in **Figure V-2**); and (3) 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 in **Figure V-2**). 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 V-17. Breeding history of MON 89034

The LH172 BC0 F_6 x F_{2H1} generation was used for all molecular analyses. Generations used for molecular stability analysis are shown in bold in the breeding tree.

[1] Recurrent parent (RP) that was used in the analyses is referred to as conventional corn A

V.E.1. Stability of the T-DNA I Insert across Generations of MON 89034

Figure V-18 shows the Southern blot analysis examined with overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18-23 in **Figure V-3**). Conventional corn control DNA digested with *Ssp* I (lane 7 and 9) showed several hybridization bands. These bands are difficult to observe in the blot of **Figure V-18**, but are visible upon longer exposure. This is not unexpected since several genetic elements comprising T-DNA I were originally derived from corn.

Conventional corn control DNA containing plasmid PV-ZMIR245 DNA digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at approximately 10.4 and 7.2 kb in addition to the expected endogenous hybridization (**Figure V-18**). The migration of the approximately 10.4 kb fragment is slightly higher than indicated by the reference DNA markers in the lanes 10 and 11. The altered migration may be due to the difference in salt concentration between the test DNA samples and the reference DNA markers (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 approximately 8.2 and 7.4 kb in addition to the expected endogenous hybridization (**Figure V-18**). The approximately 8.2 kb band is the expected size for the 5' border fragment and the approximately 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 V-5** (lanes 3 and 7).

Two faint unexpected bands of approximately 15.6 and 12.0 kb were observed in lanes 3 and 4 of **Figure V-18**. These bands are likely the result of partial digestion because they are not seen in prior or subsequent generations that were produced by self pollination. The presence of the 15.6 kb band is consistent with the possibility that internal *Ssp* I site was not digested. As a result, a combination of the 7.4 and 8.2 kb bands produced 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 unexpected bands were detected, indicating that the single copy of T-DNA I in MON 89034 is stable across the generations tested.

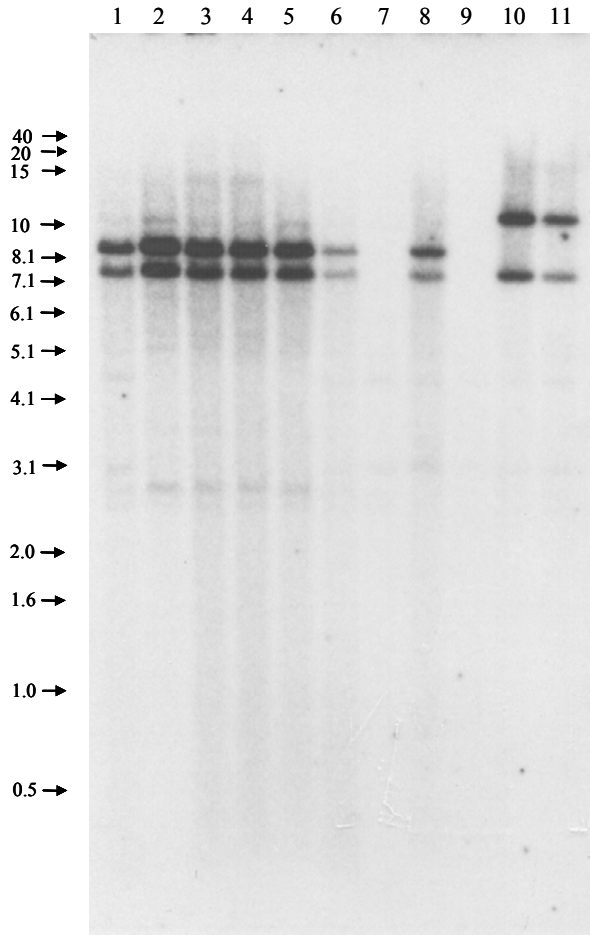


Figure V-18. Insert stability of MON 89034 – T-DNA I

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 – 23 in **Figure V-3**). The gel side arrow symbols denote the sizes of DNA (kb) based on the reference DNA size markers on the ethidium bromide stained gel. Each lane contains approximately 10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172 BC0 F_6xF_{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 (*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]

[1] Monsanto proprietary conventional corn hybrid

V.E.2. Confirmation of the Absence of T-DNA II in Multiple Generations of MON 89034

Figure V-19 shows the Southern blot analysis examined with three overlapping probes spanning T-DNA II (probes 11, 12, and 13 in **Figure V-2**). Conventional corn control DNA digested with *Ssp* I (lane 7 and 9) 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 approximately 10 and 7.2 kb. The migration of the approximately 10 kb fragment is slightly higher than indicated by the reference DNA size markers in the lanes 10 and 11. The altered migration may be due to the difference in salt concentration between the test DNA samples and the reference DNA size markers (Sambrook and Russell, 2001). The overlapping probes spanning T-DNA II contains the *35S* promoter, *nos* 3' end, and the Left Border which are also contained on T-DNA I. Therefore, the T-DNA II probe is expected to hybridize to fragments which are common to 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 approximately 8.2 and 7.4 kb (**Figure V-19**), which are consistent with those observed with the overlapping T-DNA I probes. The approximately 8.2 kb band is the expected size for the 5' border fragment and the approximately 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 unexpected bands of approximately 15.6 and 12.0 kb were observed in lanes 3 and 4 (not obvious in **Figure V-19**). These bands are consistent with the bands observed in **Figure V-18** (lanes 3 and 4) and are likely the result of partial digestion.

No additional unexpected bands were detected, indicating that different generations of MON 89034 do not contain any additional detectable T-DNA II elements other than those which are common to T DNA I.

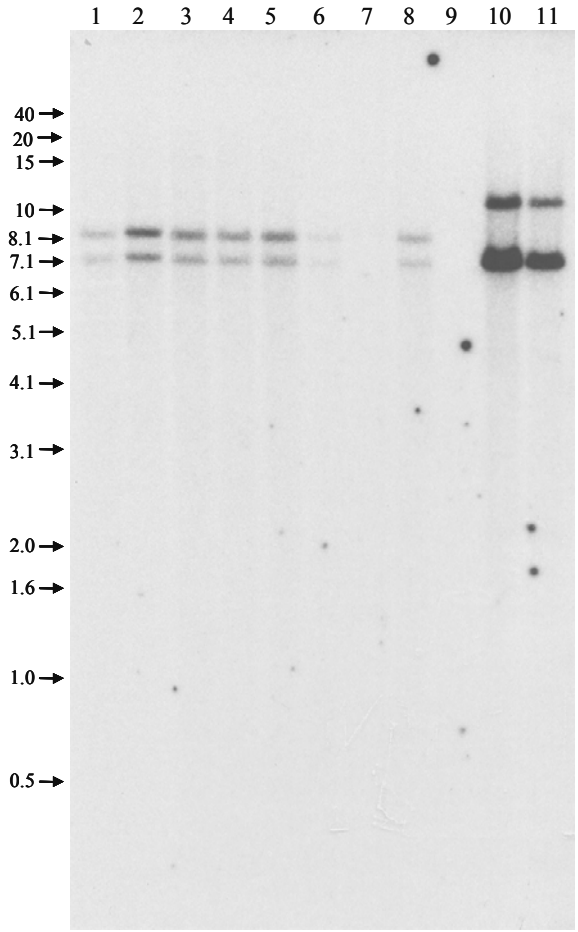


Figure V-19. T-DNA II analysis in multiple generations of MON 89034 – T-DNA II

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13 in **Figure V-2**). The gel side arrow symbols denote the sizes of DNA (kb) based on the reference DNA size markers on the ethidium bromide stained gel. Each lane contains approximately 10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172BC0F_6xF_{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 (*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]

[1] Monsanto proprietary conventional corn hybrid

V.E.3. Confirmation of the Absence of Plasmid PV-ZMIR245 Backbone Sequence in Multiple Generations of MON 89034

Figure V-20 shows the Southern blot analysis examined with four overlapping probes spanning the PV-ZMIR245 backbone sequence (probes 14-17 in **Figure V-2**). Conventional corn control DNA obtained from LH172, digested with *Ssp* I (lane 7 and 9) showed no detectable hybridization bands. Although difficult to observe in **Figure V-20**, overexposures of Southern blots showed that conventional corn A control DNA, digested with *Ssp* I (lane 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 and digested with *Ssp* I (lanes 10 and 11) produced the two expected size bands at approximately 10.4 and 7.2 kb.

DNA extracted from seven generations of MON 89034 digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) produced no hybridization bands. This indicates that MON 89034 does not contain any PV-ZMIR245 backbone elements across the different generations tested.

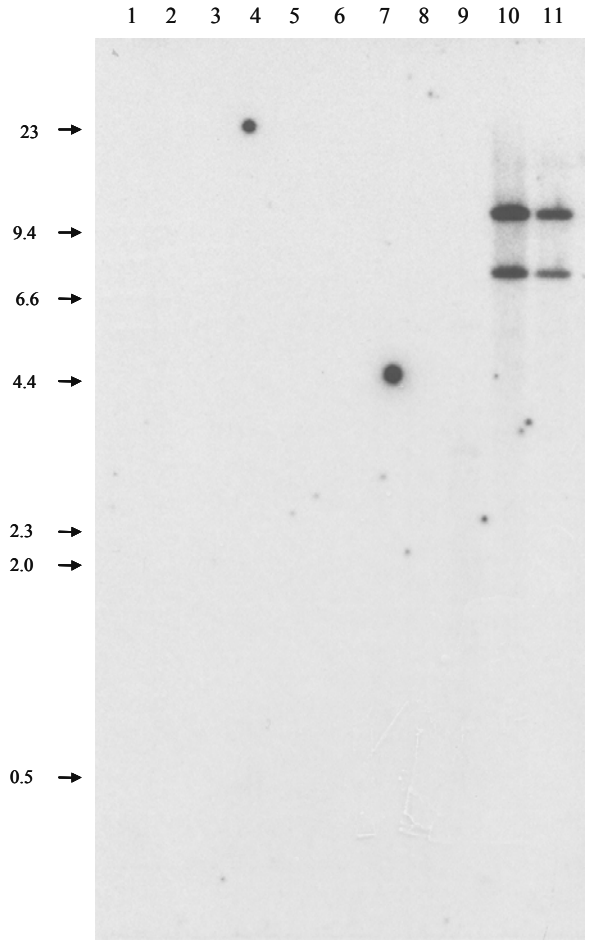


Figure V-20. Backbone analysis in multiple generations of MON 89034 – PV-ZMIR245 backbone sequence

The blot was examined with overlapping ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17 in **Figure V-2**). The gel side arrow symbols denote the sizes of DNA (kb) based on the reference DNA size markers on the ethidium bromide stained gel. Each lane contains approximately 10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: MON 89034 (LH172BC0F$_6$xF$_{2H1}$, *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 (*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]

[1] Monsanto proprietary conventional corn hybrid

V.F. Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 89034

The 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 (9317 bp), and the amplified DNA fragments were subjected to DNA sequencing analyses (for more details refer to **Appendix A**). The results confirmed that the sequence of the DNA insert in MON 89034 matched the designed, corresponding sequences in PV-ZMIR245 with one exception. This exception is 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 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 V-1**). 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.

V.G. 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 the *cry2Ab2* genes, and Cry1A.105 and Cry2Ab2 proteins) (**Table V-2**). The confirmation of the presence of the gene and stability of the trait was based on one of several assays: 1) ELISA to detect Cry2Ab2 protein; 2) ELISA to detect Cry1A.105 protein; 3) GeneCheck® immunoassays to detect Cry2Ab2 protein (Cry2A QuickStix Lateral Flow test strips, Envirologix Inc., Portland, MN); and 4) PCR assay to detect the presence of the *cry* genes.

As described earlier, MON 89034 was developed using a 2T-DNA vector transformation system that employed two separate T-DNAs. One T-DNA (T-DNA I) contained the *cry1A.105* and *cry2Ab2* genes while the other T-DNA (T-DNA II) contained the *npt*II gene. F_1 plants were generated in a LH172 germplasm by making crosses of the R_0 plant with the LH172 inbred. From the population of produced F_1 plants, selections were made for the absence of *nptII* gene, and the 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 V-17**). 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 V-2**).

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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 V-17**).
[c] To confirm segregation, The LH172 $BC1F_1$ plants were selfed to produce two different plant populations of this generation (not shown on the breeding tree, **Figure V-17**).

The Chi-square test was computed as:

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

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 V-2**. 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 V-2. Segregation analyses of MON 89034

Generation	Number of plants	Observed Positives [a]	Observed Negatives	Expected Positives [a]	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	0.5	>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.

V.H. Conclusions for the Genetic Characterization of MON 89034

MON 89034 was produced by *Agrobacterium*-mediated transformation of corn with the PV-ZMIR245, which is a binary vector containing 2T-DNAs. 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. During transformation, both T-DNAs were inserted into the genome. Traditional breeding was used to isolate plants that only contain the *cry1A.105* and *cry2Ab2* expression cassettes (T-DNA I) and do not contain the *nptII* expression cassette (T-DNA II), thereby, producing marker-free corn MON 89034.

Molecular characterization of MON 89034 by Southern blot analyses 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 *cry2Ab2* expression cassettes. All expression elements are shown to be present in the inserted DNA as expected with the exception of a modified e35S promoter regulating the *cry1A.105* gene, and there are no other elements, either full length or partial, present other other than those associated with the intended insert. 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 sequence of the DNA insert in MON 89034 matched the designed, corresponding sequences of T-DNA I in PV-ZMIR245 with one exception in 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 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. 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 originally-designed *e35S* promoter in PV-ZMIR245. Despite this modification, 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.

The stability of the integrated DNA was demonstrated by the fact that the Southern blot fingerprint of MON 89034 was maintained in the tested generations of the breeding history. Additionally, T-DNA II analysis of multiple generations from the MON 89034 breeding history indicated that there were no T-DNA II elements present other than those (i.e., *35S* promoter, *nos* 3′ end sequence, Left Border sequence) which are common to T-DNA I. Furthermore, these generations have been shown not to contain any backbone sequence from plasmid PV-ZMIR245. This stability was further confirmed by the fact that the inheritance of the lepidopteran protection trait in MON 89034 follows Mendelian segregation principle.

V.I. References

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VI. Characterization of the Introduced Cry1A.105 and Cry2Ab2 Proteins

This section summarizes the evaluation of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 including: (a) equivalence of the *in planta*-produced proteins to the recombinant *E. coli* produced proteins used in protein safety studies; (b) the expression levels of the proteins determined in corn tissues; and (c) a summary of the food and feed safety assessment of the Cry1A.105 and Cry2Ab2 proteins.

VI.A. The Cry1A.105 Protein

VI.A.1. Identity and Function of the Cry1A.105 Protein

Cry1A.105 is a full-length, insecticidal protein consisting of 1177 amino acids with a molecular weight (MW) of 133 kDa. It is a chimeric protein that consists of domains I and II from Cry1Ab or Cry1Ac[1], domain III from Cry1F, and the C-terminal domain 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). Cry1A.105 was designed using domain exchange strategy to achieve high levels of activity against target lepidopteran insect pests. The domains I and II of Cry1A.105 are 100% identical to the respective domains of Cry1Ab or Cry1Ac. The domain III of Cry1A.105 is 99% identical to the domain III of Cry1F. The C-terminal region of Cry1A.105 is 100% identical to that of Cry1Ac. 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 due to high degree of homology (Crickmore, 2004; **Figure VI-2**).

Domain exchange is a well known occurring mechanism in nature, resulting in Bt protein diversities, which has been described extensively in the literature (De Maagd et al.; 2001; 2003; Masson et al., 2002). Domain exchange strategy with modern molecular biological tools has been used to switch the functional domains of Cry1 proteins to develop microbial biopesticides with improved specificity to lepidopteran insect pests. Microbial pesticides that contain Cry1Ac/Cry1F chimeric protein have been used for control of lepidopteran pests since 1997 (Baum, 1998; 1999), and a biotechnology-derived cotton 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

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

formation. Domain II is involved in specific receptor recognition and binding. Domain III has been suggested to maintain the structural integrity of the protein molecule (Li et al., 1991) and also to contribute to specificity (De Maagd et al., 2000; 2001). The C-terminal domain is implicated in crystal formation which does not directly contribute to the insecticidal activity (De Maagd et al., 2001). C-terminal domain is cleaved upon entry into the insect midgut or by certain proteases *in vitro*. Domains I, II, and III are retained during the formation of active toxins (from the protoxins) in insect guts. Only those 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 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).

VI.A.2. Characterization of Cry1A.105 Protein Produced in MON 89034

The expression levels of Cry1A.105 protein in different tissue of MON 89034 are relatively low. Therefore, it was necessary to produce the protein in a high-expressing, recombinant microorganism in order to obtain sufficient quantities of the protein for safety studies. A recombinant Cry1A.105 protein was produced in *Escherichia coli*, the sequence of which was engineered to match that of Cry1A.105 protein produced in MON 89034. The equivalence of the physicochemical characteristics and functional activity between the MON 89034-produced and *E. coli*-produced Cry1A.105 protein was confirmed by a panel of analytical techniques, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), glycosylation analysis, and assay of biological activity. The details of the materials, methods, and results are described in **Appendix B,** while the conclusions are summarized as follows.

The Cry1A.105 protein isolated from MON 89034 was purified and characterized, and results confirmed the equivalence of MON 89034-produced and *E. coli*-produced Cry1A.105 protein. SDS-PAGE demonstrated that the MON 89034-produced Cry1A.105 co-migrated to the same position on the gel as the *E. coli*-produced Cry1A.105 protein, indicating the protein from both sources was equivalent in molecular weight. 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 those of the *E. coli*-produced Cry1A.105 protein. The intactness of the N-terminus for the MON 89034-produced Cry1A.105 protein was confirmed with an antibody which is specific to the N-terminal peptide. 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 sequence. In addition, the MON 89034- and the

E. coli-produced Cry1A.105 protein was found to be equivalent based on functional activities and the lack of glycosylation. Taken together, these data provide a detailed characterization of the Cry1A.105 protein isolated from MON 89034 and were used in establishing its equivalence to the *E. coli*-produced Cry1A.105 protein.

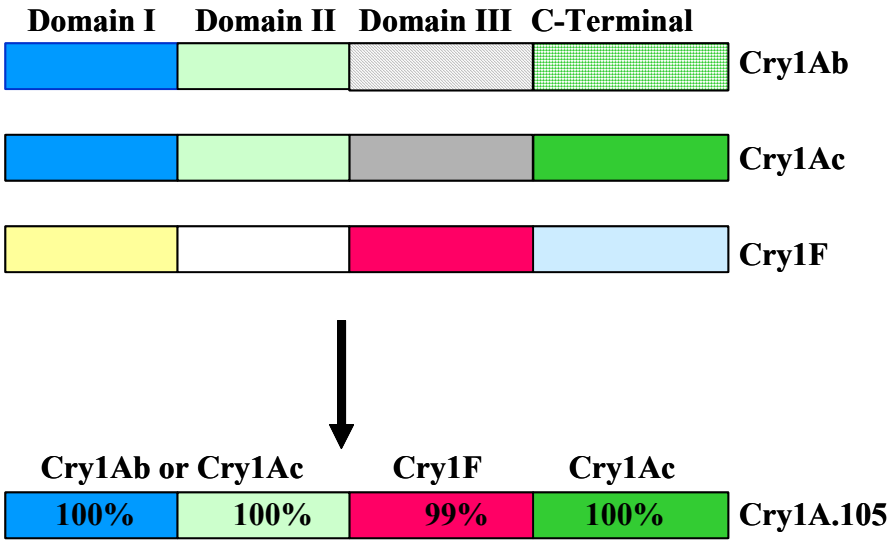

Figure VI-1. Schematic representation of the origin of Cry1A.105 protein domains

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

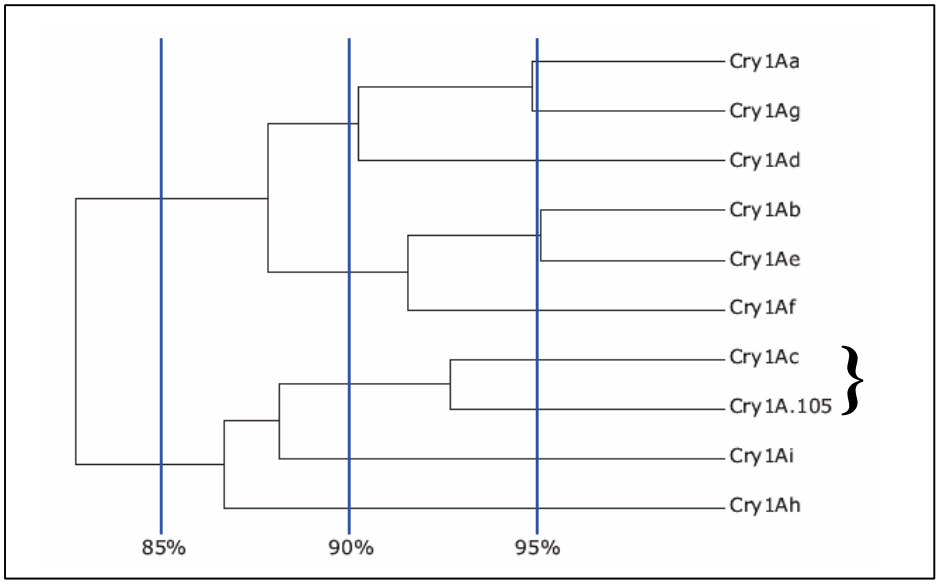

Figure VI-2. Phylogram showing Cry1A.105 and Cry1Ac proteins in the same cluster based on amino acid sequence identity

VI.B. The Cry2Ab2 Protein

VI.B.1. Identity and Function of the Cry2Ab2 Protein

The Cry2Ab2 is a protein derived from *Bacillus thuringiensis* subsp. *kurstaki* (Btk). Btk has been used as an active ingredient in many commercial microbial pesticide products such as DiPel® and Cutlass® (EPA, 2005). Cry2Ab protein has been listed as one of the proteins in Cutlass®, and Cry2Aa as one of the proteins in DiPel® (Betz et al., 2000). Cry2Ab and Cry2Aa share 88% amino acid sequence identity (Dankocsik et al., 1990; Widner and Whiteley, 1989).

Like the Cry2Ab2 protein produced in the biotechnology-derived cotton Bollgard® II, the Cry2Ab2 protein produced in MON 89034 is a slight variant of the wild-type Cry2Ab2 protein. The Cry2Ab2 produced in MON 89034 is identical to the Cry2Ab2 produced in Bollgard II cotton, and they differ from the wild-type Cry2Ab2 of Bt by only one amino acid (**Figure VI-3**). Accumulation of the Cry2Ab2 protein in corn MON 89034 is targeted to the chloroplasts using a chloroplast transit peptide (CTP). CTPs facilitate the intracellular transport of proteins from the cytoplasm and the plastids (Bruce, 2000). CTPs are typically cleaved from the mature protein on uptake into the chloroplasts, and then rapidly degraded. To allow targeting of the Cry2Ab2 protein to the plastids in MON

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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 plastids through a CTP. The attempts to determine the N-terminal sequence of the Cry2Ab2 in Bollgard® II (after the CTP was cleaved) indicated that the N-terminus was blocked, therefore, the exact excision site of the CTP is not known. Attempts to determine the N-terminal sequence of the full-length Cry2Ab2 protein produced by MON 89034 indicated that the protein was also blocked at the N-terminus. Consequently, the cleavage site in the CTP that is processed in the chloroplast could not be experimentally determined. The CTP used in MON 89034 has a preferred cleavage site (methionine) located three amino acids upstream from the start of the Cry2Ab2 protein sequence (**Figure VI-3**). The next preferred cleavage site would have resulted in a protein approximately 2.8 kDa larger than what was actually detected. Since the MON 89034-produced Cry2Ab2 co-migrated to the similar position as the Cry2Ab2 produced by a recombinant *E. coli*, which has the three extra amino acids (**Figure VI-3**)**,** very likely the CTP in MON 89034 was cleaved at the preferred cleavage site located three amino acids upstream from the start of the Cry2Ab2 protein sequence. 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.

Cry2Ab2 protein is active against corn and cotton 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), cutworms (e.g., *Agrotis ipsilon*, black cutworm), tobacco budworm (*Heliothis virescens*) and pink bollworm (*Pectinophora gossypiella*).

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 the chloroplast transit peptide (CTP)
[2] **D** – an additional amino acid included for the ease of cloning

Figure VI-3. Comparison of the putative N-terminal sequence of Cry2Ab2 protein produced in MON 89034, the recombinant *E. coli*, and *Bacillus thuringiensis*

VI.B.2. Characterization of the Cry2Ab2 Protein Produced in MON 89034

Similar to the case for Cry1A.105, the expression level of Cry2Ab2 protein in the tissue of MON 89034 is relatively low. Therefore, it was necessary to express the protein in a high-expressing, recombinant host system in order to obtain sufficient quantities of the Cry2Ab2 protein for safety studies. Thus, a recombinant Cry2Ab2 protein was produced in *E. coli*, the sequence of which was engineered to match the Cry2Ab2 protein produced in MON 89034. The equivalence of the physicochemical characteristics and functional activity between the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein was confirmed by a panel of analytical techniques, including SDS-PAGE, densitometry, Western blot analysis, MALDI-TOF MS, N-terminal sequencing, glycosylation analysis, and functional bioassay with corn earworm (CEW) (*Helicoverpa zea*). The details of the materials, methods, and results are described in **Appendix C,** while the conclusions are summarized as follows.

The Cry2Ab2 protein from MON 89034 was purified and characterized, and the results confirmed the equivalence between MON 89034- and *E. coli*-produced Cry2Ab2 protein. 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 protein for the antibody used. The N-terminus of the Cry2Ab2 derived from MON 89034 was blocked but the peptide mapping analysis by MALDI-TOF MS yielded peptide masses consistent with the expected peptide masses based on the trypsin cleavage sites in the sequence of Cry2Ab2 protein, confirming the identity of the protein. In addition, the MON 89034- and the *E. coli*-produced Cry2Ab2 protein was 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.

VI.C. Expression Levels of Cry1A.105 and Cry2Ab2 Proteins in MON 89034

The levels of the Cry1A.105 and Cry2Ab2 proteins in various tissues of MON 89034 were determined using enzyme-linked immunosorbent assays (ELISA). The materials and methods for the ELISA analysis, as well as a description of the tissue types, are provided in **Appendix D**. To produce the tissues for analysis, MON 89034 and conventional corn were planted at five field locations during the 2005 growing season. The sites were located in the major corn-growing regions of the United States. A randomized complete block design with three replications was used at all sites. Forage, stover, silk, pollen, and grain samples were collected at appropriate times of plant development. Leaf, root, and whole plant samples were collected multiple times over the season. The expression levels of Cry1A.105 and Cry2Ab2 proteins in these tissues are shown in **Tables VI-2** to **VI-4**.

The mean Cry1A.105 protein levels across all sites were 5.9 µg/g dwt in grain, 42 µg/g dwt in forage, 12 µg/g dwt in pollen, 520 µg/g dwt in leaves of plants at V2-V4 stage[1], 120 µg/g dwt in leaves of plants at pre-VT stage, 12 µg/g dwt in forage root, and 50 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry1A.105 protein levels across all sites varied from 72-520 µg/g dwt in leaf, 11-79 µg/g dwt in root, and 42-380 µg/g dwt in whole plant. In general, levels of the Cry1A.105 protein declined over the growing season.

The mean Cry2Ab2 protein levels across all sites were 1.3 µg/g dwt in grain, 38 µg/g dwt in forage, 0.64 µg/g dwt in pollen, 180 µg/g dwt in leaves of plants at V2-V4 stage, 160 µg/g dwt in leaves of plants at pre-VT stage, 21 µg/g dwt in forage root, and 62 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry2Ab2 protein levels across all sites varied from 130-180 µg/g dwt in leaf, 21-58 µg/g dwt in root, and 38-130 µg/g dwt in whole plant. In general, levels of the Cry2Ab2 protein declined over the growing season.

[1] Corn growth stages are divided into vegetative (V) and reproductive (R) stages. Corn growth staging begins with VE, or emergence of seedlings above ground. The second growth stage is V1, and is given to plants that display their first collared leaf. Subsequent stages run from V2, V3 ... V(n), each stage corresponding to the number of leaves displaying collars. V(n) refers to the last leaf stage, and will vary depending on the hybrid in consideration. The final vegetative stage is VT, or tassling stage when last branch of the tassel is completely visible and the silks have not yet emerged. VT begins approximately 2-3 days before silk emergence. Reproductive (R) stages range from R1 to R6. Stage R1 is the silking stage, when silk is visible outside the husk. Stage R2 is the blister stage and occurs 10-14 days after silking. In this stage kernels are white and small, resembling blisters. Milking stage (R3) occurs 18-22 days after silking. Kernels are yellow and contain white fluid, due to accumulating starch. In the dough stage (R4), this fluid begins to thicken into a white paste within the kernels, and the shelled cob takes on a pinkish hue. Stage R5 is the dent stage, where kernels begin drying and exhibit a dent on their top. Around 55-65 days after silking the ears are physiologically mature (R6). In this stage, all kernels have attained their maximum dry weight. Black zones have formed internally at the base of kernels, indicating that kernel growth has ceased. Also, husks and leaves are no longer green at this stage, though stems may still be pigmented. Source: Iowa State University (maize.agron.iastate.edu/corngrows.html. Accessed on July 17, 2006.

Table VI-2. Cry1A.105 and Cry2Ab2 protein expression levels in MON 89034 tissues

Tissue Type	Growth Stage	Cry1A.105 Mean[2] (SD), Range, n=15		Cry2Ab2 Mean[2] (SD), Range, n=15	
		µg/g fwt	µg/g dwt	µg/g fwt	µg/g dwt
Young leaf [1]	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-3. Cry1A.105 proteins expression levels in overseason tissues of MON 89034

Over-Season Tissue (n = 15)	Units	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[2] (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[2] (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[2] (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-2**. SD = standard deviation.

Table VI-4. Cry2Ab2 proteins expression levels in overseason tissues of MON 89034

Over-Season Tissue (n = 15)	Units	Plant growth stages[1] V2-V4 (21-29 DAP) µg/g dwt	V2-V4 (21-29 DAP) µg/g fwt	V6-V8 (28-43 DAP) µg/g dwt	V6-V8 (28-43 DAP) µg/g fwt	V10-V12 (41-53 DAP) µg/g dwt	V10-V12 (41-53 DAP) µg/g fwt	pre-VT (56-68 DAP) µg/g dwt	pre-VT (56-68 DAP) µg/g fwt	R4-R6 (100-120 DAP) µg/g dwt	R4-R6 (100-120 DAP) µg/g fwt	R6 (130-160 DAP) µg/g dwt	R6 (130-160 DAP) µg/g fwt
Leaf	Mean[2] (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[2] (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[2] (SD)	56 (17)	6.4 (1.6)	58 (18)	7.6 (4.2)	35 (17)	5.0 (2.7)	26 (7.7)	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 **Table VI-2**. SD = standard deviation.

VI.D. Food and Feed Safety Assessment Summary of the Cry1A.105 and Cry2Ab2 Proteins

Numerous factors have been considered in the safety assessment of the Cry1A.105 and Cry2Ab2 proteins that are expressed in MON 89034. A comprehensive assessment of safety of these proteins was submitted to the EPA and FDA. The assessment leads to the following conclusions, which are consistent with the conclusions reached for other Cry proteins produced in a number of insect-protected crops currently on the market.

a) The donor organism, *Bacillus thuringiensis,* has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activities. The extremely low mammalian toxicity of Bt-based insecticide products has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived Bt products during the past 45 years of use.

b) Cry1A.105 is a chimeric Cry1A protein of Bt that is comprised of three N-terminal domains (I, II, and III) and the C-terminal region. The domains I and II of Cry1A.105 are 100% identical to the respective domains of Cry1Ab or Cry1Ac. The domain III of Cry1A.105 is 99% identical to the domain III of Cry1F. The C-terminal region of Cry1A.105 is 100% identical to that of Cry1Ac. The overall amino acid sequence identity of 93.6%, 90.0%, and 76.7% to Cry1Ac, Cry1Ab, and Cry1F proteins, respectively. Cry2Ab2 is a Bt (subsp. *kurstaki*) protein. Bt strains producing Cry1Ac, Cry1Ab, Cry1F, and Cry2A proteins have been used for decades as biopesticides, and Bt pesticides that contain Cry1Ac/Cry1F chimeric protein have been used since 1997. Bt corn and cotton expressing Cry1Ac, Cry1Ab, Cry1F, or Cry2Ab2 proteins have been cultivated in large areas in the U.S. and other countries for up to a decade. There are no known reports of allergy or toxicity to Bt or to these Cry proteins.

c) A dietary safety assessment to evaluate the risks to humans and animals from the Cry1A.105 and Cry2Ab2 proteins present in the foods and feeds derived from MON 89034. Risks are quantified as a margin of exposure (MOE), which is defined as the ratio of the No Observable Effect Level (NOEL) from an acute mouse gavage study to estimates of the dietary intake of the respective Cry protein. Mice acute oral toxicity studies demonstrated that the two proteins are not acutely toxic and do not cause any adverse effects even at the higest dose levels test, which are 2072 and 2198 mg/kg body weight for Cry1A.105 and Cry2Ab2 proteins, respectively. The dietary safety assessment showed that 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, 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.

d) Cry1A.105 and Cry2Ab2 proteins are rapidly digestible in simulated gastric fluids. Greater than 95% to 99% of the proteins were digested in simulated gastric fluids in less than 30 seconds. Proteins that are rapidly digestible in mammalian gastrointestinal systems are unlikely to be allergens when consumed.

e) Cry1A.105 and Cry2Ab2 proteins do not share any amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins which have adverse effects to mammals. This has been shown by extensive assessments with bioinformatic tools, such as FASTA sequence alignment tool and an eight-amino acid sliding window search.

Using the guidance provided by the FDA, a conclusion of "no concern" is reached for the donor organisms and the Cry1A.105 and Cry2Ab2 proteins. The food and feed products containing MON 89034 or made of MON 89034 are safe for human and animal consumption. The EPA has issued a time limted tolerance exemption for 3 years starting from July 17, 2007 for the two proteins while the review for a full registration is under review.

VI.E. References

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VII. Phenotypic, Agronomic, Ecological Interactions and Compositional Assessment

This section provides an assessment of the phenotypic and agronomic characteristics, the ecological interactions, and compositional analyses of MON 89034. Phenotypic and agronomic characteristics of MON 89034 were evaluated relative to an appropriate control to assess plant pest potential and meaningfully altered ecological impact. These assessments included 14 plant growth and development characteristics, five seed germination parameters, two pollen characteristics, and more than 70 observations for each of the plant-insect, plant-disease and plant responses to abiotic stressor interactions. Results from the phenotypic and agronomic assessments indicate that MON 89034 does not possess characteristics that would confer a plant pest risk or increased ecological risk compared to conventional corn. Data on ecological interactions indicate that MON 89034 does not confer any increased susceptibility or tolerance to specific diseases, insects (except for the target lepidopteran species), or abiotic stressors. Data from compositional analyses demonstrated compositional equivalence in the levels of nutritional, anti-nutritional, and secondary metabolites between MON 89034 and conventional corn grain and forage. Taken together, these data support a conclusion that MON 89034 is not likely to pose an increased plant pest risk or result in meaningfully altered ecological impact compared to conventional corn.

VII.A. Phenotypic, Agronomic, and Ecological Interactions Assessment

VII.A.1. Phenotypic and Agronomic Characteristics and Ecological Interactions

In the phenotypic, agronomic and ecological interactions assessment of MON 89034, data were collected to evaluate specific ecological risks regarding altered pest potential based on requirements of USDA-APHIS. The assessment encompasses five general data categories: 1) germination, dormancy and emergence; 2) vegetative growth; 3) reproductive growth (including pollen characteristics); 4) seed retention on the plant; and 5) plant interactions with insects, diseases and abiotic stressors. An overview of the characteristics assessed is presented in **Table VII-1**.

The phenotypic, agronomic and ecological interactions evaluations are based on a combination of laboratory experiments and field studies conducted by scientists who are familiar with the production and evaluation of corn. In each of these assessments, the regulated article, MON 89034 (referred to as test substance or material), was compared to an appropriate conventional corn (referred to as control substance or material) which has a genetic background similar to MON 89034 but does not possess the lepidopteran-protection trait. In addition, multiple commercial corn hybrids (referred to as reference substances or materials) were also employed to provide a range of baseline values that are common to the existing commercial corn hybrids for each measured phenotypic, agronomic, and ecological interaction characteristic. Further descriptions of the test, control and reference materials are provided in **Appendices E** and **F**.

Table VII-1. Phenotypic, agronomic and ecological interaction characteristics measured for MON 89034

General characteristics	Characteristics measured	Evaluation timing	Evaluation description
Dormancy / Germination	Dormancy, Germination[1]	After 4, 7, and 12 days	Percent normally germinated, abnormally germinated, viable hard (dormant), dead, and viable firm swollen seed
Emergence	Seedling vigor	Stage V2 – V4	Rated on a 0-9 scale, where 0 = dead, and 9 = above average vigor
	Early stand count	Stage V2 - V4	Number of emerged plants per plot
Vegetative Growth	Final stand count	Pre-harvest	Number of plants per plot
	Stay green	Maturity	Rated on a 0-9 scale, where 0 = entire plant is dried, and 9 = entire plant is green
	Ear height	Maturity	Distance from the soil surface at the base of the plant to the ear attachment node
	Plant height	Maturity	Distance from the soil surface at the base of the plant to the flag leaf collar
	Stalk lodged plants	Pre-harvest	Number of plants per plot broken below the ear
	Root lodged plants	Pre-harvest	Number of plants per plot leaning at the soil surface at >30° from the vertical
Reproductive Growth	Days to 50% pollen shed	Pollen shed	Days from planting until 50% of the plants have begun to shed pollen
	Days to 50% silking	Silking	Days from planting until 50% of the plants have silks exposed
	Pollen viability[1]	Tasseling	Viable and nonviable pollen based on pollen grain staining characteristics
	Pollen morphology[1]	Tasseling	Diameter of viable pollen grains
	Grain moisture	Harvest	Moisture percentage of harvested shelled grain
	Test weight (lb/bu)	Harvest	Test weight of harvested shelled grain
	Yield (bu/ac)	Harvest	Harvested shelled grain, adjusted to 15.5% moisture
Seed Retention	Dropped ears	Pre-harvest	Number of mature ears dropped from plants
Plant-ecological interactions	Insect, disease and abiotic stressors	Variable, from planting to harvest	Qualitative assessment of each plot, with rating on a 0-9 scale for plant-insect, plant-disease, and plant response to abiotic stressor interactions

[1] These characteristics were measured in the laboratories.

VII.A.2. Interpretation of Phenotypic, Agronomic and Ecological Interaction Data

The concept of familiarity is useful when evaluating the potential environmental impact of a biotechnology-derived plant. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant variety whose biological properties and plant pest potential are known to experts. Familiarity considers the biology of the crop, the introduced trait, the receiving environment and the interaction among these factors, and provides a basis for comparative risk assessment between a biotechnology-derived plant and its conventional counterpart. An expert's knowledge and experience with a crop is the basis for selecting appropriate endpoints and estimating the range of responses that would be considered familiar. Thus, assessment of phenotypic and agronomic characteristics and ecological interactions can be used to compare the biotechnology-derived plant to the conventional counterpart, and a subset of the data (e.g., certain dormancy, lodging or pre-harvest seed loss characteristics) can be used to assess whether there is an enhanced weediness potential. Based on all of the data collected, an assessment can be made whether the biotechnology-derived plant is likely to pose an increased plant pest potential or have a meaningfully altered ecological impact compared to the conventional counterpart.

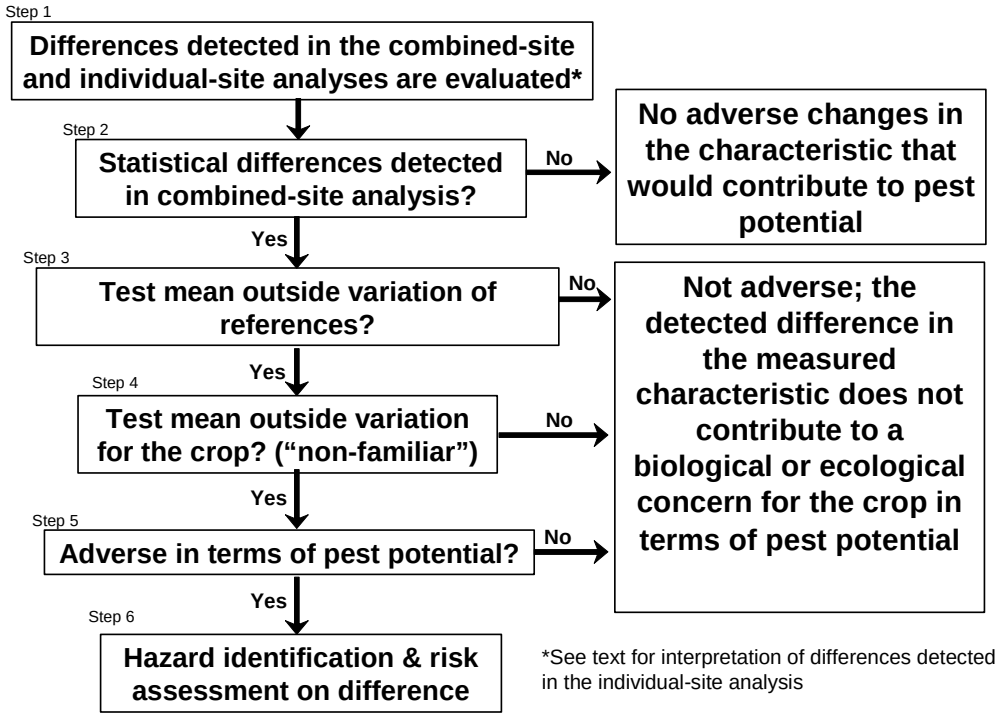
Agronomists familiar with the experimental design, evaluation criteria, and expected outcomes were involved in all steps of data collection, summarization and analyses. This oversight ensured that measurements were taken properly, data were consistent with expectations based on experience with the crop, and the experimental and field sites were carefully monitored. These scientists were expected to report any unexpected observations or issues during the course of the studies. The overall dataset was evaluated for evidence of biologically relevant changes and for any evidence of an unexpected plant response, and the data were subsequently submitted for statistical analysis.

Comparative plant characterization data between a biotechnology-derived crop and the control are considered in the context of contributions to increased pest/weediness potential. Characteristics for which no differences are detected support a conclusion of no increased pest potential of the biotechnology-derived crop compared to the conventional crop. Characteristics for which differences are detected are considered in the step-wise method (**Figure VII-1)**. Any detected difference for a characteristic is considered in the context of whether or not the difference would increase pest/weediness potential of the biotechnology-derived crop. Ultimately, a weight of evidence approach considering all characteristics and studies is used for the overall risk assessment of differences and their significance in terms of increased pest potential. In detail, **Figure VII-1** illustrates the stepwise assessment process employed:

- Steps 1 & 2. Combined-site and individual-site statistical analyses are conducted and evaluated on each measured characteristic. Differences detected in the individual-site analysis must be observed in the combined-site analysis to be considered further for potential adverse effects in terms of pest/weediness potential. A difference in the combined-site analysis is further assessed

regardless of whether or not the difference is detected in the individual-site analysis.

- Step 3. If a difference is detected in the combined-site analysis across multiple environments, then the test substance mean value is assessed relative to the range of values of the reference substances.
- Step 4. If the test substance mean is outside the variation of the reference substances (e.g., reference range or tolerance interval), the test substance mean is considered in the context of known values common for the crop.
- Step 5. If the test substance mean is outside the range of values common for the crop, the test substance is considered "non-familiar" for that characteristic. The detected difference is then assessed for whether or not it is adverse in terms of pest/weediness potential.
- Step 6. If an adverse effect (hazard) is identified, risk assessment on the difference is conducted. The risk assessment considers contributions to enhanced pest potential of the crop itself, the impact of differences detected in other measured characteristics, and potential for, and effects of trait transfer to feral populations of the crop or a sexually compatible species.



Note: A "no" answer at any step indicates that the characteristic does not contribute to a biological or ecological concern for the crop in terms of pest/weediness potential and further steps are not considered.

Figure VII-1. Flow chart for interpretation of detected differences

VII.A.3. Evaluation of Phenotypic and Agronomic Characteristics and Ecological Interactions

VII.A.3.a. Evaluation of Seed Dormancy and Germination

Seed dormancy (e.g., hard seed) is an important characteristic that is often associated with plants that are considered as weeds (Anderson, 1996; Lingenfelter and Hartwig, 2003). Dormancy mechanisms, including hard seed, vary with species and tend to involve complex processes. For corn, the number of hard seed is negligible or nonexistent. Standardized germination assays are routinely used to measure the germination characteristics of corn seed. The Association of Official Seed Analysts (AOSA) recommends a temperature range of 20-30°C as optimal for germination of corn (AOSA, 2002).

The seed materials for the evaluation of MON 89034 were produced in 2004 at three production locations (Dayton, IA; Jerseyville, IL; and Monmouth, IL) that are representative of the environmental variability found in corn producing regions. Seed materials included MON 89034, the conventional control corn (H1325023) which has a genetic background similar to MON 89034, and four reference commercial corn hybrids from each production location.

The dormancy and germination characteristics evaluated, temperature regimes and the evaluation description are presented in **Table VII-2**. The tests were conducted in temperature-controlled growth chambers using the rolled towel test method. Four replicates of MON 89034, the control, and the four references were tested in seven growth chambers, each maintained in the dark under one of the following temperature regimes (**Table VII-2**): (1) constant target temperature of approximately 5, 10, 20, or 30°C; and (2) alternating target temperatures of approximately 10/20, 10/30, or 20/30°C. In these alternating temperature regimes, the lower temperature was maintained for 16 h and the higher temperature for 8 h. Counts for characteristics were made four and seven days after planting. A statistical comparison between the MON 89034 and the control was conducted using a SAS (Statistical Analysis System) program (SAS Release 9.1, 2002-2003). Statistical significance was set at $p \leq 0.05$. A summary of the results is provided below, while the details of the materials, methods and results from individual production sites are provided in **Appendix E**.

A total of 29 comparisons were made between MON 89034 and the conventional control seed germination parameters across three seed production sites (**Table VII-3**). No statistically significant differences were detected between MON 89034 and the control for any seed characteristic measured in the AOSA temperature regime (20/30°C) or the additional temperature regimes (5, 10, 20, 30, 10/20, 10/30°C).

The observation of no viable hard seed in any temperature regime indicates that the introduction of the lepidopteran-protection trait and the expression of the Cry1A.105 and Cry2Ab2 proteins did not alter dormancy and germination of the seed of MON 89034 compared to the conventional corn seed. Thus, it is concluded that there was no change

in the pest potential of MON 89034 compared to conventional corn based on the germination and dormancy characteristics assessed.

VII.A.3.b. Evaluation of Pollen Morphology and Viability

The purpose of this evaluation was to assess whether the introduction of the lepidopteran-protection trait and the expression of the Cry1A.105 and Cry2Ab2 proteins altered the pollen characteristics of MON 89034 compared to the conventional corn. The test material was corn pollen collected from MON 89034 plants. The control material was corn pollen collected from the conventional corn plants (H1325023) which has a genetic background similar to MON 89034. The reference materials were pollen collected from plants of commercially available conventional corn hybrids that were used to demonstrate a range of morphology and viability characteristics for corn pollen.

MON 89034, the conventional control (H1325023), and three references (DKC61-42, RX740, and Burrus 576) were grown in a randomized complete block design with three replications in Carlyle, IL, under similar agronomic conditions. Pollen collected from five plants per plot were fixed and stained with 1:5 diluted Alexander stain (Alexander, 1980) and evaluated for viability. When exposed to the staining solution, viable pollen grains stained red (due to the the presence of vital cytoplasmic content), while dead pollen grains stained light blue. Ten representative viable pollen grains collected from one of the five plants per plot were measured for pollen diameter, which were observed at 200X magnification and were also evaluated for general morphology. Data analysis compared the mean of MON 89034 to the mean of the control corn for average pollen diameter and arcsine transformed percent viable pollen. No statistically significant differences were detected at $p \leq 0.05$ between MON 89034 and the control for pollen diameter or viability (**Table VII-4**). No statistically differences between MON 89034 and the control were observed in pollen general morphology.

These results demonstrated that the introduction of the lepidopteran-protection trait and the expression of the Cry1A.105 and Cry2Ab2 proteins did not alter the overall morphology or viability of MON 89034 pollen compared to the conventional corn. The lack of differences between the pollen collected from MON 89034 compared to the conventional control for the assessed characteristics demonstrate that the observed values were within the range of responses familiar to what is expected for corn.

Table VII-2. Seed dormancy and germination parameters evaluated

Germination characteristic	Temperature regime (°C)	Evaluation description
Normally Germinated	20/30	Seedlings that exhibited normal developmental characteristics and possessed both a root and a shoot.
Abnormally Germinated	20/30	Germinated, but with insufficient root or shoot development, may have possessed a hollow coleoptile, or exhibited mechanical damage.
Total Germinated	5, 10, 20, 30, 10/20, 10/30	Seedlings that had germinated.
Dead	5, 10, 20, 30, 10/20, 10/30, 20/30	Seeds that had visibly deteriorated and had become soft to the touch.
Viable Hard	5, 10, 20, 30, 10/20, 10/30, 20/30	Seeds that did not imbibe water and remained hard to the touch.
Viable Firm Swollen	5, 10, 20, 30, 10/20, 10/30, 20/30	Seeds that had visibly swollen (imbibed water) and were firm to the touch but lacked any evidence of growth.

Note: Dormancy and germination tests were conducted in temperature-controlled growth chambers using the rolled towel test method to measure dormancy and germination characteristics. Four replicates of MON 89034, the control, and the four references were tested in seven growth chambers, each maintained in the dark under one of the following temperature regimes: (1) constant target temperature of approximately 5, 10, 20, or 30°C; and (2) alternating target temperatures of approximately 10/20, 10/30, or 20/30°C. In these alternating temperature regimes, the lower temperature was maintained for 16 h and the higher temperature for 8 h. Counts for characteristics were made four and seven days after planting.

Table VII-3. Germination of MON 89034 and a conventional control corn

AOSA Temp. Regime[1] (°C)	Starting Seed Type[2]	Mean (%)				
		Normally Germinated	Abnormally Germinated	Viable Hard	Dead	Viable Firm Swollen
20/30	Test	94.2	0.1	0.0	5.8	0.0
	Control	95.3	0.2	0.0	4.5	0.0
	Ref. Range	78.0 – 100.0	0.0 – 4.0	0.0 – 0.0	0.0 – 22.0	0.0 – 1.0

Additional Temp. Regime[1] (°C)	Starting Seed Type	Mean (%)			
		Germinated	Viable Hard	Dead	Viable Firm Swollen
5	Test	0.0	0.0	5.7	94.3
	Control	0.0	0.0	6.0	94.0
	Ref. Range	0.0 – 0.0	0.0 – 0.0	0.0 – 19.0	81.0 – 100.0
10	Test	96.0	0.0	4.0	0.0
	Control	94.3	0.0	5.7	0.0
	Ref. Range	82.0 – 100.0	0.0 – 0.0	0.0 – 17.0	0.0 – 1.0
20	Test	95.9	0.0	4.1	0.0
	Control	95.2	0.0	4.8	0.0
	Ref. Range	85.0 – 100.0	0.0 – 0.0	0.0 – 15.0	0.0 – 0.0
30	Test	95.6	0.0	4.4	0.0
	Control	95.5	0.0	4.5	0.0
	Ref. Range	80.0 – 100.0	0.0 – 0.0	0.0 – 20.0	0.0 – 0.0
10/20	Test	95.9	0.0	4.1	0.0
	Control	96.3	0.0	3.8	0.0
	Ref. Range	81.0 – 100.0	0.0 – 0.0	0.0 – 20.0	0.0 – 0.0
10/30	Test	95.3	0.0	4.7	0.0
	Control	96.8	0.0	3.3	0.0
	Ref. Range	81.0 – 100.0	0.0 – 0.0	0.0 – 19.0	0.0 – 0.0

[1] In alternating temperature regimes the lower temperature was maintained for 16 h and the higher temperature for 8 h. Counts for characteristics were made four and seven days after planting.
[2] Test = MON 89034; Control = conventional control corn (H1325023); Ref. Range = Minimum and maximum values among four replications of four references at each of three production sites (except H4242304 produced at IA and HEXP1184 produced at JR with three replications).

The data in this table are the combined-site results of the seeds from the three production sites. The results for seeds from each individual production site are included in **Appendix E**.

Table VII-4. Diameter and viability of pollen collected from MON 89034 and conventional control corn

Characteristic	Mean (SE)[1]		Reference Range[3]	
	MON 89034	Control[2]	Minimum	Maximum
Diameter (µm)	91.8 (1.29)	90.4 (0.18)	85.2	95.5
Viability (%)	96.8 (0.42)	96.4 (1.14)	90.5	99.0

[1] SE = standard error.
[2] The control corn was H1325023 which has a genetic background similar to MON 89034.
[3] References were three commercial corn hybrids: DKC61-42, RX740, and Burrus 576. Minimum and maximum mean diameter values of nine reference plants (three different reference corn × three replications). Each replication was a mean of ten viable pollen grains per plant. Minimum and maximum observed viability values were based on 45 reference plants (three reference corn × three replications × five plants per replication).

VII.A.3.c. Evaluation of Phenotypic and Agronomic Characteristics under Field Conditions

Phenotypic and agronomic characteristics encompassing plant growth, development and yield characteristics were assessed under field conditions. The purpose of these evaluations was to identify any unintended changes to the phenotypic and agronomic characteristics of MON 89034 relative to conventional corn. In each study, reference corn hybrids were included to provide a range of values for the assessed characteristics that are common to corn. In addition, certain growth, reproduction, and pre-harvest seed loss characteristics (such as lodging and ear drop) can be used for an assessment of any altered weediness potential for MON 89034. The characteristics assessed are well known to experts familiar with corn breeding and agronomic performance.

Phenotypic and agronomic data were collected from 18 field locations over two consecutive years: nine locations in 2004 and nine locations in 2005. **Table VII-5** provides a list of the test site locations, soil description, cropping history, and planting information. These locations provided a range of environmental and agronomic conditions representative of major U.S. corn-growing regions where commercial production of MON 89034 would be expected. Plots were established at each of the field sites in a randomized complete block design with three replications. Each plot consisted of two to six rows of corn spaced approximately 30 inches apart and approximately 17.5-20 ft in length.

The following 14 phenotypic and agronomic characteristics were evaluated during the 2004 and 2005 field trials: seedling vigor, early stand count, days to 50% pollen shed, days to 50% silking, stay green, ear height, plant height, dropped ears, stalk lodging, root lodging, final stand count, grain moisture, test weight, and yield. The evaluations and

timing of plant assessments are already described in **Table VII-1**. The phenotypic and agronomic data were analyzed using SAS. Means were calculated across-sites (referred to as combined-site or across-site analysis, in which data were pooled from all test sites and analyzed statistically) for each characteristic, and MON 89034 was compared to the control. Differences were considered significant at the 5% level ($p \leq 0.05$). The following is a summary of the results from the two years of phenotypic and agronomic evaluations. It is noted that only the combined-site data are presented in this section, while the results from individual sites are provided in **Appendix F**.

In the 2004 field trials, comparative assessments of phenotypic and agronomic characteristics were conducted on MON 89034 and a conventional control (H1325023). In addition, 23 commercially available corn hybrids were included as references to provide a range of values common to the crop. The 2004 field trials included nine test sites or locations.

For the combined-site analyses, no significant differences were detected between MON 89034 and the control corn for seedling vigor, early stand count, final stand count, days to 50% pollen shed, days to 50% silking, stay green, ear height, dropped ears, root lodged plants, grain moisture, test weight and yield (**Table VII-6**). Two differences were detected between MON 89034 and the control, i.e., plant height was lower for MON 89034 compared to control (84.1 vs. 85.4 inches), and MON 89034 had fewer stalk lodged plants than the control (0.8 vs. 2.4) (**Table VII-6**).

Although plant height for MON 89034 was reduced compared to the control, the mean value observed for MON 89034 falls within the range of values observed for the commercial reference corn hybrids (**Table VII-6**). Furthermore, the magnitude of the difference in plant height is small (approximately 1.5%), and the difference was not detected in 2005 trials (see below). Therefore, the slightly decreased plant height is unlikely to contribute to increased weediness potential. Likewise, the mean value for stalk lodged plants observed for MON 89034 falls within the range of values observed for the commercial corn hybrids. Furthermore, fewer stalk lodged plants would be agronomically desirable, the difference was not detected in 2005 trials (see below), and fewer stalk lodged plants would not contribute to increased weediness potential. Therefore, the differences detected in plant height and in the number of stalk lodged plants during the 2004 field trials are unlikely to be biologically meaningful in terms of increased weediness potential.

In the 2005 field trials, two separate field studies were conducted with Study-1 involving four test sites and Study-2 involving five test sites for a total of nine test sites. In the two studies, MON 89034 hybrids were bred to adapt to different environmental conditions (northern regions vs the Midwest). Accordingly, two different control hybrids were employed in the two studies, and each had a genetic background similar to the MON 89034 hyrbids, respectively.

In Study-1, the control corn was conventional corn hybrid DKC51-43. The background germplasm for MON 89034 hybrid and DKC51-43 hybrid used in this study was adapted

for northern corn growing regions in the U.S. In addition, 12 commercially available corn hybrids were included as references to provide a range of values common to the crop. A total of 14 different phenotypic and agronomic characteristics were evaluated. In the combined-site analysis, no differences were detected between MON 89034 and the conventional control for any of the assessed phenotypic and agronomic characteristics (**Table VII-7**).

In Study-2, the control corn was conventional corn hybrid H1325023. In addition, 14 commercially available corn hybrids were included as references to provide a range of values common to the crop. A total of 14 different phenotypic and agronomic characteristics were evaluated. In the combined-site analysis, no differences were detected between MON 89034 and the control for any of the assessed phenotypic and agronomic characteristics (**Table VII-8**).

The results of both 2004 and 2005 field trials demonstrate that the observed values of phenotypic and agronomic characteristics for MON 89034 were within the range of responses familiar to what is expected for corn. This supports a conclusion that the measured phenotypic and agronomic characteristics were not altered for MON 89034 as a result of the introduction of the lepidopteran-protection trait and the expression of the Cry1A.105 and Cry2Ab2 proteins, and there is no altered pest potential for MON 89034 compared to the conventional corn.

Table VII-5. Test site locations, planting information, soil description, and cropping history

2004 field trials

Study Site[1]	Planting Date (mm/dd/yy)	Planting Rate[2] (seed/plot)	Planting depth (in)	Plot size (ft)	Reps[3]	Soil series description; organic matter (%); and pH	2003 crop	2002 crop
IA	05/29/04	35	1.5	15 x 20	3	Silty clay loam; 3.9%; 6.8	Soybean	Soybean
IL-1	06/02/04	35	1.3	15 x 20	3	Silt loam; 2.0%; 6.3	Soybean	Sorghum
IL-2	05/24/04	35	2.0	15 x 20	3	Silty clay loam; 4.3%; 7.0	Soybean	Corn
IN-DA	06/21/04	40	1.0	5 x 17.5	3	Crosby silt loam; 2.7%; 5.8	Corn	Soybean
MN-GE	05/16/04	40	1.5	5 x 20	3	Hamel loam; 5.4%; 7.9	Soybean	Corn
MO-BE	05/17/04	40	1.0	5 x 20	3	Putnam silt loam; 1.8%; 6.9	Soybean	Soybean
MO-CL	05/20/04	40	1.0	5 x 20	3	Putnam silt loam; 1.0%; 6.1	Soybean	Corn
NE	05/21/04	35	1.5	15 x 20	3	Silt loam; 3.0%; 6.8	Soybean	Soybean
OH	06/03/04	35	1.5	15 x 20	3	Loam; 2.2%; 6.8	Non-crop	Soybean

[1] Study sites: IA = Jefferson County, IA; IL-1 = Jersey County, IL; IL-2 = Warren County, IL; IN-DA = Hendricks County, IN; MN-GE = Freeborn County, MN; MO-BE = Shelby County, MO; MO-CL = 2nd site at Shelby County, MO; NE = York County, NE; OH = Fayette County, OH.
[2] All plots were thinned to a uniform plant density after seedling vigor and early stand count data were collected at each site.
[3] Reps = replications.

Table VII-5 continues on the next page.

Table VII-5 (continued). Test site locations, planting information, soil description, and cropping history

2005 field trials, Study-1

Study Site[1]	Planting Date (mm/dd/yy)	Plot size (ft)	Rows/ plot (#)	Row spacing (in)	Planting rate[2] (seed/row)	Planting depth (in)	Soil series description; organic matter (%); and pH	2004 crop	2003 crop
MI	05/12/05	5 x 20	2	30	40	1.5	Nester loam; 2.2%; 6.5	Cabbage	N/A[3]
MN	05/17/05	5 x 20	2	30	40	1.5	Linder sandy loam; 2.0%; 7.2	Corn	Soybean
NY	05/20/05	5 x 20	2	30	40	1.5	Collamer silt loam; 4.0%; 6.0	Soybean	Soybean
WI	05/21/05	5 x 20	2	30	40	1.0	McHenry silt loam; 1-3%; 5.6-7.3	Soybean	Corn

[1] Study sites: MI = Ottawa County, MI; MN = Freeborn County, MN; NY = Wayne County, NY; WI = Walworth County, WI.
[2] All plots at each site were thinned to a uniform plant density after seedling vigor and early stand count data were collected.
[3] N/A = Not available.

Table VII-5 continues on the next page.

Table VII-5 (continued). Test site locations, planting information, soil description, and cropping history

2005 field trials, Study-2

Study site[1]	Planting date (mm/dd/yy)	Plot size (ft)	Rows/plot (#)[2,3]	Planting rate (seed/row)[4]	Planting depth (in)	Soil series description; organic matter (%); and pH	2004 crop	2003 crop
IA	05/17/05	15 x 20	6	40	1.75	Taintor silty clay loam; 3.9%; 6.8	Sorghum	Soybean
IL-N	05/17/05	15 x 20	6	40	2.0	Sable silty clay loam; 4.3%; 7.0	Soybean	Corn
IL-S	06/17/05	5 x 17.5	2	40	2.5	Cisne silt loam; 2.1%; 7.0	Corn	Soybean
MO	05/20/05	15 x 20	6	40	1.0	Putnam silt loam; 1.8%; 6.9	Soybean	Corn
NE	05/16/05	15 x 20	6	40	2.0	Hastings silt loam; 3.0%; 6.8	Corn	Soybean

[1] Study sites: IA = Jefferson County, IA; IL-N -= Warren County, IL; IL-S = Clinton County, IL; MO = Shelby County, MO; NE = York County, NE

[2] The IL-S site was originally planted on May 20, 2005 with six-row plots. Dry conditions at planting, however, resulted in poor seedling emergence across all plots. The study area was destroyed and a new study area was established on June 17, 2005 with two-row plots.

[3] All rows were spaced 30 inches apart.

[4] All plots at each site were thinned to a uniform plant density after seedling vigor and early stand count data were collected.

Table VII-6. Phenotypic and agronomic comparison of MON 89034 to the control in the combined-site analysis for 2004 field trials

Phenotypic and agronomic characteristic (units)	MON 89034	Control	References			
			Range[1]		99% Tolerance interval[2]	
			Min.	Max.	Lower limit	Upper limit
Seedling vigor	6.9	6.9	4.3	9.0	1.7	9.0
Early stand count (#/plot)	68.3	67.8	58.3	78.7	49.5	89.4
Days to 50% pollen shed	61.9	61.5	55.3	74.7	44.8	81.6
Days to 50% silking	60.8	60.4	52.0	74.7	40.8	83.1
Stay green	6.0	5.8	4.7	7.7	3.1	8.4
Ear height (in)	39.9	40.5	34.6	54.6	27.2	59.9
Plant height (in)	84.1*	85.4	76.8	106.6	67.0	120.4
Dropped ears (#/plot)	0.1	0.2	0.0	1.0	0.0	1.2
Stalk lodged plants (#/plot)	0.8*	2.4	0.0	6.0	0.0	6.7
Root lodged plants (#/plot)	1.0	0.5	0.0	27.0	0.0	28.0
Final stand count (#/plot)	60.5	60.5	53.3	66.3	50.9	73.2
Grain moisture (%)	23.4	22.9	12.6	41.7	0.0	47.7
Test weight (lb/bu)	52.6	53.3	42.8	57.7	39.4	67.6
Yield (bu/ac)	192.9	191.3	92.8	290.8	17.9	373.5

[1] Reference range: minimum and maximum mean values among the 23 reference corn.
[2] 99% tolerance interval with 95% confidence.
* Indicates a statistically significant difference between the test and control at $p \leq 0.05$.

Table VII-7. Phenotypic and agronomic comparison of MON 89034 to the control in combined-site analysis for Study-1 of 2005 trials

Phenotypic and agronomic characteristic (units)	MON 89034	Control	References			
			Range[1]		99% Tolerance Interval[2]	
	Mean	Mean	Min.	Max.	Lower limt	Upper limit
Seedling vigor	6.7	6.6	5.0	8.0	1.8	9.0
Early stand count (#/plot)	77.1	77.4	66.7	82.3	55.4	95.8
Days to 50% pollen shed	68.0	67.6	64.3	71.0	56.7	79.2
Days to 50% silking	66.9	66.3	62.0	71.0	52.8	80.5
Stay green	6.3	6.2	3.7	9.0	0.0	9.0
Ear height (in)	41.9	42.1	38.0	59.9	13.9	77.8
Plant height (in)	91.6	89.8	82.0	112.3	43.8	142.2
Dropped ears (#/plot)	0.0	0.1	0.0	0.3	0.0	0.4
Stalk lodged plants (#/plot)	0.1	0.3	0.0	2.3	0.0	3.4
Root lodged plants (#/plot)	0.0	0.0	0.0	0.0	nv	nv
Final stand count (#/plot)	61.3	61.8	59.0	64.0	53.5	69.7
Grain moisture (%)	18.9	18.6	17.3	22.2	12.7	25.1
Test weight (lb/bu)	55.9	56.1	54.0	57.0	52.0	59.4
Yield (bu/ac)	205.5	195.1	171.0	220.0	124.4	258.5

[1] Reference range: minimum and maximum values among the 12 individual reference means.
[2] 99% tolerance interval with 95% confidence.
No statistically significant differences were detected between MON 89034 and the control at $p \leq 0.05$.

Table VII-8. Phenotypic and agronomic comparison of MON 89034 to the control in the combined-site analysis for Study-2 of 2005 trials

Phenotypic and agronomic characteristic (units)	MON 89034	Control	References			
			Range[1]		99% Tolerance Interval[2]	
	Mean	Mean	Min.	Max.	Lower limit	Upper limit
Seedling vigor	6.4	6.3	5.7	8.0	3.7	9.0
Early stand count (#/plot)	69.4	72.3	67.0	78.7	63.1	88.3
Days to 50% pollen shed	60.2	59.6	52.3	67.0	44.3	79.7
Days to 50% silking	60.9	60.2	52.3	69.0	42.5	82.3
Stay green	5.0	5.2	3.0	7.0	0.0	9.0
Ear height (in)	38.0	39.4	32.2	50.3	23.6	59.8
Plant height (in)	80.3	82.0	77.1	99.1	60.3	120.1
Dropped ears (#/plot)	1.4	0.9	0.0	2.0	0.0	3.0
Stalk lodged plants (#/plot)	9.6	5.4	0.0	49.0	0.0	66.6
Root lodged plants (#/plot)	0.6	0.6	0.0	7.3	0.0	12.2
Final stand count (#/plot)	58.9	59.8	52.7	63.0	48.8	71.2
Grain moisture (%)	19.6	19.4	16.3	25.8	7.0	33.5
Test weight (lb/bu)	56.0	55.7	49.1	63.7	40.5	71.8
Yield (bu/ac)	126.8	125.7	31.7	203.5	0.0	374.8

[1] Reference range = Minimum and maximum values among the 14 individual reference means.
[2] 99% tolerance interval with 95% confidence.
No statistically significant differences were detected between MON 89034 and the control at $p \leq 0.05$.

VII.A.3.d. Evaluation of Ecological Interactions

In the two years of field trials for evaluation of phenotypic and agronomic characteristics of MON 89034, observational data on the presence of and differential response to biotic (insects, diseases) and abiotic (drought, wind, nutrient deficiency etc.) stressors were also collected to examine the ecological interactions of MON 89034 compared with those of the conventional control corn. The observed stressors were "natural" (i.e., no artificial infestation or interference was used). Therefore, the same stressors were not necessarily observed at each field site.

Ecological interactions were assessed qualitatively. Observation of plant interactions with insect pests and diseases, and plant responses to abiotic stressors were collected from each of the 18 field locations in 2004 and 2005. The purpose of these evaluations was to assess whether plant-insect or plant-disease interactions, or plant response to abiotic stressors of MON 89034 were altered compared to the conventional control corn. For the plant-insect interactions, plant-disease interactions, and plant responses to abiotic stressors, the reported values represent the range of ratings observed across the three replications at each site. MON 89034 and the control were considered qualitatively different in response to a stressor if the ratings between MON 89034 and the control corn did not overlap across all three replications for that particular stressor (e.g. "none" rating vs "slight-moderate" rating). The ratings observed among the commercial reference hybrids provide qualitative assessment data common to the crop for each stressor assessed.

In the 2004 field trials, across the nine field locations, 11 insect and other animal categories (species or group), 12 disease categories (species or group), and eight abiotic stressor categories were evaluated (**Table VII-9**). For the plant-insect or plant-other animal interactions, no qualitative differences were observed between MON 89034 and the control among the 37[*] plant-insect or plant-other animal interactions evaluated (**Table VII-9**). It should be noted that in these field trials, efficacy testing of MON 89034 was not the objective. The entire plot area at each location would have been treated with insecticides if needed, as indicated by the insect pressure in the control and reference plots observed. Thus, it is not unexpected when no difference was observed for the plant response to lepidopteran insects like corn borer between MON 89034 and the control corn. For plant-disease interaction, no qualitative differences were observed between MON 89034 and the control for 52 out of the 54 interactions assessed (**Table VII-9**). At the IA and IN-DA locations a slight incidence of ear rot was observed in the control and no ear rot was observed in MON 89034. This difference was not observed at the other 7 locations where ear rot data were recorded.

For the plant-abiotic stressor interactions, no qualitative differences were observed between MON 89034 and the control for 35 out of the 38 interactions assessed (**Table VII-9**). At the seedling stage at MO-BE, excess moisture stress was not observed in

[*] The number of interactions is on a per stressor/test location/plant growth stage basis. For example, aphids were assessed at the IA and MO-BE sites, both during the reproductive stage. This was counted as two interactions.

MON 89034, while a slight incidence of excess moisture stress was observed in the control. Excess moisture stress was assessed at various growth stages at four sites, but differences between MON 89034 and the control were only observed at MO-BE. This qualitative difference was likely due to the lack of uniformity of moisture (i.e., standing water) across plots at MO-BE. Wind damage was assessed at various growth stages at six sites, but differences between MON 89034 and the control were only observed at IL-2 and MO-BE. At IL-2, during harvest, wind damage was not observed in MON 89034, while slight wind damage was observed in the control. At the reproductive stage at MO-BE, slight wind damage was observed in MON 89034, while no wind damage was observed in the control. This qualitative difference was not considered biologically meaningful since there was no consistent trend observed between MON 89034 and the control regarding to the response to wind damage across sites where differences were observed. Furthermore, no differences were observed between MON 89034 and the control at four additional sites where wind damage was assessed (**Table VII-9**).

In Study-1 of the 2005 field trials, a total of 6 insect categories (species or group), 8 disease categories (species or group), and 7 abiotic stressors were evaluated (**Table VII-10**). Within these categories, 16 plant-insect interactions, 24 plant-disease interactions, 16 plant responses to abiotic stressors were evaluated. For the plant-disease interactions or plant-abiotic stressor interactions, no qualitative differences were observed in MON 89034 plant responses compared to the control (**Table VII-10**). For the 16 plant-insect interactions evaluated, four qualitative differences were observed between MON 89034 and the control for corn borer and corn earworm damage (**Table VII-10**). Specifically, corn borer and corn earworm damages were not observed on MON 89034 while slight damage was observed in the control at MI and WI. This was expected because of the introduced lepidopteran-protection trait for MON 89034. If there was lepidopteran insect pressure (such as corn borer or corn earworm) and if insecticide application was not conducted in a timely fashion, one would expect to see the difference between MON 89034 (resistant to lepidopteran insects) and the control corn (susceptible to lepidopteran insects).

In Study-2 of 2005 field trials, a total of 9 insect and other animal categories (species or group), 7 disease categories (species or group), and 5 abiotic stressors were evaluated (**Table VII-11**). Within these categories, 21 plant-insect and other animal interactions, 30 plant-disease interactions and 19 plant responses to abiotic stressors were evaluated and, no qualitative differences were observed in the ecological interactions of MON 89034 compared to those of the control (**Table VII-11**).

In summary, based on 255 comparative observations recorded over 2 years, no consistent differences were observed across sites between MON 89034 and the control in their susceptibility or tolerance to the ecological stressors assessed during 2004 and 2005 field trials. These results support the conclusion that compared to the conventional corn, the ecological interactions between MON 89034 and insects, diseases, and abiotic stressors were not altered except for the introduced lepidopteran-protection trait.

Table VII-9. Ecological stressor incidence of MON 89034, the control, and references in 2004 field trials

		Seedling			Vegetative			Reproductive			Harvest		
Stressors	**Sites[4]**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**
Insects													
Aphids	IA	—	—	—	—	—	—	NO	NO	NO-MO	—	—	—
	MO-BE	—	—	—	—	—	—	SL	NO-SL	NO-SL	—	—	—
Armyworm[1]	IL-1	—	—	—	—	—	—	NO	NO	NO	—	—	—
	IL-2	NO	NO	NO	—	—	—	—	—	—	—	—	—
	MO-CL	—	—	—	NO-SL	NO	NO-SL	—	—	—	NO	NO	NO
Corn borer[2]	IL-1	—	—	—	—	—	—	—	—	—	NO	NO	NO-SL
	IL-2	NO	NO	NO	NO	NO	NO	—	—	—	NO-SL	SL	NO-SL
	MN-GE	NO	NO	NO	NO	NO-SL	NO	NO	NO-SL	NO-SL	NO-SL	NO-SL	NO-SL
	MO-BE	—	—	—	NO-SL	NO	NO-SL	—	—	—	—	—	—
	NE	—	—	—	NO	NO	NO	—	—	—	NO	NO-MO	NO-MO
	OH	—	—	—	NO	NO	NO	NO	NO	NO	NO	NO	NO
Corn earworm	IN-DA	—	—	—	—	—	—	—	—	—	NO-SL	SL	SL
Corn rootworm	IN-DA	SL-MO	SL-MO	SL-MO	SL-MO	SL-MO	SL-MO	SL	SL	SL-MO	—	—	—
	NE	—	—	—	—	—	—	NO	NO	NO	—	—	—
Cutworms[3]	IA	NO	NO	NO	—	—	—	—	—	—	—	—	—
	IN-DA	NO	NO	NO	—	—	—	—	—	—	—	—	—
	MO-BE	NO-SL	NO-SL	NO-SL	—	—	—	—	—	—	—	—	—
	NE	NO	NO	NO	—	—	—	—	—	—	—	—	—
Flea beetles	MO-CL	NO	NO-SL	NO-SL	—	—	—	—	—	—	—	—	—
	OH	NO	NO	NO	—	—	—	—	—	—	—	—	—
Grasshoppers	IA	—	—	—	NO	NO-SL	NO-SL	—	—	—	—	—	—
	MO-BE	—	—	—	—	—	—	—	—	—	NO	NO-SL	NO-SL
	MO-CL	—	—	—	—	—	—	NO	NO	NO	—	—	—
Japanese beetles	IL-1	—	—	—	NO	NO	NO	—	—	—	—	—	—
Wireworms	IL-1	NO	NO	NO	—	—	—	—	—	—	—	—	—

Seedling, vegetative, reproductive, and harvest refer to different growth stages. Test = MON 89034; Contr. = Control corn; Ref. = Reference corn; NO = None; SL = Slight; MO = Moderate; SE = Severe. Dash (—) denotes no observations taken. The severity of stressor symptoms per plot were rated using the following scale: 0 = none (no symptoms observed), 1–3 = slight (symptoms observed but not detrimental to plant growth and development), 4–6 = moderate (intermediate between slight and severe), and 7–9 = severe (symptoms observed and detrimental to plant growth and development). [1] Including fall armyworm. [2] Including European corn borer. [3] Including black cutworm. [4] Refer to **Table VII-5** for test site descriptions. **Table VII-9** continues on next page.

Table VII-9 (continued). Ecological stressor incidence of MON 89034, the control, and references in 2004 field trials

		Seedling			Vegetative			Reproductive			Harvest		
Stressors	**Sites**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**
Other animals													
Rodents	IL-1	NO-SL	NO-MO	NO-MO	—	—	—	—	—	—	—	—	—
Diseases													
Anthracnose	MO-CL	—	—	—	NO	NO	NO	—	—	—	—	—	—
Ear rot[5]	IA	—	—	—	—	—	—	—	—	—	NO	SL	NO-MO
	IL-1	—	—	—	—	—	—	—	—	—	NO-SL	SL	SL-MO
	IL-2	—	—	—	—	—	—	—	—	—	NO	NO-SL	NO-SL
	IN-DA	—	—	—	—	—	—	—	—	—	NO	SL	SL
	MN-GE	—	—	—	—	—	—	—	—	—	NO	NO	NO
	MO-BE	—	—	—	—	—	—	—	—	—	SL	NO-SL	NO-SL
	MO-CL	—	—	—	—	—	—	—	—	—	NO-SL	SL	NO-SL
	NE	—	—	—	—	—	—	—	—	—	NO	NO	NO
	OH	—	—	—	—	—	—	—	—	—	NO	NO	NO-SL
Eyespot	MO-CL	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL	—	—	—
Fusarium	MN-GE	NO	NO	NO	—	—	—	—	—	—	—	—	—
Gray leaf spot	IL-2	—	—	—	NO	NO	NO	SL	SL	SL	—	—	—
	IN-DA	—	—	—	—	—	—	—	—	—	SL	SL	SL
	MO-BE	—	—	—	NO-SL	NO-SL	NO-SL	NO-SL	NO-SL	NO-SL	—	—	—
	MO-CL	—	—	—	—	—	—	—	—	—	SL	SL	SL
Leaf blight	IA	—	—	—	SL	NO-SL	SL	—	—	—	—	—	—
	IN-DA	—	—	—	—	—	—	SL	SL	SL	—	—	—
	MN-GE	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	NE	—	—	—	—	—	—	NO	NO	NO	—	—	—
	OH	—	—	—	—	—	—	—	—	—	NO	NO	NO

[5] Ear rot data collected on 5 plants/plot at all sites.

Table VII-9 continues on next page.

Table VII-9 (continued). Ecological stressor incidence of MON 89034, the control, and references in 2004 field trials

		Seedling			Vegetative			Reproductive			Harvest		
Stressors	**Sites**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**
Diseases													
Pythium	IA	NO	NO	NO	—	—	—	—	—	—	—	—	—
	IL-2	NO	NO	NO	—	—	—	—	—	—	—	—	—
	IN-DA	NO	NO-SL	NO-SL	—	—	—	—	—	—	—	—	—
	MO-BE	NO-SL	SL	NO-SL	—	—	—	—	—	—	—	—	—
Root rot	IL-1	NO	NO	NO	—	—	—	—	—	—	—	—	—
Rust	IA	—	—	—	—	—	—	NO	NO	NO-SL	SL-SE	SL-SE	SL-MO
	IL-1	—	—	—	SL	SL	SL	—	—	—	—	—	—
	IL-2	—	—	—	—	—	—	SL	SL	SL	—	—	—
	IN-DA	—	—	—	SL	SL	SL-MO	—	—	—	—	—	—
	MN-GE	—	—	—	NO	NO	NO-SL	SL	SL	SL	SL	SL	SL
	MO-BE	—	—	—	—	—	—	—	—	—	SL-MO	MO	SL-MO
	MO-CL	—	—	—	—	—	—	—	—	—	SL-MO	SL-MO	SL-MO
	NE	—	—	—	NO	NO	NO	—	—	—	—	—	—
	OH	—	—	—	NO	NO-SL	NO-SL	NO	NO-SL	NO-SL	—	—	—
Seedling blight	MO-CL	NO	NO	NO	—	—	—	—	—	—	—	—	—
	OH	NO	NO	NO	—	—	—	—	—	—	—	—	—

Table VII-9 continues on next page.

Table VII-9 (continued). Ecological stressor incidence of MON 89034, the control, and references in 2004 field trials

		Seedling			Vegetative			Reproductive			Harvest		
Stressors	**Sites**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**
Diseases													
Stalk rot[6]	IA	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	IL-1	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	IL-2	—	—	—	—	—	—	—	—	—	NO-SL	SL-MO	SL-MO
	IN-DA	—	—	—	—	—	—	—	—	—	NO	NO	NO
	MN-GE	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	MO-BE	—	—	—	—	—	—	—	—	—	NO-SL	SL	NO-SL
	MO-CL	—	—	—	—	—	—	—	—	—	SL	SL-MO	SL
	NE	—	—	—	—	—	—	—	—	—	NO	NO	NO
	OH	—	—	—	—	—	—	—	—	—	SL	SL	SL
Stewart's wilt	IL-1	—	—	—	—	—	—	NO	NO	NO	—	—	—
	NE	NO	NO	NO	—	—	—	—	—	—	—	—	—
Abiotic													
Cold weather	MO-CL	NO	NO	NO	—	—	—	—	—	—	—	—	—
Compaction	OH	NO	NO	NO	NO	NO-SL	NO-SL	—	—	—	—	—	—
Drought-Heat	IA	—	—	—	—	—	—	NO	NO	NO	—	—	—
	IL-1	—	—	—	NO	NO	NO	NO	NO	NO	—	—	—
	IL-2	NO	NO	NO	NO	NO	NO	—	—	—	—	—	—
	IN-DA	—	—	—	—	—	—	NO	NO	NO	—	—	—
	MO-CL	—	—	—	—	—	—	NO	NO	NO	—	—	—
	OH	—	—	—	—	—	—	NO	NO	NO	—	—	—
Frost	IN-DA	—	—	—	—	—	—	—	—	—	MO	MO	MO
Hail	IL-1	NO	NO	NO	—	—	—	—	—	—	—	—	—
	NE	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO

[6] Stalk rot data collected on 5 plants/plot at all sites. The ranges of the mean plot ratings are presented in this table. At IL-1, IL-2, and NE, stalk rot data were collected both on a per plot basis and on 5 plants/plot. For these sites, the mean ratings from the 5 plants/plot were combined with the overall plot ratings and the ranges are presented in this table.

Table VII-9 continues on next page.

Table VII-9 (continued). Ecological stressor incidence of MON 89034, the control, and references in 2004 field trials

		Seedling			Vegetative			Reproductive			Harvest		
Stressors	**Sites**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**	**Test**	**Contr.**	**Ref.**
Abiotic													
Nutrient	MN-GE	NO	NO	NO	—	—	—	SL	SL	SL	NO-SL	NO-SL	NO-SL
Excess moisture	IL-2	NO	NO	NO	—	—	—	—	—	—	—	—	—
	IN-DA	—	—	—	SL	NO-SL	NO-SL	—	—	—	—	—	—
	MN-GE	NO	NO	NO	NO-SL	NO	NO-SL	—	—	—	—	—	—
	MO-BE	NO	SL	NO-SL	NO	NO	NO-SL	NO	NO	NO-SL	—	—	—
Wind	IA	SL	SL	SL	NO	NO	NO-SL	—	—	—	NO	NO	NO
	IL-1	NO	NO	NO	—	—	—	—	—	—	NO	NO	NO
	IL-2	—	—	—	—	—	—	—	—	—	NO	SL	SL-MO
	MO-BE	—	—	—	—	—	—	SL	NO	NO-SL	NO-SL	NO	NO-SL
	MO-CL	—	—	—	NO	NO	NO	—	—	—	NO	NO	NO
	OH	—	—	—	—	—	—	—	—	—	NO	NO	NO

Table VII-10. Ecological stressor incidence of MON 89034, the control, and references in Study-1 of 2005 trials

Stressors	Sites[3]	Seedling			Vegetative			Reproductive			Harvest		
		Test	Contr.	Ref.	Test	Contr.	Ref.	Test	Contr.	Ref.	Test	Contr.	Ref.
Insects													
Corn borer[1]	MI	—	—	—	NO	SL	NO-SL	NO	SL	SL	—	—	—
	MN	—	—	—	NO	NO	NO	NO	NO	NO	NO	NO	NO
	NY	—	—	—	—	—	—	—	—	—	NO	NO	NO
	WI	—	—	—	NO	SL	NO-SL	—	—	—	NO	NO-SL	NO-SL
Corn earworm	MI	—	—	—	—	—	—	—	—	—	NO	SL	SL
Corn rootworm	NY	NO	NO	NO	—	—	—	NO	NO	NO	—	—	—
	WI	NO	NO	NO	—	—	—	—	—	—	—	—	—
Cutworm[2]	MI	NO	NO	NO	—	—	—	—	—	—	—	—	—
	MN	NO	NO	NO	—	—	—	—	—	—	—	—	—
Grasshopper	WI	—	—	—	—	—	—	NO	NO	NO	—	—	—
Stalk borer	NY	—	—	—	NO	NO	NO	—	—	—	—	—	—

Seedling, vegetative, reproductive, and harvest refer to different growth states. Test = MON 89034; Contr. = Control; Ref. = References; NO = none; SL = slight; MO = moderate; SE = severe. Dashes (—) indicate no observations taken. The severity of stressor symptoms per plot were rated using the following scale: 0 = none (no symptoms observed), 1–3 = slight (symptoms observed but not detrimental to plant growth and development), 4–6 = moderate (intermediate between slight and severe), and 7–9 = severe (symptoms observed and detrimental to plant growth and development).

[1] Includes corn borer and European corn borer.

[2] Includes cutworm and black cutworm.

[3] Refer to **Table VII-5** for test site descriptions.

Table VII-10 continues on next page.

Table VII-10 (continued). Ecological stressor incidence of MON 89034, the control, and references in Study-1 of 2005 trials

Stressors	Sites	Seedling			Vegetative			Reproductive			Harvest		
		Test	Contr.	Ref.	Test	Contr.	Ref.	Test	Contr.	Ref.	Test	Contr.	Ref.
Diseases													
Ear rot[4]	MI	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	MN	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	NY	—	—	—	—	—	—	—	—	—	NO	NO	NO
	WI	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
Eyespot	WI	—	—	—	—	—	—	NO	NO	NO	—	—	—
Northern leaf blight	NY	—	—	—	NO	NO	NO	NO	NO	NO	NO	NO	NO
Pythium	MI	NO	NO	NO	—	—	—	—	—	—	—	—	—
	NY	NO	NO	NO	—	—	—	—	—	—	—	—	—
Rust[5]	MI	—	—	—	NO	NO	NO	NO	NO	NO	—	—	—
	MN	—	—	—	NO	NO	NO	SL	SL	NO-SL	SL	SL	SL
	WI	NO	NO	NO	—	—	—	—	—	—	—	—	—
Seedling blight	MN	NO	NO	NO	—	—	—	—	—	—	—	—	—

[4] Ear rot data collected from 5 plants/plot at each site. The ranges of the individual plant ratings at each site are presented in this table.
[5] Includes rust and leaf rust.

Table VII-10 continues on next page.

Table VII-10 (continued). Ecological stressor incidence of MON 89034, the control, and references in Study-1 of 2005 trials

Stressors	Sites	Seedling			Vegetative			Reproductive			Harvest		
		Test	Contr.	Ref.	Test	Contr.	Ref.	Test	Contr.	Ref.	Test	Contr.	Ref.
Diseases													
Smut	MI	—	—	—	—	—	—	—	—	—	NO	NO	NO-SL
	WI	—	—	—	NO	NO	NO	—	—	—	NO-SL	NO	NO-SL
Stalk rot[6]	MI	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL
	MN	—	—	—	—	—	—	—	—	—	NO-SL	NO-MO	NO-MO
	NY	—	—	—	—	—	—	—	—	—	NO	NO	NO
	WI	—	—	—	—	—	—	—	—	—	NO-MO	NO-SL	NO-SL
Abiotic													
Cold stress	NY	NO	NO	NO	—	—	—	—	—	—	—	—	—
Drought[7]	MI	—	—	—	NO	NO	NO	NO	NO	NO	—	—	—
	NY	—	—	—	—	—	—	NO	NO	NO	NO	NO	NO
	WI	NO	NO	NO	NO	NO	NO	—	—	—	NO-SL	NO-SL	NO-SL
Frost	MI	—	—	—	—	—	—	—	—	—	NO	NO	NO
Heat stress	NY	—	—	—	NO	NO	NO	—	—	—	—	—	—
Herbicide injury	MI	NO-SL	NO	NO-SL	—	—	—	—	—	—	—	—	—
	MN	NO	NO	NO-SL	—	—	—	—	—	—	—	—	—
Nutrient deficiency[8]	MN	—	—	—	NO	NO	NO	SL	SL	SL	SL	SL	SL
Wind	WI	—	—	—	—	—	—	NO	NO	NO	—	—	—

[6] Data collected from 5 plants/plot at each site. The ranges of the individual plant ratings at each site are presented in this table.
[7] Includes drought and drought/heat.
[8] Includes nutrient deficiency and nitrogen deficiency.

Table VII-11. Ecological stressor incidence of MON 89034, the control, and references in Study-2 of 2005 trials

Stressors	Sites[3]	Seedling			Vegetative			Reproductive			Harvest		
		Test	Contr	Ref	Test	Contr	Ref	Test	Contr	Ref	Test	Contr	Ref
Insects													
Aphids	IL-S	—	—	—	—	—	—	MO	MO	MO-SE	NO-SL	NO	NO-SL
	MO	—	—	—	—	—	—	SL	SL	SL	—	—	—
Black cutworm	IA	NO-SL	SL	NO-SL	—	—	—	—	—	—	—	—	—
	IL-N	NO	NO	NO	—	—	—	—	—	—	—	—	—
	MO	NO-SL	NO-SL	NO-SL	—	—	—	—	—	—	—	—	—
	NE	NO	NO	NO	—	—	—	—	—	—	—	—	—
Corn borer[1]	IA	—	—	—	—	—	—	—	—	—	SL	SL-MO	SL-SE
	IL-N	—	—	—	SL	SL	SL	SL	SL	NO-SL	SL	SL-MO	NO-MO
	MO	—	—	—	NO-SL	NO-SL	NO-SL	—	—	—	—	—	—
	NE	—	—	—	NO	NO	NO	—	—	—	—	—	—
Corn earworm	MO	—	—	—	—	—	—	—	—	—	SL-MO	MO	SL-MO
Corn rootworm[2]	IA	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL	—	—	—
	NE	—	—	—	—	—	—	NO	NO	NO-SL	NO	NO	NO
Grasshopper	IL-S	—	—	—	—	—	—	NO-MO	NO-SL	NO-MO	—	—	—
Japanese beetle	IL-S	MO	SL-MO	SL-MO	—	—	—	—	—	—	—	—	—
Leaf hopper	IA	—	—	—	SL	SL	NO-SL	—	—	—	—	—	—

Seedling, vegetative, reproductive, and harvest refer to different growth stages. Contr = Control; Ref = References; NO = none; SL = slight; MO = moderate; SE = severe. Dashes (—) indicate no observations taken on stressor. The severity of stressor symptoms per plot were rated using the following scale: 0 = none (no symptoms observed), 1–3 = slight (symptoms observed but not detrimental to plant growth and development), 4–6 = moderate (intermediate between slight and severe), and 7–9 = severe (symptoms observed and detrimental to plant growth and development).

[1] Including European corn borer.

[2] Including corn rootworm adults.

[3] Refer to **Table VII-5** for test site descriptions.

Table VII-11 continues on next page.

Table VII-11 (continued). Ecological stressor incidence of MON 89034, the control, and references in Study-2 of 2005 trials

Stressors	Sites	Seedling			Vegetative			Reproductive			Harvest		
		Test	Contr	Ref	Test	Contr	Ref	Test	Contr	Ref	Test	Contr	Ref
Diseases													
Anthracnose	IA	—	—	—	NO	NO-SL	NO-SL	SL	SL	SL	SL-MO	SL	SL-MO
	IL-S	—	—	—	—	—	—	—	—	—	SE	MO-SE	MO-SE
	MO	—	—	—	SL	SL	SL	—	—	—	—	—	—
Blight[4]	IA	NO-SL	NO-SL	NO-SL									
	IL-N	NO	NO	NO	—	—	—	—	—	—	—	—	—
	IL-S	NO	NO	NO	—	—	—	SL	SL-MO	SL-MO	—	—	—
	NE	—	—	—	—	—	—	NO	NO	NO	—	—	—
Ear/kernel rot[5]	IA	—	—	—	—	—	—	—	—	—	NO	NO-SL	NO-SL
	IL-N	—	—	—	—	—	—	—	—	—	NO-SL	NO-MO	NO-MO
	IL-S	—	—	—	—	—	—	—	—	—	NO-SE	NO-MO	NO-SE
	MO	—	—	—	—	—	—	—	—	—	NO-SE	SL-SE	SL-SE
	NE	—	—	—	—	—	—	—	—	—	NO	NO	NO
Fusarium	MO	NO-SL	NO-SL	NO	—	—	—	—	—	—	—	—	—
	NE	NO	NO	NO	NO	NO	NO	—	—	—	—	—	—
Gray leaf spot	IA	—	—	—	—	—	—	—	—	—	MO	MO	MO-SE
	IL-N	—	—	—	—	—	—	NO	NO	NO	SL	SL	SL
	MO	—	—	—	—	—	—	SL	SL	SL	SL-MO	MO	SL-MO
	NE	—	—	—	—	—	—	—	—	—	SL	SL	SL-MO
Rust	IL-N	—	—	—	SL	SL	SL	—	—	—	—	—	—
Stalk rot[5]	IA	—	—	—	—	—	—	—	—	—	NO-SL	NO-SE	NO-SL
	IL-N	—	—	—	—	—	—	—	—	—	NO-MO	NO-SE	NO-MO
	IL-S	—	—	—	—	—	—	—	—	—	SL-SE	SL-SE	SL-SE
	MO	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-MO
	NE	—	—	—	—	—	—	—	—	—	NO-SL	NO-SL	NO-SL

[4] Including seedling blight, pythium, southern leaf blight, and northern corn leaf blight. [5] Data collected on 5 plants/plot at each site. The ranges of the individual plant ratings are presented in this table.

Table VII-11 continues on next page.

Table VII-11 (continued). Ecological stressor incidence of MON 89034, the control, and references in Study-2 of 2005 trials

Stressors	Sites	Seedling			Vegetative			Reproductive			Harvest		
		Test	Contr	Ref	Test	Contr	Ref	Test	Contr	Ref	Test	Contr	Ref
Abiotic													
Drought[6]	IA	—	—	—	NO	NO	NO	SL	SL	SL-NO	MO	MO	MO-SE
	IL-N	NO	NO	NO	MO	MO	MO	MO	MO	MO	—	—	—
	IL-S	MO-SE	MO-SE	MO-SE	—	—	—	SE	MO-SE	SE	—	—	—
	MO	—	—	—	MO-SE	MO-SE	MO-SE	SE	MO-SE	MO-SE	MO-SE	MO-SE	MO-SE
	NE	—	—	—	—	—	—	NO	NO	NO	NO	NO	NO
Nutrient deficiency	IL-N	—	—	—	—	—	—	—	—	—	NO	NO	NO
Soil compaction	NE	SL	SL	SL	SL	SL	SL	—	—	—	—	—	—
Wet soil	MO	SL	SL	NO-MO	—	—	—	—	—	—	—	—	—
Wind	IA	SL	SL	SL	—	—	—	—	—	—	—	—	—
	IL-S	—	—	—	—	—	—	—	—	—	MO-SE	SL-SE	MO-SE
Other													
Deer damage	NE	—	—	—	—	—	—	—	—	—	NO	NO-SL	NO-SE

[6] Including drought/heat.

VII.B. Compositional Assessment of MON 89034

Compositional analyses were conducted to assess whether the nutrient, anti-nutrient and secondary metabolite levels in the grain and forage tissues derived from MON 89034 are comparable to those in the conventional control corn which has a genetic background similar to MON 89034. Additional conventional corn hybrids (references) currently in the marketplace were also included in the analysis to establish a range of natural variability for each analyte, where the range of variability is defined by a 99% tolerance interval for that particular analyte. Results of the comparisons indicate that MON 89034 is compositionally and nutritionally equivalent to conventional corn hybrids currently in commerce.

In 2004, grain and forage tissues of MON 89034 and conventional control corn were harvested from plants grown in three replicated plots at each of five field sites. 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. The five test 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. Fifteen conventional corn hybrids were also included as references by growing three different hybrids at each of five sites for a total of 15 references. These reference corn hybrids were included to provide data for the development of a 99% tolerance interval for each component analyzed. This tolerance interval is expected to contain, with 95% confidence, 99% of the values obtained from the population of commercial corns. 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 from 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 (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. Methods for analysis were based on internationally-recognized procedures and published literature.

Statistical analyses of the compositional data were conducted using a mixed model analysis of variance with data from each of the five sites, and a combination of all five

field sites. 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 of the five sites. 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 (LOD), and were not included in the statistical analyses.

Statistical analyses of the remaining 61 components 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 combination of all five sites (combined-site) indicated that there were no statistical differences for 58 of the 61 components. Details of the combined-site analysis results are provided in **Appendix G**. Analyses using data from the five single sites indicated that there were no statistically significant differences in 261 out of the 305 comparisons made between MON 89034 and the control.

Table VII-12 shows all the statistical differences in both the combined-site and individual site (total of 5 sites) analyses. 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. The differences observed are generally small (3.4 – 19.2%) considering the natural variability, and the mean levels and ranges of these analytes of MON 89034 are well within the 99% tolerance intervals for commercial corn. The levels 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 (ILSI) Crop Composition Database (ILSI-CCD, 2006), as well within published literature ranges. Therefore, it is concluded that MON 89034 and the 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 **Appendix G**.

The reproducibility and trends at individual 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 (out of the total of 5 sites). There were no consistent trends and 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 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 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. 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 differences for MON 89034 were higher than the control at both sites. 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.

Table VII-12. Summary of differences (p<0.05) for the comparison of corn component levels for MON 89034 vs the control and commercial references

Analytical Component (units)[1]	MON 89034 Mean	Control Mean	Mean Difference (Test minus Control) % of Control	Mean Difference (Test minus Control) Significance (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	0.01	(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	83.8	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	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]
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]

[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-12 continues on the next page.

Table VII-12 (continued). Summary of differences (p<0.05) for the comparison of corn component levels for MON 89034 vs the control and commercial references

Analytical Component (units)[1]	MON 89034 Mean	Control Mean	Mean Difference (Test minus Control) % of Control	Mean Difference (Test minus Control) Significance (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
Statistical Differences Observed in One Site Only						
Amino Acid						
Site IA Grain Alanine (% DW)	0.88	0.81	7.83	0.03	(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	0.02	(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	11.2	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]
Site IA Grain Proline (% DW)	1.05	0.98	7.29	0.028	(1.04 - 1.05)	[0.68,1.21]
Site IA Grain Serine (% DW)	0.6	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	17.5	0.006	(0.42 - 0.43)	[0.17,0.52]
Fatty Acid						
Site IA Grain 18:3 Linolenic (% Total FA)	1.21	1.34	-9.4	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	-1.5	0.03	(0.28 - 0.28)	[0.15,0.39]

[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-12 continues on the next page.

Table VII-12 (continued). Summary of differences (p<0.05) for the comparison of corn component levels for MON 89034 vs the control and commercial references

Analytical Component (units)[1]	MON 89034 Mean	Control Mean	Mean Difference (Test minus Control) % of Control	Mean Difference (Test minus Control) Significance (p-Value)	MON 89034 (Range)	Commercial Tolerance Int.[2]
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]
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)	3.2	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]
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.

VII.C. Conclusions of Phenotypic, Agronomic, Ecological Interactions and Compositional Assessment of MON 89034

An extensive set of information and robust data were used to assess whether the introduction of the lepidopteran-protection trait and the expression of the Cry1A.105 and Cry2Ab2 proteins altered the plant pest potential of MON 89034 compared to the control. The assessment was based on thorough phenotypic, agronomic, ecological interactions and compositional characterization and comparison of MON 89034 to control and conventional reference corn hybrids. Data were collected for five seed dormancy/germination parameters, two pollen characteristics, 14 phenotypic and agronomic characteristics during plant growth and development, more than 70 observations for each of the plant-insect, plant disease and plant-abiotic stressor interactions, and 305 comparisons in compositional analyses.

Results from the phenotypic and agronomic assessments indicate that MON 89034 does not possess characteristics that would confer a plant pest risk or result in meaningfully altered ecological impact compared to conventional corn. Data on ecological interactions also indicate that MON 89034 does not confer any increased susceptibility or tolerance to specific disease, abiotic stressors, or insects except for the introduced lepidopteran-protection trait. Data from composition analyses support the conclusion of compositional equivalence between MON 89034 and conventional corn in nutritional, anti-nutritional, and secondary metabolite levels in grain and forage. Taken together, these data support a conclusion that MON 89034 is not likely to pose increased plant pest risk or result in meaningfully altered ecological impact compared to conventional corn.

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VIII. Environmental Assessment and Impact on Agronomic Practices

This section provides an environmental assessment of MON 89034 and considers the impact of the introduced Cry1A.105 and Cry2Ab2 proteins on non-target organisms (NTOs) and endangered species, the potential for gene flow, and the weediness potential of MON 89034. The section also provides a brief summary of the current production and the agronomic practices for corn in the U.S., and an assessment of the impact from the introduction of MON 89034 on the agronomic practices and insect resistance management (IRM).

The assessment of the impact of MON 89034 and the introduced proteins on NTOs and endangered species concludes that risk to these organisms from the use of MON 89034 is negligible. This risk assessment took into consideration several components, including familiarity with the mode of action of Cry proteins; the activity spectra of the Cry1A.105 and Cry2Ab2 proteins; the expression levels of the two proteins in MON 89034; the environmental fate of the proteins; the lack of synergistic or antagonistic interaction between the two proteins; and feeding tests with each of the two proteins or MON 89034 corn materials to representative non-target organisms.

The evaluation of weediness potential and gene flow concludes that MON 89034 is no more likely to become a weed than conventional corn, and MON 89034 is expected to be similar to conventional corn regarding the potential for gene flow. Pollen-mediated gene flow would be expected to occur only within cultivated corn in the U.S. The probability for horizontal gene flow is exceedingly small. Even if it were to occur, the consequences would be negligible since the genes introduced into MON 89034 are of bacterial origin and the two Cry proteins produced have no meaningful toxicity to humans and other NTOs under the conditions of use.

An assessment of current corn agronomic practices indicates that the introduction of MON 89034 will not impact the current U.S. corn cultivation practices and the management of weeds, diseases and insects except for the control of lepidopteran insect pests. In addition, MON 89034 will provide an effective IRM tool due to the production of both the Cry1A.105 and Cry2Ab2 proteins in a single plant.

VIII.A. Environmental Assessment of the Cry1A.105 and Cry2Ab2 Proteins

VIII.A.1. Mode of Action of Cry1A.105 and Cry2Ab2 Proteins

The history of safe use of Bt microbial pesticides and Bt crops and the well understood mode of action of Bt Cry proteins are important considerations in the environmental safety assessment of these proteins. Bt microbial pesticides have been used for several decades. Bt corn producing Cry1Ab or Cry1F (YieldGard®, Herculex® I) and Bt cottons

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producing Cry1Ac, Cry1F, and Cry2Ab2 (Bollgard®, Bollgard® II, WideStrike®) have been in commerce for up to a decade.

As discussed in **Section VI**, MON 89034 produces two Cry proteins – Cry1A.105 and Cry2Ab2. Cry1A.105 is a full-length Cry1 protein (protoxin) with a molecular weight of 133 kDa. It is a chimeric protein which consists of domains I and II from Cry1Ab or Cry1Ac, domain III from Cry1F, and the C-terminal domain from Cry1Ac. The Cry2Ab2 protein produced in MON 89034 is also derived from Bt and shares 99.8% sequence identity with the wild-type Cry2Ab2 differing by one single amino acid.

The general mode of action of Bt Cry proteins is well understood. The bacterially-produced crystal proteins are first solubilized in the insect midgut, followed by activation of the protoxins (full-length proteins) to active toxins (proteolytic resistant cores) by midgut proteases. A similar process occurs when Cry proteins are expressed in plants. The activated proteins then bind to midgut membrane receptors in susceptible insects, insert into the apical membrane and form pores. Formation of the pores causes loss of osmotic regulation, and eventually leads to cell lysis, which is thought to be responsible for insect death (Gill et al., 1992; Schnepf et al., 1998; Zhuang and Gill, 2003).

Cry1 protoxins (such as Cry1Ab, Cry1Ac, and Cry1F) are 130 to 140 kDa in size, and are activated by proteases to active cores of 65 to 70 kDa. The crystal solubilization is facilitated by an alkaline pH. The typical midgut pH is between 9-11 in lepidopteran larvae. During the solubilization and activation of Cry1 proteins, an N-terminal peptide of 25-30 amino acids and approximately half of the sequences from the C-terminus are cleaved (Choma et al., 1990; Gill et al., 1992; Schnepf et al., 1998; Bravo et al., 2002; Zhuang and Gill, 2003). The role of the C-terminal domain is believed to be in the formation of crystalline inclusion bodies within the Bt bacterium and is not required for insecticidal activity (Park et al., 2000; De Maagd et al., 2001). The 25-30 amino acid residues at the N-terminus play a role in promoting crystallization of the protoxin in the bacterium, but do not contribute to toxicity to insects (Choma et al., 1990; Gill et al., 1992; Schnepf et al., 1998). In fact, it was shown with Cry1Ac that proteolytic removal of the N-terminal peptide is essential before the protein becomes fully active (Bravo et al., 2002). Cry2, Cry3, Cry10, Cry11 and Cry20 proteins appear to be naturally truncated, and they are present in crystals as 65 to 86 kDa proteins. Although naturally truncated, these 65 to 86 kDa proteins also require proteolytic activation, resulting in 60 to 65 kDa active cores (Lee and Gill, 1997; Zhuang and Gill, 2003).

The 3-dimensional structures of three members of the Cry protein family, which may well prove to be representative of all Cry proteins, reveal the presence of three structural domains (Li et al., 1991; Grochulski et al., 1995; Morse et al., 2001). Domain I, consisting of seven α-helices, is involved in membrane insertion and pore formation. Domain II, consisting of three β-sheets in a Greek key conformation, is involved in specific receptor recognition and binding. Domain III, which consists of two β-sheets in a jellyroll conformation, has been suggested to maintain the structural integrity of the protein molecule (Li et al., 1991) and also to contribute to specificity (De Maagd et al., 2000; 2001). All three domains are included in the N-terminal portion of the protoxins

during the formation of active toxins in insect guts. Since domains II and III can both contribute to the specificity, the difference in these domains among different Cry proteins would account for the diversity of insecticidal activities. These domains may dictate whether and how binding occurs between the Cry proteins and the insect midgut. Only those insects with specific receptors are affected by Cry proteins and no toxicity is observed in species that lack these receptors (Crickmore et al., 1998; De Maagd et al., 2001). Monsanto's research data indicates that there are important differences between the Cry1A.105 and Cry2Ab2 proteins regarding their binding to the lepidopteran midgut. The data demonstrate that Cry1A.105 and Cry2Ab2 proteins bind to some unique components on European corn borer brush border membrane. They also share many common binding sites. By screening a limited number of glycosylated bovine serum albumins, it was shown that galactosamine is recognized by Cry1A.105 only, while Cry2Ab2 demonstrated a high affinity for N-acetylglucosamine and galactosamine. These differences are advantageous to insect resistant management for MON 89034 because they result in a low probability of insect cross-resistance between the two proteins (see **Section VIII.F.**).

VIII.A.2. Activities against Target Insects

VIII.A.2.a. Laboratory Tests on Activities against Target Insect Pests

Lepidopteran pest larvae feed on leaves, stalks, silk and ears of corn and can significantly affect yield. Furthermore, feeding can also affect the overall quality of the crop by providing an opportunity for infection by fungi which produce mycotoxins. The major lepidopteran insect pests include corn borers, such as European corn borer (ECB, *Ostrinia nubilalis*), southwestern corn borer (SWCB, *Diatraea grandiosella*), sugarcane borer (SCB, *Diatraea saccharalis*), African stalk borer (*Busseola fusca*), and Asian corn borer (ACB, *Ostrinia funicalis*). ECB is the primary pest in the U.S. In addition, corn leaves, silks and ears are the primary food source for fall armyworm (FAW, *Spodoptera frugiperda*), corn earworm (CEW, *Helicoverpa zea*), and black cutworm (BCW, *Agrotis ipsilon*) and feeding damage from these pests negatively affects growth and development of the plants.

Studies were conducted to evaluate the spectra of the insecticidal activities of the Cry1A.105 and Cry2Ab2 proteins with a variety of agronomically-important insects from three major taxa. Insect species tested included four representative lepidopterans: ECB, CEW, FAW, and BCW; 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 for either 5 or 7 days to a range of concentrations of Cry1A.105 or Cry2Ab2 protein up to 80 or 100 µg of protein per ml of insect diet using either diet-incorporation or diet-overlay bioassay procedures. The results showed that Cry1A.105 and Cry2Ab2 proteins had activities against all four representative lepidopteran insects. There was no indication of activity of these two proteins against the two coleopteran or two hemipteran species evaluated. The results

from these assays confirm that, under expected agricultural use, these proteins have the targeted range of insecticidal activity against lepidotperan insect pests.

VIII.A.2.b. Field Efficacy Trials

The field efficacy of MON 89034 against major lepidopteran pests of corn was evaluated at multiple locations in the U.S. and Puerto Rico during 2004 and 2005. The 2004 field trials were conducted at 10 locations in Illinois, Tennessee, Mississippi, Alabama, Arkansas, Oregon, and Puerto Rico. The 2005 field trials were conducted at 18 locations in Wisconsin, Illinois, Indiana, Missouri, Arkansas, Tennessee, Mississippi, Alabama, Louisiana, Nebraska, and Colorado. MON 89034 was tested along with a conventional control corn and one current Bt corn (Yieldgard® corn borer, i.e., MON 810) as a reference. The experimental design for all locations was a randomized complete block with four replications. Experimental units were either one-, two- or four-row plots, ranging from 5.3 to 6.1 m in length depending upon location. Each row was thinned to 35 plants, and row spacing was 0.76 m. The entire trial areas were surrounded by at least two conventional corn border rows and buffered by a complete range of hybrid plants to further eliminate border effects.

Seven insect pests were tested during the two years of field trials, including ECB SWCB, FAW, CEW, SCB, BCW and western bean cutworm (WBCW, *Striacosta albicosta*). The infestations were either artificially introduced to the plants at appropriate growth stages (using neonate larvae for ECB, SWCB, FAW, CEW and BCW; and eggs for WBCW) or relied on heavy natural insect pressure (for SCB). Insect damage was assessed on 10-24 plants per plot (damage rating systems varied by insect species). Mean separation tests were performed at $\alpha = 0.05$ level to assess treatment differences.

The results demonstrate that the high level of efficacy of MON 89034 against ECB and SWCB was equivalent to the commercial reference Bt corn, MON 810. MON 89034 provided outstanding control of FAW, which was superior to that of MON 810. MON 89034 was also superior to MON 810 for the control of CEW. SCB control by MON 89034 was comparable to that of MON 810. MON 89034 appeared to be efficacious against BCW and have potential for the control of WBCW. However, since the pressure was light and the number of locations limited for these two pests, additional testing is needed to confirm this activity. The performance of MON 89034 to reduce ear damage was most evident under combined infestations of both WBCW and CEW. In conclusion, the broader spectrum of activity of MON 89034 compared to MON 810 provides greater insurance against yield loss from lepidopteran pests, and further enhances agronomic qualities, ease of harvest, and grain quality.

VIII.A.3. Impact on Non-target Organisms

Evaluation of the potential risks to NTOs is an important component of an overall environmental risk assessment of a biotechnology-derived crop. Assessment of the potential risks to NTOs associated with the introduction of a biotechnology-derived crop producing insecticidal proteins is based on the characteristics of the crop and introduced

proteins. Since risk is a function of hazard and exposure, it is critical to determine the potential hazards and exposure scenarios that are most likely and that require evaluation through experimental studies. Selection of the test organisms and test material are important decisions that are based on the characteristics of the trait and the product. In the U.S., regulatory guidelines for NTO risk assessment of insect-protected crops were developed by the EPA. The testing is conducted according to a tier-based system (EPA, 1996; 2001a-d; 2004a-b). Additionally, the EPA has convened several Scientific Advisory Panel (SAP) meetings to make recommendations and provide guidance for NTO testing and risk assessment for agricultural products produced by methods of biotechnology (EPA, 2001a-b; 2002; 2004a-b).

For the Cry proteins tested in laboratory assays to date, potentially significant adverse effects have been observed for only a very few NTO species that are closely related to the target species (Mendelsohn et al., 2003; Romeis et al., 2006). However, field studies conducted over the past decade by industry and the academic community on registered insect-protected crops that produce a variety of Cry proteins have demonstrated that these crops have no adverse effects on the diversity and abundance of non-target arthropod communities including predators, parasitoids and other ecologically important non-target arthropods (Bitzer et al., 2005; Bhatti et al., 2005; Daly and Buntin, 2005; Dively, 2005; Head et al., 2005; Lopez, et al., 2005; Pilcher et al., 2005; Torres and Ruberson, 2005; Whitehouse et al., 2005; Naranjo et al., 2005; Naranjo, 2005a-b; Lozzia et al., 1998; Orr and Landis, 1997; Pilcher et al., 1997). Additionally, Cry1 and Cry2 proteins have been shown to pose low risk to populations of non-target lepidopterans (Hellmich et al., 2001; Mattila et al., 2005; Pleasants et al., 2001). For example, the impact of exposure to pollen containing Cry1 and Cry2 proteins on populations of lepidopteran species has been evaluated in a number of empirical studies and several risk assessments (Mattila et al., 2005; Mendelsohn et al., 2003; Wolt et al., 2005), and the risk has been shown to be negligible.

The NTO risk assessment of Cry1A.105 and Cry2Ab2 proteins was a multi-step process taking into consideration hazard identification and characterization, exposure assessment, and risk characterization. The hazard identification and characterization included testing the spectra of insecticidal activities of the Cry1A.105 and Cry2Ab2 proteins, efficacy of MON 89034 against target corn insect pests, and the potential for interaction between the Cry1A.105 and Cry2Ab2 proteins. These studies demonstrated that the Cry proteins produced in MON 89034 perform in the expected manner based on the extensive knowledge and experience on Cry proteins. The insecticidal activity spectra of the Cry1A.105 and Cry2Ab2 proteins were found to be typical for the Cry1 and Cry2 classes of Bt proteins. The activity was most evident against insects within the order *Lepidoptera*. An assessment of the potential for interaction between Cry1A.105 and Cry2Ab2 proteins demonstrated that there was no interaction between the two proteins. The Cry1A.105 and Cry2Ab2 proteins were tested alone and in combination against two sensitive lepidopteran species – ECB and 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. The results demonstrated that these proteins only showed additive effect in insecticidal activity, and

no synergistic or antagonistic effects were observed. The result is consistent with the findings in an earlier study that demonstrated no interaction between the Cry1Ac and Cry2Ab2 proteins (Greenplate et al., 2003). The lack of interaction between Cry1A.105 and Cry2Ab2 allows for each protein to be tested and evaluated independently.

The exposure assessment of the NTO risk assessment is comprised of three components: estimation of the expression levels of the Cry1A.105 and Cry2Ab2 proteins in tissues from MON 89034; a conservative calculation of margins of exposure based on the maximum amount of corn tissue that might be exposed to NTOs; and assessment of the environmental fate of the Cry1A.105 and Cry2Ab2 proteins in soil. Expression values from several tissue types were used to calculate the doses used in the NTO toxicity tests, while the results from soil degradation studies were used to characterize the potential exposure to soil organisms and the likelihood that each protein could persist and accumulate in agricultural soils.

Dosing in the NTO tests was based on the estimated maximum expected environmental concentrations (MEEC) of the Cry1A.105 and/or Cry2Ab2 protein present in the tissue(s) likely to be ingested by the representative NTO. A targeted margin of exposure (MOE) of at least 10-times greater than the MEEC was used in the tests. The maximum expression levels for the Cry1A.105 (8.8 µg/g fwt) and Cry2Ab2 (0.47 µg/g fwt) proteins in pollen were used to determine the dose levels for honeybee, ladybird beetle, a parasitic wasp, and minute pirate bug, for which pollen represents the major route of exposure.

The principal route of exposure for soil-dwelling organisms such as Collembola and earthworms was assumed to be from decomposing plant tissue containing the Cry1A.105 and Cry2Ab2 proteins. The use of a soil MEEC to calculate MOEs is appropriate because these organisms feed on detritus that is made up of soil and decaying plant and other material. Therefore, the maximum amount of plant tissue entering the soil environment and the maximum concentration of the Cry1A.105 and Cry2Ab2 proteins in the plant tissue were considered in determining the MEEC for the Cry1A.105 and Cry2Ab2 proteins in the top 15 cm of soil. The EPA model assumes that corn plants in the field are tilled into the top 6 inches (15 cm) of soil, and the plants uniformly express the introduced proteins at the maximum concentration. However, because expression of Cry proteins varies in a tissue-specific and temporal manner this hazard assessment was generated based on the maximum *in planta* expression of the Cry1A.105 (240 µg Cry1A.105/g dwt) and the Cry2Ab2 (210 µg Cry2Ab2/g dwt) proteins at a late developmental stage (pre-tasseling) of corn. The protein levels in late plant developmental stage represent a probable exposure scenario. These data as well as additional parameter estimates[1] were used to calculate the soil MEECs for Collembola and earthworm.

[1] The soil MEEC for Collembola and earthworm was calculated using the following parameter assumptions: 25,000 corn plants/acre; a corn plant dry weight is 650 g/plant; a bulk density of soil of 1500 kg/cubic meter; a soil depth is 0.15 m (about 6 inches) and a soil volume in a one-hectare 0.15 m layer is 1500 cubic meters. The Cry1A.105 and Cry2Ab2 maximum expression values were taken for leaves at the pre-tasseling stage and were 240 and 210 µg/g dwt, respectively.

The August 2002 EPA SAP reports (EPA, 2002) recommended that nontarget testing be focused on species exposed to the crop being registered (i.e., beneficial insects and avian species). The exposure of fish to Cry proteins in corn is negligible in agricultural settings. According to EPA "There is no evidence of sensitivity of aquatic (including endangered) species to Cry proteins. Toxicity studies with aquatic organisms show very limited hazard for fish or invertebrates exposed to either corn pollen or bacterially-expressed Cry protein" (EPA, 2001d). The requirements for a freshwater fish were waived for a number of Bt corn products based on a lack of any substantial exposure of fish to Bt proteins produced in corn (EPA, 2003). Therefore, a static renewal freshwater fish toxicity study was not performed. The exposure of aquatic invertebrates to corn pollen, if it occurs at all, would be of a very short duration and at extremely low levels that are not expected to produce any adverse effects. Nevertheless, an acute aquatic invertebrate species test was performed with *Daphnia magna* using pollen from MON 89034. No treatment mortality or other adverse effects were observed at the concentration of 100 mg pollen/l over a 48-hour exposure period. Under a well accepted toxicity classification system for freshwater ecotoxicology, if no adverse effects are observed at a concentration of 100 mg/l, the tested material is classified as practically non-toxic (EPA, 1985). Therefore, no hazard is anticipated to aquatic invertebrates from exposure to the registered uses of MON 89034.

Based on the results from the product characterization and exposure assessment, an evaluation of the potential toxicity to selected NTOs (hazard assessment) was conducted. The detailed hazard assessment included toxicity testing against one mammalian species (mice), two avian species (broiler chicken and bobwhite quail), one aquatic species (Daphnia), two species of soil decomposers (Collembola and earthworm), and four beneficial insect species [honeybee (*Apis mellifera*); minute pirate bugs (*Orius insidiosus*), ladybird beetle (*Coleomegilla maculata*); and parasitic wasp (*Ichneumon promissorius*)]. The test materials were selected for each study based on the species being evaluated and whether more ecologically relevant exposures (plant tissues) could be achieved without compromising the performance of the study. In many cases, pure proteins were used because ingestion of the material could be ensured using artificial diets containing high levels of either Cry1A.105 or Cry2Ab2 protein. When tissues were used (e.g. Collembola), the expression levels for both Cry1A.105 and Cry2Ab2 proteins were estimated to calculate the doses. The NOECs (no observed effect concentrations) of Cry1A.105 and Cry2Ab2 proteins determined for each of the tested NTOs are summarized in **Table VIII-1**.

MOEs for the non-target arthropods were also calculated based on the ratio of the NOECs to the MEECs. The result showed that the MOEs were ≥14 fold of the potential maximum exposure level for these NTOs (**Table VIII-2**). MOEs that exceed 10 are considered as indicative of minimal risk by many regulatory authorities. Therefore, as with other Cry proteins, Cry1A.105 and Cry2Ab2 are not likely to produce adverse effects at field exposure levels on tested representative terrestrial beneficial invertebrate species. This conclusion is in agreement with prior published literature which reported no adverse effects on non-target organisms from insect-protected crops that produce Cry1

or Cry2 proteins (Mendelsohn et al., 2003; Daly and Buntin, 2005; Dively, 2005; Naranjo et al., 2005, Pilcher et al., 2005).

Table VIII-1. No observed effect concentrations (NOECs) of Cry1A.105 and Cry2Ab2 proteins for non-target organisms

Test organism	NOECs for Cry1A.105	NOECs for Cry2Ab2
Collembola	80 µg/g	70 µg/g
Earthworm	178 mg/kg dry soil	330 mg/kg dry soil
Honeybee larvae	1100 µg/ml as a single dose[1]	100 µg/ml as a single dose
Honeybee adult	550 µg/ml	68 µg/ml
Minute pirate bugs	120 µg/g	100 µg/g
Ladybird beetle	240 µg/g	120 µg/g
Parasitic wasp	240 µg/ml	100 µg/ml
Mouse	2072 mg/kg	2198 mg/kg
Quail	50% corn grain from MON 89034 in diet	
Broiler	50% to 55% corn grain from MON 89034 in diet	
Daphnia	100 mg/l pollen from MON 89034	

[1] The NOEC for the honeybee larval assay is based on the concentration of the dosing solution.

Table VIII-2. Estimated margins of exposure (MOEs) to non-target arthropods for the Cry1A.105 and Cry2Ab2 proteins

Test organism	Cry1A.105				Cry2Ab2			
	Source	MEEC[1]	NOEC	MOE[2]	Source	MEEC	NOEC	MOE
Collembola	Leaf	4.3 mg/kg dry soil [3]	≥80 µg/g	≥19	Leaf	3.7 mg/kg dry soil[3]	≥70 µg/g	≥19
Earthworm	*E. coli*	4.3 mg/kg dry soil	≥178 mg/kg dry soil	≥41	Bt	3.7 mg/kg dry soil	≥330 mg/kg dry soil	≥89
Honeybee larvae	*E. coli*	8.8 µg/g fw (pollen level)	≥1100 µg/ml as a single dose [4]	≥125	Bt	0.47 µg/g fw (pollen level)	≥100 µg/ml as a single dose[4]	≥213
Honeybee adult	*E. coli*	8.8 µg/g fw (pollen level)	≥550 µg/ml	≥63	Bt	0.47 µg/g fw (pollen level)	≥68 µg/ml	≥145
Minute pirate bugs	*E. coli*	8.8 µg/g fw (pollen level)	120 µg/g	14	*E. coli*	0.47 µg/g fw (pollen level)	≥100 µg/g	≥213
Ladybird beetle	*E. coli*	8.8 µg/g fw (pollen level)	≥240 µg/g	≥27	*E. coli*	0.47 µg/g fw (pollen level)	≥120 µg/g	≥255
Parasitic wasp	*E. coli*	8.8 µg/g fw (pollen level)	≥240 µg/ml	≥27	*E. coli*	0.47 µg/g fw (pollen level)	≥100 µg/ml	≥213

[1] Expression levels determined from MON 89034.

[2] Margins of Exposure (MOE) were calculated based on the ratio of the NOEC to MEEC. The MOE was determined based on the expression level of the Cry1A.105 and Cry2Ab2 proteins in the tissue from MON 89034 deemed most relevant to the NTO exposure.

[3] The MEEC for Collembola and earthworm was calculated using the following parameter assumptions: 25,000 corn plants/acre; corn plant dry weight is 650 g /plant; the bulk density of soil is 1500 kg/cubic meter; soil depth is 0.15 m (about 6 inches); soil volume in a one-hectare 0.15 m layer is 1500 cubic meters. The Cry1A.105 and Cry2Ab2 expression values were taken for leaves at the pre-tasseling stage and were 240 and 210 µg/g dwt, respectively.

[4] The NOEC for the honeybee larval assay is based on the concentration of the dosing solution.

VIII.A.4. Impact on Endangered Species

As discussed in the above sections, Cry proteins are known to have biological activity exclusively toward insect species. Extensive literature references support the observation that Cry proteins have a high degree of specificity and will not pose a significant hazard to non-insect animals (Federici, 2002; Romeis *et al*., 2006). This observation has been confirmed through testing with a standard battery of terrestrial and aquatic non-target organisms, including mammals, birds, water fleas and earthworms and beneficial insects, for Bt crop registrations (Mendelsohn et al., 2003). These data establish that the Cry proteins pose negligible risk to non-insect animals and the vast majority of non-target insects. Based on the demonstrated low hazard of Cry proteins to non-insect animals, no adverse effects are expected for endangered mammals, birds, non-insect aquatic animals, and non-insect soil organisms.

Monsanto has conducted extensive studies testing the Cry1A.105 and Cry2Ab2 proteins for activity against a range of both target and non-target insect species (refer to **Sections VIII.A.2** and **VIII.A.3**). Experiments were also conducted to examine any potential interaction between the two proteins, and the results concluded the activities of Cry1A.105 and Cry2Ab2 on representative lepidopteran species are additive. There are no synergistic or antagonistic interactions between the two proteins. The results also show that both the Cry1A.105 and Cry2Ab2 proteins are highly specific in insecticidal activity against lepidopteran insects and have little activity against non-lepidopteran insects. These data indicate that the only potential risk to endangered species resides with endangered butterflies and moths in the order *Lepidoptera*.

Risk assessments of potential effects of Bt crops on the federally listed threatened or endangered lepidopterans began with an examination of an overlay map showing the county level distribution of endangered lepidopteran species as listed by the U.S. Fish and Wildlife Service, relative to corn production counties in the U.S. The result showed that most endangered lepidopteran species do not occur in agricultural counties where corn is grown. Of the 11 endangered lepidopteran species that do occur in counties that also produce corn, an analysis of the endangered species critical habitat showed that only the habitat of the Karner blue butterfly (*Lycaeides melissa samuelis*) has the potential to occur in proximity to corn fields. A recent academic analysis of Karner blue butterfly phenology and corn pollen shed showed that there are only two Wisconsin counties in the Midwestern corn belt where there is a potential for temporal overlap between corn pollen shed and the presence of Karner blue larvae. At present there is no evidence that Karner blue larvae are exposed to corn pollen in these two locations (Peterson et al., 2006). Based on its feeding habits and phenology, Karner blue larvae will have a low likelihood of exposure to corn pollen from MON 89034.

Although the likelihood of exposure of Karner blue larvae to corn pollen from MON 89034 is low, a risk analysis based on conservative hazard using the most sensitive species approach and exposure estimates was used to determine the margin of safety. Since Karner blue larvae use wild lupine plant (*Lupinus perennis*) exclusively as a host

plant, the only route of exposure of Cry1A.105 and Cry2Ab2 proteins to Karner blue larvae is through deposition of pollen from MON 89034 onto lupine plants. The interactions among Karner blue, its lupine host, and corn pollen are very similar to those among the monarch butterfly, milkweed plant, and corn pollen. Therefore, the approaches used for evaluating potential effects of corn pollen on Monarch butterfly (Pleasants et al., 2001; Stanley-Horn et al., 2001, Sears et al., 2001; Dively et al., 2004) were used for assessing the effects of MON 89034 pollen on Karner blue. The exposure level of Karner blue to MON 89034 pollen was estimated based on the potential pollen density on the lupine plants and the combined maximum expression level of Cry1A.105 and Cry2Ab2 proteins in the pollen. Since toxicity testing of Karner blue larvae directly is not possible due to its endangered status, the LC_{50} value of Cry1A.105 and Cry2Ab2 proteins for larvae of a most sensitive lepidopteran insect – European corn borer (ECB) – was used in the risk analysis. The results of this risk analysis showed that there was a minimum 12-fold margin of safety for Karner blue larvae in the event that they would be exposed to the highest possible pollen concentration from MON 89034. The margin of safety was calculated as the quotient of the LC_{50} value for ECB divided by the maximum exposure level of the two proteins. Therefore, it can be concluded that pollen from MON 89034 is not likely to adversely affect Karner blue larvae.

VIII.A.5. Environmental Fate of Cry1A.105 and Cry2Ab2 Proteins and Impact on Soil-dwelling Organisms

Soil organisms may be exposed to the Cry1A.105 and Cry2Ab2 proteins by contact with roots, the above-ground plant residues after falling or tilling into the soil, or by pollen deposited on the soil. Microbial exposure may occur by feeding on living or dead corn biomass or by ingestion or absorption of the Cry proteins after their release into the soil. Several soil factors (e.g., pH and clay content) can influence the degradation rate of Cry proteins. Published studies suggest that Cry proteins may bind to the clay components of soil and become more resistant to degradation by soil microorganisms (Tapp and Stotzky, 1998). However, soil pH near or above neutrality substantially increases the degradation rate of Cry proteins. Under most corn production conditions, corn would be grown on soils that are near neutral pH (Wright et al 2004), i.e., under conditions favorable to degradation of Cry proteins. Indeed, published field monitoring studies have shown no persistence or accumulation of Cry proteins in fields where corn expressing Cry1Ab protein (Dubelman et al., 2005) or Cry3Bb1 protein (Ahmad et al., 2005), and cotton expressing Cry1Ac protein (Head et al., 2002) were grown continuously for several years.

A laboratory aerobic soil degradation study showed that the Cry1A.105 and Cry2Ab2 proteins dissipated rapidly in soils from several sources (MO, IL and ND) that contained significant proportions of clay (up to 41%), organic matter (up to 4.5%) and a range of soil pH (5.9-8.0). Purified Cry1A.105 and Cry2Ab2 proteins were added to each soil in a large excess relative to the maximum (worst-case) concentration possible under corn growing conditions. The large excess was used to improve the accuracy of the analytical quantifications, and to ensure that the DT_{50} and DT_{90} (time to 50% and 90% dissipation of the proteins) could be calculated from the measured values. This excess also provided

an increased margin of environmental safety, including possible exudation of the Cry proteins through the roots into soil. A sensitive soil insect bioassay showed that the DT_{50} ranged 2 to 7 days, and the DT_{90} ranged 7 to 19 days for the Cry1A.105 protein. The DT_{50} ranged 0.5 to 3 days and the DT_{90} ranged 3 to 13 days for the Cry2Ab2 protein.

Published studies on the effect of Cry proteins on soil-dwelling organisms show little or no impact on the soil microflora from the use of biotechnology-derived plants producing Bt proteins. For example, a season-long field study conducted with the Cry3A protein expressed in biotechnology-enhanced potato, showed no adverse effects towards soil-dwelling microorganisms (Donegan et al., 1996). In a study conducted in Kansas during the 2000 and 2001 growing seasons, the numbers of soil mites, Collembola and nematodes observed in plots planted with Cry3Bb1-producing corn were similar to those observed in plots planted with conventional corn (Al-Deeb et al., 2003). Other published reports showed that Cry proteins had no microbiocidal or microbiostatic activity *in vitro* against selected bacteria, fungi, and algae (Koskella and Stotzky, 2002), and had no apparent effect on earthworms, nematodes, protozoa, bacteria and fungi (Saxena and Stotzky, 2001).

These results suggest that there will be negligible risk to soil-dwelling organisms from their exposure to MON 89034 which expresses Cry1A.105 and Cry2Ab2 proteins.

VIII.B. Weediness Potential of MON 89034

In the United States, corn is not listed as a weed in the major weed references (Crockett, 1977; Holm et al., 1979; Muenscher, 1980), nor is it present on the lists of noxious weed species distributed by the federal government (7 CFR Part 360). Furthermore, corn has been grown throughout the world without any report that it is a serious weed. Modern corn cannot survive as a weed because of past selection in the evolution of corn. During domestication of corn, traits often associated with weediness such as seed dormancy, a dispersal mechanism, or the ability to form reproducing populations outside of cultivation have not been selected. For example, the corn ear is enclosed with husks. Consequently, seed dispersal of individual kernels is limited. Even if individual kernels of corn were distributed within a field or along transportation routes from the fields to storage or processing facilities, sustainable volunteer corn populations are not found growing in fence rows, ditches, and road sides. Corn is poorly suited to survive without human assistance and is not capable of surviving as a weed (Baker, 1965; Keeler, 1989; Galinat, 1988). Although corn seed can overwinter into a crop rotation with soybeans, mechanical and chemical measures can be used to control volunteers.

In comparative studies conducted between MON 89034 and a conventional control, dormancy and germination, growth and development, and reproductive characteristics were evaluated for changes that would impact plant pest potential, and in particular, plant weediness potential (see **Section VII**). The results of these studies support a conclusion that the introduction of the insect protection trait did not unexpectedly alter the assessed characteristics compared to the control. Thus, the results support a conclusion of no increased weediness potential of MON 89034 compared to the conventional corn.

Furthermore, extensive postharvest monitoring of field trial plots planted with MON 89034 under USDA-APHIS notifications (see **Appendix H** for a complete list of USDA notifications approved for MON 89034) did not reveal any differences in survivability or persistence relative to other corn. These data suggest that MON 89034 is no more likely to become a weed than conventional corn.

VIII.C. Gene Flow

Gene flow (often used synonymously with the term "outcrossing" or "cross pollination") is a natural biological process that occurs in most crop species including corn. Pollen-mediated gene flow is a term used to describe the movement of plant genes from one plant to another via pollen. The rate of pollen-mediated gene flow depends on biotic and abiotic factors such as plant biology, pollen biology/volume, plant phenology, overlap of flowering times, proximity of the pollen source and sink, ambient conditions such as temperature and humidity, and field architecture.

VIII.C.1. Vertical Gene Flow – Transfer of Genetic Information among Species with which Corn can Interbreed

Corn morphology fosters cross-pollination; therefore, high levels of pollen-mediated gene flow can occur in this species. Researchers also recognize that 1) the amount of gene flow that occurs can be high because of open-pollination; 2) the percent gene flow will vary by population, hybrid or inbred; 3) the level of gene flow decreases with greater distance between the source and recipient plants; 4) environmental factors affect the level of gene flow; 5) corn pollen is viable for a short period of time under field conditions; 6) corn produces ample pollen over an extended period of time; and, 7) there are no purposeful insect pollinators of corn but that pollinating insects, especially bees, are occasional visitors to the tassels but rarely visit silks of corn.

For gene flow to occur by normal sexual transmission, certain conditions must exist: 1) the two parents must be sexually compatible; 2) there must be overlapping phenology; and 3) a suitable factor (such as wind or insects) must be present and capable of transferring pollen between the two parents.

Several studies have been conducted on the extent of pollen-mediated gene flow in corn. As expected, results were found to vary depending on the experimental design, environmental conditions and detection method. In general, percent gene flow was found to diminish with increasing distance from the source field. It was previously reported that corn cross-pollination rate fell below 1% at distances >200 m (Jemison and Vayda, 2000; Luna et al., 2001). A more recent research showed that corn cross pollination rate decreased below 0.9% level beyond 15 m (Bannert, 2006)

As discussed in **Section II.G**, corn and annual teosinte (*Zea mays* subsp. *mexicana* Schrad.) are genetically compatible, wind-pollinated and, in areas of Mexico and Guatemala, freely hybridize when in close proximity to each other. Corn easily crosses with teosinte; however, teosinte is not present in the U.S. other than as an occasional

botanical garden specimen. These specimens would only flower at the same time as corn if they were subject to artificial day length shortening for several weeks at a time (Wilkes, 1967). Differences in factors such as flowering time, geographical separation and development factors make natural crosses in the U.S. highly unlikely.

In contrast with corn and teosinte, which easily hybridize, it is only with extreme difficulty and special techniques that corn and *Tripsacum* hybridize. Furthermore, the offspring of the cross show varying levels of sterility (Galinat, 1988; Mangelsdorf, 1974; Russell and Hallauer, 1980). (see **Section II.G.**). No cases of gene flow between corn and its wild relatives are known to occur naturally in the U.S.

VIII.C.2. Horizontal Gene Flow – Transfer of Genetic Information to Species with which Corn Cannot Interbreed

Monsanto is aware of no reports of the transfer of genetic material from corn to other species with which corn cannot sexually interbreed. The probability for horizontal gene flow to occur is judged to be exceedingly small. Even if it were to occur, the consequences would be negligible since the genes introduced into MON 89034 are of bacterial origin and the two Cry proteins produced have no meaningful toxicity to humans and other NTOs under the conditions of use.

VIII.D. Production and Current Agronomic Practices for U.S. Field Corn

This section provides a review of U.S. agronomic practices in corn and the anticipated environmental consequences from the commercialization of MON 89034.

VIII.D.1. Production

Corn is the largest U.S. crop in terms of acreage planted and net crop value. In the past 10 years (1996-2005), total annual corn acrerage planted varied from approximately 76 to 82 million acres, total annual production ranged from 9 to 11.8 billion bushels, and total annual value fluctuated from 17 to 25 billion dollars depending on the production output and commodity prices (**Table VIII-3**).

Corn is planted in almost every state in the continental U.S. For convenience the states can be grouped into the following regions (number of states): Midwest (12), Northeast (8), Mid-Atlantic (5), Southeast (9), Great Plains (5), Northwest (4) and Southwest (5). However, the majority of the corn (86%) is grown in the Midwest region (**Table VIII-4**). Yields vary considerably from region to region because of the diversity in rainfall/irrigation, climatic conditions and soil productivity.

Table VIII-3. Field corn production in the U.S. (1996 – 2005)

Year	Acres planted (x 1000)	Acres harvested (x 1000)	Yield (bu/acre)	Production (x 1000 bu)	Price ($/bu)	Value ($ billion)
1996	79,229	72,644	127.1	9,232,557	2.71	25.15
1997	79,537	72,671	126.7	9,206,832	2.43	22.35
1998	80,165	72,589	134.4	9,758,685	1.94	18.92
1999	77,386	70,487	133.8	9,430,612	1.82	17.10
2000	79,551	72,440	136.9	9,915,051	1.85	18.50
2001	75,702	68,768	138.2	9,502,580	1.97	18.88
2002	78,894	69,330	129.3	8,966,787	2.32	20.88
2003	78,603	70,944	142.2	10,089,222	2.42	24.48
2004	80,929	73,631	160.4	11,807,086	2.06	24.38
2005	81,759	75,107	147.9	11,112,072	1.90	21.04

Source: USDA-NASS, 2006c

Table VIII-4. Field corn production by regions and states in the U.S. (2005)

State	Acres planted (x 1000)	Acres harvested (x1000)	Yield (bu/acre)	Production (x1000 bu)	% to national total production
Midwest region					
Illinois	12,100	11,950	143	1,708,850	15.38
Indiana	5,900	5,770	154	888,580	8.00
Iowa	12,800	12,500	173	2,162,500	19.46
Kentucky	1,250	1,180	132	155,760	1.40
Michigan	2,250	2,020	143	288,860	2.60
Minnesota	7,300	6,850	174	1,191,900	10.73
Missouri	3,100	2,970	111	329,670	2.97
Nebraska	8,500	8,250	154	1,270,500	11.43
North Dakota	1,410	1,200	129	154,800	1.39
Ohio	3,450	3,250	143	464,750	4.18
South Dakota	4,450	3,950	119	470,050	4.23
Wisconsin	3,800	2,900	148	429,200	3.86
Regional total	**66,310**	**62,790**		**9,515,420**	**85.63**
Northest region					
New York	990	460	124	57,040	0.51
Pennsylvania	1,350	960	122	117,120	1.05
Connecticut	28				
Maine	26				
Massachusetts	20				
New Hampshire	15				
Rhode Island	2				
Vermont	95				
Regional total	**2,526**	**1,420**		**174,160**	**1.57**
Mid-Atlantic region					
Delaware	160	154	143	22,022	0.20
Maryland	470	400	135	54,000	0.49
New Jersey	80	62	122	7,564	0.07
Virginia	490	360	118	42,480	0.38
West Virginia	45	28	109	3,052	0.03
Regional total	**1,245**	**1,004**		**129,118**	**1.16**

Source: USDA-NASS, 2006c

Table VIII-4 continues on the next page.

Table VIII-4 (continued). Field corn production by regions and states in the U.S. (2005)

State	Acres planted (x 1000)	Acres harvested (x1000)	Yield (bu/acre)	Production (x1000 bu)	% to national total production
Southeast region					
Alabama	220	200	119	23,800	0.21
Arkansas	240	230	131	30,130	0.27
Florida	65	28	94	2,632	0.02
Georgia	270	230	129	29,670	0.27
Louisiana	340	330	136	44,880	0.40
Mississippi	380	365	129	47,085	0.42
North Carolina	750	700	120	84,000	0.76
South Carolina	300	285	116	33,060	0.30
Tennessee	650	595	130	77,350	0.70
Regional total	**3,215**	**2,963**		**372,607**	**3.35**
Great plains region					
Colorado	1,100	950	148	140,600	1.27
Kansas	3,650	3,450	135	465,750	4.19
Oklahoma	290	250	115	28,750	0.26
Texas	2,050	1,850	114	210,900	1.90
Wyoming	80	49	140	6,860	0.06
Regional total	**7,170**	**6,549**		**852,860**	**7.68**
Northwest region					
Idaho	235	60	170	10,200	0.09
Montana	65	17	148	2,516	0.02
Oregon	53	25	160	4,000	0.04
Washington	150	80	205	16,400	0.15
Regional total	**503**	**182**		**33,116**	**0.30**
Southwest region					
Arizona	50	22	195	4,290	0.04
California	540	110	172	18,920	0.17
Nevada	5				0.00
New Mexico	140	55	175	9,625	0.09
Utah	55	12	163	1,956	0.02
Regional total	**790**	**199**		**34,791**	**0.31**

VIII.D.2. Corn Cultivation and Management

The key considerations for corn production include soil quality, tillage practices, hybrid selection, moisture, nutrients, and the management of insects, weeds and diseases. A summary of the corn cultivation and management practices is provided below.

VIII.D.2.a. Soil Quality and Tillage Practices

Corn is grown in a variety of soils in the U.S. ranging from the sandhills of Nebraska and Colorado to the clays of delta regions, from strongly acidic to strongly alkaline soils, and from shallow soils on residual material to deep soils in loess, till or alluvium. Within these ranges, however, there is a corresponding variation in the crop's productivity. Ideal soils are those that have intermediate textures of loam to silt loam in the surface horizon and somewhat higher clay content as silt loam to silty clay loam in the subsoil. This combination, complemented with good structural properties, allows good storage of water and nutrients and a degree of permeability favorable to water intake and air exchange. Corn growth is optimal on soils with pH levels ranging between 6.0 and 7.0 and moderate-to-high fertility (Jones, 2003).

There are three main tillage practices employed in corn production: conventional tillage, reduced tillage, and conservation tillage. Conventional tillage practices leave <15% crop residue cover after planting and involve the use of a moldboard plow or other intensive tillage procedure. Reduced tillage practices leave between 15-30% crop residue cover after planting and exclude the use of a moldboard plow or other intensive tillage procedure. Conservation tillage is a system that covers 30% or more of the soil surface with crop residue after planting to reduce soil erosion by water, and consists of three subtypes: no-till, ridge-till and mulch-till. These subtypes differ in the timing of cultivation of the seedbed and type of equipment used. In recent years, there has been a trend toward the increased use of conservation tillage practices. In 1996, the total acres planted to corn were tilled as follows: conservation tillage (38%), reduced tillage (32%), and conventional tillage (30%) (USDA-ERS, 2002).

VIII.D.2.b. Corn Hybrids

In the U.S. hundreds of corn hybrids are marketed by seed companies. Their selection is primarily based on yield potential, climatic environment, and disease/pest resistance for that locale. One key determinant is the selection of seed with the appropriate maturity group for the local region (see **Section II.E**). Corn hybrids with relative maturities of 100 to 115 days are typically grown in the U.S. corn belt. To maximize yield, planting of corn on 30-in rows with a density of between 28,000 to 32,000 kernels per acre is typically recommended for the corn belt (Aldrich et al., 1986; Iowa State University, 2002).

VIII.D.2.c. Moisture and Nutritional Requirements

In the five major corn belt states, corn uses more moisture for evapotranspiration than is provided by rainfall, especially during the critical months of July and August (Shaw, 1988). The additional moisture requirement can be supplemented by irrigation, which is practiced on 10-11 million acres or approximately 15% of the total corn acreage (USDA-ERS, 2002). Irrigation practices also facilitate the application of agricultural chemicals and fertilizers.

Corn, like all higher plants, requires at least 13 elements from the soil for growth and development (Olson and Sander, 1988). The 13 elements include the primary elements (nitrogen, phosphorus, and potassium), secondary elements (calcium, magnesium, sulphur), and micronutrient elements (iron, manganese, zinc, copper, boron, molybdenum and chlorine). By far the most important are the primary elements, which are depleted in the soil as the corn plant develops. Whereas nitrogen and phosphate uptake continues until maturity, potassium absorption is largely completed by the silking stage. This is why fertilization of corn fields is essential to ensure production and profitability. In 2005, nitrogen was applied to 96% of the planted corn acreage at an average usage rate of 138 pounds of nitrogen per acre per crop year. Phosphate was applied to 81% of the corn acreage at an average rate of 58 pounds per acre per crop year. Potassium, applied at 84 pounds per acre per crop year, was applied to 65% of the acreage planted to corn (USDA-NASS, 2006b).

VIII.D.2.d. Management of Diseases and Insects

Management of diseases and insects during corn growth and development is essential for protecting the yield of the harvested grain. Estimates for annual yield losses because of diseases have ranged from 7 to 17% (Shurtleff, 1980). Incidence of disease infestation is highly variable and depends on many factors such as location, climate, and other environmental factors. Most corn hybrids on the market today have acceptable levels of resistance to common diseases. The diseases found to occur in corn grown in the U.S. are summarized in **Table VIII-5**. In addition, several nematode species have been known to cause diseases in corn (Dicke and Guthrie, 1988). The use of fungicides in corn is limited because the incidence and severity of most diseases tends to be low and quite variable. The fungicides currently used on corn plants in the U.S. include azoxystrobin, chlorothalonil, and propiconazole (USDA-NASS, 2006b).

The corn crop is subject to attack by a complex of insects from the time it is planted until it is used as food and feed. The economically important insect pests in North America include wireworms, the black cutworm, European corn borer, Southwestern corn borer, the corn rootworms, grasshoppers, fall armyworm, and corn earworm. **Table VIII-6** lists the insect pests in corn grown in the U.S. Approximately 27 active ingredients are registered for use in corn for the control of insect pests. In its annual survey of agricultural chemical usage, USDA determined that 23% of the corn acreage was treated with insecticides in 2005 (USDA-NASS, 2006b). Tefluthrin, cyfluthrin, and

tebupirimphos were the most widely applied insecticides, at 7, 7, and 6%, respectively, to the acres planted to corn. Chlorpyrifos was only applied to 2% of the acres, but total quantity applied is more than 3 times greater than next highest insecticide at 2.0 million pounds.

The introduction of biotechnology-derived Bt corn has offered growers an alternative and effective solution for the control of major insect pests in corn. The Bt corn products which have been commercialized in the U.S. include YieldGard® Corn Borer corn (producing Cry1Ab), YieldGard® Rootworm corn (Cry3Bb1), Herculex® I (Cry1F), Herculex® RW (Cry34Ab1 and Cry35Ab1), and Bt11 (Cry1Ab). These Bt corn products provide control of a spectrum of lepidopteran and coleopteran insect pests. In 2006, approximately 40% of the total corn acreage in the U.S. was planted with hybrids possessing insect protection traits (USDA-NASS, 2006a).

VIII.D.2.e. Weed Management

Weeds cause significant losses and require careful management by the growers because they interfere with corn plants by competing for available resources including water, nutrients and light. Economically damaging weeds in corn include annuals and perennials, grasses, broadleaf and sedge species. Some weeds can tolerate cold, wet conditions better than corn, and can get a head start prior to planting. Fields infested with perennial weeds present special problems for corn growers. Like annual weeds, perennials can reproduce by seeds, but they also regrow and spread vegetatively. This means that their rhizomes, thickened roots or tubers propogate new shoots, usually soon after corn is planted. Unless effectively controlled, perennial weeds can quickly gain a season-long advantage over the corn crop.

Corn yield loss is generally proportional to the amount of weeds present. While the ratio is not always one-to-one, some studies suggest that for every pound of weed dry matter, there is a reduction of approximately one pound of corn plant dry matter (Gianessi et al., 2002). Competition for light, nutrients, and moisture resources by the crop and weeds can lead to proportional reductions in yield (Knake et al., 1990). Numerous studies have shown that weed control early in the growing season is necessary to reduce yield losses in corn. Weed species such as giant foxtail, barnyardgrass and pigweed can reduce corn yields by up to 13, 35 and 50% respectively (Bosnic and Swanton, 1997; Fausay et al., 1997; Knake and Slife, 1965). In a study of mixed weed populations competing with corn, corn yields were reduced by up to 20% when the weed plants reached a height of eight inches (Carey and Kells, 1995).

Corn is typically planted in wide rows (30 inches) and has an upright leaf orientation. As a result, corn is not successful in competing with weeds early in the growing season. Corn is also usually planted early when soil temperature and weather conditions favor weed over corn growth. A survey of Extension Service weed scientists solicited estimates of the percent of corn acreage infested with individual weed species by state or region, as well as the the potential impact on corn yields if the species were left uncontrolled. In this survey, 12 annual broadleaf, nine annual grass, and seven perennial

species were identified as troublesome weeds (**Table VIII-7**) (Gianessi et al., 2002). Estimates of yield loss ranged from a low of 15% due to wirestem muhly and sandburs to a high of 48% from burcucumber.

Pigweed is the most widespread weed species, infesting corn fields throughout the U.S. despite the fact that it is readily controlled by most corn herbicides. This is because it is a prolific seed producer and those seeds remain viable in the soil for years. Some weed species are problems at a regional level, e.g., hemp dogbane is a problem in Missouri and Illinois, wirestem muhly is a problem in Pennsylvania, and woolly cupgrass is a problem in Wisconsin and Iowa.

Until the early 1950s, tillage and cultivation practices were primarily used for weed control in corn, but since then they have been largely replaced by the use of herbicides. Herbicide use in corn became widespread by the end of the 1970s. In 2005, herbicides were applied to 97% of the corn planted acreage (USDA-NASS 2006b). Atrazine continues to be the most widely applied herbicide with 66% of the planted acreage being treated. It was applied at an average rate of 1.133 pounds per acre. Glyphosate isopropylamine salt was applied to 31% of planted acres, up from 19% in 2003, at an average rate of 0.963 pounds per acre. In terms of area applied, that was followed closely by S-metolachlor and acetochlor, at 23% of the planted corn acreage treated. Glyphosate (Roundup) is the most effective herbicide in corn. Roundup Ready corn developed by Monsanto makes the application of glyphosate easy for control of a broad spectrum of weeds.

Table VIII-5. Diseases of corn

Common Name	Causative Agent [transmittal agent]
Seed rots and seedling blights	*Fusarium moniliform, Pythium* spp.
Foliar Diseases	
Bacterial leaf blight and stalk rot	*Pseudomonas avenae*
Bacterial stripe	*Pseudomonas andropogonis*
Stewart's wilt	*Erwinia stewartii*
Chocolate spot	*Pseudomonas coronafaciens*
Goss's wilt	*Clavibacter michiganense*
Holcus spot	*Pseudomonas syringe*
Anthracnose	*Colletotrichum graminicola*
Eyespot	*Kabatiella zeae*
Gray leaf spot	*Cercospora zeae-maydis*
Northern leaf spot	*Bipolaris zeicola*
Northern corn leaf blight	*Exserohilum turcicum*
Physoderma brown spot	*Physoderma maydis*
Southern corn leaf blight	*Bipolaris maydis*
Yellow leaf blight	*Phyllosticta maydis*
Common rust	*Puccinia sorghi*
Southern corn rust	*Puccinia polysora*
Common corn smut	*Ustilago maydis*
Systemic Diseases	
Head smut	*Sphacelotheca reiliana*
Crazy top	*Sclerophthora macrospora*
Sorghum downy mildew	*Peronosclerospora sorghi*
Maize dwarf mosaic virus	[aphids]
Maize chlorotic dwarf virus	[leafhoppers]
Corn lethal necrosis	[chrysomelid beetles]
Maize white line mosaic virus	[not identified]
Corn stunt	[leafhoppers]
Maize bushy stunt	[leafhoppers]
Stalk and root rots	
Gibberella stalk rot	*Gibberella zeae*
Diplodia stalk rot	*Stenocarpella maydis*
Anthracnose stalk rot	*Colletotrichum graminicola*
Charcoal rot	*Macrophomina phaseolina*
Fusarium stalk rot	*Fusarium moniliforme*
Pythium stalk rot	*Pythium aphanidermatum*
Bacterial stalk rot diseases	*Erwinia chrysanthemi*
Root rots	*Pythium* spp.
Ear rots and storage molds	
Fusarium ear rot	*Fusarium moniliforme*
Gibberella ear rot	*Gibberella zeae*
Diplodia ear rot	*Diplodia maydis*
Aspergillus ear and kernel rot	*Aspergillus flavus*
Storage molds	*Penicillium* spp., *Aspergillus* spp.

Source: Smith and White, 1988

Table VIII-6. Insect pests of corn

Common Name	Latin name
Soil Insects	
Northern corn rootworm	*Diabrotica barberi*
Western corn rootworm	*Diabrotica virgifera virgifera*
Southern corn rootworm	*Diabrotica undecimpunctata*
Black cutworm	*Agrotis ipsilon*
Wireworms	*A. mancus, Horistonotus uhlerii, Melanotus cribulosus,* others
Billbugs	*Sphenophorus* spp.
White grubs	*Phyllophaga* spp.
Corn root aphid	*Anuraphis maidiradicis*
Seedcorn maggot	*Delia platura*
Grape colaspis	*Colaspis brunnea*
Seed corn beetle	*Stenolophus lecontei*
Insects attacking the leaf, stalk, and ear	
Corn earworm	*Helicoverpa zea*
European corn borer	*Ostrinia nubilalis*
Corn leaf aphid	*Rhopalosiphum maidis*
Fall armyworm	*Spodoptera frugiperda*
Stalk borers	*Diatraea spp.*
Armyworm	*Pseudaletia unipuncta*
Lesser stalk borer	*Elasmopalpus lignosellus*
Chinch bug	*Blissus leucopterus leucopterus*
Grasshoppers	*Melanoplus differentialis*
Corn flea beetle	*Chaetocnema pulicaria*
Japanese beetle	*Popillia japonica*
Other insects	
Thrips	*Anaphothrips* spp., *Frankliniella* spp.
Leafhoppers	*Trigonotylus brevipes*, others
Western bean cutworm	*Striacosta albicosta*
Corn blotch leaf miner	*Agromyza parvicornis*
Spider mites	*Oligonychus* spp., *Tetranychus* spp.
Pink scavenger caterpillar	*Pyroderces rileyi*
Garden symphlan	*Scuttigerella immaculata*
Hop-vine borer	*Hydraecia immanis*
Sod webworms	Subfamily Cramdinae
Leaf rollers	
Stink bugs	
Insect disease vectors	Several

Sources: Dicke and Guthrie, 1988; and University of Missouri, 1998.

Table VIII-7. Troublesome weeds in U.S. corn field

Weed Species	Area Infested [1]	Acreage Infested (%)	Potential Yield Loss (%)
Annuals			
Broadleaves			
Burcucumber	PA/OH/TN/SE	5-10	48
Cocklebur	MW/NP/SE	20-60	33
Jimsonweed	MW/CO	5-20	17
Kochia	NP/NW	10-70	33
Lambsquarters	MW/SE/NE/CA	15-80	33
Morningglory	MW/SE/SP	20-75	33
Nightshade	MW/NP/CA	25-50	26
Pigweeds/Waterhemp	US	30-90	36
Ragweed, Common	MW/SE/NE	20-70	30
Ragweed, Giant	MW/NP	10-45	28
Smartweeds	MW/SD/NE/SE	30-70	22
Velvetleaf	MW/NE/NP	25-70	28
Grasses			
Barnyardgrass	SP/NW/CA	80-90	23
Bermudagrass	MD/SE/UT/CA	10-20	47
Crabgrass spp.	MW/SE/NE	20-80	29
Cupgrass, Woolly	IA/WI	15-20	29
Foxtail spp.	MW/NE/NP	50-90	31
Millet, Wild-Proso	UT/WY/CO/ID	15-40	31
Panicum, Fall	MW/SE/NE/NP	15-80	30
Sandburs	NP/UT/WY	5-30	15
Shattercane	MW/SP	5-40	33
Perennials			
Bindweed, Field	ND/SW/CA	40-80	18
Dogbane, Hemp	IL/MO	2-20	21
Johnsongrass	MW/SE/SW/CA	20-60	45
Muhly, Wirestem	PA	2	15
Nutsedge, Yellow	MW/SE/NE/NP/CA	10-70	21
Quackgrass	MW/NE/UT	10-70	27
Thistle, Canada	NE/MW/NP/CO	5-25	26

Source: Gianessi et al., 2002.

[1] ***Regions***

MW: Midwest
NE: Northeast
NP: Northern Plains
NW: Northwest
SE: Southeast
SW: Southwest
SP: Southern Plains
US: United States

States

CA: California
CO: Colorado
ID: Idaho
IA: Iowa
MD: Maryland
MO: Missouri
ND: North Dakota
OH: Ohio
PA: Pennsylvania
SD: South Dakota
TN: Tennessee
UT: Utah
WI: Wisconsin
WY: Wyoming

VIII.D.2.f. Volunteer Management

Volunteer corn commonly occurs in rotational crops in the season following corn cultivation regardless of whether or not the corn was conventional or biotechnology-derived. Volunteers in rotational crops do not occur with corn grown for silage (approximately 9% of the US corn acres) since corn harvested for silage does not produce grain. In the warmer climates of the Southeast and Southwest, the occurrence of volunteer corn is rare because any corn grain remaining after harvest is likely to germinate in the fall and the resulting plants can usually be controlled by tillage or by freezing temperatures in the winter. In the Northern corn-growing regions, volunteer corn does not always occur in the rotational crop because of seed decomposition over the winter, efficient harvest procedures, and tillage prior to planting rotational crops.

The first step to manage volunteer corn in rotational crops is to minimize or reduce the potential for volunteers. The following practices should be implemented to reduce volunteer corn in rotational crops: 1) adjust harvest equipment to minimize the amount of corn grain lost in the field, 2) plant corn hybrids that reduce the extent of ear drop, 3) choose corn hybrids with superior stalk strength and reduced lodging, and 4) practice no-till production to significantly reduce the potential for volunteer growth in the rotational crop. If volunteer corn does occur in subsequent crops, preplant tillage or in-crop cultivation is very effective in managing it. Selective herbicides labeled for the control of volunteer corn in the particular rotational crop are available. Assure II® (quizalofop), Fusilade® DX (fluazifop), Fusion® (fluazifop + fenoxaprop), Poast® (sethoxydim), and Select® 2EC (clethodim) provide effective postemergence control of volunteer corn in labeled crops. These products are labeled for use in eight field crops, including soybeans, cotton, sugar beet and alfalfa, and eleven vegetable crops identified as rotational crops for corn.

VIII.E. Impact of the Introduction of MON 89034 on Agronomic Practices

No negative impact is expected from the introduction of MON 89034 on current cultivation and management practices for corn. MON 89034 has been shown to be no different from conventional corn in its phenotypic, ecological, and compositional characteristics (refer to **Section VII**). Thus, MON 89034 is expected to be similar in its agronomic characteristics, and have the same levels of resistance to insects and diseases as current commercial corn, except for the introduced trait of enhanced protection from feeding damage caused by lepidoperan pests. Biotechnology-derived Bt corn has been cultivated and consumed in the US since 1997 and has proven to have a positive impact on agricultural practices. These products have provided simple, inexpensive and highly effective means for controlling lepidopteran and coleopteran pests. They are

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environmentally benign so that they preserve beneficial insects, decrease cultivation input, and require less chemical pesticide applications. MON 89034, with two Cry proteins in a single plant, will provide an even more effective tool for corn farmers to control lepidopteran pests compared with Bt corn which only produces one Cry protein.

VIII.F. Insect Resistance Management

A critical component for the long-term use of biotechnology-derived Bt crops containing insecticidal proteins is to implement Insect Resistance Management (IRM) programs to prevent or delay the onset of resistance in the target insect species. Research by industry as well as academic scientists over the past decade has improved understanding and gained broad agreement for the major elements of IRM plans for Bt crops. The core element of an IRM plan is the use of a refuge to ensure an adequate population of susceptible insects of the target species is available to mate with any resistant insects that survive exposure to the Cry protein produced by the crop. This refuge may include wild host plants, other crops, or non-Bt plantings of the crop in question. Monsanto has developed an IRM plan for MON 89034, which has been submitted separately to the EPA. A summary of the plan is included as follows.

The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant MON 89034 provides both better insect control than current Bt corn products and a more effective approach for IRM. Mathematical modeling conducted by Monsanto indicates that biotechnology-derived plants expressing two Cry proteins will have significantly greater durability than plants producing either of the single proteins if: (1) cross-resistance between the Cry proteins is low; and (2) the mortality of susceptible insects caused by each of the individual proteins is at least 90%, and preferably >95%. Several lines of evidence indicate that the Cry1A.105 and Cry2Ab2 proteins have important differences in the way in which they bind to the lepidopteran midgut. Therefore, the probability of cross-resistance between these proteins is very low. Comparable biophysical studies also indicate that Cry1A.105 differs from Cry1Ab in the mode of action with regard to binding to the insect midgut. Therefore, Cry1A.105 and Cry2Ab2 have distinct modes of action regarding to receptor binding. Furthermore, *in vitro* and *in planta* studies with Cry1A.105 and Cry2Ab2 demonstrate that both proteins are highly active against the primary lepidopteran pests of corn (ECB, SWCB, CEW, and FAW), particularly ECB, achieving close to or greater than the critical 95% level of control in all cases. With these properties, MON 89034 should be durable with a significantly smaller structured refuge than is necessary for a Bt corn product containing only a single insecticidal protein. Based on these data and appropriate mathematical models, a 5% structured refuge in the U.S. corn belt (where current Bt corn products have a 20% refuge), and a 20% structured refuge in cotton growing regions (where current Bt corn products have a 50% refuge), is predicted to preserve the durability of MON 89034 for more than 20 years.

The specific requirements of the IRM plan for MON 89034 are described below.

1) Refuge Requirements - Planting of a 5% non-B.t. corn refuge in the U.S. corn belt and a 20% non-B.t. corn refuge in cotton growing regions.

2) Grower Agreements - Requirement for the growers to sign a contractual agreement, which imposes an obligation on each grower to comply with the refuge requirements.
3) Grower Education – Requirement to develop, implement, and report to EPA on a program to educate growers about IRM requirements for MON 89034.
4) Compliance Assurance Program - Requirement to develop, implement, and report to EPA on a program to evaluate and promote grower compliance with IRM requirements.
5) Monitoring - Requirement to develop, implement, and report to EPA on programs to evaluate whether there are statistically significant and biologically relevant changes in target insect susceptibility to the Cry1A.105 or Cry2Ab2 proteins.
6) Mitigation - Requirement to develop and, if triggered, to implement a remedial action plan in the event that any insect resistance is detected.
7) Reporting to EPA - Submit annual reports on sales of MON 89034 seed, IRM grower agreement results, compliance assurance, and educational program.

VIII.G. Conclusions of the Environmental and Agronomic Practice Assessment

An environmental assessment of MON 89034 was conducted to assess the potential impact of the introduced Cry1A.105 and Cry2Ab2 proteins on non-target organisms, endangered species and soil-dwelling organisms, the potential for gene flow, and the weediness potential of MON 89034. Based on the results of this assessment, it has been concluded that the potential risk of MON 89034 and the Cry1A.105 and Cry2Ab2 proteins to cause adverse effects on NTOs and endangered species is negligible. MON 89034 is no more likely to become a weed than conventional corn, and MON 89034 is also expected to be similar to conventional corn regarding to the potential for gene flow. Introduction of MON 89034 will not impact current corn cultivation practices and the management of weeds, diseases and insects except for the control of lepidopteran insect pests. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides not only a wider spectrum of pest control but also a more effective insect resistance management tool.

VIII.H. References

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IX. Adverse Consequences of Introduction

Monsanto knows of no study results or observations associated with MON 89034 that would be anticipated to result in adverse environmental consequences from its introduction. MON 89034 provides protection from feeding damage caused by lepidopteran insect pests. As demonstrated by field results and laboratory tests, the only phenotypic difference between MON 89034 and conventional corn is the presence of the Cry1A.105 and Cry2Ab2 proteins.

The data and information presented in this petition demonstrate that MON 89034 is unlikely to pose an increased plant pest potential or to have an adverse environmental consequence compared to conventional corn. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional corn, followed by risk assessment on detected differences. The characterization studies included molecular and protein analyses, which confirmed the insertion of a single functional copy of *cry1A.105* and *cry2Ab2* expression cassettes at a single locus within the corn genome and that the two proteins were expressed in tissues at levels that are efficacious for the control of target insect pests. Extensive characterization of the plant phenotype including compositional analysis of key nutrient and antinutrients also indicated that MON 89034, with the exception of intended modification, was unchanged compared to conventional corn. Allergenicity assessment concluded that Cry1A.105 and Cry2Ab2 proteins are unlikely to be allergens for humans. Toxicity tests including an acute mouse oral gavage and other selected non-target organisms with either equivalent proteins produced by recombinant strains of *E. coli* or MON 89034 tissues showed no signs of adverse effects at high doses. An endangered species risk assessment also concluded that MON 89034 is unlikely to have adverse effects on these organisms, including endangered lepidopteran insects. Therefore, the risks for humans, animals, and other non-target organisms from MON 89034 are negligible under the conditions of use.

The introduction of MON 89034 is expected to increase economic, environmental and health benefits due to the protection of corn yields, decrease of chemical insecticide usage, reduction of mycotoxin levels in corn grain, and increase of Bt corn product durability. The introduction of MON 89034 will not impact cultivation practices and the management of weeds, diseases and insects except for the control of lepidopteran insect pests. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides not only a wider spectrum of pest control but also a more effective insect resistance management tool.

Appendix A. Materials and Methods for Molecular Characterization and Results of PCR Analysis on MON 89034 Insert

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.

A.1. Test Material

The test material was grain, leaf or seed from corn MON 89034.

A.2. Control Material

The control material was conventional corn with a genetic background similar to MON 89034. Where applicable, a second conventional corn with a different genetic background to MON 89034 was also included to provide an additional negative control.

A.3. Reference Materials

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

A.4. Characterization of Test and Control Materials

Event specific PCR assays were used to confirm the identity of test and control materials. The test and control materials were considered stable during storage if they did not appear visibly degraded on ethidium bromide-stained gels and/or yielded interpretable signals on the Southern blots.

A.5. Genomic DNA Isolation

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. For leaf tissue, genomic DNA was extracted using a Sarkosyl extraction method

(Fulton et al, 1995). 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.

A.6. Quantitation 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.

A.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 using 100 units of the restriction enzyme. For positive hybridization controls, approximately one or half genomic equivalent of PV-ZMIR245 was spiked into conventional corn DNA prior to digestion.

A.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 a '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 gel and approximately 10 µg on the short run gel. The long run electrophoresis 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.

A.9. 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).

A.10. Southern Blot Analyses

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

A.11. 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. The products A-G are illustrated in **Figure A-1**.

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.

A.12. PCR Analysis Result across the Insert in MON 89034

The organization of the elements within the insert in MON 89034 was confirmed using PCR analysis by amplifying seven overlapping regions of DNA that span the entire length of the insert. The location of the PCR products generated in relation to the insert, as well as the results of the PCR analyses, are shown in **Figure A-1**. The control reactions containing no template DNA (lanes 2, 5, 8, 12, 16, 20, and 23) and the conventional corn control reactions (lanes 3, 6, 9, 13, 17 and 21) did not generate PCR products with any of the primer sets, as expected. The conventional corn control reaction in lane 24 produced a product of equal size to that of MON 89034 (lane 25) because both primer sequences are located in the corn genomic flanking sequence adjacent to the 3' end of the insert in MON 89034. Additionally, the products generated using plasmid PV-ZMIR245 DNA as a template (lanes 11 and 15) appear overloaded in comparison to the MON 89034 genomic DNA samples which likely contributes to the intensity of the products observed in these lanes.

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 V-4** (see **Section V**). The results of DNA sequencing on the amplified DNA fragments confirmed that the sequence of the DNA insert in MON 89034 matched the designed, corresponding sequences in PV-ZMIR245 with one exception. This exception is 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 was replaced by a Left Border sequence in MON 890343 (see **Section V** for more discussion).

A.13. References

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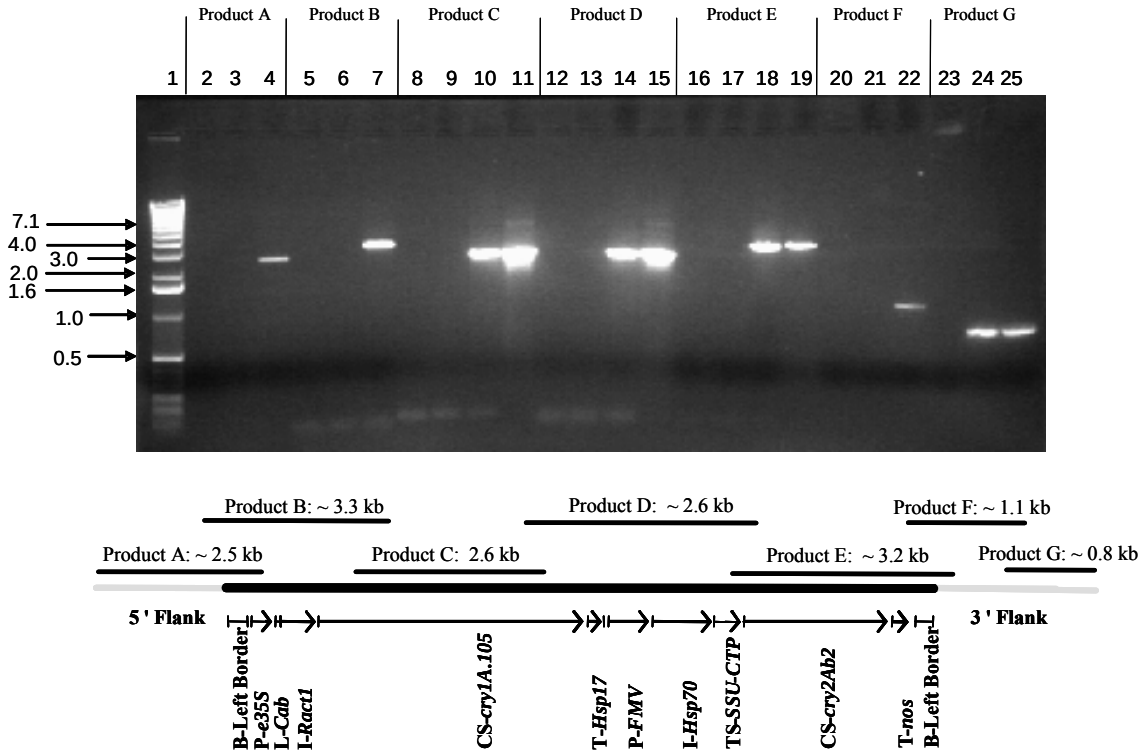

Figure A-1. Overlapping PCR analysis across the insert in MON 89034

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.

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, Methods, and Results for Characterization of Cry1A.105 Protein

The Cry1A.105 protein was purified from the grain of MON 89034 as well as from a recombinant *E. coli* culture. A panel of analytical tests was used to identify, characterize and compare the MON 89034-produced and *E. coli*-produced Cry1A.105 protein including: (1) Western blot analysis; (2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry; (3) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); (4) glycosylation analysis; and (5) insect activity bioassay. The materials, methods and the results are described below.

B.1. Materials and Methods

B.1.1. Purification of MON 89034-Produced Cry1A.105 Protein

The MON 89034 produced Cry1A.105 protein was purified from grain of MON 89034. The identity of the MON 89034 grain was confirmed by event-specific PCR. The purified MON 89034-produced Cry1A.105 protein was stored in a 4ºC 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.

The purification procedure was conducted 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 (phosphate buffered saline) extraction buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) for 2.5 h at approximately a 1:10 ratio of sample weight (g) to buffer volume (ml). The slurry was filtered using an Ertel Alsop filter press (Kingston, NY) and the PBS-washed filter cakes were retained. The cakes were resuspended in approximately 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 h. 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 min. The extract was clarified by filtration using the Ertel Alsop filter press and concentrated using a hollow fiber cartridge (Amersham Biosciences, Piscataway, NJ). The plant genomic 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 DNA. An ammonium sulfate pellet (35% saturation) was prepared by the addition of ammonium sulfate salt to the clarified extract and was allowed to settle 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 then 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 column, which was equilibrated with a loading buffer (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 proteins were eluted with a linear salt gradient that increased from 0 M to 0.20 M NaCl over 12 L and then maintained a 0.20 M NaCl for 4 L. Next, the salt gradient increased to 0.65 M NaCl over 21 L, and finally elevated to 1.0 M NaCl 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 a 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 column, which was equilibrated with another loading buffer (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 proteins were eluted with a linear salt gradient that increased from 0 M to 0.25 M NaCl over 4 L and then held at 0.25 M NaCl for 10 L. Next, the salt gradient increased to 0.65 M NaCl over 21 L and finally increased to 1.0 M NaCl over 4 L and held constantly for 8 L. The fractions containing Cry1A.105 protein were pooled to a final volume of 10 L. These fractions were diafiltrated into a 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 then concentrated to 2 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 to 1 L. 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 proteins were 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. After analysis of fractions by lateral flow test strips, SDS-PAGE gel and Western blot, fraction #25 was determined to contain the majority of the full-length Cry1A.105. This fraction was used for further characterization tests.

B.1.2. *Escherichia coli*-Produced Cry1A.105 Protein

E. coli-produced Cry1A.105 protein which was produced and purified previously was used as a reference standard for determination of protein concentration of the MON 89034-produced Cry1A.105 protein. This protein was also used as a reference standard to evaluate equivalence between MON 89034- and *E. coli*-produced Cry1A.105 protein for immunoreactivty (Western blot), molecular weight (SDS-PAGE) and functional activity assay. The *E. coli*-produced 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.

B.1.3. Determination of MON 89034-Produced Cry1A.105 Protein Concentration

The concentration of the full-length Cry1A.105 protein in the purified sample from MON 89034 grain was estimated using quantitative immunoblot analysis. The *E. coli*-produced Cry1A.105 protein was used to create a standard curve. Aliquots of the MON 89034-produced Cry1A.105 protein and reference standard were diluted in deionized water and 5x Laemmli buffer (5x LB), heated at approximately 95.5°C for 5 min, and applied to a pre-cast Tris-glycine 4→20% polyacrylamide gradient gel. Three different amounts of the MON 89034-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 the completeness of electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands observed. The 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 h with 5% (w/v) NFDM (non-fat dry milk) in 1×PBST (phosphate buffered saline with Tween 20). 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 washes (5 min each) with PBST. Finally, the membrane was probed with HRP (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 min. Excess HRP-conjugate was removed using three washes (5 min each) 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 MON 89034-produced Cry1A.105 protein in each lane migrating at the same distance as the full-length reference standard Cry1A.105 protein of *E. coli* 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 with a Bio-Rad GS-800 densitometer. The concentration of the MON 89034-produced Cry1A.105 was determined by dividing the amount of protein in each lane by the volume of protein loaded in the respective lanes.

B.1.4. Western Blot Analysis

Western blot or immunoblot analysis was performed to confirm the identity of the Cry1A.105 protein, the intactness of its N-terminus, and compare immunoreactivity of the MON 89034- and *E. coli*-produced Cry1A.105 protein.

Western blot analysis using anti-Cry1A.105 antibody

The Western blot (see above **Section B.1.3**) was used to establish the identity of the MON 89034-produced protein and to compare the immunoreactivity of the MON 89034-produced and *E. coli*-produced Cry1A.105 protein. Equivalence was demonstrated for bands representing full-length Cry1A.105 protein that was identified by the anti-Cry1A.105 antibody and showed similar mobility with the *E. coli*-produced reference standard on the gel/blot.

Western blot analysis using the anti-N-terminal peptide antibody

Western blot analysis using the anti-N-terminal peptide antibody was performed to confirm the intactness of the N-terminus of the MON 89034-produced Cry1A.105 protein. The anti-N-terminal peptide antibody was produced with a synthetic peptide consisting of the first 14 amino acids of the Cry1A.105 protein N-terminus as the antigen. The trypsin resistant core of Cry1A.105 was used as a negative control because its N-terminus was removed by trypsin. The MON 89034-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 reference standard, and the Cry1A.105 trypsin-resistant core were each loaded on gels at 20 ng and 40 ng per lane. Each protein was mixed with 5× LB, heated at 96°C for 5 min, and applied to a pre-cast Tis-glycine 4→20% polyacrylamide gradient gel. The eletrophoresis 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 rabbit anti-N-terminal peptide antibody in 1% (w/v) NFDM in PBST for 60 min. Excess antibody was removed using three washes (5 min each) 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 min. Excess HRP-conjugate was removed using three washes (5 min each) with PBST. Immunoreactive bands were visualized using the ECL detection system and exposed for 1 min, 2 min, 5 min, and 10 min to ECL high performance chemiluminescence film. Films were developed using a Konica SRX-101A automated film processor.

B.1.5. Molecular Weight and Purity Estimation by SDS-PAGE

Aliquots of the *E. coli*-produced reference standard and MON 89034-produced Cry1A.105 protein were mixed with 5× LB to a final protein concentration of 10 ng/µl and 2.4 ng/µl, respectively. The MON 89034-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 for the 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 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 (Invitrogen, Carlsbad, CA). The gel was fixed for 10 min in 200 ml of fixing solution (90 ml ultra pure water, 100 ml methanol, and 20 ml acetic acid). This was followed by 10 min 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 washes (5 min each) in 200 ml ultra pure water. The gel was stained for 15 min 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 washes (5 min each) of 200 ml ultra pure water. Development occurred in 100 ml of solution (5 ml developer and 95 ml ultra pure water) for 3-15 min, and was stopped by addition of 5 ml stopping solution for 10 min. The gel was washed three times for 10 min 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 MON 89034-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 approximately 56 kDa to 130 kDa were included in the purity calculation for the protein of interest as they represent various lengths (proteolytic fragments to full-length) of the Cry1A.105 protein.

B.1.6 Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 89034-produced Cry1A.105 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 89034-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 reference standard, and the positive control glycoproteinss, 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 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 h, and then the solution was changed once and the membrane was incubated overnight. Two 15 min washes (50 ml each) of 3% (v/v) glacial acetic acid (wash solution), were followed by a 20 min oxidation in 25 ml of the kit supplied oxidizing solution. After oxidation, three 15 min 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 h of staining in the dark, two 30 min, 50 ml wash cycles were followed by two 45 min, 50 ml wash cycles. The final wash cycles included two 25 ml, 1 min deionized water washes followed by three 25 ml, 5 min methanol washes. The blot was then scanned using the Bio-Rad Molecular Imager FX with the Alexa 488 illumination setting (Qunatity One software; version 4.6, build 036) in order to visualize the fluorescing glycosylated proteins.

B.1.7. Tryptic Peptide Mapping Analysis by MALDI-TOF MS

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to further confirm the identity of the MON 89034-produced Cry1A.105 protein.

The purified MON 89034-produced protein (4.5 ml) was diluted to 9.0 ml with a concentration buffer [25 mM CAPS, 0.02% (w/v) SDS] followed by concentration 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 min at 95°C. Sixteen microliters of this 5× LB was then used as the loading buffer for the Cry1A.105 sample for SDS-PAGE. The broad- range molecular weight markers (Bio-Rad) were used to estimate molecular weights. MON 89034-produced Cry1A.105 protein and molecular weight markers were heated at 95°C for 5 min and then applied to a pre-cast Tris-glycine 4→20% polyacrylamide gradient 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 h, and destained by washing with 1× destain solution (Bio-Rad) for 2 h with one change of the destain solution.

The bands representing the full-length Cry1A.105 protein 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 h 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 then 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 min. 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 slices 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 h at 37°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.

An aliquot (4 µl) of the digested sample was desalted (Bagshaw *et al*., 2000) using Millipore (Bedford, MA) ZipTip C18 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].

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, Foster City, CA). 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 Da range using 100 shots at a laser intensity setting of 2781 (a unit-less MALDI-TOF instrument specific value). Protonated (MH^+) peptide masses were measured monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate expected tryptic peptide masses based on the expected cleavage sites in Cry1A.105 protein sequence. The *in silico* generated masses were compared to the raw experimental masses obtained. 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 Da from the mass analyzed.

B.1.8. Functional Activity Biossay

Aliquots of the MON 89034-produced and *E. coli*-produced Cry1A.105 protein were tested for biological activity in a diet-incorporation insect bioassay to compare the equivalence of the biological activity of the Cry1A.105 protein from the two sources.

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 mg/ml. The Cry1A.105 protein concentration of the purified MON 89034-produced Cry1A.105 protein aliquots was 3 μg/ml. The control substances used in the bioassays were buffers of the same composition used for formulating the *E. coli*-produced and MON 89034-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 buffer composition for the MON 89034-produced protein 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. The MON 89034-produced and *E. coli*-produced Cry1A.105 proteins were stored at 4°C and -80°C, respectively. The control buffers for both proteins were stored at 4°C.

The test system was corn earworm (CEW, *Helicoverpa zea*) which was obtained from Benzon Research Inc (Carlisle, PA). Insect eggs were incubated at a temperatures ranging from 10°C to 27°C, to achieve the desired hatch time. The bioassay was replicated three times on separate days with separate batches of insects. The MON 89034-produced and *E. coli*-produced protein treatments were run in parallel during each bioassay. 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. 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 diluted solution into an agar-based insect diet (Southland Corp., Lake Village, AR). Diet mixture was then dispensed in 1 ml aliquots into a 128 well tray (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 (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 14 h:10 h (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.

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 (Statistical Analysis System):

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).

B.2. Results

B.2.1 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 B-1**). As expected, the immunoreactive signal increased with increasing levels of loading for both MON 89034- and *E. coli*-produced proteins. The immunoreactive band with lower molecular weight most likely represents a a proteolytic fragment 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 had equivalent immunoreactive properties for the antibody used for the antibody used.

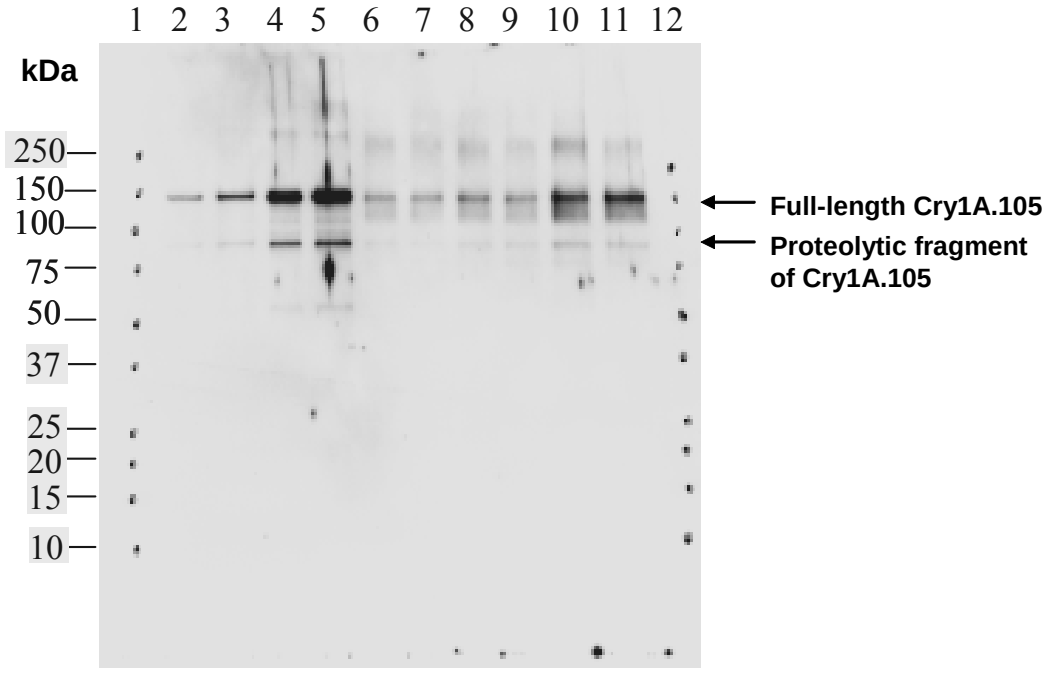

Figure B-1. Western blot analysis of MON 89034-produced and *E. coli*-produced Cry1A.105 protein

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 incubated with rabbit polyclonal anti-Cry1A.105 antibody and developed using an ECL system. 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	—	—

B.2.2. Cry1A.105 Protein Molecular Weight

The equivalence in apparent molecular weight of the purified MON 89034- and the *E. coli*-produced Cry1A.105 protein was demonstrated using SDS-PAGE stained using the SilverXpress® silver staining kit (Invitrogen). The full-length MON 89034-produced Cry1A.105 protein migrated to a position similar to that of the *E. coli*-produced protein standard which was analyzed concurrently (**Figure B-2**). Based on the comparable electrophoretic mobility, the MON 89034- and *E. coli*-produced Cry1A.105 protein was determined to have equivalent molecular weight.

B.2.3. Confirmation of the N-terminus Intactness Using Immunoblot Analysis

The N-terminus of the plant-produced proteins could be blocked by chemical modifications. 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.

The *E. coli*-produced Cry1A.105 containing the intact N-terminal sequence, 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 B-3**, 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 protein. Additionally, a fragment of lower molecular weight (approximately 85 kDa) was observed in both protein preparations (**Figure B-3**, lanes 2-5). This fragment represents a proteolytic product of C-terminal degradation of Cry1A.105 protein. As expected, the intensity of the bands increased in a manner dependent of the loading quantities. These results are consistent with published literature that the intermediate proteolytic fragments of Cry1 proteins have intact N-terminus, while the N-terminal peptide (approximately 25-30 amino acids) was cleaved for the trypsin-resistant core (Gao et al., 2006).

In conclusion, the intactness of the N-terminus of the MON 89034-produced Cry1A.105 protein was confirmed.

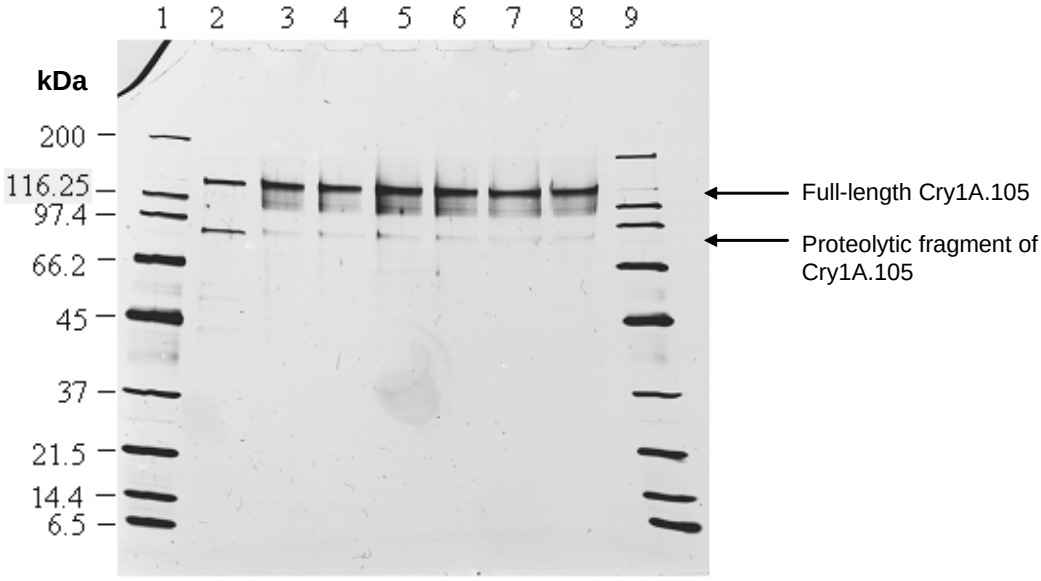

Figure B-2. SDS-PAGE of MON 89034-produced and *E. coli*-produced Cry1A.105 protein

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)	—

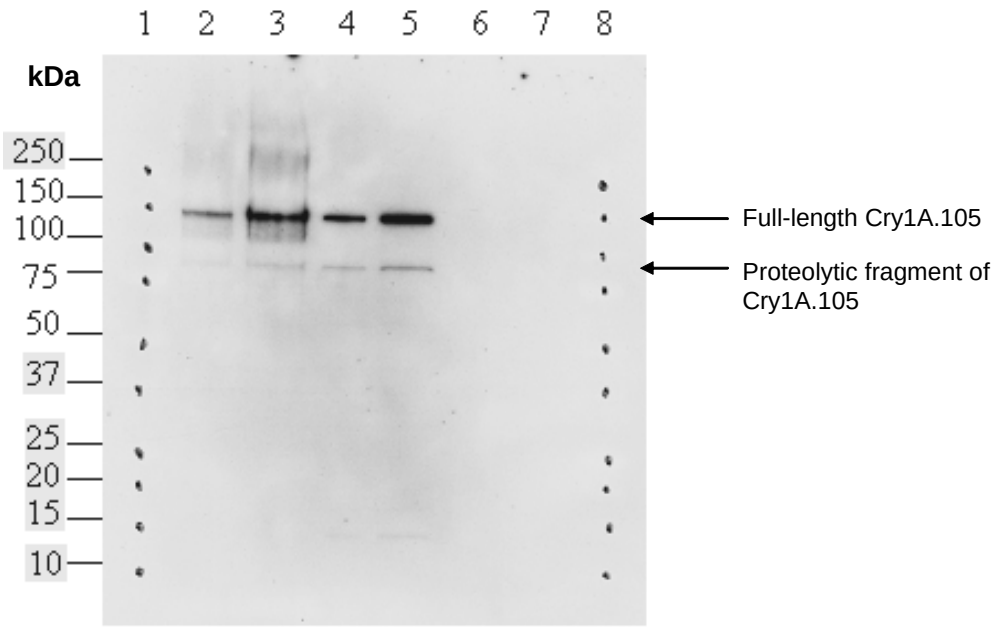

Figure B-3. Examination of the intactness of N-terminus by immunoblot analysis using anti-N-terminal peptide antibody

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	—

B.2.4. 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 B-4**). Overall, the confirmed sequence accounts for 43.8% (516 out of 1177 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 MON 89034-produced Cry1A.105 protein was confirmed.

B.2.5. Lack of Glycosylation for Cry1A.105 Protein

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 synthetic capacity required for protein glycosylation.

To examine 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 control was represented by transferrin and horseradish peroxidase (HRP) proteins that are known to have multiple covalently-linked carbohydrate modifications. The transferrin, 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 this analysis are shown in **Figure B-5**. The positive controls were detected at the expected molecular weights in a concentration-dependent manner (**Figure B-5**, lanes 2-5). No detectable signal was observed for the MON 89034-produced and *E. coli*-produced Cry1A.105 protein (**Figure B-5**, lanes 6-9).

Therefore, 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.

```
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
1151TDKVWIEIGE TEGTFIVDSV ELLLMEE
```

Figure B-4. Sequence coverage in the tryptic peptide mapping analysis of MON 89034-produced full-length Cry1A.105 with MALDI-TOF MS

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

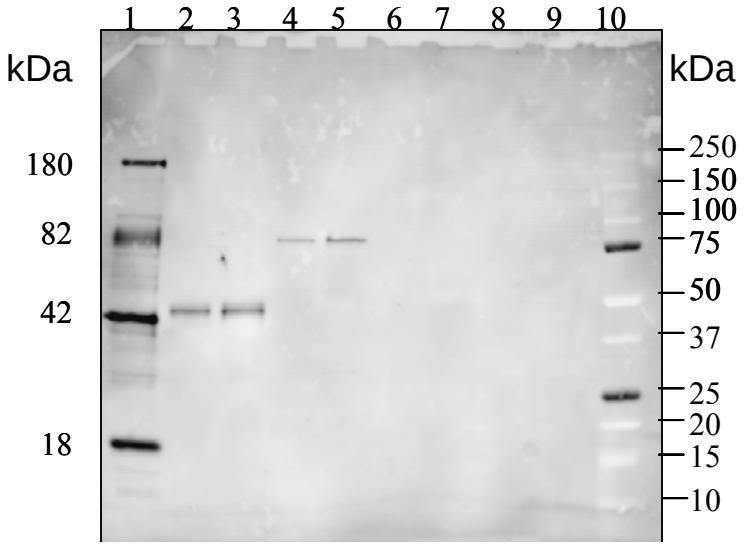

Figure B-5. Glycosylation analysis of the MON 89034-produced Cry1A.105 protein

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	—

B.2.6. Cry1A.105 functional activity

The biological activities of *E. coli*-produced and MON 89034-produced Cry1A.105 protein 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 to larval growth.

The EC_{50} values for each replicate bioassay are summarized in **Table B-1**, and the dose response relationships for MON 89034- and *E. coli*-produced Cry1A.105 are illustrated in **Figure B-6**. 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 B-6** shows an equivalent slope for the dose-response relationship for the *E. coli*- and MON 89034-produced Cry1A.105 protein in the CEW bioassay. These results clearly demonstrate that the Cry1A.105 protein derived from MON 89034 and *E. coli* has equivalent functional activity.

Table B-1. EC_{50} values of *E. coli*-produced and MON 89034-produced Cry1A.105 protein in a corn earworm diet-incorporation bioassay

		EC_{50} (µg Cry1A.105/ml diet)[1]	
		E. coli-produced	MON 89034-produced
Replicate[2]	1	0.0150 ± 0.0025	0.0055 ± 0.0014
	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.

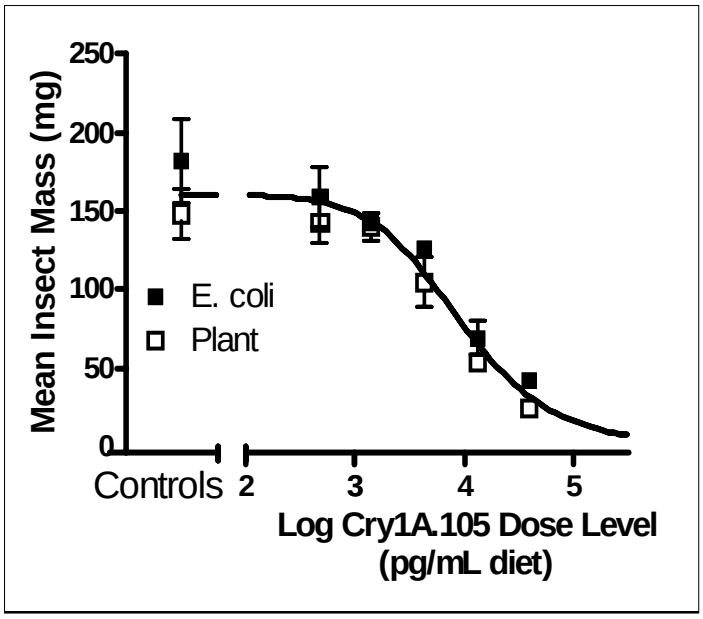

Figure B-6. Functional equivalence of the *E. coli*-produced and the MON 89034-produced Cry1A.105 protein against corn earworm (CEW)

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 Cry1A.105 protein in the CEW bioassay. 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.

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Appendix C. Materials, Methods, and Results for Characterization of Cry2Ab2 Protein

The Cry2Ab2 protein was purified from the grain of MON 89034 as well as from a recombinant *E. coli* culture. A panel of analytical tests was used to identify, characterize and compare the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein including: (1) Western blot analysis; (2) SDS-PAGE and densitometry; (3) MALDI-TOF MS; (4) N-terminal sequence analysis; (5) glycosylation analysis; and (6) insect activity bioassay. The materials, methods and the results are described below.

C.1. Materials and Methods

C.1.1. Purification of MON 89034-Produced Cry2Ab2 Protein

The MON 89034 produced Cry2Ab2 protein was purified from grain of MON 89034. The identity of the grain containing MON 89034 was confirmed by event-specific PCR. The isolated MON 89034-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.

The purification was performed using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

The isolation of Cry2Ab2 protein from ground corn grain was started from two 10 kg batches. Each batch was extracted with 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 h in a 4°C cold room at approximately 1:10 ratio of sample weight (g) to buffer volume (ml). 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 ratio of sample weight (g) to buffer volume (ml) for 2-3 h. During extraction, lipids were removed from the extract by adding CelPure P65 diatomaceous earth (Advanced Minerals Corp, Goleta, CA) to the homogenate at approximately 7.5% (w/v) and allowed to mix for 10 min. 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 (nominal molecular weight cutoff) hollow fiber cartridge (Amersham Biosciences, Piscataway, NJ) to a final volume of 35 L. To remove plant genomic DNA, 10% (w/v) polyethyleneimine, was added 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 with 35% ammonium sulfate saturation. The precipitate was pelleted 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 a buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 (loading buffer) 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 (6 L) and Run 2 (7 L). The elution parameters were identical for both runs and thus only the Run 1 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 column, which was equilibrated with the loading buffer. The bound proteins were eluted with step gradients as follows: 0-20% buffer B (loading buffer plus 1M NaCl) in 10 column volumes (CV), 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 for 2 CV. Fractions, each of 4 L, containing Cry2Ab2 protein were identified by Cry2A QuickStix™ lateral flow test kit (EnviroLogix, Portland, ME) for further analysis. Based on SDS-PAGE and Western blot analysis results, fraction 7 from Run 2 was selected for affinity purification.

The 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 h at 100 ml/h. 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 an EPPS buffer with a different pH (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was then eluted with 100 mM triethylamine, pH 11.4, in five fractions (1.5 ml each). Based on SDS-PAGE analysis, fractions containing Cry2Ab2 protein from batches 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 the fraction 7 from Run 2. As previously described, the sample was concentrated and buffer-exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.1) to a final volume of 50 ml using centrifuge concentrators (30 kDa MWCO). This sample was re-circulated for 2 h (60 ml/h) through the affinity column equilibrated with an EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.1). The column was then washed with 15 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.1) followed by 5 CV of another EPPS buffer with a different pH (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM triethylamine, pH 11.4, in five fractions (1.5 ml each).

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 sample and the sample from the first runs (2 ml) were pooled, resulting in a final volume of 4.4 ml. This sample was a clear, colorless solution and was used for further characterization tests.

C.1.2. *Escherichia coli*-Produced Cry2Ab2 Protein

E. coli-produced Cry2Ab2 protein produced and purified previously was used as a reference standard for determination of protein concentration of the MON 89034-produced Cry2Ab2 protein. This protein was also used as a reference standard to evaluate equivalence between MON 89034-produced and *E. coli*-produced Cry2Ab2 proteins for immunoreactivity (Western blot), molecular weight (SDS-PAGE) and functional activity assay.

C.1.3. Determination of MON 89034-Produced Cry2Ab2 Protein Concentration

The total protein concentration of the purified MON 89034-produced Cry2Ab2 protein was estimated using a Bio-Rad protein assay. The *E. coli*-produced Cry2Ab2 reference standard protein (concentrations range from 0.05 to 0.5 mg/ml) was used to prepare a standard curve. The MON 89034-produced Cry2Ab2 total protein concentration was estimated by comparison of absorbance values obtained for the sample to the values of the standard curve.

C.1.4. Western Blot Analysis

Aliquots of the stock solutions of the MON 89034-produced Cry2Ab2 and *E. coli*-produced reference standard were diluted to a final 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) SDS, 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 gel. The MON 89034-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. The pre-stained MW markers (Bio-Rad 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 µm 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 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 HRP-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 HRP-conjugated antibody was removed using three washes (10 min each) 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.

C.1.5. Molecular Weight and Purity 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. MW markers (Bio-Rad broad-range, Hercules, CA) that were used to estimate the apparent MW of the test protein were diluted to a final concentration of 0.9 µg/µl. The MON 89034-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 per lane. All samples were heated at approximately 102°C for 5 min and applied to a pre-cast Tris-glycine 4→20% polyacrylamide gradient 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 for 16 h 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 h.

Analysis of the proteins in the stained gel was performed using a Bio-Rad 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 MW of each observed band. All visible bands within each lane were quantified using Quantity One software. For the MON 89034-produced Cry2Ab2 protein, purity was calculated as the percent optical density of the subject protein band relative to all bands combined a given lane.

C.1.6 Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 89034-produced Cry2Ab2 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 89034-produced Cry2Ab2 protein, the *E. coli*-produced Cry2Ab2 reference standard (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 concentration of 50 ng/µl. These samples were heated at 100°C for 5 min, and loaded along with Precision Plus Dual Color pre-stained protein MW markers (Bio-Rad, Hercules, CA) on a pre-cast Tris-glycine 4→20% polyacrylamide gradient gel. All three samples were loaded at 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 µm PVDF membrane for one hour at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane 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 min. The oxidation solution was removed from the membrane by two brief rinses followed by three sequential washes (10 min each) 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 min. Biotin hydrazide solution was removed by washing in PBS as described above. The membrane was blocked with 5% blocking agent in PBS for 60 min. 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 described above. 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. Films were developed using a Konica SRX-101A automated film processor.

C.1.7. N-terminal Sequencing

An aliquot of the MON 89034-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final concentration of 80 ng/µl. Molecular weight markers (Bio-Rad Precision Plus Dual Color, Hercules, CA) were used to confirm the transfer of protein to the PVDF membrane. The MON 89034-produced Cry2Ab2 protein was electrophoresed in eight lanes at 2 µg per lane. The Cry2Ab2 containing samples were heated to approximately 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 200 V for 43 min. The gel was then electroblotted to a 0.2 µm 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 (Bio-Rad) and visualized by destaining with a Coomassie R-250 destain solution (Bio-Rad).

The protein bands of interest 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 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 from Applied Biosystems) was analyzed before and after the analysis of each different protein band to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

C.1.8. Tryptic Peptide Mapping Analysis by MALDI-TOF MS

MALDI-TOF mass spectrometry was used to confirm the identity of the MON 89034-produced Cry2Ab2 protein. An aliquot of the MON 89034-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final concentration of 80 ng/µl, and 2 µg was electrophoresed in each of five lanes. The broad-range MW markers (Bio-Rad, Hercules, CA) were used to estimate molecular weights. Samples were heated to approximately 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 h 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 h.

Two protein bands, identified as band-1 and band-2, were subjected to the tryptic peptide mapping analysis. The protein bands of interest were excised, destained, reduced, alkylated, and subjected to an in-gel trypsin 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 h 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 then 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 min. 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 h 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.

A portion (5 µl) of the digested samples was desalted (Bagshaw *et al*., 2000) using Millipore (Bedford, MA) ZipTip C18 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].

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 the peptide masses, *in silico*, based on the trypsin cleavage sites in the Cry2Ab2 protein sequence. Experimental peptide 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 Da from the mass analyzed.

C.1.9. Functional Activity Biossay

The purpose of this assay was to compare the biological activity of the MON 89034-produced and the *E. coli*-produced Cry2Ab2 proteins by determining EC_{50} values in a corn earworm (CEW, *Helicoverpa zea)* diet-incorporation bioassay.

The test system was corn earworm (CEW) which was obtained from Benzon Research Inc (Carlisle, PA). Insect eggs were incubated at temperatures ranging from 10°C to 27°C, to achieve the desired hatch time. The bioassay was replicated three times on separate days with separate batches of insects. The MON 89034-produced and *E. coli*-produced proteins were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and MON 89034-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. The Cry2Ab2 protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into an agar-based insect diet (Southland Corp. Lake Village, AR). 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 (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 (CD International, Pitman, NJ) and the insects were allowed to feed for a period of 7 days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 h:10 h (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 7-day incubation period. Dose response modeling was the same as described in **Section B.1.8** of **Appendix B**.

C.2. Results

C.2.1. Cry2Ab2 Protein Immunoreactivity

Western blot analysis using a polyclonal, goat 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 C-1**, 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 C-1**, band-2) was observed in the partially purified MON 89034-produced Cry2Ab2 sample, which represents a proteolytic fragment of the Cry2Ab2 protein.

The above Western blot result confirmed the identity of Cry2Ab2 protein produced by MON 89034, and demonstrated that MON 89034- and *E. coli*-produced Cry2Ab2 had equivalent immunoreactivity for the antibody used for the antibody used.

C.2.2. Cry2Ab2 Protein Molecular Weight Equivalence

The equivalence in apparent molecular weight of the purified MON 89034- and the *E. coli*-produced Cry2Ab2 protein was further demonstrated using SDS-PAGE stained with Brilliant Blue G-Colloidal stain. The MON 89034-produced, full-length Cry2Ab2 protein (band-1) migrated to a position comparable to that of the *E. coli*-produced protein standard which was analyzed concurrently (**Figure C-2**). Based on the comparable electrophoretic mobility, the MON 89034- and *E. coli*-produced, full-length Cry2Ab2 protein (band-1) was determined to have equivalent molecular weight.

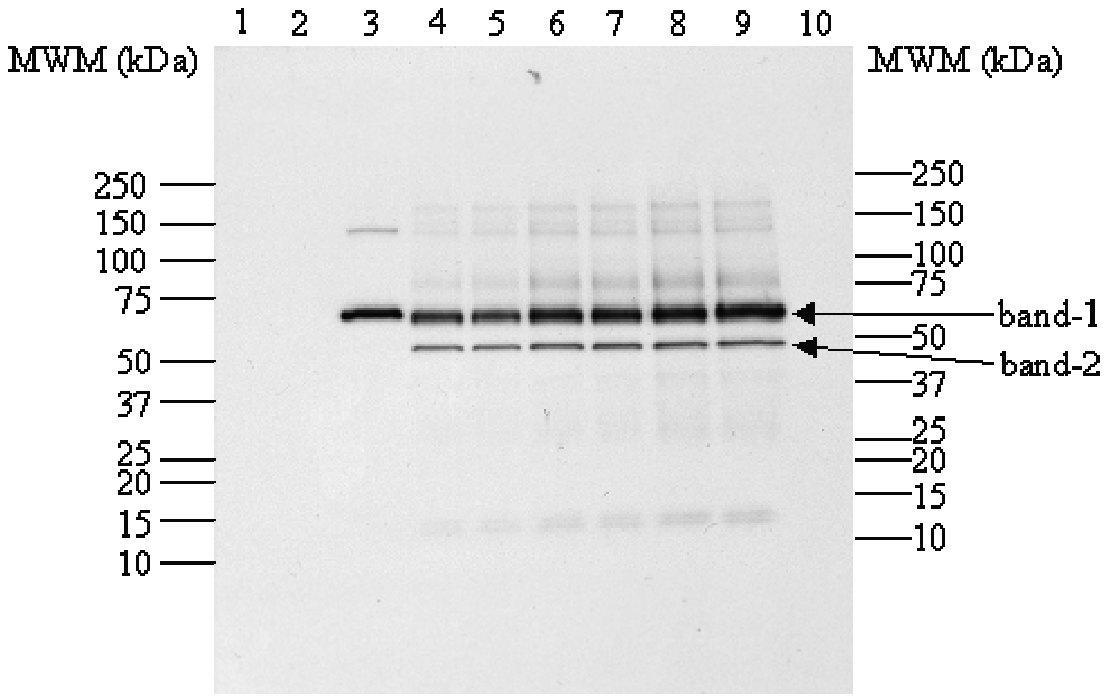

Figure C-1. Western blot analysis of MON 89034-produced and *E. coli*-produced Cry2Ab2 protein

Samples of the partially purified MON 89034-produced and *E. coli*-produced Cry2Ab2 protein 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

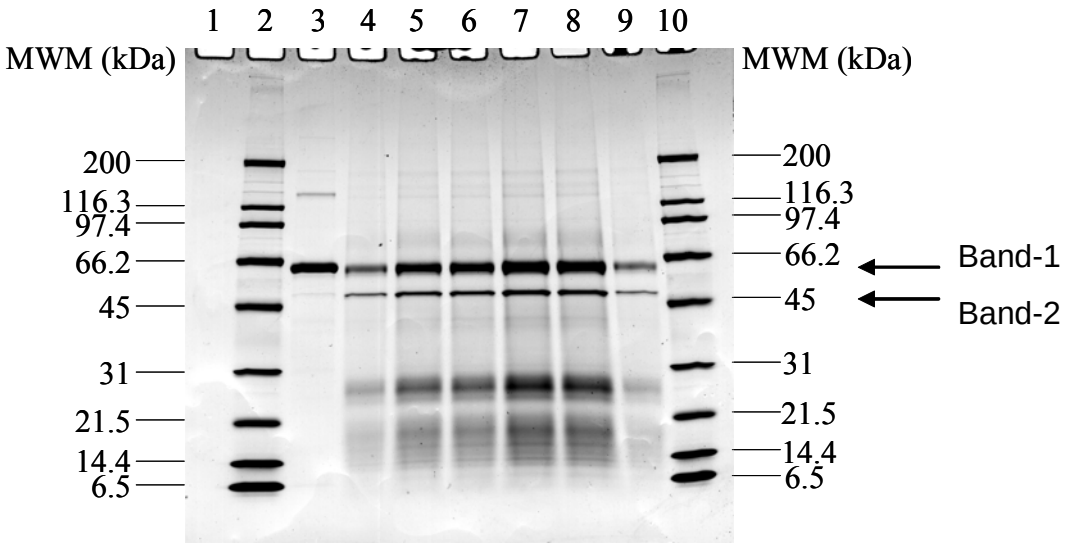

Figure C-2. SDS-PAGE of the MON 89034- and *E. coli*-produced Cry2Ab2 protein

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

C.2.3. Analysis of the N-terminal Sequence

N-terminal sequencing analysis demonstrated that the MON 89034-produced, full-length Cry2Ab2 (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 No. 24. N-terminal sequence analysis of the lower molecular weight proteolytic fragment (band-2) revealed that this fragment starts from amino acid residue No. 145. With *E. coli*-produced Cry2Ab2 (band-1) the N-terminus sequence was determined as MQAMDN, as expected. 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 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.

C.2.4. Tryptic peptide mapping by MALDI-TOF MS

The MON 89034-produced, full-length Cry2Ab2 (band-1) and the proteolytic fragment (band-2) 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. These identified peptides were used to assemble a coverage map in the Cry2Ab2 protein sequence (**Figure C-3**). 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 C-4**).

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 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 QNNTTARYTL
541 RGNGNSYNLY LRVSSIGNST IRVTINGRVY TATNVNTTTN NDGVNDNGAR FSDINIGNVV
601 ASSNSDVPLD INVTLNSGTQ FDLMNIMLVP TNISPLY
```

Figure C-3. Peptide mass coverage map of the MON 89034-produced full-length Cry2Ab2 protein in MALDI-TOF MS analysis

Shaded regions correspond to 32 fragments of tryptic peptide masses that were identified from the band-1 of the isolated Cry2Ab2 protein from MON 89034. The N-terminal 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
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Figure C-4. Peptide mass coverage map of the MON 89034-derived proteolytic fragment of Cry2Ab2 protein in MALDI-TOF MS analysis

Shaded regions correspond to 24 tryptic peptide masses that were identified from the band-2 (proteolytic fragment of Cry2Ab2) 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 band-2 started from amino acid residue No. 145.

C.2.5. Lack of Glycosylation of MON 89034-Produced Cry2Ab2

To test 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 C-5,** 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 C-5,** lanes 5-8).

Since no positive signal was seen at the band position for Cry2Ab2 protein, it was evident that the MON 89034-produced Cry2Ab2 protein is not glycosylated and is equivalent to the *E. coli*-produced Cry2Ab2 protein regarding to the lack of glycosylation.

C.2.6. Functional activity of the Cry2Ab2 protein

The functional activity of the MON 89034-produced and *E. coli*-produced Cry2Ab2 protein was compared by determining EC_{50} values in a corn earworm 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 C-1** and the dose response relationships for the Cry2Ab2 protein from the two sources are illustrated in **Figure C-6**. The mean EC_{50} values for the *E. coli*- and the MON 89034-produced proteins were similar and were determined to be 0.16 µg Cry2Ab2/ml diet, with standard deviations of 0.04 and 0.01 µg Cry2Ab2/ml diet, respectively. **Figure C-6** 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 MON 89034- and *E. coli*-produced Cry2Ab2 protein has equivalent functional activity.

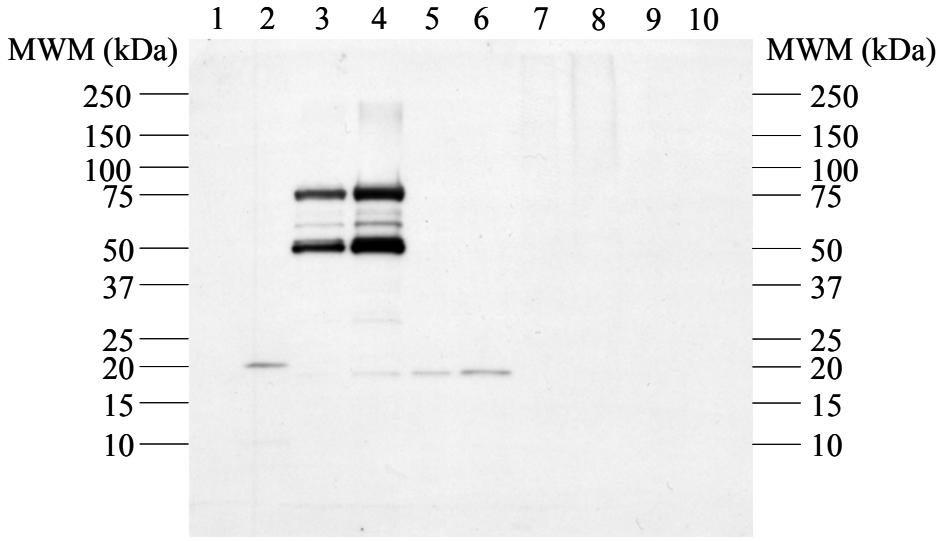

Figure C-5. Glycosylation analysis of the MON-89034-produced Cry2Ab2 Protein

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 C-1. EC_{50} values of *E. coli*-produced and MON 89034-produced Cry2Ab2 proteins in a corn earworm diet-incorporation bioassay

		EC_{50} (µg Cry2Ab2/ml diet)[1]	
		E. coli-produced	MON 89034-produced
Replicate[2]	1	0.13 ± 0.03	0.17 ± 0.03
	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.

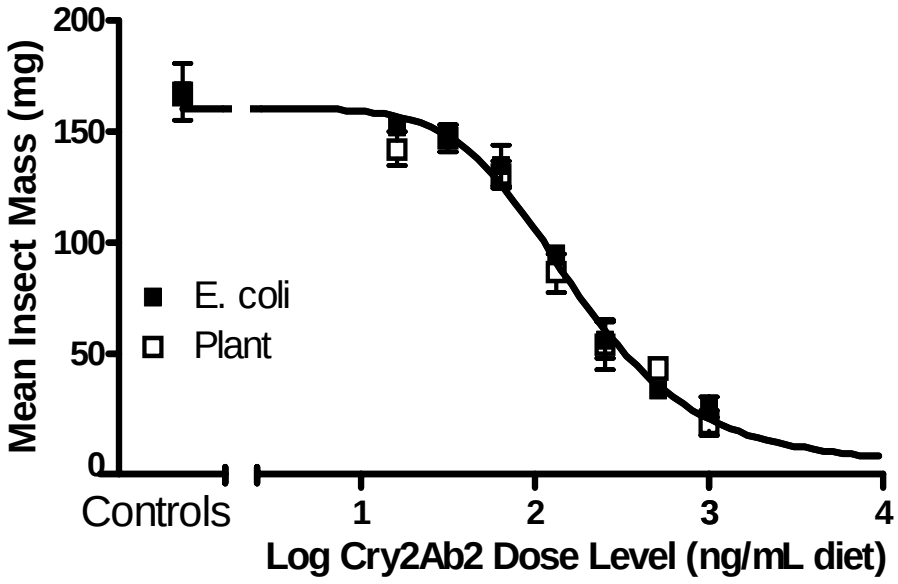

Figure C-6. Functional equivalence of the *E. coli*-produced and the MON 89034-produced Cry2Ab2 protein against corn earworm (CEW)

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 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.

C.3. References

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Appendix D. Materials and Methods for the Determination of Cry1A.105 and Cry2Ab2 Proteins

D.1. Test, Control, and Reference Materials

The test material was MON 89034 grown in 2005 U.S. field trials. The control material was a conventional corn with a similar genetic background to the MON 89034 plants. The identities of the test and control corn seeds were confirmed by event-specific PCR analyses. In addition, the identities of grain samples harvested from the field were also verified by PCR analyses.

The protein reference standards of Cry1A.105 and Cry2Ab2 were produced with recombinant *E. coli.* The Cry1A.105 standard (lot 20-100086) had a purity-corrected concentration of 1.0 mg/ml by amino acid composition analysis. The purity was 80% as determined SDS-PAGE and densitometry. The Cry2Ab2 standard (lot 20-100071) had a purity-corrected concentration of 0.4 mg/ml by amino acid composition analysis. The purity was 87% as determined by SDS-PAGE and densitometry.

D.2. Generation of Plant Samples

D.2.1. Field Design

The field production of the test materials was initiated during the 2005 planting season to generate test and control materials at various corn-growing locations in the U.S. The field sites were as follows: 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 and the conventional control corn were planted using a randomized complete block design. Over season leaf (OSL 1-4), over season root (OSR 1-4), over season whole plant (OSWP 1-4), forage, stover, forage root, senescent root, pollen, silk, and grain were collected from each replicated plot at all field sites. All 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.

D.2.2. Description of the Collected Tissues

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

Silks. Silks were collected from the ears of five plants in each plot, 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 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 and dried to a moisture content of 12-15%.

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

Leaf. The youngest immature whorl leaf was collected from 15 plants in each plot, which were 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 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

D.3. Tissue Processing and Protein Extraction

All tissue samples produced at the field sites were shipped to Monsanto's processing facility. Processed tissue samples were stored in a -80°C freezer until shipped on dry ice to Monsanto's analytical facility. All processed tissue samples were stored in a -80°C freezer during the study.

For all tissue types except pollen, Cry1A.105 protein was extracted with a Tris-borate buffer (pH 7.8) containing 100 mM Tris, 100 mM $Na_2B_4O_7 \cdot 10\ H_2O$, 5 mM $MgCl_2 \cdot 6\ H_2O$, 0.05% (v/v) Tween 20, 0.2% (w/v) L-ascorbic acid, 2 M urea, and 100 mM dithiothreitol. For pollen, Cry1A.105 protein was extracted with 50 mM $Na_2B_4O_7 \cdot 10\ H_2O$, 750 mM KCl, 0.075% (v/v) Tween 20, 0.2% (w/v) L-ascorbic acid, and 10 mM N-lauroylsarcosine, pH 10.5.

For all tissue types except pollen, Cry2Ab2 protein was extracted with a Tris-borate buffer (pH 7.8) containing 100 mM Tris, 100 mM $Na_2B_4O_7 \cdot 10\ H_2O$, 5 mM $MgCl_2 \cdot 6\ H_2O$, and 0.05% (v/v) Tween 20. For pollen, Cry2Ab2 protein was extracted with a buffer with the same compositions except the pH of 7.4.

All tissue samples were extracted using a Harbil mixer, and tissue debris 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 analysis.

D.4. ELISA Reagents and Methods

D.4.1 Cry1A.105 Antibodies and ELISA.

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 1 mM KH_2PO_4, 10 mM $Na_2HPO_4 \cdot 7H_2O$, 137 mM NaCl, and 2.7 mM 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).

The goat anti-Cry1A.105 antibody was 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.

D.4.2. Cry2Ab2 Antibodies and ELISA

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 20 mM $Na_2HPO_4 \cdot 7H_2O$ and 150 mM 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.

The mouse anti-Cry2Ab2 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 $\geq$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.

Appendix E: Materials, Methods and Individual Site Results for Seed Dormancy and Germination

E.1. Materials

The MON 89034, the conventional control corn, and reference corn seeds were produced in Dayton, Webster Co., IA (IA); Jerseyville, Jersey Co., IL (JR); and Monmouth, Warren Co, IL (MN) in 2004.

Material	Material Type	Genotype	Locations
MON 89034	Test	Lepidopteran-protected	IA, JR, MN
H1325023	Control	Conventional	IA, JR, MN
HEXP1184	Reference	Conventional	IA, JR, MN
H4242304	Reference	Conventional	IA, JR, MN
H1198600	Reference	Conventional	IA, JR, MN
H1200203	Reference	Conventional	IA, JR, MN

E.2. Characterization of the Materials

The presence or absence of MON 89034 insert was verified by event-specific PCR analysis for the MON 89034 and control corn seeds. The results of these verifications were as expected.

E.3. Performing Facility and Experimental Methods

Dormancy and germination evaluations were conducted at BioDiagnostics, Inc. in River Falls, WI. The principal investigator was certified to conduct seed dormancy and germination testing consistent with the standards established by the Association of Official Seed Analysts (AOSA), a seed trade association (AOSA, 2000; AOSA, 2002).

Seven germination chambers were used in the study and each chamber was maintained dark under one of the following seven temperature regimes: constant temperature of approximately 5, 10, 20 or 30^{o}C or alternating temperatures of approximately 10/20, 10/30, or 20/30^{o}C. The alternating temperature regimes were maintained at the lower temperature for 16 h and the higher temperature for 8 h. The temperature inside each germination chamber was monitored and recorded throughout the duration of the study.

Germination towels for MON 89034, control, and reference materials were prepared per the facility SOPs. Each germination towel represented one replication. The types of data collected depended on the temperature regime. Each rolled germination towel in the AOSA-recommended temperature regime (i.e., 20/30°C) was assessed periodically during the study for normally germinated, abnormally germinated, hard (viable and nonviable), dead, and firm swollen (viable and nonviable) seed as defined by AOSA guidelines (AOSA, 2002). Each rolled germination towel in the additional temperature regimes (i.e., 5, 10, 20, 30, 10/20 and 10/30^{o}C) was assessed periodically during the

study for germinated, hard (viable and nonviable), dead, and firm swollen (viable and nonviable) seed.

E.4. Statistical Analysis

Statistical analyses were performed by the Monsanto Statistics Technology Center. Analysis of variance was conducted according to a completely randomized design using the Statistical Analysis System (SAS® Version 9.1, SAS Institute, Inc. 2002-2003) to compare the MON 89034 to the control material for each temperature regime.

For each temperature regime, test material was separately compared to the control material at each site (individual-site or by-site analysis) and pooled across all sites (referred to as combined-site). No statistical comparisons were made between the test and reference materials. Minimum and maximum values for the reference materials were determined. An analysis of variance was conducted according to a completely randomized design with four replications using SAS. The level of statistical significance was predetermined to be 5% ($p \leq 0.05$). Dormancy and germination characteristics that were analyzed included: percent germinated seed (categorized as normally germinated and abnormally germinated seed for AOSA-recommended temperature regimes), percent viable hard seed, percent dead seed, and percent viable firm swollen seed.

E.5. Individual Site Seed Dormancy and Germination Results

MON 89034, the control, and reference seed materials were produced at three sites to assess germination characteristics of seed grown under various environmental conditions. No viable hard seed were detected in any temperature regime with seed produced from any location, indicating no increased weediness potential of MON 89034 from changes in seed dormancy. Furthermore, of the 87 comparisons over the germination characteristics from the three seed production locations, only two differences were detected between MON 89034 and the control. MON 89034 from the JR site had higher percentage of germinated seed and a lower percentage of dead seed at the 10^{o}C temperature regime than the control (**Table E-1**). No differences were detected between MON 89034 and the control at the IA or MN site in any temperature regime. The significant differences detected in the by-site analysis were not detected in the combined-site analyses (see **Section VII** for the combined-site result). Therefore, the differences detected in the by-site analysis were not indicative of a consistent trend and are not likely to be biologically meaningful in terms of altered seed dormancy or germination of MON 89034 compared to the conventional corn.

E.6. References

AOSA. 2000. Tetrazolium Testing Handbook. Association of Official Seed Analysts. Lincoln, NE.

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AOSA. 2002. Rules for Testing Seeds. Association of Official Seed Analysts. Lincoln, NE. 166 pp.

Table E-1. Germination for MON 89034

AOSA Temp. Reg.[1] (°C)	Prod. Site[2]	Mean (%)									
		Normally Germinated		Abnormally Germinated		Viable Hard		Dead		Viable Firm Swollen	
		T[3]	C[4]	T	C	T	C	T	C	T	C
20/30	IA	99.5	99.0	0.0	0.0	0.0	0.0	0.5	1.0	0.0	0.0
	JR	83.3	87.0	0.3	0.5	0.0	0.0	16.5	12.5	0.0	0.0
	MN	99.8	100.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0

Additional Temp. Regime (°C)	Prod. Site	Mean (%)							
		Germinated		Viable Hard		Dead		Viable Firm Swollen	
		T	C	T	C	T	C	T	C
5	IA	0.0	0.0	0.0	0.0	3.5	2.8	96.5	97.3
	JR	0.0	0.0	0.0	0.0	13.0	12.8	87.0	87.3
	MN	0.0	0.0	0.0	0.0	0.5	2.5	99.5	97.5
10	IA	98.0	98.5	0.0	0.0	2.0	1.5	0.0	0.0
	JR	90.5*	84.5	0.0	0.0	9.5*	15.5	0.0	0.0
	MN	99.5	100.0	0.0	0.0	0.5	0.0	0.0	0.0
20	IA	99.3	98.5	0.0	0.0	0.8	1.5	0.0	0.0
	JR	89.3	87.5	0.0	0.0	10.8	12.5	0.0	0.0
	MN	99.3	99.5	0.0	0.0	0.8	0.5	0.0	0.0
30	IA	98.3	98.5	0.0	0.0	1.8	1.5	0.0	0.0
	JR	88.8	88.0	0.0	0.0	11.3	12.0	0.0	0.0
	MN	99.8	100.0	0.0	0.0	0.3	0.0	0.0	0.0
10/20	IA	99.5	98.8	0.0	0.0	0.5	1.3	0.0	0.0
	JR	88.8	90.0	0.0	0.0	11.3	10.0	0.0	0.0
	MN	99.5	100.0	0.0	0.0	0.5	0.0	0.0	0.0
10/30	IA	98.8	99.5	0.0	0.0	1.3	0.5	0.0	0.0
	JR	87.5	90.8	0.0	0.0	12.5	9.3	0.0	0.0
	MN	99.8	100.0	0.0	0.0	0.3	0.0	0.0	0.0

* Indicates that a significant difference between MON 89034 and the control was detected at $p \leq 0.05$ for seed produced at a given site within a given temperature regime.

[1] In alternating temperature regimes the lower temperature was maintained for 16 hours and the higher temperature for eight hours.

[2] Prod. Site = site where seed lot was produced; IA = Dayton, Iowa; JR = Jerseyville, Illinois; MN = Monmouth, Illinois.

[3] T = MON 89034

[4] C = Control corn (H1325023)

Appendix F: Phenotypic and Agronomic Assessment: Individual Site Results from 2004 and 2005 Field Trials

The methods, test sites, and the combined-site results for the phenotypic and agronomic assessments are described in **Section VII**. This appendix provides the details of the test, control, and reference corn materials and the results for each individual test site.

F.1. Materials

The materials for phenotypic, agronomic, and ecological interaction assessments studies are included in **Tables F-1**, **F-2**, and **F-3**. The presence or absence of MON 89034 insert in the test and control materials was confirmed by event-specific PCR analyses.

F.2. Test Sites

2004 field trials included nine (9) test sites (site codes in parenthesis): Hendricks County, IN (IN-DA); Freeborn County, MN (MN-GE); Shelby County, MO (MO-BE and MO-CL); Jefferson County, IA (IA); Jersey County, IL (IL-1); Warren County, IL (IL-2); York County, NE (NE); and Fayette County, OH (OH).

Study-1 of the 2005 field trials included four (4) test sites: Ottawa County, MI (MI); Freeborn County, MN (MN); Wayne County, NY (NY); and Walworth County, WI (WI).

Study-2 of the 2005 field trials included five (5) test sites: Jefferson County, IA (IA); Warren County, IL (IL-N); Clinton County, IL (IL-S); Shelby County, MO (MO), and York County, NE (NE).

F.3. Individual Field Site Results

Table F-4 shows the results of the 14 phenotypic or agronomic characteristics evaluated at 9 sites in 2004. No differences between MON 89034 and the control were detected for seedling vigor, ear height, plant height, dropped ears, root lodged plants, and final stand count. A total of 13 out of 123 site-by-characteristic comparisons were significantly different between MON 89034 and the control. The significant differences were distributed among eight of the fourteen phenotypic characteristics. Except for plant height and number of stalked lodged plants, all of the significant differences detected in the individual-site analysis were not detected in the combined-site analyses (see **Section VII** for combined-site analysis result). Therefore, the differences detected in the individual-site analysis were not indicative of a consistent, trait-induced response, and are not likely to be biologically meaningful in terms of increased weediness potential of MON 89034 compared to the control.

Table F-5 shows the results of 14 phenotypic or agronomic characteristics for the four test sites in Study-1 of the 2005 trials. No differences were detected between MON 89034 and the control for the following ten characteristics: seedling vigor, early stand

count, days to 50% pollen shed, stay green, ear height, dropped ears, stalk lodged and root lodged plants, grain moisture and test weight. A total of four differences out of 56 site-by-characteristic comparisons were significantly different between MON 89034 and the control. The significant differences were distributed among four of the fourteen phenotypic characteristics. None of the significant differences detected in the individual-site analysis were detected in the combined-site analyses (see **Section VII** for combined-site analysis result). Therefore, the differences detected in the individual-site analysis were not indicative of a consistent, trait-induced response, and are not likely to be biologically meaningful in terms of increased weediness potential of MON 89034 compared to the control.

Table F-6 shows the results of the 14 phenotypic or agronomic characteristics for the five test sites in Study-2 of the 2005 trials. For the individual-site analyses, no differences were detected between MON 89034 and the control for the following nine characteristics: seedling vigor, days to 50% pollen shed and 50% silking, plant height, dropped ears, stalk and root lodged plants, final stand count and grain moisture. A total of five out of 65 site-by-characteristic comparisons were significantly different between MON 89034 and the control. The significant differences were distributed among five of the fourteen phenotypic characteristics. None of the significant differences detected in the individual-site analysis were detected in the combined-site analyses (see **Section VII** for combined-site analysis result). Therefore, the differences detected in the individual-site analysis were not indicative of a consistent, trait-induced response, and are not likely to be biologically meaningful in terms of increased weediness potential of MON 89034 compared to the control.

Table F-1. Test, control, and reference corn used in the 2004 field trials

Corn Hybrid Names	Seed Types	Site
MON 89034	Test	All
H1325023	Control	All
Golden Harvest-H8751	Reference	IA
Golden Harvest-H9231	Reference	IA
Northrup King-N60-N2	Reference	IA
Burris-590	Reference	IL-1
Mycogen-2784	Reference	IL-1
DKC 62-15	Reference	IL-1
Pfister-2730	Reference	IL-2
Mycogen-2E685	Reference	IL-2
DKC 61-42	Reference	IL-2
DK611	Reference	IN-DA
DKC59-08	Reference	IN-DA
RX708	Reference	IN-DA
DKC51-45	Reference	MN-GE
DKC57-01	Reference	MN-GE
DKC60-15	Reference	MN-GE
DKC61-42	Reference	MO-BE
DKC62-15	Reference	MO-BE
RX690	Reference	MO-BE
DKC60-17	Reference	MO-CL
RX742RR2	Reference	MO-CL
RX772	Reference	MO-CL
DKC 60-15	Reference	NE
Mycogen-2P682	Reference	NE
Mycogen-2A791	Reference	NE
Seed Consultants-SC1124A	Reference	OH
Crows-4908	Reference	OH
RX708	Reference	OH

Table F-2. Test, control and reference corn used in the Study-1 of 2005 field trials

Corn hybrid Names	Seed Types	Sites
MON 89034	Test	All
DKC51-43	Control	All
DKC48-15	Reference	MI
DK537	Reference	MI
Pioneer 36K67	Reference	MI
DKC51-45(RR2)	Reference	MN
DKC53-33(RR2)	Reference	MN
Pioneer 36N70	Reference	MN
DKC51-88	Reference	NY
Garst 8779	Reference	NY
Garst 8715	Reference	NY
RX508	Reference	WI
N45-T5	Reference	WI
N48-K2	Reference	WI

Table F-3. Test, control, and reference corn used in the Study-2 of 2005 field trials

Corn Hybrid Names	Seed Types	Sites
MON 89034	Test	All
H1325023	Control	All
DKC59-08	Reference	IA
DKC60-15	Reference	IA
H-8991	Reference	IA
DKC61-42	Reference	IL-S
RX740	Reference	IL-S
Mycogen 2E685	Reference	IL-S
RX708	Reference	IL-N
DKC61-50	Reference	IL-N
Pioneer 34H31	Reference	IL-N
DKC61-44(RR2)	Reference	MO
Pfister 2760	Reference	MO
Pioneer 34B23	Reference	MO
DKC60-17(RR2)	Reference	NE
RX715	Reference	NE
Mycogen 2E685	Reference	NE

Table F-4. Phenotypic comparison of MON 89034 to the control at each site in 2004 field trials

	Phenotypic Characteristic (units)											
	Seedling vigor		Early stand count (#/plot)		Days to 50% pollen shed		Days to 50% silking		Stay green[1]		Ear height (in)	
Site	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
IA	6.7	6.3	65.7	64.3	59.0	59.3	58.3	58.7	4.7	4.7	45.3	45.7
IL-1	5.3	5.0	60.0	56.0	53.3	53.7	50.7*	50.0	—	—	38.9	42.3
IL-2	8.0	8.5	68.3	68.3	59.4	58.9	56.2	56.7	7.6*	6.6	44.5	41.0
IN-DA	5.0	6.0	72.3*	76.7	66.0*	64.3	66.0*	64.3	—	—	31.9	36.1
MN-GE	8.0	7.7	75.0	77.3	73.3	73.3	73.0	73.3	6.0	6.0	42.5	41.2
MO-BE	7.3	7.3	75.3	72.3	58.3	58.3	57.3	57.0	5.0	4.7	40.3	40.1
MO-CL	6.0	5.7	70.7	68.0	59.0	58.3	57.7	57.3	6.3	6.3	39.1	38.8
NE	8.0	8.0	63.7	63.0	67.0	66.7	67.0	66.3	6.7*	5.7	40.1	41.1
OH	7.7	7.7	63.7	64.0	61.0	60.0	61.0	60.0	6.0	7.0	37.1	37.5

* Indicates a statistically significant difference between the test and control at $p \leq 0.05$.
[1] Dashes indicate data that are missing or excluded from the statistical analysis.

Table F-4 continues on the next page.

Table F-4 (continued). Phenotypic comparison of MON 89034 to the control at each site in 2004 field trials

	Phenotypic Characteristic (units)											
	Plant height (in)		Dropped ears (#/plot)		Stalk lodged plants (#/plot)[1]		Root lodged plants (#/plot)		Final stand count (#/plot)		Grain moisture (%)	
Site	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
IA	85.2	84.8	0.0	0.0	0.0	0.3	0.0	0.0	56.0	59.0	28.5	28.0
IL-1	81.8	83.1	0.0	0.0	—	—	0.0	0.0	54.3	52.7	14.1	13.1
IL-2	90.6	91.7	0.0	0.0	2.3*	8.3	11.1	4.6	66.2	64.2	25.3	23.5
IN-DA	69.6	74.8	0.0	0.0	0.0	1.3	0.3	0.3	65.7	61.7	40.1	38.4
MN-GE	102.1	102.8	0.0	0.0	0.0	0.3	0.0	0.0	62.7	63.7	30.5	30.5
MO-BE	83.8	86.1	1.0	1.0	1.7	1.7	0.0	0.0	63.7	64.7	16.8*	19.6
MO-CL	85.7	85.6	0.0	0.0	1.0	1.3	0.0	0.0	53.3	57.3	15.9	16.1
NE	80.2	81.0	0.0	0.0	0.0	1.3	0.0	0.0	62.0	62.0	19.3*	17.6
OH	78.6	79.1	0.0	0.7	1.0*	4.7	0.0	0.0	60.7	59.3	20.2	18.9

* Indicates a statistically significant difference between the test and control at $p \leq 0.05$.
[1] Dashes indicate data that are missing or excluded from the statistical analysis.

Table F-4 continues on the next page.

Table F-4 (continued). Phenotypic comparison of MON 89034 to the control at each site in 2004 field trials

	Phenotypic characteristic (units)			
	Test weight (lbs/bu)		Yield (bu/ac)	
Site	Test	Control	Test	Control
IA	54.3	54.7	258.8*	234.8
IL-1	54.4	54.8	207.0	197.0
IL-2	48.6	52.0	224.9	205.8
IN-DA	43.3	44.2	96.7	100.2
MN-GE	53.0	53.0	171.1	187.0
MO-BE	53.7	53.1	236.3	235.4
MO-CL	55.0	55.0	177.5	202.0
NE	55.1*	57.2	205.3*	185.5
OH	56.5	57.0	159.3	172.0

* Indicates a statistically significant difference between the test and control at $p \leq 0.05$.

Table F-5. Phenotypic comparison of MON 89034 to the control at each site in Study-1 of 2005 field trials

	Phenotypic Characteristic Means									
	Seedling Vigor		Early stand count (#/plot)		Days to 50% pollen shed		Days to 50% silking		Stay green	
Site	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control
MI	6.7	6.7	76.7	79.7	69.3	68.7	69.3	70.0	5.7	6.0
MN	6.0	5.7	76.0	72.0	64.7	64.3	66.3	66.3	5.0	5.0
NY	6.0	6.0	79.3	80.0	71.0	71.0	62.0	62.0	9.0	9.0
WI	8.0	8.0	76.3	78.0	67.0	66.3	70.0*	67.0	5.7	4.7

	Phenotypic Characteristic Means									
	Ear height (in)		Plant height (in)		Dropped ears (#/plot)		Stalk lodged plants (#/plot)		Root lodged plants (#/plot)	
Site	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control
MI	38.7	37.3	82.1	81.7	0.0	0.3	0.0	0.7	0.0	0.0
MN	36.0	38.8	82.7	82.7	0.0	0.0	0.3	0.3	0.0	0.0
NY	48.7	48.7	103.9*	100.5	0.0	0.0	0.0	0.0	0.0	0.0
WI	44.2	43.6	97.7	94.1	0.0	0.0	0.0	0.3	0.0	0.0

* Indicates a statistically significant difference between MON 89034 and the control at $p \leq 0.05$.

Table F-5 continues on the next page.

Table F-5 (continued). Phenotypic comparison of MON 89034 to the control at each site in Study-1 of 2005 field trials

	Phenotypic Characteristic Means							
	Final stand count (#/plot)		Grain moisture (%)		Test weight (lbs/bu)		Yield (bu/ac)	
Site	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control
MI	64.0	64.0	21.7	21.2	55.7	55.3	205.8*	175.6
MN	61.7	61.3	17.5	17.8	56.5	56.4	204.3	197.7
NY	58.7	58.7	19.0	18.7	55.5	55.7	174.1	175.6
WI	60.7*	63.3	17.4	16.7	55.8	56.8	237.7	231.6

* Indicates a statistically significant difference between MON 89034 and the control at $p \leq 0.05$.

Table F-6. Phenotypic comparison of MON 89034 to the control at each site in Study-2 of 2005 field trials

	Phenotypic Characteristic Means									
	Seedling Vigor		Early stand count (#/plot)		Days to 50% pollen shed		Days to 50% silking		Stay green	
Site	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control
IA	6.0	4.7	67.0*	73.0	57.7	58.3	58.3	59.0	6.7*	7.3
IL-N	7.3	7.3	72.0	71.3	65.0	64.7	64.3	63.7	6.3	6.3
IL-S	6.3	7.0	75.0	73.7	51.3	50.3	52.0	51.3	3.3	3.3
MO	6.0	6.5	64.7	70.5	65.0	64.0	67.0	66.0	4.7	5.0
NE	6.0	6.0	67.5	72.5	63.0	63.5	64.0	64.0	3.5	3.0

	Phenotypic Characteristic Means									
	Ear height (in)		Plant height (in)		Dropped ears (#/plot)		Stalk lodged plants (#/plot)		Root lodged plants (#/plot)	
Site	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control
IA	39.9	39.8	83.0	84.3	1.7	0.0	0.0	0.3	0.0	0.0
IL-N	35.2	36.1	85.7	86.0	0.0	0.7	0.0	0.0	0.0	0.0
IL-S	42.3*	46.0	87.1	89.9	1.3	2.3	40.0	19.0	2.0	2.7
MO	35.9	37.2	68.9	69.8	3.3	1.0	4.3	3.0	1.0	0.0
NE	35.8	35.9	75.1	73.2	0.0	0.5	1.0	3.0	0.0	0.0

* Indicates a statistically significant difference between MON 89034 and the control at $p \leq 0.05$.

Table F-6 continues on the next page.

Table F-6 (continued). Phenotypic comparison of MON 89034 to the control at each site in Study-2 of 2005 field trials

	Phenotypic Characteristic Means							
	Final stand count (#/plot)		Grain moisture (%)		Test weight (lbs/bu)		Yield (bu/ac)	
Site	MON 89034	Control	MON 89034	Control	MON 89034	Control	MON 89034	Control
IA	58.3	58.3	26.0	26.2	55.9	55.3	164.3	154.9
IL-N	63.3	63.0	18.7	17.6	59.1	59.4	183.3	164.5
IL-S	52.0	56.7	16.2	16.5	55.3	55.7	83.9	100.6
MO	60.0	60.0	19.3	17.7	53.3*	51.9	47.2	40.1
NE	62.0	62.0	16.4	18.0	56.5	55.1	169.7*	146.9

* Indicates a statistically significant difference between MON 89034 and the control at $p \leq 0.05$.

Appendix G: Forage and Grain Compositional Analysis

G.1. Materials

MON 89034, control corn (LH198 × LH172), and commercial reference corns were grown at five U.S. locations in 2004. The control material, LH198 × LH172, has a genetic background similar to MON 89034 but does not possess the lepidopteran protection trait. In addition, 15 conventional corn hybrids produced along side of MON 89034 were included for the generation of 99% tolerance interval. The reference corn hybrid varieties, locations, and seed lot numbers are listed below:

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

The identities of the MON 89034, the control, and reference corn hybrids were verified prior to the study by examination of the chain-of-custody documentation supplied with the samples collected from the field. Additionally, the grain samples of the test and control corn were further characterized by an event-specific PCR analysis to confirm the presence or absence of the MON 89034 insert.

G.2. Field Trial Description

Seed was planted in a randomized complete block design with three replicates per block. All the samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. The five 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. The samples were 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.

G.3. Analytical Methods

A total of 90 ground forage and grain samples were analyzed by Covance Laboratories, Inc. Analyses were performed using methods that are currently used to evaluate the nutritional quality of food and feed. Specifically, standard AOAC (Association of Official Analytical Chemists, 2000) methods were used to determine amino acid composition, fat, minerals, total dietary fiber, moisture, folic acid, niacin, pyridoxine (Vitamin B6), riboflavin (Vitamin B2), and thiamin (Vitamin B1). The protein content was determined based on both AOAC (2000) methods and two literature methods (Bradstreet, 1965; Kalthoff and Sandell, 1948). Acid detergent fiber was analyzed based on the method of USDA Agriculture Handbook No. 379 (1970). Carbohydrates were measured based on an USDA Agriculture Handbook No. 74 (1973). Fatty acids were analyzed based on an AOCS (American Oil Chemists Society, 1997) method. Neutral detergent fiber was determined based on an AACC (American Association of Cereal Chemists, 1998) method and an USDA Agriculture Handbook No. 379 (1970) method. In addition, other literature methods were used to measure furaldehyde (Albala-Hurtado et al., 1997), p-coumaric and ferulic acids (Hagerman and Nicholson, 1982). phytic acid (Lehrfeld 1989; 1994), raffinose (Mason and Slover, 1971; Brobst, 1972), and Vitamin E (Cort et al., 1983; McMurray et al., 1980; Speek et al., 1985).

G.4. Statistical Analysis

At the field sites, the test (MON 89034), control, and reference corns were grown in single plots randomly assigned within each of three replication blocks. The compositional components for the test and control corn 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 MON 89034 was compared to the conventional control.

A range of observed values from the reference corns was determined for each analytical component. Additionally, the reference corn 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., 2002b). 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).

G.5. Compositional Analysis Results

The results of compositional analysis, including a summary of statistical differences, are discussed in **Section VII**. **Tables G-1** to **G-6** of this appendix provide a summary of the combined site analysis, and **Table G-7** lists the literature and historical ranges for the components analyzed.

Table G-1. Comparison of fiber, mineral, and proximate content in forage from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (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	0.17	(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	0.01	(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	0.22	(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; SE = 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 G-2. Comparison of amino acid content in grain from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE[1] (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (p-Value)	Commercial (Range) [99% Tolerance Int.[2]]
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]
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	0.7	(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]

[1] DW = dry weight; SE = 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 G-2 continues on the next page.

Table G-2 (continued). Comparison of amino acid content in grain from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE[1] (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (p-Value)	Commercial (Range) [99% Tolerance Int.[2]]
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]
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; SE = 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 G-3. Comparison of fatty acid content in grain from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE[1] (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (p-Value)	Commercial (Range) [99% Tolerance Int.[2]]
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; SE = 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 G-4. Comparison of mineral content in grain from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE[1] (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (p-Value)	Commercial (Range) [99% Tolerance Int.[2]]
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	0.18	(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	0.25	(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	0.45	(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; SE = 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 G-5. Comparison of proximate and fiber content in grain from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE[1] (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (p-Value)	Commercial (Range) [99% Tolerance Int.[2]]
Proximate						
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	0.41	(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	0.37	(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; SE = 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 G-6. Comparison of vitamin, antinutrient and secondary metabolite content in grain from MON 89034 and control corn in combined-site analysis

Analytical Component (units)[1]	MON 89034 Mean ± SE[1] (Range)	Control Mean ± SE[1] (Range)	Difference (MON 89034 minus Control) Mean ± SE (Range)	95% CI[1] (Lower,Upper)	Significance (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]
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	0.74	(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; SE = 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 G-7. Literature and historical 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 G-7 (continued). Literature and historical ranges of components of corn forage and grain

Tissue/ Component[1]	Literature Range[2]	ILSI Range[3]
Grain		
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	-	0.170 – 1.917
22:0 Behenic	-	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 G-7 (continued). Literature and historical 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*., 2002a. [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

G.6. References

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Appendix H: USDA Notifications Approved for MON 89034

Field trials of MON 89034 were conducted in the U.S. since 2001. The protocols for these trials include field performance, agronomics, and generation of field materials and data necessary for this petition. In addition to the phenotypic assessment data provided for MON 89034, observational data on pest and disease stressors were collected from these product development trials. The majority of the final reports have been submitted to the USDA. However, some final reports, mainly from the 2005-2006 seasons, are still in preparation. A list of trials conducted under USDA notification and the status of the final reports for these trials are provided in **Table H-1**.

Table H-1. USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial Status
2001 Field Trials			
01-348-04n	1/13/2002	PR (2)	Submitted to USDA
01-348-07n	1/11/2002	HI (6)	Submitted to USDA
2002 Field Trials			
02-009-12n	2/8/2002	IL (3)	Submitted to USDA
02-009-15n	2/8/2002	IA (6)	Submitted to USDA
02-017-12n	3/20/2002	AL, MS, OR, TN, WA	Submitted to USDA
02-242-10n	10/1/2002	PR (2)	Submitted to USDA
2003 Field Trials			
03-024-06n	2/23/2003	AL MS	Submitted to USDA
03-034-25n	3/5/2003	OR, TN (2)	Submitted to USDA
03-034-27n	3/5/2003	IL (2)	Submitted to USDA
03-042-06n	3/13/2003	MO	Submitted to USDA
03-043-04n	3/17/2003	IL (11)	Submitted to USDA
03-043-05n	3/17/2003	IA (11)	Submitted to USDA
03-052-57n	3/23/2003	TN	Submitted to USDA
03-161-05n	8/5/2003	HI (5)	Submitted to USDA
03-219-04n	9/5/2003	PR (2)	Submitted to USDA
03-265-09n	11/21/2003	HI (6)	Submitted to USDA
03-280-01n	11/6/2003	PR (3)	Submitted to USDA
2004 Field Trials			
04-009-01n	3/25/2004	HI (5)	Submitted to USDA
04-009-02n	3/25/2004	PR (4)	Submitted to USDA
04-014-05n	2/26/2004	GA	Submitted to USDA
04-014-08n	2/13/2004	TN (2)	Submitted to USDA
04-021-02n	2/20/2004	AL, MS	Submitted to USDA
04-021-09n	2/20/2004	IA (2), IL (4), IN, KS, NE, OH	Submitted to USDA
04-021-11n	2/20/2004	AR	Submitted to USDA
04-021-14n	2/20/2004	TN	Submitted to USDA
04-023-09n	2/25/2004	IA (12)	Submitted to USDA
04-023-10n	2/25/2004	IA (8)	Submitted to USDA
04-023-11n	2/25/2004	WI (3)	Submitted to USDA
04-028-10n	3/24/2004	MN (5)	Submitted to USDA
04-028-23n	2/27/2004	TN	Submitted to USDA
04-028-24n	2/27/2004	CO (3), NE (4)	Submitted to USDA
04-028-25n	2/27/2004	IA (4)	Submitted to USDA
04-028-26n	2/27/2004	OR (2), WA	Submitted to USDA
04-028-27n	2/27/2004	IL (15)	Submitted to USDA
04-028-28n	2/27/2004	KS (2)	Submitted to USDA

Table H-1 continues on next page.

Table H-1 (continued). USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial Status
04-028-29n	2/27/2004	IN (4)	Submitted to USDA
04-028-30n	2/27/2004	MI (2)	Submitted to USDA
04-028-31n	2/27/2004	MO (2)	Submitted to USDA
04-028-32n	2/27/2004	NE (4)	Submitted to USDA
04-028-33n	2/27/2004	OH (2)	Submitted to USDA
04-040-01n	4/2/2004	MD (2)	Submitted to USDA
04-040-02n	3/12/2004	NE	Submitted to USDA
04-040-03n	3/10/2004	IL (6)	Submitted to USDA
04-041-03n	3/11/2004	IL (10)	Submitted to USDA
04-041-04n	3/11/2004	KS (2)	Submitted to USDA
04-044-07n	3/14/2004	IA (6)	Submitted to USDA
04-056-06n	3/26/2004	IA (2), IL (2), IN, MO (3)	Submitted to USDA
04-079-02n	4/26/2004	IN, MN, MO, WI	Submitted to USDA
04-085-15n	4/24/2004	NE (3)	Submitted to USDA
04-090-03n	6/18/2004	HI (5)	Submitted to USDA
04-093-03n	5/2/2004	IL	Submitted to USDA
04-093-04n	5/2/2004	IA	Submitted to USDA
04-189-03n	9/27/2004	HI (15)	Submitted to USDA
04-217-02n	9/20/2004	PR (2)	Submitted to USDA
04-231-03n	9/27/2004	PR (3)	Submitted to USDA
04-260-01n	11/1/2004	HI	Submitted to USDA
04-273-01n	10/18/2004	IA, IL (2), NE	Submitted to USDA
04-274-08n	10/28/2004	PR (2)	Submitted to USDA
04-309-03n	1/10/2005	HI (9)	Submitted to USDA
04-309-04n	1/10/2005	PR (3)	Submitted to USDA
04-337-02n	12/16/2004	MS	Submitted to USDA
04-337-03n	12/20/2004	AL	Submitted to USDA
04-358-20n	1/25/2005	GA (2)	Submitted to USDA
04-358-21n	1/24/2005	IA (14)	Submitted to USDA
04-358-22n	1/24/2005	IA (15)	Submitted to USDA
04-358-23n	1/24/2005	IL (11)	Submitted to USDA
04-358-24n	1/25/2005	IN (3)	Submitted to USDA
04-358-25n	1/24/2005	KS (2)	Submitted to USDA
04-358-26n	2/7/2005	MD	Submitted to USDA
04-358-27n	1/26/2005	MI (3)	Submitted to USDA
04-358-28n	2/9/2005	MN (8)	Submitted to USDA
04-358-29n	1/24/2005	MO (2)	Submitted to USDA
04-358-30n	1/25/2005	NE (5)	Submitted to USDA
04-358-31n	1/25/2005	OH (2)	Submitted to USDA
04-358-32n	1/27/2005	TN (2)	Submitted to USDA
04-358-33n	1/25/2005	WI (3)	Submitted to USDA
04-362-09n	1/18/2005	TN (3)	Submitted to USDA
04-362-11n	1/18/2005	AR	Submitted to USDA

Table H-1 continues on next page.

Table H-1 (continued). USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial Status
2005 Field Trials			
05-025-04n	2/22/2005	IA, IL (3), IN, KS, NE, OH (2)	In Progress
05-026-19n	2/14/2005	IL (8), IN, WI (3)	In Progress
05-028-02n	2/14/2005	CO (5), KS, NE (3)	In Progress
05-028-11n	2/22/2005	IA (4), IL (9)	In Progress
05-028-12n	2/22/2005	HI (4)	In Progress
05-039-09n	3/10/2005	NE, OR, WA	In Progress
05-039-11n	3/28/2005	IA (2) IL (2), IN (3), MI, MN, MO (2), NE, NY, WI	In Progress
05-040-15n	3/10/2005	LA	Submitted to USDA
05-049-13n	3/24/2005	IA (5), IL (2), IN (2), NE (4), TX (2)	In Progress
05-055-03n	4/11/2005	IL (2), MN (5), MO (2)	In Progress
05-056-10n	4/1/2005	MO	In Progress
05-063-06n	4/1/2005	IL (2)	Submitted to USDA
05-104-03n	5/17/2005	HI (10)	In Progress
05-129-01n	5/31/2005	NE (6)	In Progress
05-131-01n	6/9/2005	LA	In Progress
05-133-05n	7/20/2005	HI (4), PR (2)	In Progress
05-201-06n	9/26/2005	PR (3)	In Progress
05-201-10n	9/6/2005	HI (5)	In Progress
05-201-11n	9/26/2005	PR (3)	In Progress
2006 Field Trials			
06-025-04n	3/30/2006	AL, AR, LA, MS (2), TN (2)	In Progress
06-027-06n	2/27/2006	GA (4)	In Progress
06-027-07n	3/6/2006	OH (2)	In Progress
06-027-08n	3/6/2006	WI (3)	In Progress
06-027-09n	3/7/2006	MN (9)	In Progress
06-027-11n	3/1/2006	MI (3)	In Progress
06-027-12n	2/27/2006	NE (4)	In Progress
06-027-13n	2/27/2006	MO (2)	In Progress
06-027-14n	2/27/2006	TN (4)	In Progress
06-027-15n	2/27/2006	KS (2)	In Progress
06-027-17n	4/5/2006	IA (5)	In Progress
06-030-01n	2/28/2006	IN (4)	In Progress
06-030-02n	2/28/2006	IL (13)	In Progress
06-030-04n	3/7/2006	IA (12)	In Progress
06-030-05n	3/7/2006	IA (13)	In Progress
06-030-06n	2/28/2006	IL (12)	In Progress
06-031-25n	2/28/2006	NE (2)	In Progress
06-032-12n	4/5/2006	IA (12)	In Progress

Table H-1 continues on next page.

Table H-1 (continued). USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial Status
06-033-07n	5/18/2006	HI (7)	In Progress
06-037-08n	5/18/2006	HI (5)	In Progress
06-037-10n	5/26/2006	PR (3)	In Progress
06-037-11n	3/16/2006	PR (3)	In Progress
06-038-05n	4/6/2006	IA, IL (3), IN (2), TX (3)	In Progress
06-046-06n	3/23/2006	CO (5), NE (2)	In Progress
06-046-10n	3/16/2006	IL (4), MO (5)	In Progress
06-046-11n	4/17/2006	IA (8), NE (6), SD	In Progress
06-052-11n	3/23/2006	MS	In Progress
06-060-09n	4/24/2006	IA (2)	In Progress
06-060-10n	4/24/2006	IA (14)	In Progress
06-073-01n	6/12/2006	IA (3), IL (4), IN (2), KS, MN	In Progress
06-073-02n	5/5/2006	IA (7), IL (5), NE, OH (2)	In Progress
06-081-02n	5/9/2006	IA	In Progress
06-082-05n	5/18/2006	HI (3), PR (2)	In Progress
06-089-11n	5/15/2006	IA (5), IL, NE	In Progress
06-090-04n	5/18/2006	HI (6)	In Progress
06-090-05n	6/12/2006	PR (3)	In Progress
06-090-06n	5/18/2006	HI (5)	In Progress
06-090-07n	5/26/2006	PR (3)	In Progress
06-108-03n	5/26/2006	PR	In Progress
06-108-04n	5/26/2006	HI, IN (2), PR	In Progress
06-108-05n	5/18/2006	HI, PR (2)	In Progress
06-108-06n	5/26/2006	HI, IN	In Progress
06-114-03n	5/26/2006	HI, IL	In Progress
06-118-02n	5/9/2006	IA	In Progress

Attachment: Study Reports Submitted to the EPA

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MONSANTO
imagine

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January 23, 2007

Dr. Michael T. Watson
Supervisory Biotechnologist
Plant Pests and Protectants Branch
USDA/APHIS/BRS
4700 River Road, Unit 147
Riverdale, MD 20737

Re: Response to USDA/APHIS/BRS Letter on Draft Review for Completeness and Acceptability of USDA Petition Numbered 06-298-01p (i.e., Monsanto Company File No. 06-CR-166U) for the Determination of Non-regulated Status for Corn MON 89034

Dear Dr. Watson:

Thank you for your letter dated January 8, 2007, requesting additional information and clarification regarding the petition indicated above. Responses by Monsanto Company are presented in the attached addendum to the petition. In addition to the paper copy, a compact disc (CD) is also included with files of this addendum and the non-CBI version of the petition attached by this addendum.

Should you have any questions regarding the addendum, please feel free to contact Dr. Russell Schneider, Monsanto Regulatory Affairs Director in Washington DC at (202) 383-2866, or me at (314) 694-2943.

Yours sincerely,

Yong Gao, PhD
Regulatory Affairs Manager

Attachment

cc: Dr. Russell Schneider
Ms. Carolyn Carrera

Addendum to USDA Petition No. 06-298-01p (i.e., Monsanto Company File No. 06-CR-166U) for Determination of Non-regulated Status for MON 89034

January 23, 2007

This addendum provides further information requested by USDA-APHIS in the Agency's letter to Monsanto Company dated January 8, 2007, concerning Monsanto's petition for the determination of non-regulated status for corn MON 89034. In the letter, USDA requested Monsanto Company to provide a short summary of a list of studies on the non-target organisms (NTOs) of MON 89034. In addition, the Agency requested Monsanto Company to provide an update on the trial status of USDA notifications approved for MON 89034.

The following provides a short summary for each NTO study with the Agency's request highlighted in bold font and the full reference of the studies in italic.

1. Evaluation of Dietary Effects of Lyophilized Leaf Tissue from Corn MON 89034 in a Chronic Exposure Study with Collembola (*Folsomia candida*).

Teixeira, D. 2006. Evaluation of Dietary Effects of Lyophilized Leaf Tissue from Corn MON 89034 in a Chronic Exposure Study with Collembola (Folsomia candida). Monsanto Technical Report MSL-20169.

The objective of this study was to determine the potential effect of chronic dietary exposure of lyophilized corn leaf tissue of MON 89034 on survival and reproduction of Collembola (*Folsomia candida*). Collembola were exposed for 28-days to lyophilized leaf tissue, combined with Brewer's yeast, from either MON 89034 or a conventional control corn with similar background genetics at 50% (w/w) of diet, yielding dose levels of 80 µg Cry1A.105/g diet and 70 µg Cry2Ab2/g diet for the MON 89034 treatments, which resulted in a safety factor (margin of exposure or MOE) of 19X for Collembola to both proteins based on the estimated maximum expected concentrations of the two proteins in the top 15 cm soil[1]. Additionally, positive controls (treatments with thiodicarb) were included to demonstrate the validity of the test system. No adverse effect on survival and reproduction was observed in the treatment containing leaf tissue from MON 89034. Therefore, the NOEC (no-observed-effect-concentration) was determined to be ≥80 µg Cry1A.105/g diet and ≥70 µg Cry2Ab2/g diet.

2. Evaluation of Exposure to MON 89034 with the Cladoceran *Daphnia magna*: An Acute Static-renewal Test with Corn Pollen.

Palmer, S.J. and H.O. Krueger. 2006. Evaluation of Exposure to MON 89034 with the Cladoceran Daphnia magna: An acute static-renewal test with corn pollen. Monsanto Study WL-2005-011.

[1] Details on assessment of the maximum expected environmental concentrations and calculation of safety factor (MOE) are presented in **Section VIII.A.3**.

The objective of this study was to determine the potential for acute effects to the cladoceran, *Daphnia magna,* during a static-renewal test with pollen from MON 89034. The test procedure followed the methodology of U.S. EPA OPPTS Guideline Number 850.1010, which provides specific guidance for testing *Daphnia magna*. Initially, a limit test was performed at a concentration of 120 mg pollen/l from MON 89034, including a control group containing 120 mg pollen/l from a conventional corn line with a similar genetic background to MON 89034, and a diluent water control. At the end of the limit test, there was 17% mortality/immobility and no mortality in the control groups. Therefore, a dose-response test was conducted under static-renewal conditions at concentrations of 6.3, 13, 25, 50, 100 and 120 mg pollen/l. There were no mortalities, immobile daphnids or signs of toxicity noted in any control or test group during the exposure in the dose-response test. Based on the combined results of the limit test and the dose-response test using pollen from MON 89034, the NOEC was determined to be ≥100 mg pollen/l. Under a well accepted toxicity classification system for freshwater ecotoxicology, if no adverse effects are observed at a concentration of 100 mg/l, the tested material is classified as practically non-toxic (EPA, 1985)[2].

3. Evaluation of Potential Effects of Exposure to Cry1A.105 Protein in an Acute Study with the Earthworm in an Artificial Soil Substrate.

Sindermann, A.B., J.R. Porch, and H.O. Krueger. 2006. Evaluation of Potential Effects of Exposure to Cry1A.105 Protein in an Acute Study with the Earthworm in an Artificial Soil Substrate. Monsanto Technical Report MSL-20147.

The objective of this study was to evaluate the potential effects of acute exposure of the Cry1A.105 protein administered to the earthworm, *Eisenia fetida*, during a 14-day exposure period when mixed in an artificial soil substrate. A single concentration of 178 mg Cry1A.105 protein/kg soil dry weight was tested, which resulted in a safety factor (margin of exposure) of 41X based on the maximum expected environmental concentration for the protein in the top 15 cm of soil. Appropriate negative and positive controls were also included in the study. The results showed that there was no mortality in the assay control group (soil only), control substance group (soil with 25 mM CAPS buffer), and the Cry1A.105 protein group during the 14-day test. The positive control groups of 15 mg and 30 mg chloroacetamide/kg soil resulted in 48% and 100% mortalities, respectively, demonstrating the validity of the test system. A slight loss in average individual earthworm body weight from test initiation to test termination was noted in all test groups, which was expected since the earthworms were not fed during the 14-day test period. There was no significant difference ($p>0.05$) in body weight losses between the Cry1A.105 protein treatment group and the control substance group. The study concluded that the NOEC for earthworms was ≥ 178 mg Cry1A.105 protein/kg dry soil.

4. Evaluation of the Dietary Effect(s) of a Cry1A.105 Protein on Honeybee Larvae (*Apis mellifera* L.). The report for this study states that honey bee larvae were dosed

[2] EPA. 1985. Hazard evaluation division, standard evaluation procedure: acute toxicity for freshwater invertebrates. PB86-129269.

with at least a 10X safety factor. In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Richards, K.B. 2006. Evaluation of the Dietary Effect(s) of a Cry1A.105 Protein on Honeybee Larvae (Apis mellifera L.). Monsanto Study CA-2005-071.

The objective of this study was to evaluate potential dietary effects of Cry1A.105 protein when administered to honeybee larvae. The protein was tested at a concentration of 1100 µg Cry1A.105/ml using a 25 mM CAPS buffer, which resulted in a safety factor of approximately 125X based on the maximum Cry1A.105 protein expression level (8.8 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative and positive controls were included in the study. The results revealed that the survival rate for the honeybee larvae in the negative controls was 92.5% for both the 25 mM CAPS buffer and water treatments. The Cry1A.105 protein treatment yielded a 95% survival rate, comparable to the negative control groups. Behavioral observations at emergence indicated no adverse behavior or morphological effects. Based on statistical analyses and behavioral observations there were no significant effects on the development or survival of honeybees treated with either the Cry1A.105 protein or the buffer/water controls. The survival rates for the positive control treatments were 26.4% and 5.0% for 200 µg and 2000 µg potassium arsenate/ml, respectively, confirming the validity of the test system. In conclusion, the NOEC of Cry1A.105 protein on honeybee larvae was ≥1100 µg/ml.

5. Evaluation of the Dietary Effect(s) of a Cry1A.105 Protein on Adult Honeybees (*Apis mellifera* L.). In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Richards, K.B. 2006. Evaluation of the Dietary Effect(s) of a Cry1A.105 Protein on Adult Honeybees (Apis mellifera L.). Monsanto Study CA-2005-072.

The objective of this study was to evaluate potential dietary effects of Cry1A.105 protein on the adult honeybee during chronic feeding. The protein was tested at a concentration of 550 µg Cry1A.105/ml in a 30% sucrose solution, which resulted in a safety factor of approximately 63X based on the maximum Cry1A.105 protein expression level (8.8 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative controls (12.5 mM CAPS buffer in 30% sucrose, and 30% sucrose in water) and a positive control (100 µg/ml potassium arsenate in 30% sucrose) were included in the study. Adult honeybees (0 to 5 days old) were exposed to the test and control solutions continually for the test period. The number of dead bees was assessed on a daily basis. The study was terminated on day 20 and all the bees in each cage were counted to determine the exact number of bees present in each cage. The potassium arsenate positive control produced 100% mortality by Day 2, confirming the validity of the test system. Based on statistical analyses and behavioral observations there were no significant effects on the development or survival of honeybees treated with either the Cry1A.105 protein or the negative controls. Thus, the NOEC of Cry1A.105 protein on adult honeybees was ≥550 µg/ml.

6. Honey bee dietary toxicity studies submitted with this petition utilized Cry1A.105 protein as the test material. Studies with Cry2Ab2 protein should also be submitted and summarized for the petition.

Studies (below) regarding the effects of Cry2Ab2 on honeybees have been previously submitted to USDA in Bollgard II cotton (MON 15985) submission (USDA Petition No. 00-342-01p). The summaries of the studies are provided below.

Maggi, V.L. 2000. Evaluation of dietary effect(s) of purified Bacillus thuringiensis Cry2Ab2 protein on honey bee larvae. Monsanto Technical Report MSL-16961.

The objective of this study was to evaluate potential dietary effects of Cry2Ab2 protein when administered to honey bee larvae. The protein was tested at two concentrations of 1 and 100 µg Cry2Ab2/ml in 10 mM sodium carbonate buffer. The concentration of 100 µg/ml resulted in a safety factor of approximately 213X based on the maximum Cry2Ab2 protein expression level (0.47 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative and positive controls were included in the study. Survival of honey bee larvae was assessed on days 7 and 12 after treatment. The results showed that mean mortality at Day 12 was 17.5% and 18.75% for honey bee larvae exposed to 1 and 100 µg Cry2Ab2/ml solutions, respectively. Mean mortality at Day 12 in the buffer (10 mM sodium carbonate) and water control groups was 21.25% and 10.0%, respectively. No significant difference was observed in mortality between the Cry2Ab2 treatments and the buffer control treatment using the Dunnett's test. The mortality in the positive control treatments was 19.89% and 76.79% for 50 µg and 1000 µg potassium arsenate/ml, respectively, confirming the validity of the test system. In conclusion, this study indicated that the NOEC of the Cry2Ab2 protein on honey bee larvae was ≥100 µg/ml.

Maggi, V.L. 2000. Evaluation of the dietary effect(s) of insect protection protein 2 on adult honey bees (Apis mellifera L.)[3]*. Monsanto Technical Report MSL-16176.*

The objective of this study was to evaluate the toxicity of the Cry2Ab2 protein administered in the diet to adult honey bees. Two levels of the Cry2Ab2 protein were evaluated at 3.4 and 68 µg/ml in 5 mM sodium carbonate solution with 30% sucrose. The concentration of 68 µg/ml resulted in a safety factor of approximately 145X based on the maximum Cry2Ab2 protein expression level (0.47 µg/g fwt) in pollen from MON 89034. In addition, three control groups were included: a buffer control (5 mM sodium carbonate buffer with 30% sucrose), an assay control (30% sucrose in water), and a positive control received 100 µg potassium arsenate/ml in 30% sucrose solution. The bees were allowed to feed *ad libitum* in cages inside a dark, environmentally controlled room. The number of dead bees in each cage was assessed on a daily basis. On Day 19, the study was terminated as the cumulative percentage of mortality in the assay control group (22.4%) exceeded the pre-determined criterion of 20%. At termination, the cumulative mortality of 3.4 and 68 µg Cry2Ab2/ml treatments was 26.1% and 19.0%, respectively. The buffer control group had a mortality of 21.7%. No significant

[3] The insect protection protein 2 referred to in the title of the report is synonymous with the Cry2Ab2 protein.

difference was noted between the Cry2Ab2 protein treatments and the control treatment. No behavioral or morphological abnormalities were observed in bees exposed to the test or negative control substance treatments. The positive control group produced 100% mortality by Day 5 of the study, confirming the validity of the test system. In conclusion, this study indicated that the NOEC of the Cry2Ab2 protein on adult honey bees was ≥68 µg/ml.

7. Evaluation of Potential Dietary Effects of Cry1A.105 Protein on the Ladybird Beetle, *Coleomegilla maculata* (Oleoptera: Coccinellidae). In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Paradise, M.S. 2006. Evaluation of Potential Dietary Effects of Cry1A.105 Protein on the Ladybird Beetle, Coleomegilla maculata (Coleoptera: Coccinellidae). Monsanto Technical Report MSL-20150.

The objective of this study was to examine the potential for dietary effects of the Cry1A.105 protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata* using an agar-based artificial diet. The Cry1A.105 protein was incorporated at 240 µg/g of diet, which resulted in a safety factor of approximately 27X based on the maximum Cry1A.105 protein expression level (8.8 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative controls (25 mM CAPS buffer, purified water) and a positive control (100 µg potassium arsenate/g diet) were included in the study. The results showed that there were no significant differences for the mean survival percentage of *C. maculata* among the Cry1A.105 protein treatment group (88.5%), buffer control group (87.5%), and the water control group (91.6%). The positive control group (potassium arsenate) only produced a survival rate of 2.08%, confirming the validity of the test system. Likewise, there were no significant differences for the mean percentage of *C. maculata* larvae that developed to adults among the Cry1A.105 protein treatment group (88.5%), buffer control group (85.4%), and the water control group (90.6%). None of the insects in the potassium arsenate positive control group developed to the adult stage. In addition, there were no significant differences in the mean weight of *C. maculata* adults among the Cry1A.105 protein treatment group (10.8 mg) and the buffer control group (11.2 mg). In conclusion, the NOEC of Cry1A.105 on ladybird beetle was ≥240 µg/g of diet.

8. Evaluation of Potential Dietary Effects of Cry2Ab2 Protein on the Ladybird Beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae).

Paradise, M.S. 2006. Evaluation of Potential Dietary Effects of Cry2Ab2 Protein on the Ladybird Beetle, Coleomegilla maculata (Coleoptera: Coccinellidae). Monsanto Technical Report MSL-20151.

The objective of this study was to examine the potential for dietary effects of the Cry2Ab2 protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata* using an agar-based artificial diet. The test substance was incorporated at 120

µg Cry2Ab2 protein/g of diet, which resulted in a safety factor of approximately 255X based on the maximum Cry2Ab2 protein expression level (0.47 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative controls (50 mM CAPS buffer, purified water) and a positive control (100 µg potassium arsenate/g diet) were included in the study. The results showed that there were no significant differences for the mean survival percentage of *C. maculata* among the Cry2Ab2 protein treatment group (94.7%), buffer control group (88.8%), and the water control group (91.6%). The positive control group (potassium arsenate) only produced a survival rate of 2.08%, confirming the validity of the test system. Likewise, there were no significant differences for the mean percentage of *C. maculata* larvae that developed to adults among the Cry2Ab2 protein treatment group (92.6%), buffer control group (85.3%), and the water control group (90.6%). None of the insects in the potassium arsenate positive control group developed to the adult stage. In addition, there were no significant differences in the mean weight of *C. maculata* adults among the Cry2Ab2 protein treatment group (10.3 mg) and the buffer control group (10.5 mg). In conclusion, the NOEC of Cry2Ab2 on ladybird beetle was ≥120 µg/g of diet.

9. Evaluation of Potential Dietary Effects of Cry1A.105 Protein on Minute Pirate Bugs, *Orius insidiosus* (Hemiptera: Anthocoridae). In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Teixeira, D. 2006. Evaluation of Potential Dietary Effects of Cry1A.105 Protein on Minute Pirate Bugs, Orius insidiosus (Hemiptera: Anthocoridae). Monsanto Technical Report MSL-20170.

The purpose of this study was to determine the potential dietary effects of the Cry1A.105 protein on mortality and development of minute pirate bugs, *Orius insidiosus*. The Cry1A.105 protein was incorporated into a pollen-based diet for treatment of the test group. A buffer control group was fed with the base pollen diet treated with 25 mM CAPS buffer. An assay control group was fed with the base pollen diet only. In addition, a positive control group was fed the base pollen diet treated with 15 µg potassium arsenate/g of diet, which demonstrated the validity of the test system. The study was first started with a test at the maximum dose level of 240 µg Cry1A.105 protein/g of diet. This treatment produced a mean survival rate of 47% for *Orius insidiosus*, while 88% survival rate was observed in the buffer control and assay control groups in this initial test. Based on the results of this maximum dose test, three dose-response tests were conducted with Cry1A.105 exposure levels at 30, 60, 120, and 240 µg/g of diet. Each dose-response test was performed independently at a different time using separate groups of *Orius*. For the three dose response replicate tests, mean survival of 55% was observed for the 240 µg Cry1A.105/g of diet treatments. In the buffer control and assay control groups, mean survival rates of 89% and 91%, respectively, were observed in the dose response tests. Based on the results of these dose response tests, the LC_{50} value was empirically determined to be >240 µg Cry1A.105/g of diet. The recorded endpoints of measurement (survival and development) of each dose-response replicate in the 30, 60, and 120 µg Cry1A.105/g of diet treated *Orius* was equal to or greater than those of the

buffer control and assay control groups. Therefore, the NOEC of Cry1A.105 to *Orius* was ≥120 µg/g of diet, which resulted in a safety factor of 14X based on the maximum Cry1A.105 protein expression level (8.8 µg/g fwt) in pollen from MON 89034.

10. Evaluation of Potential Dietary Effects of Cry2Ab2 Protein on Minute Pirate Bugs, *Orius insidiosus* (Hemiptera: Anthocoridae). In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Teixeira, D. 2006. Evaluation of Potential Dietary Effects of Cry2Ab2 Protein on Minute Pirate Bugs, Orius insidiosus (Hemiptera: Anthocoridae). Monsanto Technical Report MSL-20171.

The purpose of this study was to determine the potential dietary effects of the Cry2Ab2 protein on mortality and development of minute pirate bugs, *Orius insidiosus*. The Cry2Ab2 protein was incorporated into a pollen-based diet at 100 µg/g of diet for treatment of the test group, which was equivalent to a safety factor of approximately 213X based on the maximum Cry2Ab2 protein expression level (0.47 µg/g fwt) in MON 89034 pollen. A buffer control group was fed with the base pollen diet treated with 50 mM CAPS buffer. An assay control group was fed with the base pollen diet only. In addition, a positive control group was fed the base pollen diet treated with 15 µg potassium arsenate/g of diet. The results demonstrated that the test substance treatment (100 µg Cry2Ab2/g of diet) produced a mean survival rate of 91% for *Orius insidiosus*, which was similar to the survival rates of the buffer control group (81%) and the assay control group (88%). Statistical analysis detected no significant differences in the survival percentage between the Cry2Ab2 protein treatment, buffer control and assay control treatments. The percent survival for the *Orius* exposed to the potassium arsenate positive control group was 40%, confirming the validity of the test system. The percent of nymphs that developed to adults for the Cry2Ab2 treatment, buffer control, and assay control treatments was 93%, 91%, and 95%, respectively. In conclusion, the NOEC of Cry2Ab2 to *Orius insidiosus* was ≥100 µg/g of diet.

11. Evaluation of Potential Effects of Exposure to Cry1A.105 Protein in an Acute Study with the Parasitic Wasp, *Ichneumon promissorius* (Hymenoptera: Ichneumonidae). In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Sindermann, A.B., J.R. Porch, and H.O. Krueger. 2006. Evaluation of Potential Effects of Exposure to Cry1A.105 Protein in an Acute Study with the Parasitic Wasp, Ichneumon promissorius (Hymenoptera: Ichneumonidae). Monsanto Technical Report MSL-20149.

The objective of this study was to evaluate the potential effects of acute exposure to the Cry1A.105 protein administered to the non-target parasitic wasp, *Ichneumon promissorius*. Parasitic wasps were exposed to the test substance Cry1A.105 protein at a concentration of 240 µg/ml in a 30% sucrose solution diet, which resulted in a safety factor of approximately 27X based on the maximum Cry1A.105 protein expression level

(8.8 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative controls (25 mM CAPS buffer in 30% sucrose and 30% sucrose alone) and positive controls (100 and 400 µg/ml potassium arsenate in 30% sucrose) were included in the study. The results showed that at test termination on Day 21, the mortality in the assay control group (30% sucrose alone), control substance group (25 mM CAPS buffer in 30% sucrose), and the Cry1A.105 protein treatment group was 10%, 8%, and 7%, respectively. There was no significant difference ($p>0.05$) in mean mortality between the Cry1A.105 protein treatment group and the control substance group. All surviving wasps were normal in appearance and behavior. The mortality rates for the positive controls of 100 and 400 µg/ml potassium arsenate in 30% sucrose were 70% and 97%, respectively, confirming the validity of the test system. In conclusion, the NOEC of the Cry1A.105 protein for the parasitic wasp, *Ichneumon promissorius*, was ≥240 µg/ml.

12. Evaluation of Potential Effects of Exposure to Cry2Ab2 Protein in an Acute Study with the Parasitic Wasp, *Ichneumon promissorius* (Hymenoptera: Ichneumonidae). In the summary of this study for the petition, explain what the safety factor is based on (e.g., 10X expression in pollen).

Sindermann, A.B., J.R. Porch, and H.O. Krueger. 2006. Evaluation of Potential Effects of Exposure to Cry2Ab2 Protein in an Acute Study with the Parasitic Wasp, Ichneumon promissorius (Hymenoptera: Ichneumonidae). Monsanto Technical Report MSL-20148.

The objective of this study was to evaluate the potential effects of acute exposure to the Cry2Ab2 protein administered to the non-target parasitic wasp, *Ichneumon promissorius*. Parasitic wasps were exposed to the test substance Cry2Ab2 protein at a concentration of 100 µg/ml in a 30% sucrose solution diet, which resulted in a safety factor of approximately 213X based on the maximum Cry2Ab2 protein expression level (0.47 µg/g fwt) in pollen from MON 89034. In addition, appropriate negative controls (50 mM CAPS buffer in 30% sucrose and 30% sucrose alone) and positive controls (100 and 400 µg/ml potassium arsenate in 30% sucrose) were included in the study. The results showed that at test termination on Day 21, the mortality in the assay control group (30% sucrose alone), control substance group (50 mM CAPS buffer in 30% sucrose), and the Cry2Ab2 protein treatment group was 10%, 3%, and 3%, respectively. There was no significant difference ($p>0.05$) in mean mortality between the Cry2Ab2 protein treatment group and the control substance group. All surviving wasps were normal in appearance and behavior. The mortality rates for the positive controls of 100 and 400 µg/ml potassium arsenate in 30% sucrose were 70% and 97%, respectively, confirming the validity of the test system. In conclusion, the NOEC of the Cry2Ab2 protein for the parasitic wasp, *Ichneumon promissorius*, was ≥100 µg/ml.

13. Evaluation of Potential Dietary Effects of MON 89034 with the Northern Bobwhite: an Eight-day Dietary Study with Corn Grain. An explanation of how this study can be used in a risk assessment for migratory birds should be included.

Gallagher, S.P. and J.B. Beavers. 2006. Evaluation of Potential Dietary Effects of MON 89034 with the Northern Bobwhite: an Eight-day Dietary Study with Corn Grain. Monsanto Technical Report WL-2005-012.

The objective of this study was to examine the potential of effects from a dietary exposure to corn grain from MON 89034 to bobwhite quail (*Colinus virginianus*). Bobwhites are a commonly used surrogate species to develop data on dietary toxicity and are one of EPA's preferred test species. The test procedure followed the methodology of U.S. EPA ecological effects test guideline OPPTS Guideline Number 850.2200, which provides specific guidance for testing bobwhite. Groups of 30 bobwhite quail, 10 days of age, were fed basal diets (with the corn component removed) mixed with either finely ground test, control, or reference corn grain at a limit dose of 50% by weight of the diet for eight days. The control substance was a corn grain from a conventional corn line that has a similar genetic background to MON 89034, and the reference substances were three conventional corn varieties. The results showed that there was no mortality in the test group fed diet containing 50% MON 89034, and all the birds in the test group were normal in appearance and behavior throughout the test. There was a single incidental mortality in the control substance group and in two of the reference groups. There were no statistically significant ($p>0.05$) treatment-related effects on body weight and feed consumption among birds fed MON 89034 when compared with the control group. Therefore, the 8-day dietary NOEC was determined to be ≥ 50% corn grain from MON 89034.

The results from the short-term study with bobwhite demonstrate that no significant risk to wild avian species is anticipated from consumption of grain from MON 89034. This study is considered to be acceptable for assessing short-term risk to wild migratory bird populations because: 1) the study followed accepted methodology for assessing short-term risk to wild avian populations and 2) juvenile birds were tested at a high dietary level of corn grain from MON 89034.

Update on USDA Notification Trials

In the Agency's letter, it was stated that "All field test reports for trials terminated six months or more ago should be submitted. Appendix H: USDA Notifications Approved for MON 89034 in the petition should be amended to indicate that all field test reports that are due have been submitted particularly those from 2005 that are outstanding".

Table H-1 has been amended to include the most recent updated trial status as of January 23, 2007 (the **Amended Table H-1** is attached, starting from the next page). All field test reports that are due have been submitted to the USDA. The USDA regulations specify that a field test report be submitted to the agency within 6 months after termination of the field test. We have noted the most recent guidance on the USDA web site, USDA-APHIS Biotechnology Regulatory Services User's Guide, "Chapter 6, Notification" which states that APHIS considers the field test report to be due no later than 6 months after expiration of the notification. Monsanto follows this most recent guidance on the due date of field test reports.

Amended Table H-1. USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial/Report Status
2001 Field Trials			
01-348-04n	1/13/2002	PR (2)	Submitted to USDA
01-348-07n	1/11/2002	HI (6)	Submitted to USDA
2002 Field Trials			
02-009-12n	2/8/2002	IL (3)	Submitted to USDA
02-009-15n	2/8/2002	IA (6)	Submitted to USDA
02-017-12n	3/20/2002	AL, MS, OR, TN, WA	Submitted to USDA
02-242-10n	10/1/2002	PR (2)	Submitted to USDA
2003 Field Trials			
03-024-06n	2/23/2003	AL MS	Submitted to USDA
03-034-25n	3/5/2003	OR, TN (2)	Submitted to USDA
03-034-27n	3/5/2003	IL (2)	Submitted to USDA
03-042-06n	3/13/2003	MO	Submitted to USDA
03-043-04n	3/17/2003	IL (11)	Submitted to USDA
03-043-05n	3/17/2003	IA (11)	Submitted to USDA
03-052-57n	3/23/2003	TN	Submitted to USDA
03-161-05n	8/5/2003	HI (5)	Submitted to USDA
03-219-04n	9/5/2003	PR (2)	Submitted to USDA
03-265-09n	11/21/2003	HI (6)	Submitted to USDA
03-280-01n	11/6/2003	PR (3)	Submitted to USDA
2004 Field Trials			
04-009-01n	3/25/2004	HI (5)	Submitted to USDA
04-009-02n	3/25/2004	PR (4)	Submitted to USDA
04-014-05n	2/26/2004	GA	Submitted to USDA
04-014-08n	2/13/2004	TN (2)	Submitted to USDA
04-021-02n	2/20/2004	AL, MS	Submitted to USDA
04-021-09n	2/20/2004	IA (2), IL (4), IN, KS, NE, OH	Submitted to USDA
04-021-11n	2/20/2004	AR	Submitted to USDA
04-021-14n	2/20/2004	TN	Submitted to USDA
04-023-09n	2/25/2004	IA (12)	Submitted to USDA
04-023-10n	2/25/2004	IA (8)	Submitted to USDA
04-023-11n	2/25/2004	WI (3)	Submitted to USDA
04-028-10n	3/24/2004	MN (5)	Submitted to USDA
04-028-23n	2/27/2004	TN	Submitted to USDA
04-028-24n	2/27/2004	CO (3), NE (4)	Submitted to USDA
04-028-25n	2/27/2004	IA (4)	Submitted to USDA
04-028-26n	2/27/2004	OR (2), WA	Submitted to USDA
04-028-27n	2/27/2004	IL (15)	Submitted to USDA
04-028-28n	2/27/2004	KS (2)	Submitted to USDA

Amended Table H-1 continues on next page.

Amended Table H-1 (continued). USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial/Report Status
04-028-29n	2/27/2004	IN (4)	Submitted to USDA
04-028-30n	2/27/2004	MI (2)	Submitted to USDA
04-028-31n	2/27/2004	MO (2)	Submitted to USDA
04-028-32n	2/27/2004	NE (4)	Submitted to USDA
04-028-33n	2/27/2004	OH (2)	Submitted to USDA
04-040-01n	4/2/2004	MD (2)	Submitted to USDA
04-040-02n	3/12/2004	NE	Submitted to USDA
04-040-03n	3/10/2004	IL (6)	Submitted to USDA
04-041-03n	3/11/2004	IL (10)	Submitted to USDA
04-041-04n	3/11/2004	KS (2)	Submitted to USDA
04-044-07n	3/14/2004	IA (6)	Submitted to USDA
04-056-06n	3/26/2004	IA (2), IL (2), IN, MO (3)	Submitted to USDA
04-079-02n	4/26/2004	IN, MN, MO, WI	Submitted to USDA
04-085-15n	4/24/2004	NE (3)	Submitted to USDA
04-090-03n	6/18/2004	HI (5)	Submitted to USDA
04-093-03n	5/2/2004	IL	Submitted to USDA
04-093-04n	5/2/2004	IA	Submitted to USDA
04-189-03n	9/27/2004	HI (15)	Submitted to USDA
04-217-02n	9/20/2004	PR (2)	Submitted to USDA
04-231-03n	9/27/2004	PR (3)	Submitted to USDA
04-260-01n	11/1/2004	HI	Submitted to USDA
04-273-01n	10/18/2004	IA, IL (2), NE	Submitted to USDA
04-274-08n	10/28/2004	PR (2)	Submitted to USDA
04-309-03n	1/10/2005	HI (9)	Submitted to USDA
04-309-04n	1/10/2005	PR (3)	Submitted to USDA
04-337-02n	12/16/2004	MS	Submitted to USDA
04-337-03n	12/20/2004	AL	Submitted to USDA
04-358-20n	1/25/2005	GA (2)	Submitted to USDA
04-358-21n	1/24/2005	IA (14)	Submitted to USDA
04-358-22n	1/24/2005	IA (15)	Submitted to USDA
04-358-23n	1/24/2005	IL (11)	Submitted to USDA
04-358-24n	1/25/2005	IN (3)	Submitted to USDA
04-358-25n	1/24/2005	KS (2)	Submitted to USDA
04-358-26n	2/7/2005	MD	Submitted to USDA
04-358-27n	1/26/2005	MI (3)	Submitted to USDA
04-358-28n	2/9/2005	MN (8)	Submitted to USDA
04-358-29n	1/24/2005	MO (2)	Submitted to USDA
04-358-30n	1/25/2005	NE (5)	Submitted to USDA
04-358-31n	1/25/2005	OH (2)	Submitted to USDA
04-358-32n	1/27/2005	TN (2)	Submitted to USDA
04-358-33n	1/25/2005	WI (3)	Submitted to USDA
04-362-09n	1/18/2005	TN (3)	Submitted to USDA
04-362-11n	1/18/2005	AR	Submitted to USDA

Amended Table H-1 continues on next page.

Amended Table H-1 (continued). USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial/Report Status
2005 Field Trials			
05-025-04n	2/22/2005	IA, IL (3), IN, KS, NE, OH (2)	Submitted to USDA
05-026-19n	2/14/2005	IL (8), IN, WI (3)	Submitted to USDA
05-028-02n	2/14/2005	CO (5), KS, NE (3)	Submitted to USDA
05-028-11n	2/22/2005	IA (4), IL (9)	Submitted to USDA
05-028-12n	2/22/2005	HI (4)	Submitted to USDA
05-039-09n	3/10/2005	NE, OR, WA	Submitted to USDA
05-039-11n	3/28/2005	IA (2) IL (2), IN (3), MI, MN, MO (2), NE, NY, WI	Submitted to USDA
05-040-15n	3/10/2005	LA	Submitted to USDA
05-049-13n	3/24/2005	IA (5), IL (2), IN (2), NE (4), TX (2)	Submitted to USDA
05-055-03n	4/11/2005	IL (2), MN (5), MO (2)	Submitted to USDA
05-056-10n	4/1/2005	MO	Submitted to USDA
05-063-06n	4/1/2005	IL (2)	Submitted to USDA
05-104-03n	5/17/2005	HI (10)	Submitted to USDA
05-129-01n	5/31/2005	NE (6)	Submitted to USDA
05-131-01n	6/9/2005	LA	Submitted to USDA
05-133-05n	7/20/2005	HI (4), PR (2)	Submitted to USDA
05-201-06n	9/26/2005	PR (3)	In progress*
05-201-10n	9/6/2005	HI (5)	In Progress*
05-201-11n	9/26/2005	PR (3)	In Progress*
2006 Field Trials			
06-025-04n	3/30/2006	AL, AR, LA, MS (2), TN (2)	In Progress
06-027-06n	2/27/2006	GA (4)	In Progress
06-027-07n	3/6/2006	OH (2)	In Progress
06-027-08n	3/6/2006	WI (3)	In Progress
06-027-09n	3/7/2006	MN (9)	In Progress
06-027-11n	3/1/2006	MI (3)	In Progress
06-027-12n	2/27/2006	NE (4)	In Progress
06-027-13n	2/27/2006	MO (2)	In Progress
06-027-14n	2/27/2006	TN (4)	In Progress
06-027-15n	2/27/2006	KS (2)	In Progress
06-027-17n	4/5/2006	IA (5)	In Progress
06-030-01n	2/28/2006	IN (4)	In Progress
06-030-02n	2/28/2006	IL (13)	In Progress
06-030-04n	3/7/2006	IA (12)	In Progress
06-030-05n	3/7/2006	IA (13)	In Progress
06-030-06n	2/28/2006	IL (12)	In Progress
06-031-25n	2/28/2006	NE (2)	In Progress
06-032-12n	4/5/2006	IA (12)	In Progress

Amended Table H-1 continues on next page.

Amended Table H-1 (continued). USDA notifications approved for MON 89034 and status of trials conducted under these notifications.

USDA No.	Effective Date (m/d/y)	Release Site (State)	Trial/Report Status
06-033-07n	5/18/2006	HI (7)	In Progress
06-037-08n	5/18/2006	HI (5)	In Progress
06-037-10n	5/26/2006	PR (3)	In Progress
06-037-11n	3/16/2006	PR (3)	In Progress
06-038-05n	4/6/2006	IA, IL (3), IN (2), TX (3)	In Progress
06-046-06n	3/23/2006	CO (5), NE (2)	In Progress
06-046-10n	3/16/2006	IL (4), MO (5)	In Progress
06-046-11n	4/17/2006	IA (8), NE (6), SD	In Progress
06-052-11n	3/23/2006	MS	In Progress
06-060-09n	4/24/2006	IA (2)	In Progress
06-060-10n	4/24/2006	IA (14)	In Progress
06-073-01n	6/12/2006	IA (3), IL (4), IN (2), KS, MN	In Progress
06-073-02n	5/5/2006	IA (7), IL (5), NE, OH (2)	In Progress
06-081-02n	5/9/2006	IA	In Progress
06-082-05n	5/18/2006	HI (3), PR (2)	In Progress
06-089-11n	5/15/2006	IA (5), IL, NE	In Progress
06-090-04n	5/18/2006	HI (6)	In Progress
06-090-05n	6/12/2006	PR (3)	In Progress
06-090-06n	5/18/2006	HI (5)	In Progress
06-090-07n	5/26/2006	PR (3)	In Progress
06-108-03n	5/26/2006	PR	In Progress
06-108-04n	5/26/2006	HI, IN (2), PR	In Progress
06-108-05n	5/18/2006	HI, PR (2)	In Progress
06-108-06n	5/26/2006	HI, IN	In Progress
06-114-03n	5/26/2006	HI, IL	In Progress
06-118-02n	5/9/2006	IA	In Progress

***** It is noted that as of January 23, 2007, when this addendum is made, there are three notifications for 2005 field trials of which the reports have not been submitted. Among the three notifications, no plantings occurred under 05-201-06n, and a field test report will be submitted to that effect. The two field trials under notifications 05-210-10n and 05-201-11n are still in progress. These two notifications involve field trials in continuous planting situations in Hawaii and Puerto Rico. We plan to submit these reports prior to the due date as defined by 6 months after notification expiration (as shown in the table below).

USDA No.	Trial/Report Status	Notification effective date	Notification Expiration	6 months after termination date[δ]	6 months after notification expiration
05-201-06n	In progress	9/26/2005	9/26/2006	Not planted	3/26/2007
05-201-10n	In progress	9/6/2005	9/6/2006	2/28/2007	3/6/2007
05-201-11n	In progress	9/26/2005	9/26/2006	3/11/2007	3/26/2007

δ Termination date refers to the destruction of the last plants in the trials.

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May 18, 2007

Dr. Neil Hoffman
Director, Environmental Risk Analysis Program
USDA/APHIS/BRS
4700 River Road, Unit 147
Riverdale, MD 20737

Re: Addendum to Section VIII (Environmental Assessment and Impact on Agronomic Practices) of USDA Petition No. 06-298-01p (i.e., Monsanto Company File No. 06-CR-166U) for Determination of Non-regulated Status for MON 89034

Dear Dr. Hoffman:

We are submitting an addendum to Section VIII of USDA Petition No. 06-298-01p (Petition) submitted by Monsanto Company (Monsanto) on October 25, 2006.

This addendum provides additional information and assessments on: (1) potential cumulative impact of MON 89034 with other Bt crops on insect pest resistance development and evolution; (2) potential cumulative impact of deregulation of MON 89034 on Bt protein accumulation in the soil environment; (3) potential cumulative impact of MON 89034 in combined trait products resulted from conventional breeding with other biotechnology-derived corn products; (4) potential impact of deregulation of MON 89034 on biodiversity, preservation of corn germplasm purity, and specialty corn production; (5) potential impact of deregulation of MON 89034 on land use and agricultural production; and (6) mitigation and remediation measures before and after registration and deregulation of MON 89034 in the U.S.

Our understanding is that the National Environmental Policy Act or NEPA[1] requires the inclusion of a detailed statement "in every recommendation or report on proposals for legislation and other major Federal actions significantly affecting the quality of the human environment."[2] The Council on Environmental Quality (CEQ) regulations define

[1] 42 U.S.C. Section 4321 et seq.
[2] 42 U.S.C. § 4332(C) (2005).

"major Federal action" to include actions with effects that may be major and which are potentially subject to Federal control and responsibility.[3] Once it is clear that a proposed action triggers the requirement of NEPA and is not categorically excluded, the federal agency must decide what NEPA document to prepare. The CEQ regulations direct agencies to determine whether, under the agency's own supplemental procedures, the proposal is one which "normally requires an environmental impact statement." If so, an environmental impact statement (EIS) is prepared. If not, the agency prepares an environmental assessment (EA).[4]

We believe an EA would satisfy the NEPA requirement for the determination of non-regulated status for MON 89034 because the action is "characterized by its limited scope (particular sites, species, or activities) and potential effect (impacting relatively few environmental values or systems). Individuals and systems that may be affected can be identified. Methodologies, strategies, and techniques employed to deal with the issues at hand are seldom new or untested. Alternative means of dealing with those issues are well established. Mitigation measures are generally available and have been successfully employed."[5]

Our understanding is that an EA leads to one of two determinations; the first possibility is that the proposed action will not result in significant impacts to the human environment, in which case a "Finding of No Significant Impact" (FONSI) is prepared which documents the agency's rationale for its decision. The second possibility is that the proposed action will result in significant impacts to the human environment, in which case the agency proceeds to prepare an EIS to inform its decision-making on the proposed action.

Monsanto believes the Petition and the 38 volumes of the attachment that Monsanto submitted to APHIS on October 25, 2006, the addendum submitted on January 23, 2007, and the addendum submitted herein collectively provide substantial data, information, and evidence that MON 89034 will not have any significant impact on the human environment, i.e., a finding of no significant impact or FONSI.

Our conclusion that there are no significant environmental impacts resulting from the deregulation of MON 89034 is based on the comprehensive assessments which have taken into consideration both the "context" and "intensity" that deregulation of MON 89034 may have on the human environment. The term "context" refers to the setting within which the proposed action takes place. We have considered the potential impacts of MON 89034 on the human environment in the following context:

(i) *The affected crop:* the biology and genetics of corn, pollination and outcrossing characteristics and gene flow potential, and assessment of weediness and plant pest potential (Petition sections II, VIII.B, and VIII.C).

[3] 40 C.F.R. § 1508.18.
[4] 40 C.F.R. § 1501.4.
[5] 7 C.F.R. § 372.5

(ii) *The target insect pests, non-target organisms, and threatened and endangered species:* (Petition sections VIII.A.2 to VIII.A.5; addendum submitted on January 23, 2007; points No. 1 and 2 of the current addendum).
(iii) *Affected agricultural practices:* (Petition sections VIII.D.2, VIII.E, and VIII.F).
(iv) *Affected regions and locality:* farms in all states where corn is grown (Petition section VIII.D.1).
(v) *Affected interests:* the interests of U.S. farmers (field corn growers, specialty corn growers, organic corn growers, and farmers specializing in other major crops such as soybeans and cotton), poultry and dairy farmers who use corn as animal feeds, and grain handlers and traders (Petition section I.C; points No. 4 and 5 of the current addendum).
(vi) *Land use and agricultural production:* U.S. cropland and uses for major crops; supply and demand of corn grain for utilizations as food, feed, production of ethanol for fuel, and for export (point No. 5 of the current addendum; Petition section I.C).

With respect to the intensity element, CEQ regulations provide ten factors to guide the analysis.[6] The term "intensity" refers to the severity of the impact.[7] We have taken into consideration the potential direct, indirect, and cumulative impacts of the deregulation of MON 89034 on the human environment.

(1) Impacts that may be beneficial or adverse with respect to the use of MON 89034 in the U.S.

a. MON 89034 is expected to protect corn yields against damage caused by lepidopteran insect pests, thus increasing cropland productivity and increasing farmers' income (point No. 5 of the current addendum).
b. MON 89034 is expected to reduce chemical pesticide use in corn and reduce the environmental impact of the conventional agricultural production of corn (point No. 5 of the current addendum).
c. MON 89034 is expected to reduce mycotoxin levels commonly found in conventional corn grain. Lower levels of mycotoxin in the grain are beneficial to human and animal health (Petition section I.C.; point No. 5 of the current addendum).
d. MON 89034 is expected to provide an effective insect resistance management (IRM) tool and increase the durability of Bt corn technology (Petition sections I.C. and VIII.F; points No. 1 and 6 of the current addendum).
e. MON 89034 is not likely to have any impact on the weediness potential of corn and raises no concerns of ourcrossing with wild relatives of corn in the U.S.; and unintended cross pollination between corn varieties targeted for different market segments is manageable under the well-established agricultural practices and marketing standards in the U.S. (Petition sections II, VIII.B and VIII.C; point No. 4 of the current addendum).

[6] 40 C.F.R. § 1508.27.
[7] 40 C.F.R. § 1508.27(b).

f. MON 89034 is not expected to have any adverse impact on current agricultural practices (Petition section VIII.D; point No. 5 of the current addendum).
g. MON 89034 is not likely to pose any risk to non-target organisms, and endangered and threatened species (Petition sections VIII.A.2 to VIII.A.5; addendum submitted January 23, 2007; points No. 1 and 2 of the current addendum). MON 89034 contains two insecticidal proteins, therefore, this product is also subject to regulatory review and oversight by EPA. Monsanto has filed applications to the EPA for the registration of the plant-incorporated protectants Cry1A.105 and Cry2Ab2 proteins, and the genetic material (vector PV-ZMIR245) necessary for their production. EPA registration processes include extensive assessments regarding potential effects of MON 89034 on non-target organisms, and threatened and endangered species. A copy of Monsanto's submission to EPA was provided to APHIS as an attachment (38 volumes in total) to the Petition on October 25, 2006. It is our understanding that APHIS consults with EPA and confirms EPA's regulatory responsibility with respect to assessment of potential effects of MON 89034 on non-target organisms including threatened and endangered species.

(2) The degree to which the proposed action affects public health or safety.

a. MON 89034 has no potential adverse effect on human and animal health. The food and feed derived from MON 89034 are at least as safe and nutritious as those derived from corn varieties currently in commerce. MON 89034 is expected to reduce mycotoxin levels in corn grain and thereby provide added benefit to human and animal health compared to conventional corn grain (Petition section I.C.; point No. 5 of the current addendum). Under the "Coordinated Framework for the Regulation of Biotechnology"[8], the responsibilities of regulatory oversight of biotechnology-derived crops fall on three lead federal agencies, including USDA, EPA, and FDA. Monsanto has submitted a premarket notification entitled "Food and Feed Safety and Nutritional Assessment of the Lepidopteran-Protected Corn MON 89034" to the FDA. The key conclusions of that assessment have been included in the Petition section VI.D. It is our understanding that APHIS consults with FDA and confirms FDA's regulatory responsibility over the food and feed safety assessment on MON 89034. FDA is responsible for all the food and feed safety issues except the safety of the two insecticidal proteins which falls into the jurisdiction of EPA.
b. Monsanto has filed applications to the EPA for the registration of the plant-incorporated protectants Cry1A.105 and Cry2Ab2 proteins, and the genetic material (vector PV-ZMIR245) necessary for their production. In July 2006, EPA established a temporary exemption from the requirement of a tolerance for Cry1A.105 protein and the genetic material necessary for its production in all corn commodities (40 CFR §174.453). Likewise, temporary exemption from the requirement of a tolerance for Cry2Ab2 protein and the genetic material necessary for its production in corn was also granted by EPA in July 2006 (40 CFR

[8] Federal Register. 1986. Coordinated framework for regulation of biotechnology; announcement of policy. 1986 June 26; 51(123):23302-50.

§174.454). Monsanto is petitioning EPA to amend 40 CFR §174.453 and 40 CFR §174.454 by removing the time limitation for the exemption from the requirement of a tolerance for the Cry1A.105 and Cry2Ab2 proteins. It is expected that the EPA review will conclude with the registration of the MON 89034 and the issuance of tolerance exemption for the two proteins relative to the food and feed safety.

(3) Unique characteristics of the geographic area such as proximity to historic or cultural resources, park lands, prime farmlands, wetlands, wild and scenic rivers, or ecologically critical areas.

Like any other corn variety, MON 89034 will be grown in existing cropland (point No. 5 of the current addendum). As such, the planting of MON 89034 is not likely to affect historic or cultural resources, park lands, wetlands, wild and scenic rivers, or ecologically critical areas in any way different from the cultivation of current corn varieties. MON 89034 is substantially equivalent to conventional corn except for the lepidopteran protection trait (Petition section VII). MON 89034 has no plant pest risk compared to conventional corn (Petition section VIII.B). MON 89034 poses no significant risk to endangered butterflies and moths in the order *Lepidoptera* (Petition section VIII.A.4).

(4) The degree to which the effects on the quality of the human environment are likely to be highly controversial.

The term "controversial" as used in the NEPA context, based on CEQ guidelines, refers not to the amount of public opposition, but to where there is a substantial dispute as to the size, nature, or effect of the major federal action.[9] Although there is general opposition from certain groups or individuals to plant biotechnology in general, there is no substantial dispute as to the size, nature, or effect of MON 89034 corn on the human environment based on scientific data and evidence.

(5) The degree to which the possible effects on the human environment are highly uncertain or involve unique or unknown risks.

MON 89034 is a lepidopteran insect protected Bt corn product. Similar Bt corn products (such as events MON 810, line 1507, and Bt11) have completed the regulatory processes by EPA, FDA, and USDA, and have been successfully cultivated by farmers in the U.S. and other countries in large areas for nearly a decade. In 2006, 40% of corn acreage in the U.S. was planted with corn varieties possessing Bt traits, including lepidopteran protection or coleopteran protection, or the combined Bt trait products. The cultivation and consumption records and experiences of Bt corn have shown, with a great degree of certainty, significant beneficial impacts and no adverse impacts on human and animal health and the environment. Similar conclusions of no significant adverse impacts on human

[9] Hanley v. Kleindienst, 471 F.2d 823 (2d Cir. 1972), cert. denied, 412 U.S. 908 (1973).

environment have been reached for MON 89034 [Petition sections VI, VII, and V.III; points No. 5 of the current addendum; also refer to the above points (1) and (2)].

(6) The degree to which the action may establish a precedent for future actions with significant effects or represents a decision in principle about a future consideration.

The review, approval and deregulation of biotechnology-derived crops have been conducted on case-by-case basis. There have been 71 deregulations by USDA-APHIS from 1992 to April 2007.[10] There is no indication that APHIS or any other federal agencies intend to use deregulation of MON 89034 as a precedent for future biotechnology-derived crop deregulations or registrations.

(7) Whether the action is related to other actions with individually insignificant but cumulatively significant impacts. Significance exists if it is reasonable to anticipate a cumulatively significant impact on the environment. Significance cannot be avoided by terming an action temporary or by breaking it down into small component parts.

"Cumulative impact" is the impact on the environment which results from the incremental impact of the action when added to other past, present, and reasonably foreseeable future actions regardless of what agency (Federal or non-Federal) or person undertakes such other actions. Cumulative impacts can result from individually minor but collectively significant actions taking place over a period of time.[11] The attached addendum provides, as indicated below, additional assessments regarding potential cumulative impacts of MON 89034, taking into considerations other biotechnology-derived crops deregulated and cultivated in the past and present, and those likely to be cultivated in the future (points No. 1, 2, 3, and 4 of the current addendum). These assessments concluded that MON 89034 is unlikely to pose a significant impact on the human environment considering the past, present, and likely future deregulations and approvals of biotechnology-derived crops.

(8) The degree to which the action may adversely affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or may cause loss or destruction of significant scientific, cultural, or historical resources.

Like any other corn variety, MON 89034 will be grown on existing cropland (point No. 5 of the current addendum). As such, it is not likely to affect districts, sites, highways, structures, or objects listed in or eligible for listing in the National Register of Historic Places or may cause loss or destruction of significant scientific, cultural, or historical resources.

[10] Petitions of Nonregulated Status Granted or Pending by APHIS.
http://www.aphis.usda.gov/brs/not_reg.html
[11] 40 C.F.R. § 1508.7

(9) The degree to which the action may adversely affect an endangered or threatened species or its habitat that has been determined to be critical under the Endangered Species Act of 1973.

The potential risks to non-target organisms, and threatened and endangered species by the introduction of MON 89034 has been assessed by Monsanto and the assessment has been submitted to EPA for review. A summary of the assessment have been included in the Petition section VIII.A. It was concluded that MON 89034 poses no significant risk to these organisms. This conclusion is consistent with other approved Bt corn products. Substantial number of studies conducted over the past decade by industry and the academic community on registered and deregulated insect-protected crops that produce a variety of Cry proteins have demonstrated that these crops have no adverse effects on biodiversity, tested populations of natural enemies, and other ecologically important non-target arthropods. Importantly, when expressed in Bt crops, even sensitive non-target lepidopteran and coleopteran species have been shown to be exposed to very low levels of the Cry proteins. The exposure levels were too low to pose a significant risk to populations of the sensitive non-target species including threatened and endangered species (Petition sections VIII.A3, VIII.A.4, and VIII.A5).

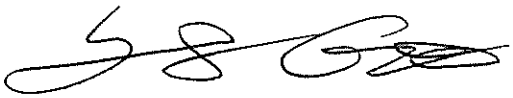
(10) Whether the action threatens a violation of Federal, State, or local law or requirements imposed for the protection of the environment.

There is no indication that this action would violate Federal, State, or local law or requirements imposed for the protection of the environment. Concurrent jurisdictions exist among federal agencies, namely, USDA, EPA, and FDA under the "Coordinated Framework for the Regulation of Biotechnology" regarding the regulation of biotechnology-derived crops. As mentioned above, Monsanto has submitted registration or premarket consultation documents to EPA and FDA, in addition to the Petition to USDA-APHIS (point No. 6 of the current addendum). Furthermore, a number of states have statutes or regulations on the cultivation and environmental release of biotechnology-derived insect protected crops, such as Bt corn. Monsanto will seek registrations and approvals from those states in which it plans to market MON 89034 prior to the commercialization. Deregulation of MON 89034 by USDA does not serve as a substitute for the approval or registration requirement of other federal or state agencies but constitutes an essential element towards overall regulatory compliance for this product.

In conclusion, the Petition, the prior submitted addendum, and the addendum attached herein, together, provide substantial evidence that MON 89034 is not likely to pose any significant impact on the human environment. Monsanto requests a determination from APHIS that MON 89034 and any progeny derived from crosses between MON 89034 and other commercial corn varieties be granted non-regulated status.

Thank you very much for your attention. In addition to the paper copy, a compact disc (CD) is also provided with files of this letter and the addendum, and the non-CBI version of the petition attached by the addendum. Should you have any questions regarding the Petition, please feel free to contact Dr. Russell Schneider, Monsanto Regulatory Affairs Director in Washington DC at (202) 383-2866, or me at (314) 694-2943.

Sincerely,



Yong Gao, PhD
Regulatory Affairs Manager

Encl.

cc: Dr. Russell Schneider
Ms. Carolyn Carrera

Addendum to Section VIII (Environmental Assessment and Impact on Agronomic Practices) of USDA Petition No. 06-298-01p (i.e., Monsanto Company File No. 06-CR-166U) for Determination of Non-regulated Status for MON 89034

May 18, 2007

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Addendum to Section VIII (Environmental Assessment and Impact on Agronomic Practices) of USDA Petition No. 06-298-01p (i.e., Monsanto Company File No. 06-CR-166U) for Determination of Non-regulated Status for MON 89034

Summary

This addendum provides additional information and assessments to **section VIII** of Monsanto Company (Monsanto) File No. 06-CR-166U for the "Determination of Non-regulated Status for MON 89034" (Petition). Specifically, the addendum addresses the following topics: (1) potential cumulative impact of MON 89034 with other Bt crops on insect pest resistance development and evolution; (2) potential cumulative impact of deregulation of MON 89034 on Bt protein accumulation in the soil environment; (3) potential cumulative impact of MON 89034 in combined trait products resulted from conventional breeding with other biotechnology-derived corn products; (4) potential impact of deregulation of MON 89034 on biodiversity, preservation of corn germplasm purity, and specialty corn production; (5) potential impact of deregulation of MON 89034 on land use and agricultural production; and (6) mitigation and remediation measures before and after registration and deregulation of MON 89034 in the U.S.

The introduction of MON 89034 in the U.S. is not expected to result in an increase in the risk of resistance development and evolution in the target pests to the Cry proteins. This conclusion takes into consideration the potential cumulative impact from Bt crops approved in the past, present, and likely to be introduced in the future. MON 89034 will be introduced with an insect resistance management (IRM) plan under the regulatory oversight of EPA. The effectiveness of this plan will be monitored through surveys of target pest susceptibility, and the implementation of the plan will be tracked through a Compliance Assurance Program (CAP) that is comparable to the CAP used for current Bt corn products. Adoption of MON 89034 should reduce the risk of the resistance evolution in lepidopteran pests towards Cry proteins because MON 89034 will provide two Bt proteins (Cry1A.105 and Cry2Ab2) that are independently active against the lepidopteran pests of corn. Also, by introducing distinctive properties in insecticidal modes of action, the selection pressure on Cry1Ab and Cry1F, the active ingredients of the existing lepidopteran protected corn products, will be reduced.

There is no significant risk of any potential cumulative adverse impact from Cry1A.105 and Cry2Ab2 proteins derived from MON 89034 to the environment and soil-dwelling organisms. Numerous studies conducted in both laboratories and fields demonstrated little or no cumulative impact on the soil microflora from the use of biotechnology-derived crops expressing Cry proteins. In the past decade, a number of Bt corn and Bt cotton products have been approved and commercialized in the U.S. and around the world. The active insecticidal Cry proteins in these crops include Cry1Ab, Cry1Ac, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, Cry35Ab1, and Cry3A proteins. Many laboratory soil degradation studies have been conducted with these Cry proteins. In most studies, a rapid initial dissipation of the Cry proteins was observed, sometimes followed by a slower decline of the remaining soil-bound trace levels of the Cry proteins. In

addition, a number of field monitoring studies have been conducted to assess the potential accumulation of Cry proteins following several years of sustained Bt corn or Bt cotton cultivation. The results showed no persistence or accumulation of Cry proteins in the fields. Given the structural and functional similarities of the Cry1A.105 and Cry2Ab2 with those Cry proteins, and the rapid degradation of both of these proteins in studies conducted and submitted in the Petition **section VIII.A.5**, the risk of any potential cumulative adverse impact from Cry1A.105 and Cry2Ab2 proteins to the environment and soil-dwelling organisms is negligible.

Crop varieties produced by conventional plant breeding have a demonstrated long history of safety. For biotechnology-derived corn products, once single events are assessed and determined to be safe for human and animal consumption and safe to the environment, then combining the unrelated single events through conventional breeding should not pose any new characteristics which would change the safety assessment conclusions. The safety assessments conducted for the single event products are sufficient to cover any event combinations that may occur for unrelated traits. Breeders have put in place standard testing and assessment procedures to examine and confirm the equivalence of the combined trait products to the single event products in phenotypes, agronomic characteristics, and the efficacy of the traits. In the case of combining closely related traits, e.g., two or more Bt proteins, any potential cumulative effects will be assessed on case-by-case basis. So far, no cumulative effects have been documented for all registered, combined trait products of Bt corn. MON 89034 may be combined with the existing approved and deregulated corn events including rootworm protection trait, herbicide tolerance trait, or high lysine corn trait by conventional breeding. The combined trait products are not expected to interact at the molecular or protein levels based on their well characterized modes of action. The assessments on any existing corn products in commerce and on MON 89034 apply to the combined trait products. No new cumulative impacts should be expected by these combinations.

There is no evidence to suggest that the introduction of MON 89034 corn will adversely impact biodiversity, genetic purity of corn germplasm, or specialty corn production even considering the potential cumulative effect of multiple biotechnology-derived traits. There has been a long history of preserving, protecting and enhancing corn germplasm. Measures of preservation of corn germplasm purity including isolation, detasseling and the use of cytoplasmic male sterility have been well established and have a track record of effectiveness for many years. The adoption and large scale cultivation of biotechnology-derived corn in the past decade did not have any negative impact on biodiversity, genetic purity of corn, or specialty corn production including organic corn in the U.S.

Introduction of MON 89034 will not have any adverse impact on agricultural land use, instead, it will help to protect corn yields and sustain agricultural land productivity. The introduction and rapid adoption of biotechnology-derived corn products in the past decade had no significant impact on the cropland use in the U.S. The total crop area in the U.S. has remained relatively steady for decades, as was the case with field corn planting acreage. From 1996 to 2006, the total annual field corn acres fluctuated around

80 million acres, while in the same time span the adoption rate for biotechnology-derived corn products increased from none to 61%. Farmers rotate crops and adjust planting acreages of different crops based on the market demand and commodity prices. U.S. and global corn market demand remains high for food, feed, ethanol production, and other industrial uses. Planted corn acres are projected to significantly increase in 2007 compared to previous years due to the increased need of corn for ethanol production. This increase is not related to biotechnology-derived corn. Corn products that maximize the yield potential may reduce the overall acres needed for such new uses. Adoption of biotechnology-derived corn has been demonstrated to protect corn yields, increase agricultural land output, and alleviate pressure on the reduction of acreages of other large crops (such as soybeans, wheat, and cotton), which translates into sustained benefits to the U.S. farmers and consumers.

Monsanto employs a rigorous product compliance and stewardship program which considers many factors. Consistent with Monsanto's commitments, MON 89034 will not be launched without regulatory approvals in the U.S. and key corn import countries to ensure global compliance with local regulations and the flow of international trade. We commit to the leading industry standards on seed quality and control to prevent adventitious presence of unapproved traits. Monsanto's stewardship policy is the shared responsibility of Monsanto, our licensees and our customers to insure that its products are used properly. We are committed to legal and ethical obligations to ensure that our products and technologies are safe and environmentally responsible, and do not pose undue risks to human health or the environment during any stage of their life cycle. In the U.S., under the Coordinated Framework for the Regulation of Biotechnology, the responsibility for regulatory oversight of biotechnology-derived crops falls on three lead federal agencies – EPA, FDA, and USDA. Prior to the commercialization of MON 89034, Monsanto will seek approvals and clearances from those three agencies and any necessary approvals at the state level as well.

1. Assessment of Potential Cumulative Impact of MON 89034 with other Bt Crops on Insect Pest Resistance Development and Evolution

The first Bt corn product expressing the Cry1Ab protein was approved for commercial use in the U.S. in 1995 (Federal Register, 1995). Since then, additional Cry1Ab expressing biotechnology-derived corn, along with Cry1F, Cry3Bb1, Cry34Ab1, Cry35Ab1, and Cry3A expressing corn, and Cry1F, Cry1Ac and Cry2Ab2 expressing cotton have been approved and cultivated in the U.S. and other countries (EPA, 2007; FDA, 2007; USDA, 2007a; James, 2006).

Currently, Bt corn products are grown on over 40 million acres in the U.S. and on substantial areas in Canada, Argentina, Spain and the Philippines, and on smaller areas in a number of other countries (James, 2006). In many parts of the northern U.S. Corn Belt, Bt corn adoption exceeds 50% of the corn acreage. The consistently high adoption of Bt corn potentially exerts selection for resistance to these Cry proteins by the targeted pests, such as European corn borer, southwestern corn borer, corn earworm, and corn rootworm.

As a mitigation measure to reduce the likelihood of the development of resistant insects, Insect Resistance Management (IRM) plans have been developed and implemented for all Bt corn and cotton products in all countries in which they have been cultivated. These IRM plans and their underlying strategies received extensive expert review and are required conditions of the EPA registration of these products (EPA, 2001a, b; 2005a, b, c, d, e, f, g; 2006). Also the effectiveness of these plans has been monitored annually through surveys of target pest susceptibility (Farino et al., 2004; ABSTC, 2006; Siegfried et al., 2006). No instances of insect resistance development to any of the Bt corn or Bt cotton events have been confirmed in any of the target pests anywhere in the world (Tabashnik et al., 2003; Farino et al., 2004; ABSTC, 2006). Even when highly sensitive methods have been used to search for Bt resistant alleles, no evidence of an increase in Bt resistant allele frequency has been observed during the eleven years of commercial Bt corn use (Bourguet et al., 2003; Stodola et al., 2006). Therefore, the risk of resistance evolving to the currently commercial Bt corn and Bt cotton products has been managed effectively through the current agricultural practices and IRM programs. This *in planta* Bt protein expression approach contrasts with the use of traditional Bt microbial pesticide spray applications where no IRM plans have been implemented and field resistance has been observed, most notably in the diamondback moth (Tabashnik et al., 1997).

As with all previously commercialized Bt corn products, MON 89034 will be introduced with an effective IRM plan. Monsanto has drafted such a plan, which is being reviewed by the EPA (Head, 2006). EPA will mandate an IRM plan as part of the EPA registration conditions for MON 89034. The components of this plan are based on the best available science and employ comparable approaches to the plans which have been successfully implemented for the current biotechnology-derived Bt corn products. The effectiveness of this plan will be monitored through annual surveys of target pest susceptibility, and the implementation of the plan will be tracked through a Compliance Assurance Program

(CAP) that is comparable to the CAP used for current Bt corn products. The CAP will employ a combination of educational programs and surveys of growers' compliance. The results of the susceptibility surveys and the CAP will be reported to EPA for review on a regular basis. Therefore, the introduction of MON 89034 in the U.S. is not expected to result in an increase in the overall risk of resistance development and evolution in the target pests to the Cry proteins.

To the contrary, the adoption of MON 89034 should reduce the overall risk of the resistance evolution in lepidopteran pests towards Cry proteins in a number of important ways. First, the introduction of MON 89034 into the marketplace will provide two Bt proteins that are independently active against the lepidopteran pests of corn. By introducing new and distinctive properties in insecticidal modes of action, the selection pressure on Cry1Ab and Cry1F, the active ingredients of the current lepidopteran protection corn, will be reduced, decreasing the risk of resistance development in European corn borer, southwestern corn borer, and corn earworm. Second, MON 89034 contains a combination of two Bt proteins – Cry1A.105 and Cry2Ab2 - each of which is highly effective against these corn pests and which differ significantly in key characteristics in their insecticidal modes of action such that this product exhibits two independent and effective modes of action.

Crops producing two or more insecticidal proteins are sometime called pyramided products (Roush, 1998) and have been shown to have a lower associated risk of insect resistance than products containing a single Bt protein for a similar spectrum of pests. Mathematical modeling (Caprio, 1998; Roush, 1998) and experimental studies in model systems (Zhao et al., 2003) have shown that insect resistance evolves many times more slowly to products with two highly effective Bt proteins than products with a single highly effective Bt protein. MON 89034 will be introduced by Monsanto as a replacement for the Cry1Ab expressing MON 810 (YieldGard® corn borer) and also may displace other lepidopteran protection, single Bt protein corn products in the marketplace. The net effect will be to convert much of the corn-growing area in the U.S. and other countries from single Bt protein products to MON 89034 and combined trait products, with a significant associated reduction in the risk of resistance evolution in the target pests.

2. Assessment of Potential Cumulative Impact of Deregulation of MON 89034 on Bt Protein Accumulation in Soil Environment

Long before biotechnology-derived Bt crops were developed, the agriculture industry had been using whole Bt microbes and spores as microbial pesticides on crops for insect control, a pest management practice still in application in organic crop production and forestry today. *Bacillus thuringiensis* is a Gram-positive, soil dwelling bacterium. It also occurs naturally in the caterpillars of some moths and butterflies as well as on the surface of plants (Madigan and Martinko, 2005). Upon sporulation, Bt produces a variety of

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crystal (Cry) proteins which display specific activities against insect species of the orders *Lepidoptera* (moths and butterflies), *Diptera* (flies and mosquitoes) and *Coleoptera* (beetles).

Spray applications with microbial pesticide formulations consisting of whole Bt cells and spores have been used in organic agriculture in the U.S. for over four decades (Betz et al.; 2002). No significant environmental impact has been reported with this practice despite findings of detectable concentrations of Cry proteins long after spraying (Smith and Barry, 1998). The soil degradation of Cry proteins from microbial Bt sprays was initially documented by radioisotope labeling and monitoring of the liberated $^{14}CO_2$ (West, 1984). The half-life of the sprayed Bt pesticides was reported to be less than six days in soil. Later studies of aerial Bt-spray applications indicated that Bt spores persisted for two years in soil and one year in leaf samples (Smith and Barry, 1998). Field studies in Italy indicated that Bt spray residues were detectable by insect bioassay for 28 months after spraying, while the bacterial spores (Btk) were detected for 88 months. However, the insecticidal activity decreased markedly between 14 and 28 months (Vettori et al., 2003).

In contrast to Bt spray applications with microbial pesticide formulations, biotechnology-derived plants have built-in protection through the expression of one or more specific Cry proteins. This approach offers some environmental advantages over the Bt microbial pesticide formulations. First, Bt crops are very selective and specific with respect to the active insecticidal ingredients, normally one, two, or three Cry proteins compared to a unknown number of Cry proteins from the Bt cells and spores in the microbial formulations. Second, Bt crops are readily degradable, exposing the Cry proteins to microbial soil degradation. As a consequence, the Cry proteins expressed in the biotechnology-derived crops have a lower tendency for accumulation in the environment. A recent study compared the concentration of Cry1Ab protein from a biotechnology-derived Bt corn and from Dipel® Bt spray formulations in soil and surface water. The report concluded that Cry1Ab from the biotechnology-derived Bt corn degraded faster in soil and water than the Cry1Ab protein contained in the Dipel® microbial spray formulations (Douville et al., 2005).

In the past decade, a number of biotechnology-derived Bt corn and Bt cotton products have been approved and commercialized in the U.S. and around the world. The active insecticidal Cry proteins in these crops include Cry1Ab, Cry1Ac, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1, Cry35Ab1, and Cry3A proteins (EPA, 2007). In 2006, there were 22 countries growing biotechnology-derived crops, and there were 19 million hectares (or 47 million acres) planted to Bt crops including corn and cotton (James, 2006). In the U.S., 40% of corn acreage and 57% of the cotton acreage were planted with varieties possessing Bt insect protection traits in 2006 (USDA-NASS, 2006). Many laboratory soil degradation studies have been conducted with various Cry proteins. In most studies, a rapid initial dissipation of the Cry proteins was observed, sometimes followed by a slower decline of the remaining soil-bound trace levels of the Cry proteins (Palm et al., 1996; Sims and Holden, 1996; Sims and Ream, 1997; Herman et al., 2001; Herman et al.,

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2002a, b; Dubelman et al., 2002a, b; Stotzky, 2004; Hopkins and Gregorich, 2005; Mueth et al.; 2006).

In addition to the laboratory studies, a number of field monitoring studies have been conducted to assess the potential accumulation of Cry proteins following several years of sustained Bt corn or Bt cotton production. These studies have shown no persistence or accumulation of Cry proteins in fields where corn expressing Cry1Ab protein (Dubelman et al., 2005) or Cry3Bb1 protein (Ahmad et al., 2005), and cotton expressing Cry1Ac protein (Head et al., 2002) were grown continuously for several years. The results of these long-term field studies suggest that there were no significant accumulation of Bt proteins from continuous cultivation of Bt crops.

Published studies show little or no cumulative impact on the soil microflora from the use of biotechnology-derived crops expressing Cry proteins. For example, a recent field study over a 3-year cropping cycle concluded that growing MON 863, which expresses the Cry3Bb1 protein, showed no adverse impact on the soil ecology (Devare et al., 2007). Another study conducted in two fields in Germany showed that corn-rhizosphere microbial communities exposed to the Cry1Ab protein from MON 810 were less affected by Cry1Ab protein than by other environmental factors, such as the age of the plants and field heterogeneities (Baumgarte and Teebe, 2005). A separate study to assess the effect of Bt-corn on soil microbial community structure concluded that Cry proteins had only minor effects on soil microbial community profiles when compared to their non-Bt isolines (Blackwood and Buyer, 2004). A season-long field study conducted with the Cry3A protein expressed in biotechnology-derived potato showed no adverse effects towards soil-dwelling microorganisms (Donegan et al., 1996). In a study conducted in Kansas during the 2000 and 2001 growing seasons, the numbers of soil mites, collembola and nematodes observed in plots planted with Cry3Bb1-producing corn were similar to those observed in plots planted with non-Bt corn (Al-Deeb et al., 2003). Other published reports showed that Cry proteins had no microbiocidal or microbiostatic activity *in vitro* against selected bacteria, fungi, and algae (Koskella and Stotzky, 2002), and had no apparent effect on earthworms, nematodes, protozoa, bacteria and fungi (Saxena and Stotzky, 2001).

MON 89034, the product of this petition, expresses two Cry proteins – Cry1A.105 and Cry2Ab2. MON 89034 is intended to replace MON 810 corn and to compete with other existing biotechnology-derived, lepidopteran protection corn products. Given the structural and functional similarities of Cry1A.105 and Cry2Ab2 with other Cry proteins, and the lack of soil accumulation of other Cry proteins derived from large scale cultivation of Bt corn and Bt cotton in the past decade, and the rapid degradation of both of these proteins in studies conducted and submitted in the Petition **section VIII.A.5**, the risk of any potential cumulative adverse impact from Cry1A.105 and Cry2Ab2 proteins to the environment and soil-dwelling organisms is negligible.

3. Assessment of Potential Cumulative Impact of MON 89034 in Combined Trait Products Resulted from Conventional Breeding with other Biotechnology-Derived Products

Different biotechnology-derived corn products have been combined through conventional breeding practices to produce combined trait corn products that meet the broader needs of corn growers. For example, insect protection traits have been combined through conventional breeding with herbicide tolerance traits, and lepidopteran insect protection traits have been combined through conventional breeding with coleopteran insect protection traits to produce corn products possessing a combination of traits in a single plant.

In the past one and a half decades, over 20 biotechnology-derived corn products have completed the regulatory process in the U.S. under the regulatory oversight of EPA, FDA, and USDA (EPA, 2007; FDA, 2007; USDA, 2007a). Three major types of biotechnology-derived corn traits have been developed and widely adopted by the U.S. corn growers, who increasingly choose to purchase and plant combined trait products. These three major types of the biotechnology-derived corn traits include (1) lepidopteran pest protection corn, (2) coleopteran pest protection corn, and (3) herbicide tolerant corn including the glyphosate tolerance trait and the glufosinate tolerance trait. In addition, other corn trait such as high lysine corn was also developed and approved in the U.S.

Different combined trait products have been produced from the approved and deregulated single event corn products through conventional breeding, and have been cultivated by corn farmers in the U.S. A survey by the National Agricultural Statistics Service showed that that 61% of total U.S. corn acreage in 2006 was planted with biotechnology-derived varieties (USDA-NASS, 2006). The percentages of insect protected corn, herbicide tolerant corn, and combined trait corn products possessing both insect protection and herbicide tolerance traits were 25%, 21% and 15%, respectively. It should be noted that in this survey the insect protected corn includes both single event and combined trait Bt corn products.

Biotechnology-derived, combined trait or combined event products produced by conventional breeding from the approved and deregulated single event products should be subject to the same regulatory oversight that is applied to conventional crop varieties that are produced by conventional plant breeding. The safety assessments conducted for the single event products are sufficient to cover any trait combinations that may occur for unrelated traits. Crop varieties produced by conventional plant breeding have a demonstrated long history of safety. For biotechnology-derived products, once single events are assessed and determined to be safe for human and animal consumption and safe to the environment, then combining the unrelated single events through conventional breeding should not pose any new characteristics which would change the safety assessment conclusions. Any assessments conducted for the single trait products can be used as a basis for any potential trait combinations based on the type of genes, the

localization of the proteins or other active ingredients, and the modes of action of the traits. Breeders have put in place standard testing and assessment procedures to further examine and confirm the equivalence of the combined trait products to the single event products in phenotypes, agronomic characteristics, and the efficacy of the traits. In the case of combining closely related traits, e.g., two or more Bt proteins, any potential cumulative effects will be assessed on case-by-case basis. No cumulative effects have been reported for all registered combined trait products of Bt corn or Bt cotton (EPA, 2007).

MON 89034 may be combined with the existing approved and deregulated corn events including rootworm protection, herbicide tolerance traits, or high lysine corn trait by conventional breeding. The combined trait products are not expected to interact at the molecular or protein levels based on their well characterized modes of action. The assessments on any existing corn products in commerce and on MON 89034 apply to the potential combined trait products. No new cumulative impacts should be expected by these combinations.

4. Assessment of Potential Impact of Deregulation of MON 89034 on Biodiversity, Preservation of Corn Germplasm Purity, and Specialty Corn Production

The Petition **section II** has discussed, in great detail, the outcrossing potential of contemporary domestic corn varieties and their wild relatives. **Section VII** presented data on the phenotypic, agronomic, ecological interactions and compositional assessment of MON 89034. **Section VIII.B** and **VIII.C** examined the potential of weediness and gene flow of MON 89034 corn. These analyses concluded that (1) MON 89034 exhibits no characteristics that would cause increased weediness and is not likely to become invasive or form self-sustaining populations outside of agricultural fields; (2) unconfined cultivation should not lead to increased weediness of sexually compatible relatives (of which there are none in the U.S.); and (3) use of MON 89034 in breeding for improved corn hybrids will not reduce or limit the genetic diversity available to corn breeders in the future. In addition, the assessment in **section VIII** also concluded that it is unlikely that MON 89034 would have long-term direct or indirect effects on non-target organisms common to agricultural production areas or on threatened and endangered species recognized by the U.S. Fish and Wildlife Service and the National Marine Fishery Service. Based on these analyses, there is negligible potential for adverse effects on biodiversity from MON 89034 compared to conventional and previously approved insect protection corn already under cultivation.

Since the development of hybrid corn systems in the 1930's, the corn industry has created and adopted systems to maintain and preserve the purity of inbred corn lines, hybrids and corn populations developed for various uses including food, feed, processing, and specialty uses such as popcorn, sweet corn, high oil corn, white corn, blue corn, high protein corn (Smith et al., 2004; Sprague and Dudley, 1988). These systems have been developed and implemented over several decades to maintain and meet industry standards on genetic purity and seed quality. Biotechnology-derived corn traits have

been incorporated into these systems while achieving accepted purity standards for the corn industry and taking into consideration the cumulative effects of biotechnology-derived corn products.

To maintain the genetic purity of inbred parents, hybrids and corn populations, production activities for each type are isolated from one another and from commercial grain production (Smith et al. 2004; Sprague and Dudley, 1988). Isolation is achieved through various means but may include physical separation to prevent cross pollination, temporal isolation by planting at different times to stagger pollination times of different materials, detasseling, and the use of cytoplasmic male sterility. Additionally, production guidelines and operating procedures are used to ensure genetic purity and quality throughout the entire production process from planting and growth of the crop, through harvesting, transport, conditioning, packaging, storage and sale. With these operating procedures being widely used in the corn industry, the industry has been able to introduce new varieties including biotechnology-derived corn into the marketplace while still maintaining the desired levels of purity and quality of individual varieties. Thus, over the last 70 years, systems have been developed to maintain the purity and quality of the diverse corn types needed to meet the needs and desires of corn farmers and consumers. These systems have allowed the introduction of biotechnology-derived traits into the desired inbred lines while at the same time allowing for the maintenance of desired conventional inbred lines. Therefore, no significant single or cumulative impact on the production of corn seeds for various desired uses has been observed. The introduction of MON 89034 is not expected to have any effect on the well established effective processes already in place to insure genetic isolation.

There has been a long history of preserving, protecting and enhancing corn germplasm. The introduction of biotechnology-derived corn has had little impact on genetic diversity and germplasm resources of corn, even considering the potential cumulative effect of multiple biotechnology-derived traits being introduced to corn. Plant breeders, institutions, government and non-government agencies take great strides to maintain corn germplasm resources. A full review of this topic is presented in Chapter 1.3 of the book *Corn: Origin, History, Technology and Production* (Smith et al., 2004). As noted in the text, corn genetic diversity is maintained through public and private plant breeding efforts, seed conservation in gene banks, germplasm collections maintained by the International Maize and Wheat Improvement Center (CIMMYT) and other governmental agencies, on-farm conservation and enhancement activities in Latin America, and conservation of corn wild relatives in Mexico and Central America. These efforts maintain and preserve corn genetic diversity necessary for improving the base genetics of corn, separate from or in combination with the breeding practices used to introduce biotechnology-derived traits into new corn varieties. Thus, the genetic diversity of corn will continue to be maintained.

The introduction and large scale cultivation of biotechnology-derived corn in the past decade did not have any negative impact on organic corn production or other specialty corn production in the U.S. It is well recognized that some corn seed companies and farmers specialize in small specialty use corn, such as organic corn, sweet corn and

popcorn. Organic farmers may prefer to use spray applications of Bt microbial pesticides over their crops for insect control instead of using the biotechnology-derived, built-in insect protection Bt traits. These seed manufacturers and growers have been using the same established processes and methods (e.g., isolation, detasseling, and use of cytoplasm male sterility) as described above to ensure the genetic purity of their corn products. Organic producers, like the producers of other specialty corn products including popcorn, use process based standards to assure that products produced meet the specifications set by the industry or the purchasers of the products.

Organic farming is a small sector of the U.S. agriculture industry with only about 0.5% of all U.S. cropland and 0.5% of all U.S. pasture being certified organic in 2005 (USDA-ERS, 2007). Only a small percentage of the top U.S. field crops (0.16% of corn, 0.17% of soybeans, and 0.48% wheat) were grown under certified organic farming systems (**Table 1**). Although small, organic production has been one of the fastest growing segments of U.S. agriculture for the past decade. The U.S. had less than one million acres of certified organic farmland when Congress passed the Organic Foods Production Act of 1990. By the time USDA implemented national organic standards in 2002, certified organic farmland had doubled, and doubled again between 2002 and 2005 (USDA-ERS, 2007). For example, from 1995 to 2005 the organic corn acreage increased by 300% in the U.S. (**Table 1**), while during the same time frame the biotechnology-derived corn acreage increased from none to 52% of total U.S. corn acreage in 2005 (USDA-NASS, 2006). These national statistics do not suggest that the increase in organic corn production and the increase in biotechnology-derived corn production are correlated, but they do indicate that the adoption of biotechnology-derived corn did not adversely affect organic corn production. Accordingly, there is no reason to expect that Monsanto's second generation lepidopteran protection corn product, MON 89034, will have any negative impact on the production of organic corn or other specialty corn.

In conclusion, measures of preservation of corn germplasm purity including isolation, detasseling and the use of cytoplasmic male sterility have been well established and have a track record of effectiveness over many years. The adoption and large scale cultivation of biotechnology-derived corn in the past decade did not have any negative impact on biodiversity, genetic purity of corn, or the production of specialty corn including organic corn in the U.S. There is no evidence to suggest that the introduction of MON 89034 corn will adversely impact biodiversity, genetic purity of corn germplasm, or small scale specialty use corn production even considering the potential cumulative effect of multiple biotechnology-derived traits. It is also concluded based on past experience and standards that the introduction of MON 89034 will not have an impact on the production of specialty corn products, including organic corn.

Table 1. Certified organic and total U.S. acreage of selected crops (USDA-ERS, 2007).

Item	Total certified organic (acres)								U.S. Cropland 2005 (acres)	Certified organic to total (%)
	1995	1997	2000	2001	2002	2003	2004	2005		
Total cropland:	638,500	850,173	1,218,905	1,302,392	1,299,632	1,451,601	1,452,353	1,723,271	340,650,083	0.51%
Grains--										
Corn	32,650	42,703	77,912	93,551	96,270	105,574	99,111	130,672	81,759,000	0.16%
Wheat	96,100	125,687	181,262	194,640	217,611	234,221	214,244	277,487	57,229,000	0.48%
Oats	13,250	29,748	29,771	33,254	53,459	46,074	42,616	46,465	4,246,000	1.09%
Barley	17,150	29,829	41,904	31,478	34,031	30,265	26,629	39,271	3,875,000	1.01%
Sorghum	--	3,075	1,602	938	3,043	4,152	4,453	6,042	6,454,000	0.09%
Rice	8,400	11,043	26,870	29,022	22,381	20,152	22,173	26,428	3,384,000	0.78%
Spelt	12,350	1,704	12,606	7,639	6,939	9,719	6,203	8,169	--	--
Millet	18,550	12,285	15,103	23,366	17,575	26,935	21,036	14,175	565,000	2.51%
Buckwheat	13,250	7,616	10,599	14,311	8,388	8,086	7,960	6,364	--	--
Rye	2,900	4,365	7,488	7,056	9,644	11,616	10,289	8,597	1,433,000	0.60%
Beans--										
Soybeans	47,200	82,143	136,071	174,467	126,540	122,403	114,239	122,217	72,142,000	0.17%
Dry beans	--	4,641	14,010	15,080	2,430	9,836	7,642	10,561	1,659,300	0.64%
Dry peas & lentils	5,900	5,187	10,144	9,362	7,476	16,188	15,893	17,757	571,000	3.11%
Oilseeds--										
Flax	5,850	8,053	25,076	20,672	20,484	14,940	35,104	30,843	983,000	3.14%
Sunflowers	14,200	10,894	19,342	15,295	7,624	7,121	9,742	6,087	2,709,000	0.22%

5. Assessment of the Potential Impact of Deregulation of MON 89034 on Land Use and Agricultural Production

The introduction and rapid adoption of biotechnology-derived corn products in the past decade had no significant impact on cropland use in the U.S. The total crop area in the U.S. remained relatively steady for decades, as was the case with field corn acreage. From 1996 to 2006, the total annual field corn acres fluctuated around 80 million acres (**Table 2**), while at the same time frame the adoption rate for biotechnology-derived corn products increased from none to 61% (USDA-NASS, 2006). Farmers rotate crops and adjust planting acreages of different crops based on the market demand and commodity prices. U.S. and global corn market demand remains high for food, feed, ethanol production, and other industrial uses. Adoption of biotechnology-derived corn has been proven to protect corn yields, increase agricultural land productivity, and alleviate pressure on the reduction of acreages of other large crops (such as soybeans, wheat, and cotton), which translates into sustained benefits to farmers and consumers. Introduction of MON 89034 will not have any adverse impact on the agricultural land use, instead it will help to protect corn yields and sustain agricultural land productivity.

According to the national census, the total harvested cropland areas from 1974 to 2002 varied annually from 295 to 326 million acres. Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2006, corn was planted on 78.3 million acres with a total production of 10.5 billion bushels of grain valued at $33.8 billion (**Table 2**), with an additional 6.5 million acres of corn crops harvested for silage use. The acreages of other major crops (in million acres) in 2006 were 75.5 for soybeans, 57.3 for wheat, 15.2 for cotton, 3.5 for barley, 2.8 for rice, 2.0 for sunflower, 1.4 for sugarbeets, 1.1 for potatoes, and 1.0 for canola (USDA-NASS, 2007).

Corn grain and its processed products have long been a very important component of the U.S. agricultural production essential to the nation's economy. Because of its high starch content, corn grain is used as a valuable energy source in animal feed for domestic livestock, such as cattle, pigs and poultry. Corn is also extensively processed to generate a wide range of food and industrial products including corn syrup, glucose, dextrose, starch, cereals, and ethanol. In addition, as the largest corn producer in the world, the U.S. also exports corn grain to a number of countries. As corn demand and supply fluctuate year over year, the average annual data of the most recent five years (2002-2006) were used to illustrate the contemporary utilization profile of U.S. corn. The average annual total corn use from 2002-2006 was 10,683 million bushels, among which, feed use is highest, taking 55.5%, followed by export shipment (18.2%), ethanol for fuel (13.6%), and food (12.6%) (**Table 3**).

Table 2. U.S. corn acreages, production, value, supply, demand, and export shipment (USDA-NASS, 2007).

Year	Planting Area (million acres)	Production (million bushels)	Yield (bushels/acre)	Price ($/bushel)	Value ($ billion)	Supply Total (million bushels)	Use Total (million bushels)	Ending stock inventory (million bushels)	Export (million bushels)
1987	66.20	7,131.30	119.80	1.94	14.11	12,016.40	7,757.32	4,259.08	1,716.43
1988	67.72	4,928.68	84.60	2.54	12.66	9,190.55	7,260.12	1,930.43	2,028.45
1989	72.32	7,531.95	116.30	2.36	17.91	9,464.28	8,119.83	1,344.45	2,367.30
1990	74.17	7,934.03	118.50	2.28	18.19	9,281.90	7,760.66	1,521.24	1,726.63
1991	75.96	7,474.77	108.60	2.37	17.86	9,015.65	7,915.34	1,100.31	1,583.92
1992	79.31	9,476.70	131.50	2.07	19.72	10,584.10	8,471.12	2,112.98	1,663.28
1993	73.24	6,337.73	100.70	2.50	16.04	8,471.53	7,621.38	850.15	1,328.32
1994	78.92	10,050.52	138.60	2.26	22.87	10,910.22	9,352.38	1,557.84	2,177.48
1995	71.48	7,400.05	113.50	3.24	24.20	8,974.38	8,548.44	425.94	2,227.82
1996	79.23	9,232.56	127.10	2.71	25.15	9,671.76	8,788.60	883.16	1,797.37
1997	79.54	9,206.83	126.70	2.43	22.35	10,098.80	8,791.00	1,307.80	1,504.43
1998	80.17	9,758.69	134.40	1.94	18.92	11,085.29	9,298.32	1,786.97	1,984.19
1999	77.39	9,430.61	133.80	1.82	17.10	11,232.33	9,514.78	1,717.55	1,936.57
2000	79.55	9,915.05	136.90	1.85	18.50	11,639.42	9,740.32	1,899.10	1,941.35
2001	75.70	9,502.58	138.20	1.97	18.88	11,411.83	9,815.40	1,596.43	1,904.77
2002	78.89	8,966.79	129.30	2.32	20.88	10,577.66	9,490.99	1,086.67	1,587.89
2003	78.60	10,089.22	142.20	2.42	24.48	11,189.97	10,231.88	958.09	1,899.82
2004	80.93	11,807.09	160.40	2.06	24.38	12,776.01	10,662.04	2,113.97	1,818.06
2005	81.78	11,114.08	148.00	2.00	22.20	13,237.00	11,269.84	1,967.16	2,147.34
2006	78.33	10,534.87	149.10	3.20	33.84	12,512.03	11,760.00	752.03	2,250.00

Table 3. Average annual U.S. corn utilization during 2002-2006.

	Mean (million bushels)	Standard deviation (million bushels)	% to total use
Feed and residual	5,926.5	224.4	55.5%
Food	1,348.6	15.6	12.6%
Ethanol	1,447.8	403.9	13.6%
Export	1,940.6	236.5	18.2%
Seed	20.3	0.4	0.2%
Total use	10,683.8	791.0	100.0%

Corn is emerging as an important renewable resource that can be processed and fermented to produce ethanol as an alternative fuel and gasoline additive. In the recent years, significantly increased oil prices and the government regulations on cleaner air and alternative fuel (e.g. Energy Policy Act of 2005) have dramatically driven the demand for ethanol production from corn grain. Many new ethanol plants are being built in the Corn Belt. Current USDA projections indicated that U.S. corn acreage will increase, while other major crops such as soybeans and wheat will either decrease or remain flat. As a result of the strong near-term economic outlook for corn, a sharp increase in corn planting is expected to occur for 2007. Corn acreage is expected to increase from 78.8 to 90.4 million acres from 2006 to 2007, representing a 15% increase in a single year. Corn production is projected to stay strong through 2016 (USDA, 2007b). This increase in projected acres of corn is independent of the biotechnology-derived traits that have been commercialized in corn. The biotechnology-derived corn traits that have been introduced or with the introduction of MON 89034 in the future, combined with advances in conventional breeding method are intended to maximize the yield protection of corn. Therefore, these traits will hopefully help to meet the growing demands for corn and reduce the number of increased acres that may need to be planted.

Higher corn yields and sustained productivity of U.S. cropland are also important to maintain the status for the U.S. as a key supplier of corn grain to the world. As the world's largest corn grain producer, the U.S. not only supplies the domestic market but also exports large quantities of corn to other countries. In the past 20 years, U.S. corn export fluctuated year over year, but overall remained flat (**Table 2**). The average of annual corn export in 1987-2006 was 1,879.6 million bushels and the average for the most recent five years (2002-2006) was 1,940.6 million bushels (**Table 3**). The lead importing countries and regions of U.S. corn include Japan, Mexico, Korea, and Taiwan. USDA projects U.S. corn export will remain flat or slightly decrease over the next five years due to the strong domestic demand for corn.

The introduction and adoption of biotechnology-derived insect protection corn products in the past decade had no adverse impact on cropland use but protected corn yields and increased agricultural land output. Corn plants are subject to damage by both insect pests and weeds. Insect pests of corn such as corn borers and corn rootworms can be controlled by chemical pesticides or plant incorporated protectants such as Bt corn. No adverse effects to human and animal health and the environment have been documented

for the adoption and cultivation of biotechnology-derived corn in the past decade. In contrast, there have been well-documented economical and environmental benefits from the adoption of Bt corn, herbicide tolerance corn, and their combined trait products. The use of these biotechnology-derived corn in the U.S. increased corn yields, reduced mycotoxin levels in corn grain, enhanced simplicity and flexibility of insect pest control and weed management, reduced chemical insecticide and herbicide use, increased no-till corn acreage which resulted in less soil erosion and less runoff of pesticides and water (Koziel, 1993; Martin and Hyde, 2001; Carpenter and Gianessi, 2001; Gianessi et al., 2002; Shelton et al., 2002; Fawcett and Towery, 2002; Hyde et al., 2003; Carpenter et al., 2004; Sankula and Blumenthal, 2004; Sankula et al., 2005; Sankula, 2006; Wu, 2006; NCGA, 2007).

Numerous studies demonstrated that lepidopteran protection corn reduces mycotoxin levels in corn grain, resulting in healthier food and feed. Lepidopteran pests such as ECB, CEW and FAW cause substantial damage to stalks, ears, and leaves of developing corn plants. Some of these pests, notably CEW and FAW, cause damage to corn ears which provide entrance of mycotoxin-producing fungi (Miller, 2001). Corn ears that are protected from feeding damage caused by CEW and FAW would have fewer ports of entry for invasion by fungi, especially those that produce mycotoxins, increasing the safety of grain for food and feed use. Results of field studies have consistently demonstrated that Bt corn had significantly lower incidence and severity of *Fusarium* ear rot and *Aspergillus* kernel rot and produced corn grains with lower levels of fumonisins and aflatoxins (Munkvold et al., 1999; Bakan et al., 2002; Hammond et al., 2003; Windham et al., 1999). The results from field trials conducted between 1997 and 2001, in Argentina, France, Italy, Turkey, and USA indicated that the overall fumonisin levels in Bt corn were reduced by 47% to 97% compared with non-Bt corn (Hammond et al., 2003). Windham et al. (1999) reported that when plants were infested with southwestern corn borers, a Bt corn hybrid provided more than 75% reduction in aflatoxin compared with its non-Bt counterpart. Most of these prior studies included MON 810 as the Bt corn. Preliminary data for MON 89034 demonstrates that MON 89034 is subject to even less insect damage than MON 810, and this lower level of insect damage tends to translate into even lower mycotoxin contamination in the corn grain. The data show there is a statistically significant reduction in aflatoxin levels in MON 89034 compared to MON 810 or non-Bt control corn.

Monsanto's first generation, biotechnology-derived, lepidopteran protection corn product MON 810 (YieldGard® corn borer) has been shown to protect corn yields and reduce chemical insecticide use. In 2005 about 34 U.S. states planted 27.9 million acres of corn possessing YieldGard® corn borer trait either as a single trait product (YieldGard® corn corer) or combined trait products (YieldGard® Plus and YieldGard® Triple), resulting in an increased production of 109.3 million bushels or 6.12 billion pounds of corn valued at $213 million (Sankula, 2006). Net returns were estimated to be $197 million. In addition, the amount of pesticide required for pest control is estimated to decrease by 4.85 million pounds per year when using corn-borer protected traits. In percentage terms, MON 810 and combined trait corn containing MON 810 planted in 2005 improved the return on

investments that translates to 24% increase in yield, 27% decrease in pesticide use, and increased monetary gain of 26% compared to 2004 (Sankula, 2006).

MON 89034 is Monsanto's second generation lepidopteran protection corn with dual effective dose that expands the pest spectrum beyond that of MON 810. MON 89034 is intended to replace MON 810, and is expected to be at least equally or even more economically advantageous depending on the level and type of pest infestation that occurs locally. There is no reason to expect that deregulation of MON 89034 will have any adverse impact on cropland use in the U.S., instead, like the existing biotechnology-derived lepidopteran protection corn, MON 89034 will protect corn yields, increase agricultural land productivity, and decrease mycotoxin levels in corn grain, which will translate into direct benefits for farmers and consumers.

6. Mitigation and Remediation Measures before and after Registration and Deregulation of MON 89034 in the U.S.

Under the Coordinated Framework for Regulation of Biotechnology, the responsibility for regulatory oversight of biotechnology-derived crops falls on three lead federal agencies: EPA, FDA, and USDA (Federal Register, 1986). Deregulation of MON 89034 from USDA constitutes only one component of the overall regulatory oversight and review of this product. MON 89034 cannot be released and marketed until all three agencies have completed their review and assessments under their respective jurisdictions.

Food and feed from biotechnology-derived crops are subject to regulatory review by FDA under the Federal Food, Drug, and Cosmetic Act [21 U.S.C. 346 a(d)]. Since 1992, FDA has used a voluntary consultation process to work together with biotechnology-derived product developers to identify and resolve any issues regarding the safety and nutritional content of food and feed from biotechnology-derived crops that would necessitate legal action by the agency if the products were introduced into commerce. Although this process is not legally binding, to date, all biotechnology-derived crops marketed in the U.S. have completed the consultation process before commercialization. Using the current process, Monsanto has initiated and will complete a consultation with FDA prior to commercial distribution of MON 89034 (refer to Petition **section I.E.2**).

Monsanto has provided FDA a comprehensive data package which included: (1) the intended technical effect of the modification of the corn plants; (2) a molecular characterization of the modification, including the identities, sources, and functions of the introduced genetic material; (3) information on the expressed protein products encoded by the introduced genes; (4) information on assessment of potential allergenicity of the introduced proteins; (5) information on assessment of potential toxicity of the introduced proteins; and (6) information on the compositional and nutritional characteristics of the food and feed derived from MON 89034. At the conclusion of the consultation process, FDA has the ability to notify the submitter if there are unresolved questions regarding the safety of the food under review, thereby effectively halting introduction of the food into commerce. As an ultimate mitigation measure, FDA has the authority to initiate a variety

of actions against food or feed in commerce if the agency has reason to believe that the food or feed is unsafe.

As presented in the Petition **section I.E.1**, because MON 89034 contains two insecticidal proteins, it is also subject to regulatory review and oversight by EPA under the Federal Insecticide, Fungicide and Rodenticide Act [7 U.S.C. §136(u)] and the Federal Food, Drug, and Cosmetic Act [21 U.S.C. 346 a(d)]. Monsanto has filed applications with EPA for the registration of the plant-incorporated protectants Cry1A.105 and Cry2Ab2 proteins, and the genetic material (vector PV-ZMIR245) necessary for their production. Monsanto has also submitted data to enable EPA to conduct a food safety review of those proteins in support of exemptions from the requirement of a tolerance. Sale or distribution of those insecticidal proteins or their presence in food or feed without the requisite EPA approvals would be a violation of federal law.

Since 1995, EPA has reviewed the safety and environmental effects and approved the use of 23 biotechnology-derived Bt protein/crop combinations (EPA, 2007). Over the past decade, many of these Bt crops, in particular, Bt corn and Bt cotton have been cultivated in the U.S. over substantial acres. No adverse effects on humans, animals, or the environment have been documented from the use of these crops (Petition **section VIII**). EPA has implemented a comprehensive data review and assessment process on the biotechnology-derived Bt crops and other plant-incorporated protectants or PIPs (EPA, 2001b). The review includes, but is not limited to, the examination of data and information on (1) the source of the genes; (2) the expression of the PIPs; (3) the modifications to the introduced trait as compared to that trait in nature; (4) the biology of the recipient crops; (5) environmental fate of the PIPs; (6) potential effects on non-target organisms, threatened and endangered species; and (7) potential toxicity and allergenicity of the PIPs on humans and animals.

As an important mitigation and remediation measure, EPA has placed time restrictions on many of the registered PIPs and requires revaluation and re-registration prior to the expiration date for those PIPs if the technology providers decide to continue to market those products (EPA, 2007). One concern with the use of Bt crops is the potential development and evolution of resistance from the insect pests as has been observed with chemical insecticides in the past. As a preventative and remedial measure, EPA has implemented conditional registrations with mandatory requirements for Bt crop developers to implement Insect Resistance Management (IRM) plans. As discussed earlier in this addendum, the IRM programs include refuge requirement, grower agreements, IRM education, and Compliance and Assurance Program, and insect resistance monitoring [refer to reference EPA (2005e) for an example of the details of these requirements]. Annual reporting to EPA is required with respect to these requirements and the sales of the product. It is expected that EPA will implement similar measures and requirements to MON 89034 and the combined trait products containing MON 89034.

Monsanto employs a rigorous product stewardship program that demonstrates respect for our customers, their markets and the environment. Our market stewardship program

considers many factors to ensure global integration and increased transparency. In keeping with past practice, we will not launch MON 89034 without regulatory approvals from key corn import countries to ensure global compliance and the flow of international trade. We commit to the industry practices on seed quality and control to prevent adventitious presence of unapproved traits. Before commercializing MON 89034, a detection method will be made available to grain producers, processors and buyers. Our stewardship policy is the shared responsibility of Monsanto, our licensees and our customers to insure that our products are used properly. We are committed to legal and ethical obligations to ensure that our products and technologies are safe and environmentally responsible, and do not pose undue risks to human health or the environment during any stage of their life cycle. As such, Monsanto has policies in place to monitor our procedures as we research, develop, design, manufacture, market and discontinue products through the product life cycle.

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