MONSANTO

VOLUME 1 OF 3

to
Application to Food Standards Australia New Zealand for the inclusion of Lepidopteran-protected maize MON 89034 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

Monsanto Australia Limited
Level 12 / 600 St Kilda Road,
Melbourne Victoria 3004

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Title

Amended Report for MSL-20072: Molecular Analysis of Corn MON 89034

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Amended Report Completed

September 5, 2006

Performing Laboratory

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Laboratory Project ID

Study 05-01-39-12
MSL-20311

The text below applies only to use of the data by the United States Environmental Protection Agency (U.S. EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

STATEMENT OF DATA CONFIDENTIALITY CLAIM

A claim of data confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C).

Information claimed confidential on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C) has been removed to a confidential appendix, and is cited by cross reference number in the body of the report.

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STATEMENT OF COMPLIANCE

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160 with the following exceptions:

Sequence information used in this study was generated by the Monsanto Genomics Sequencing Center, which does not generate its data in compliance with the GLP regulations

Submitter	Date

Sponsor Representative	5 - Sept - 2006
	Date

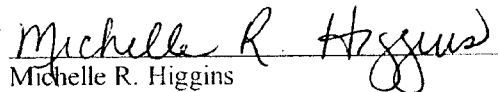|	
Study Director	9/5/06
	Date

QUALITY ASSURANCE STATEMENT

Study Title: Amended Report for MSL-20072: Molecular Analysis of Corn
MON 89034

Study Number: 05-01-39-12

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates Of Inspection / Audit	Phase	Date Reported To Study Director	Date Reported To Management
06/24/2005	Southern Blot	01/24/2006	01/24/2006
10/18/2005	PCR/Sequence	10/26/2005	10/26/2005
10/19/2005	PCR/Sequence	11/02/2005	11/02/2005
02/10/2006	Raw Data Audit	02/20/2006	02/20/2006
02/10/2006	Draft Report Review	02/20/2006	02/20/2006
08/28/2006	Draft Report Amendment Review	08/31/2006	08/31/2006

Michelle R. Higgins
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

09-05-06
Date

STUDY CERTIFICATION PAGE

This report is an accurate and complete representation of the study/project activities.

_____

James D. Masucci, Ph.D.
Study Director
Product Characterization Center

Date

STUDY INFORMATION PAGE

Study Number(s): 05-01-39-12

Title: Amended Report for MSL-20072: Molecular Analysis of Corn MON 89034

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Study Initiation Date: April 4, 2005

Original Study Completion Date: February 22, 2006

Amended Report Completion Date: September 5, 2006

Records Retention: All study-specific raw data (including data rejected and data not reported), protocol, final report and facility records will be retained at Monsanto St. Louis.

Sample Storage: Any study samples that are to be retained will be stored at Monsanto, St. Louis.

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ABBREVIATIONS AND DEFINITIONS [1]

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
aadA	bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
B-Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
B-Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
Cab	the 5' untranslated leader of the wheat chlorophyll a/b-binding protein
CaMV	cauliflower mosaic virus
Cry1A.105	protein comprised of naturally occurring Cry1Ab, Cry1F, and Cry1Ac proteins from *Bacillus thuringiensis*
cry1A.105	coding sequence for a protein comprised of naturally occurring Cry1Ab, Cry1F, and Cry1Ac proteins from *Bacillus thuringiensis*
Cry2Ab2	Cry2Ab protein from *Bacillus thuringiensis*
cry2Ab2	coding sequence for a synthetic version of Cry2Ab protein from *Bacillus thuringiensis*
CS-*cry1A.105*	coding sequence for a protein comprised of naturally occurring Cry1Ab, Cry1F, and Cry1Ac proteins from *Bacillus thuringiensis*
CS-*cry2Ab2*	coding sequence for a synthetic version of Cry2Ab protein from *Bacillus thuringiensis*
CS-*nptII*	coding sequence of neomycin phosphotransferase II gene that confers resistance to neomycin and kanamycin
CS-*rop*	coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
CTAB	hexadecyltrimethylammonium bromide
CTP	chloroplast transit peptide
dNTP	deoxynucleoside triphosphate
e35S	The promoter and leader from cauliflower mosaic virus (CaMV) 35S RNA containing the duplicated enhancer region
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FMV	the Figwort Mosaic Virus 35S promoter

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

ABBREVIATIONS AND DEFINITIONS (CONTINUED)

Hsp17	the 3' nontranslated region of the gene for wheat heat shock protein 17.3 which ends transcription and directs polyadenylation
Hsp70	the first intron from the maize heat shock protein 70 gene
I-*Hsp70*	the first intron from the maize heat shock protein 70 gene
I-*Ract1*	intron from the rice actin gene
kb	kilobase
LB	left border
L-*Cab*	the 5' untranslated leader of the wheat chlorophyll a/b-binding protein
Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
MgSO₄	Magnesium Sulphate
nos	the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*
nptII	coding sequence of neomycin phosphotransferase II gene that confers resistance to neomycin and kanamycin
OR-*ori-PBR322*	origin of replication from pBR322 for maintenance of plasmid in *E. coli*
OR-*ori V*	origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2
P-*35S*	the promoter and leader from the Cauliflower mosaic virus (CaMV) 35S RNA
P-*e35S*	The promoter and leader from cauliflower mosaic virus (CaMV) 35S RNA containing the duplicated enhancer region
P-*FMV*	the Figwort Mosaic Virus 35S promoter
PCR	polymerase chain reaction
PV-ZMIR245	plasmid vector used to develop MON 89034
rop	coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
Ract1	the rice actin gene
Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
RB	right border
SDS	sodium dodecyl sulfate
SOP	standard operating procedure
sp.	species
SSU-CTP	the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit including the first intron

ABBREVIATIONS AND DEFINITIONS (CONTINUED)

T-DNA	transferred DNA
T-DNA I	transferred DNA containing the *cry1A.105* and *cry2Ab2* expression cassettes
T-DNA II	transferred DNA containing the *nptII* gene cassette
T-*Hsp17*	the 3' nontranslated region of the gene for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation
T-*nos*	the 3' nontranslated region of the nopaline synthase (*nos*) gene from *Agrobacterium tumifaciens* which terminates transcription and directs polyadenylation
TS-*SSU-CTP*	the transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit including the first intron
VOI	Verification of Identity

1.0 SUMMARY

Monsanto has developed MON 89034 corn, 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* (*B.t.*) Cry1A protein. Cry2Ab2 is a *B.t.* (subsp. *kurstaki*) protein. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides better insect control and offers an additional insect-resistance management tool.

MON 89034 was produced using the 2T-DNA transformation plasmid PV-ZMIR245 which contains two separate T-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 contain the *cry1A.105* and the *cry2Ab2* expression cassettes (T-DNA I) and do not contain the *nptII* expression cassette (T-DNA II) producing marker-free, lepidopteran-protected corn.

In this study, the integrated DNA in MON 89034 was characterized by Southern blot analyses. The insert number (number of integration sites within the corn genome), the copy number (the number of copies of the T-DNA I within one locus), the integrity of the inserted *cry1A.105* and *cry2Ab2* expression cassettes, and the presence or absence of transformation vector backbone sequences and selectable marker sequences were assessed. The data indicate that MON 89034 contains one copy of the insert at a single integration locus and all expression elements are present. These data also demonstrated that MON 89034 does not contain detectable backbone or selectable marker sequence from plasmid PV-ZMIR245.

Southern blot analysis demonstrated that the Southern blot fingerprint of MON 89034 has been maintained through seven generations of breeding, thereby confirming the stability of the insert over multiple generations. These generations did not contain any detectable T-DNA II elements other than those associated with T-DNA I nor did they contain any detectable backbone sequence from plasmid PV-ZMIR245.

Finally, the complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 89034 was determined, which confirmed the reported organization of the elements within the insert and identified the 5' and 3' insert-to-genomic DNA junctions.

2.0 INTRODUCTION

2.1 Background

Monsanto has developed MON 89034 corn, 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* (*B.t.*) Cry1A protein. Cry2Ab2 is a *B.t.* (subsp. *kurstaki*) protein. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides better insect control and offers an additional insect-resistance management tool.

MON 89034 was produced by *Agrobacterium*-mediated transformation of embryonic corn cells with the 2T-DNA transformation plasmid PV-ZMIR245 (Figures 1 and 2). PV-ZMIR245 contains two separate T-DNAs. A description of the elements located within PV-ZMIR245 is located in Table 1. The first T-DNA, designated as T-DNA I, contains the *cry1A.105* and the *cry2Ab2* expression cassettes. An expression cassette, or gene cassette, is defined in this report 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 contains the *cry1A.105* coding sequence under the regulation of the *e35S* promoter, *Ract1* intron, and the *Hsp17* 3' end sequence. The second cassette contains the *cry2Ab2* coding sequence under the regulation of the *FMV* promoter, the *Hsp70* intron, a chloroplast transit peptide (TS-*SSU-CTP*), 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 confers 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) and do not contain the *nptII* expression cassette (T-DNA II) producing marker-free, lepidopteran-protected corn. The presence of the *cry1A.105* and the *cry2Ab2* coding sequences and the absence of the *nptII* coding sequence were confirmed by Southern blot analyses.

2.2 Purpose

The purpose of this study was to characterize the integrated DNA in MON 89034. Genomic DNA was analyzed using Southern blot analyses to determine the insert number (number of integration sites within the corn genome), the copy number (the number of copies of the integrated DNA within one locus), the integrity of the inserted *cry1Ab.105* and *cry2Ab2* gene cassettes (T-DNA I), and evaluate the presence or absence of plasmid backbone and selectable marker sequences (T-DNA II). Additionally, PCR and DNA

sequence analyses were performed which confirmed the organization of the elements within the insert, determined the 5' and 3' insert-to-genomic DNA junctions, and determined the complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 89034. Also, insert stability analysis was performed to confirm the stability of the transgene insertion in multiple generations of MON 89034.

3.0 MATERIALS AND METHODS

3.1 Test substances
The test substance was MON 89034. Grain from production plan 04-01-50-02 (generated from seed lot# GLP-0404-14916-S, LIMS ID 04ZMGRO00067) was used in this study.

Additional test substances from the MON 89034 breeding tree were used to assess the stability of MON 89034 across generations. For these analyses, DNA was isolated from leaf material (lot GLP-0509-16598-S), as well as from seed material (lot GLP-0503-15974-S, GLP-0503-15975-S, GLP-0505-16269-S, GLP-0503-15977-S, and GLP-0411-15624-S).

3.2 Control substances
The control substance is conventional corn with the same genetic background as the test substance. Grain from Production Plan 04-01-50-02 (generated from seed lot# GLP-0404-14928-S, LIMS ID 04ZMGRO00087) was used in this study.

An additional conventional corn control substance with the same genetic background as seed lot GLP-0503-15977-S was used in experiments to assess the stability of MON89034 across generations. For these analyses DNA was isolated from seed (lot# GLP-0504-16176-S).

3.3 Reference substances
PV-ZMIR245 was used as a positive hybridization control for Southern blots and when applicable as a template control for PCR analyses. The plasmid was isolated prior to the start of the study according SOP BR-ME-0920-01, and its identity confirmed by restriction enzyme digestion.

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

3.4 Characterization of test, control and reference substances

The identity of the test and control substances was determined by event-specific PCR. Grain from MON 89034 (LIMS 04ZMGRO00067) and conventional corn (LIMS 04ZMGRO00087) was tested, and the chain-of-custody documentation and the identity of these samples were verified by the study director prior to initiation of the study. The verification of identity certificates (VOI) will be archived with production plan 04-01-50-02. For the characterization of the additional test and control substances, pools of 300 kernels were not tested as specified by SOP BR-PO-0573-02. However, the Study Director reviewed the chain-of-custody documentation to confirm the identity of these test and control substances, and event specific PCR assays were used to confirm the identity of these test substances and the absence of MON 89034 for control substances. The test and control substances were considered stable during storage if they did not appear visibly degraded on ethidium-stained gels and/or yielded interpretable signals on the Southern blot.

3.5 Genomic DNA isolation for Southern blot and PCR analyses

Grain and seed samples were processed according to SOP BR-ME-0878-01 and genomic DNA was extracted using a CTAB (hexadecyltrimethylammonium bromide)-based method according to SOP BR-ME-0094-01. For a single DNA extraction, chloroform was used rather than chloroform:isoamyl alcohol. Genomic DNA was extracted from leaf tissue using a Sarkosyl DNA extraction method according to SOP BR-ME-0626-01. For two Sarkosyl DNA extractions DNA was hooked out rather than using centrifugation. DNA extractions were stored in a 4°C refrigerator and/or a -20°C freezer.

3.6 Genomic DNA quantification

Extracted DNA was quantified using Hoefer's DyNA Quant 200 Fluorometer according to SOP BR-EQ-0065-02. Molecular size marker IX (Roche, Indianapolis, IN) was used as the calibration standard.

3.7 Restriction enzyme digestion

Approximately 10 or 20 µg of genomic DNA extracted from the test and control substances were digested according to SOP BR-ME-0316-01 in a total volume of approximately 500 µl using 100 units of the restriction enzyme. For positive hybridization controls, approximately one or one-half genomic equivalent of PV-ZMIR245 was spiked into conventional corn DNA prior to digestion.

3.8 Agarose gel electrophoresis

Approximately 10 µg of digested DNA were separated using 0.8% agarose gels according to SOP BR-ME-0315-01 or BR-ME-0315-02. For insert number, copy number, and cassette intactness experiments, a 'long run' and 'short run' were

performed during the gel electrophoresis. Approximately 20 μg of digested test and control substance DNA was divided in half to load approximately 10 μg on the long run and approximately 10 μg on the short run. The long run enabled greater separation of the higher molecular weight restriction fragments while the short run allowed the smaller molecular weight restriction fragments to be retained on the gel.

3.9 Probe preparation
Probe templates (Figures 1 and 2) were prepared by PCR amplification according to SOP BR-ME-0486-01. Approximately 12.5-27 ng of each probe template were radiolabeled with ^{32}P-dCTP (6000 Ci/mmol) using the random priming method (except probe 10 which was labeled by PCR) according to SOP BR-ME-0611-01.

3.10 Southern blot analyses
Digested genomic DNA isolated from test and control materials was evaluated using Southern blots, according to SOP BR-ME-0317-02.

3.11 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.

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.

3.12 Data rejected or not reported

Some Southern blot analyses conducted as part of this study were not reported because the results were not of report quality. Southern blot analyses were rejected if high levels of background signal hindered the ability to draw accurate conclusions. PCR analyses were rejected if the amplification was weak or if the expected amplification product was not produced. Sequencing electropherograms were rejected if they were of unacceptable quality, particularly with respect to peak shape and intensity. Nothing in the rejected or not reported data was inconsistent with the conclusions presented in this report.

3.13 Changes to the study protocol

During the course of the study, several changes to the original protocol were required. These changes were documented as either protocol amendments or protocol deviations and are summarized below. The changes included a typo in a sample identification number, an alternate source for a reference standard, a restriction enzyme change, several test or control substances were replaced or removed, the study title was updated, a DNA extraction procedure was added, the timing of identity confirmation was clarified, and a DNA digestion procedure was clarified. None of these changes had any negative impact on the study conclusions.

4.0 RESULTS AND DISCUSSION

Genomic DNA from MON 89034 was digested using restriction enzymes and subjected to Southern blot analyses. Maps of plasmid vector PV-ZMIR245 annotated with the probes used in the Southern analysis are presented in Figures 1 and 2. A linear map depicting the restriction sites within the insert DNA sequence, as well as within the corn genomic DNA immediately flanking the insert in MON 89034 is shown in Figure 3. The generations used in this study are depicted in Figure 4. Additionally, Southern blot analysis was used to demonstrate the absence of the *nptII* selectable marker cassette and elements associated with T-DNA II which contains the *nptII* selectable marker cassette. The Southern blot figures (Figures 5 – 19) presented in this report are representative of the data generated in the study. For estimating the sizes of bands present in the long-run lanes of Southern blots, the molecular weight markers on the left of the figure were used.

For estimating the sizes of bands present in the short-run lanes, the molecular weight markers on the right of the figure were used.

4.1 Southern blot analyses of MON 89034

4.1.1 Southern blot analyses to examine insert and copy number (Figure 5)
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, plant genomic DNA. The blot was examined with overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18 – 23 in Figure 2). The results of this analysis are shown in Figure 5.

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 material, and therefore they are considered to be endogenous background. Plasmid PV-ZMIR245 DNA mixed with conventional corn control DNA and digested with Ssp I (lanes 9 and 10) produced the expected bands at 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 13.0 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 (Figure 3). 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.

The results presented in Figure 5 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.

4.1.2 Southern blot analyses to confirm the presence of the elements comprising the *cry1A.105* and *cry2Ab2* expression cassettes in MON 89034

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 *Bst*E II.

Digestion with *Ssp* I releases two border fragments with the expected size of approximately 8.2 and greater than 4.3 kb (Figure 3). 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 (Figure 3).

Digestion of the test substance with *Nco* I releases two internal restriction fragments and two border fragments. 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. 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 *Ract*I intron, the *cry1A.105* coding sequence, the *Hsp17* 3' end sequence, the *FMV* promoter, the *Hsp70* intron, the *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence, and the *nos* 3' end sequence. (probes 1-10, Figure 1).

4.1.2.1 *e35S* promoter/*Cab* leader (Figure 6)

Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) probed with the *e35S* promoter and *Cab* leader probe (probe 1, Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional corn control DNA containing 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.

4.1.2.2 *Ract1* intron (Figure 7)

Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) probed with the *Ract1* intron probe (probe 2, Figure 1) showed no detectable hybridization bands, as expected for the negative control.

Conventional corn control DNA containing plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band at approximately 10.0 kb. The migration of the approximately 10 kb fragments is slightly higher than indicated by the molecular weight marker band sizes. The altered migrations may be due to the difference in salt concentrations between the DNA sample and the molecular weight marker (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.

4.1.2.3 *cry1A.105* coding sequence (Figure 8)
Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I
(lanes 4 and 8) hybridized simultaneously with overlapping probes spanning the
cry1A.105 coding sequence (probe 3 and 4, Figure 1) showed no detectable
hybridization bands as expected for the negative control. Conventional DNA
containing 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 fragments is slightly higher than indicated by the molecular
weight marker band sizes. The altered migrations may be due to the difference in

salt concentrations between the DNA sample and the molecular weight marker
(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.

4.1.2.4 *Hsp17* 3' end sequence (Figure 9)
Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I
(lanes 4 and 8) examined with the *Hsp17* 3' end sequence probe (probe 5, Figure
1) showed no detectable hybridization bands, as expected for the negative control.
Conventional corn control DNA containing 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.

4.1.2.5 *FMV* promoter (Figure 10)
Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *FMV* promoter probe (probe 6, Figure 1) showed no detectable hybridization bands, as expected for the negative control.

Conventional corn control DNA containing 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 fragments is slightly higher than indicated by the molecular weight marker band sizes. The altered migrations may be due to the difference in salt concentrations between the DNA sample and the molecular weight marker (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.

4.1.2.6 *Hsp70* intron (Figure 11)
Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *Hsp70* intron probe (probe 7, Figure 1) produced several hybridization signals. This is not unexpected since the *Hsp70* intron was originally derived from corn. These hybridization signals result from the probe hybridizing to endogenous sequences residing in the corn genome and are not specific to the inserted DNA. These signals were produced in both test and control lanes and therefore the bands are considered to be endogenous background.

Conventional corn control DNA containing 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 fragments is slightly higher than indicated by the molecular weight marker band sizes. The altered migrations may be due to the difference in salt concentrations between the DNA sample and the molecular weight marker (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 (see Figure 3). 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. 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 *Hsp70* intron elements other than those associated with the *cry2Ab2* cassette.

4.1.2.7 *SSU-CTP* targeting sequence/*cry2Ab2* coding sequence (Figure 12)
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 *SSU-CTP*/*cry2Ab2* coding sequence probe (probes 8 and 9, Figure 1) produced several hybridization signals. This is not unexpected since the *SSU-CTP* targeting sequence was originally derived from corn. These hybridization signals result from the probes hybridizing to endogenous targets residing in the corn genome

and are not specific to the inserted DNA. These signals were produced with both test and control material, therefore they are considered to be endogenous background. Endogenous bands were not detected in the long runs of the *Nco* I digests because they were run off the gel.

Conventional corn control DNA containing 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 greater than 4.3 kb (see Figure 3). 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 molecular weight marker band sizes 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 test DNA sample and the molecular weight marker (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.

4.1.2.8 *nos* 3' end sequence (Figure 13)

Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Bst*E II (lanes 4 and 8) examined with the *nos* 3' end sequence probe (probe 10, Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional corn control DNA containing plasmid PV-ZMIR245 DNA digested with *Bst*E 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 greater than 4.3 kb (see Figure 3). MON 89034 DNA digested with *Bst*E 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, detectable T-*nos* elements other than those associated with the *cry2Ab2* cassette.

4.1.3 Southern blot analysis to determine the presence or absence of plasmid PV-ZMIR245 backbone (Figure 14)

Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with overlapping probes spanning the vector backbone of PV-ZMIR245 (probes 14 - 17, Figure 1) showed no detectable hybridization bands as expected for the negative control.

Conventional corn control DNA containing plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of approximately 10.0 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 detectable PV-ZMIR245 backbone elements.

4.1.4 Southern blot analysis to determine the presence or absence of the *nptII* coding sequence (Figure 15)

Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) or *Nco* I (lanes 4 and 8) examined with the *nptII* coding sequence probe (probe 12, Figure 1) showed no detectable hybridization bands, as expected for the negative control.

Conventional corn control DNA containing plasmid PV-ZMIR245 DNA digested with *Nco* I (lanes 9 and 10) produced the expected size band of approximately 10.0 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 detectable CS-*nptII*-derived elements.

4.1.5 Southern blot analysis to determine the presence or absence of T-DNA II (Figure 16)

Conventional corn control DNA digested with *Ssp* I (lanes 2 and 6) and *Bst*E II (lanes 4 and 8) examined with overlapping probe spanning T-DNA II (probes 11, 12, and 13, Figure 1) showed no detectable hybridization bands.

Conventional corn control DNA containing plasmid PV-ZMIR245 DNA digested with *Bst*E II (lanes 9 and 10) produced the two expected size bands at approximately 7.8 and 2.0 kb. The overlapping probe spanning T-DNA II contains the *35S* promoter, *nos* 3' end sequence, and Left Border sequence which are contained on T-DNA I . Therefore, the T-DNA II probe is expected to hybridize to fragments derived from 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 and 7.4 kb bands are consistent with the 8.2 and 7.4 kb bands detected with the *e35S* promoter/*Cab* leader region (Figures 6, lanes 1 and 5) and *nos* 3' end sequence (Figure 13, lanes 1 and 5) probes.

MON 89034 DNA digested with *Bst*E 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 greater than 2.8 kb expected band for T-DNA I DNA digested with *Bst*E II (Figure 3), and the approximately 1.7 kb band is consistent with the T-DNA I specific band observed in Figure 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 associated with T-DNA I.

4.1.6 Southern blot analyses to examine insert stability in multiple generations of MON 89034

In order to demonstrate the stability of MON 89034 across generations, additional Southern blot analyses were performed using DNA obtained from multiple generations of the MON 89034 breeding history. For reference, the breeding history of MON 89034 is presented in Figure 4. The specific generations tested are indicated in the legends of Figures 17, 18, and 19. For these analyses, DNA samples

were digested with the restriction enzyme *Ssp* I. *Ssp* I 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 greater than 4.3 kb (Figure 3). 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 (Figure 3).

Plasmid PV-ZMIR245 DNA was combined with conventional corn control DNA, digested with *Ssp* I, and loaded on the gel to serve as a positive hybridization control. Individual Southern blots were examined with three probe sets. The stability of the MON89034 insert across generations was confirmed using overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18 – 23 in Figure 2). The absence of the *nptII* selectable marker and unique T-DNA II genetic elements not contained in T-DNA I was confirmed using overlapping probes spanning T-DNA II (probes 11, 12, and 13, Figure 1), and the absence of plasmid PV-ZMIR245 backbone sequence across generations was confirmed using overlapping probes spanning the vector backbone of PV-ZMIR245 (probes 14 - 17,

Figure 1). 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.

4.1.6.1 Southern blot analyses to examine insert stability in multiple generations of MON 89034 (Figure 17)

Conventional corn control DNA digested with *Ssp* I (lane 7 and 9) examined with overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 18-23, Figure 2) showed several hybridization bands (Figure 17). These bands are difficult to observe in Figure 17, 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. The migration of the approximately 10.4 kb fragment is slightly higher than indicated by the molecular weight marker band sizes 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 molecular weight marker (Sambrook and Russell, 2001).

DNA extracted from MON 89034, LH172 BC0 F$_3$, LH172 BC0 F$_4$, LH172 BC0 F$_5$, LH172 BC0 F$_6$, [LH172 BC0 F$_7$ x LH198]F$_{1H}$, and T1:BC1:F$_1$ x RP 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. 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 greater than 4.3 kb for the 3' border fragment. These bands are consistent with the bands detected in Figure 5 (lanes 3 and 7). Two faint unexpected bands of approximately 15.6 and 12.0 kb were observed in lanes 3 and 4. These bands are likely the result of partial digestion because they are not seen in prior or subsequent generations (These are self pollinated generations). In support of this conclusion the 15.6 kb band is consistent with the internal *Ssp* I site not digesting resulting in a combination of the 7.4 and 8.2 kb bands. 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 (sequence data not reported). No additional unexpected bands were detected, indicating that the single copy of T-DNA I in MON 89034 is stable across the selected generations.

4.1.6.2 Southern blot analysis to confirm the presence or absence of T-DNA II in multiple generations of MON 89034 (Figure 18)
Conventional corn control DNA digested with *Ssp* I (lane 7 and 9) examined with three overlapping probes spanning T-DNA II (probes 11, 12, and 13, Figure 1) showed no detectable hybridization bands. 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 and 7.2 kb. The migration of the approximately 10 kb fragment is slightly higher than indicated by the molecular weight marker band sizes 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 molecular weight marker (Sambrook and Russell, 2001). The overlapping probes spanning T-DNA II contains the *35S* promoter, *nos* 3' end, and the Left Border which are contained on T-DNA I. Therefore, the T-DNA II probe is expected to hybridize to fragments derived from T-DNA I.

DNA extracted from MON 89034, LH172 BC0 F$_3$, LH172 BC0 F$_4$, LH172 BC0 F$_5$, LH172 BC0 F$_6$, [LH172 BC0 F$_7$ x LH198]F$_{1H}$, and T1:BC1:F$_1$ x RP 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 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 greater than 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. These bands are consistent with the bands observed in Figure 17 (lanes 3 and 4) and are likely the result of partial digestion. No additional unexpected bands were detected, indicating that MON 89034, LH172 BC0 F₃, LH172 BC0 F₄, LH172 BC0 F₅, LH172 BC0 F₆, [LH172 BC0 F₇ x LH198]F₁H, and T1:BC1:F₁ x RP do not contain any additional detectable T-DNA II elements other than those associated with T-DNA I.

4.1.6.3 Southern blot analysis to confirm the absence of plasmid PV-ZMIR245 backbone sequence in multiple generations of MON 89034 (Figure 19)

Conventional corn control DNA obtained from LH172, digested with *Ssp* I (lane 7 and 9), and examined with four overlapping probes spanning the PV-ZMIR245 backbone sequence (probes 14, 15, 16, and 17, Figure 1) showed no detectable hybridization bands. Although difficult to observe in Figure 19, 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 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.

DNA extracted from MON 89034, LH172 BC0 F₃, LH172 BC0 F₄, LH172 BC0 F₅, LH172 BC0 F₆, [LH172 BC0 F₇ x LH198]F₁H, and T1:BC1:F₁ x RP digested with *Ssp* I (lanes 1, 2, 3, 4, 5, 6, and 8) produced no hybridization bands. This indicates that MON 89034, LH172 BC0 F₃, LH172 BC0 F₄, LH172 BC0 F₅, LH172 BC0 F₆, [LH172 BC0 F₇ x LH198]F₁H, and T1:BC1:F₁ x RP do not contain any additional detectable PV-ZMIR245 backbone elements.

4.2 Organization and sequence of the insert and adjacent genomic DNA in MON 89034 (Figure 20)

The reported 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 20. The control reactions containing no template DNA (lanes 2, 5, 8, 12, 16, 20, and 23) and the conventional corn control reactions in 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 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 non-specific 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 3. The PCR products that appear on the gel in Figure 20 were generated for the purpose of creating Figure 20. They were generated under the same conditions and are representative of the PCR products that were sequenced.

PCR products generated from MON 89034 were subjected to DNA sequencing to further confirm the organization of the elements within the insert. The consensus sequence representing the insert in MON 89034, including the genomic DNA flanking the ends of

the insert, is shown in Figure 21 and described in Table 2. This consensus sequence was generated by compiling numerous sequencing reactions performed on the PCR products which spanned the length of the insert and the 5' and 3' junctions with the flanking corn genomic DNA.

The insert is 9317 bases long and matches the sequence of PV-ZMIR245 in two places:

1) The 5' end of the insert, beginning at base 2061 and ending at base 2384 in Figure 21 matches the sequence of PV-ZMIR245 between bases 14696 and 14373 in Figure 2. It is possible that a crossover event occurred during the transformation resulting in a left border and modified e35S promoter at the 5' end of the insert.

2) The remainder of the insert, beginning at base 2385 and ending at base 11377 in Figure 21, matches PV-ZMIR245 between bases 2083 and 11075 in Figure 2.

In addition to the insert sequence, 2060 base pairs of corn genomic DNA flanking the 5' end of the insert and 905 base pairs of corn genomic DNA flanking the 3' end of the insert are reported in Figure 21.

5.0 CONCLUSIONS

Molecular characterization of MON 89034 by Southern blot analyses demonstrated that the introduced DNA inserted into the corn genome at a single locus and contains one copy each of *cry1A.105* and *cry2Ab2* expression cassettes. All expression elements are shown to be present in the inserted DNA and there are no other elements detectable other than those associated with the respective insert. No backbone plasmid DNA or *nptII* sequences were detected.

Analysis of stability of integrated DNA demonstrated that an expected Southern blot fingerprint of MON 89034 has been 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 detectable T-DNA II elements other than those associated with T-DNA I. Furthermore, these generations have been shown not to contain any detectable backbone sequence from plasmid PV-ZMIR245.

PCR and DNA sequence analyses confirmed the reported organization of the elements within the insert, determined the 5' and 3' insert-to-plant junctions, and determined the complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 89034.

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Table 1. Summary of 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 and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985) (GenBank accession X03043)
Intervening Sequence	1147-1261	Sequences used in DNA cloning
T-DNA I		
B[2]-Right Border	1262-1618	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	1619-1728	Sequences used in DNA cloning
P[3]-*e35S*	1729-2349	The promoter and 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[4]-*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[5]-*Ract1*	2453-2932	Intron from the rice actin gene (McElroy et al., 1991)
Intervening Sequence	2933-2941	Sequences used in DNA cloning
CS[6]-*cry1A.105*	2942-6475	Coding sequences for a protein comprised of naturally occurring Cry1Ab, Cry1F, and Cry1Ac proteins from *Bacillus thuringiensis* (Monsanto unpublished data)

[2] B -- border region
[3] P - promoter
[4] L - leader
[5] I - intron
[6] CS -- coding sequence

Table 1 (cont.) Summary of genetic elements in plasmid vector PV-ZM(R)45

Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	6476-6506	Sequences used in DNA cloning
T[7]-*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[8]-*SSU-CTP*	8190-8590	The transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit including the first intron. (Matsuoka et al., 1987)
CS-*cry2Ab2*	8591-10498	Coding region for a synthetic version of Cry2Ab protein from *Bacillus thuringiensis* (Widner and Whitely, 1989; Donovan, 1991)
Intervening Sequence	10498-10524	Sequences used in DNA cloning
T-*nos*	10525-10777	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	10778-10844	Sequences used in DNA cloning
B-Left Border	10845-11286	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)

[7] T – transcript termination sequence
[8] TS – targeting sequence

Table 1 (cont.) Summary of 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 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' nontranslated region 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	Neomycin phosphotransferase II gene 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 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-*ori V*	14986-15382	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2 (Stalker et al., 1981)

[9] OR-origin of replication

Table 1 (cont.) Summary of genetic elements in plasmid vector PV-ZMIR245

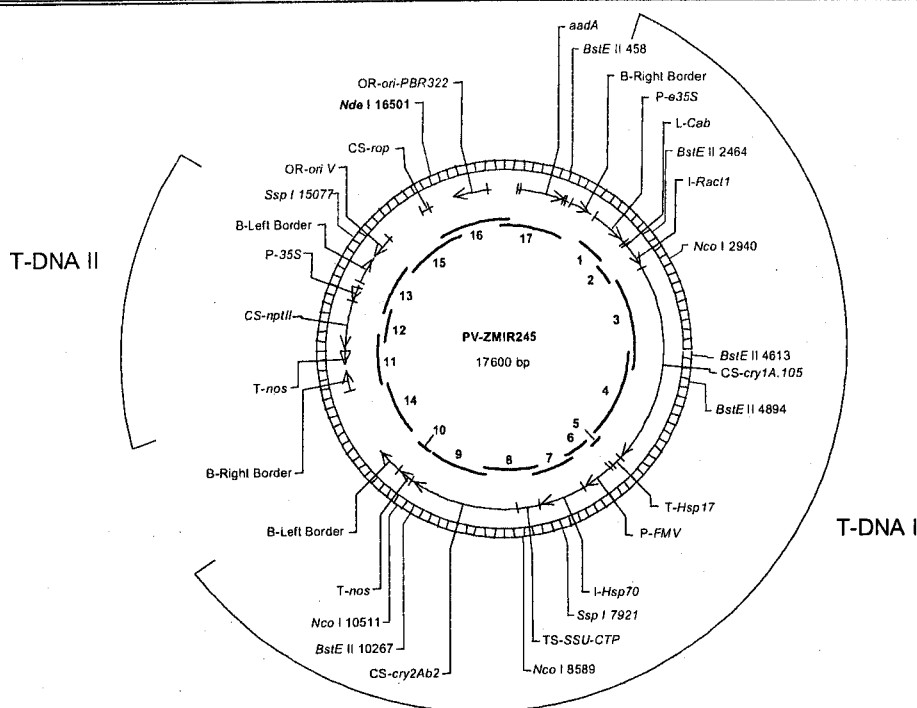| Genetic Element	Location in Plasmid	Function (Reference)
Intervening Sequence	15383- 16119	Sequences used in DNA cloning
CS-*rop*	16120- 16311	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	16312- 16738	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

Table 2. 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[10]-Left Border	2061-2299	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	2300-2349	Sequences used in DNA cloning
P_p[11]-*e35S*	2350-2651	A modified promoter and leader for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987)
Intervening	2652-2677	Sequences used in DNA cloning
L[12]-*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[13]-*Ract1*	2755-3234	Intron from the rice actin gene (McElroy et al., 1991)
Intervening Sequence	3235-3243	Sequences used in DNA cloning
CS[14]-*cry1A.105*	3244-6777	Coding sequences for a protein comprised of naturally occurring Cry1Ab, Cry1F, and Cry1Ac proteins from *Bacillus thuringiensis* (Monsanto unpublished data)
Intervening Sequence	6778-6808	Sequences used in DNA cloning
T[15]-*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

[10] B – border region
[11] P_p –modified promoter
[12] L - leader
[13] I - intron
[14] CS – coding sequence
[15] T – transcript termination sequence

Table 2 (cont.) Summary of genetic elements in MON 89034

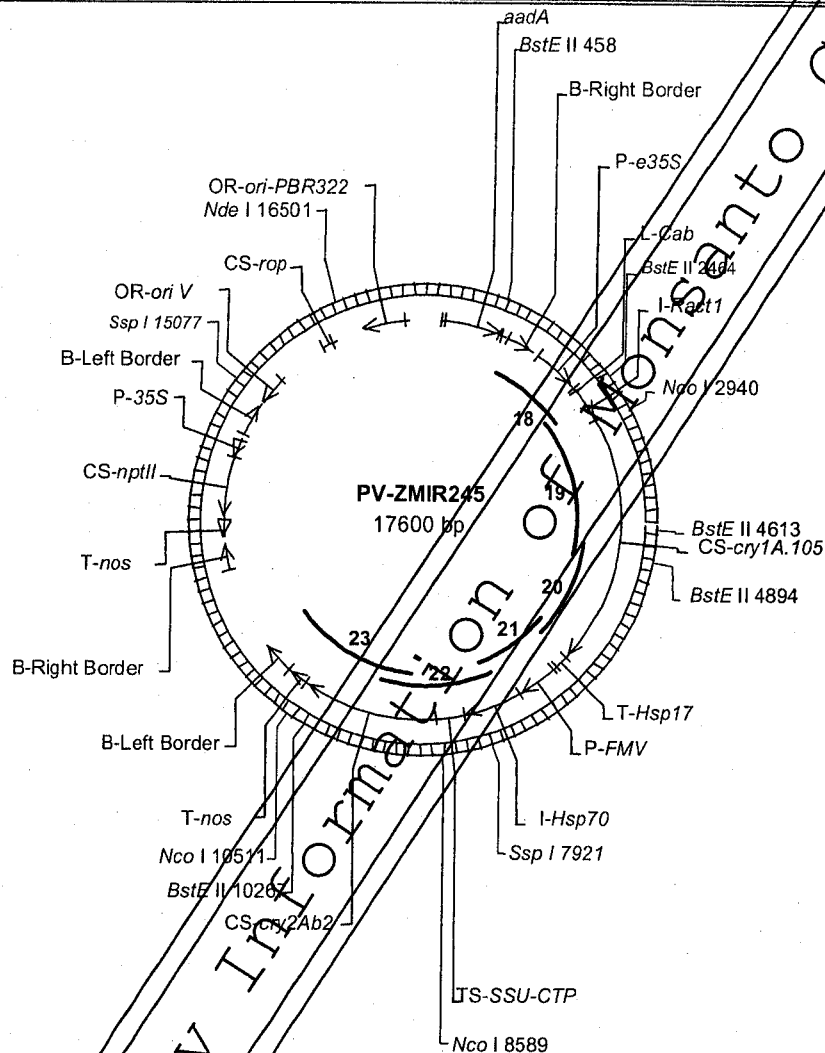| Genetic Element	Location in Sequence	Function (Reference)
P[16]-*FMV*	7086-7649	The Figwort Mosaic Virus 35S promoter (Rogers, 2000)
Intervening Sequence	7650-7671	Sequences used in DNA cloning
I-*Hsp70*	7672-8475	The first intron from the maize heat shock protein 70 gene (Brown and Santino, 1995)
Intervening Sequence	8476-8491	Sequences used in DNA cloning
TS[17]-*SSU-CTP*	8492-8892	The transit peptide region of maize ribulose 1,5-bisphosphate carboxylase small subunit including the first intron (Matsuoka et al., 1987)
CS-*cry2Ab2*	8893-10800	Coding region for a synthetic version of Cry2Ab protein from *Bacillus thuringiensis* (Widner and Whitely, 1989; Donovan, 1991)
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	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Sequence flanking the 3' end of the insert	11378-12282	Corn genomic DNA

[16] P – promoter
[17] TS – targeting sequence

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

Figure 1. Plasmid map of vector PV-ZMIR245 showing probes 1-17
A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown.
Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of
the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analysis
are shown on the interior of the map. PV-ZMIR245 contains two T-DNA regions designated as T-
DNA I and T-DNA II above.

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

Figure 2. Plasmid map of vector PV-ZMIR245 showing probes 18 - 23

A circular map of the plasmid vector PV-ZMIR245 used to develop corn MON 89034 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The overlapping T-DNA I probes used in the Southern analysis are shown on the interior of the map.
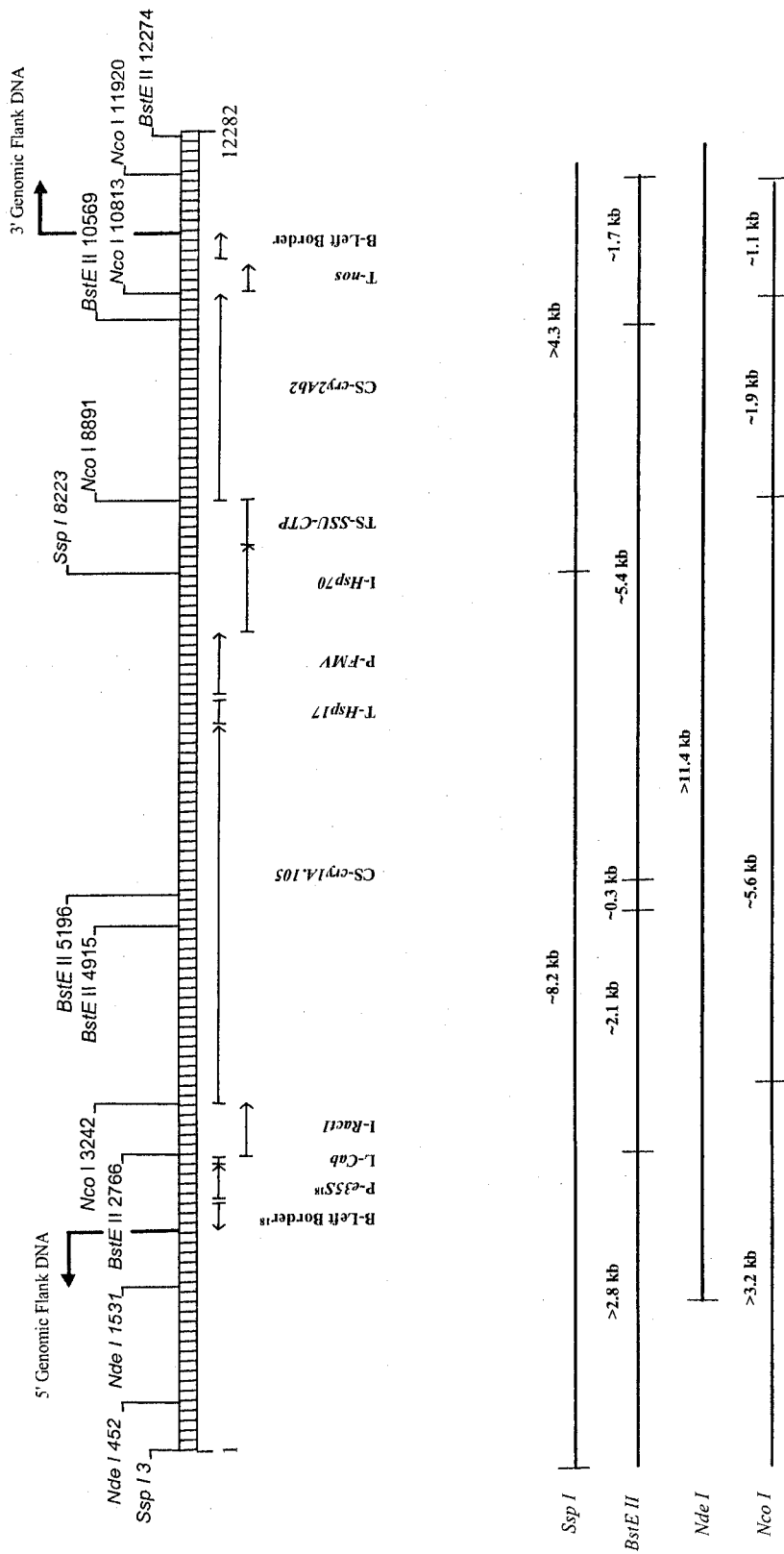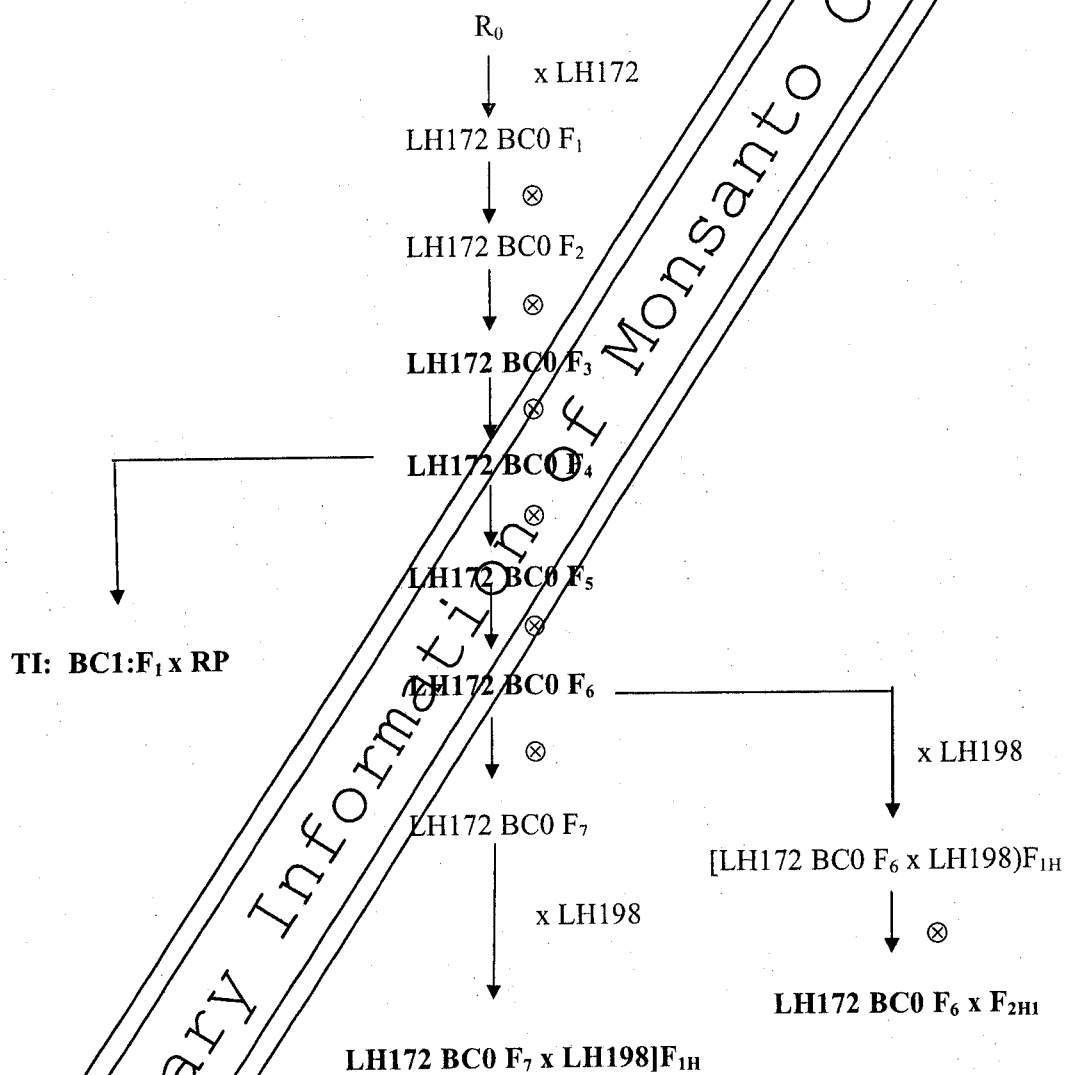
5' Genomic Flank DNA

3' Genomic Flank DNA

Nde I 452
Ssp I 3
Nde I 1531
BstE II 2766
Nco I 3242
BstE II 4915
BstE II 5196

Ssp I 8223
Nco I 8891
BstE II 10569
Nco I 10813
Nco I 11920
BstE II 12274

12282

B-Left Border[18]
P-e35S[18]
L-Cab
I-RacI1

CS-cry1A.105

T-Hsp17
P-FMV
I-Hsp70
TS-SSU-CTP

CS-cry2Ab2

T-nos
B-Left Border

Ssp I
~8.2 kb   >4.3 kb

BstE II
>2.8 kb   ~2.1 kb   -0.3 kb   ~5.4 kb   ~1.7 kb

Nde I
>11.4 kb

Nco I
>3.2 kb   ~5.6 kb   ~1.9 kb   ~1.1 kb

Figure 3. Schematic representation of the insert and genomic flanking sequences in MON 89034
Linear DNA derived from T-DNA I of vector PV-ZMIR245 expected to be incorporated into MON 89034. 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[18], as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analyses.

---

[18] A portion of Left Border sequence and a modified *e35S* promoter sequence is present at the 5' insert-to-flank junction in MON 89034.

R_0

| x LH172

LH172 BC0 F_1

| $\otimes$

LH172 BC0 F_2

| $\otimes$

LH172 BC0 F_3

| $\otimes$

LH172 BC0 F_4

| $\otimes$

TI: BC1:F_1 x RP

LH172 BC0 F_5

| $\otimes$

LH172 BC0 F_6 ————

x LH198

[LH172 BC0 F_6 x LH198)F_{1H}

| $\otimes$

LH172 BC0 F_7

LH172 BC0 F_6 x F_{2H1}

x LH198

LH172 BC0 F_7 x LH198]F_{1H}

R0 = transformed plant; F(#) = filial generation; $\otimes$ = self-pollination; BC(#) = backcross generation; RP = recurrent parent

Figure 4. Breeding history for MON 89034
The LH172 BC0 F_6 x F_{2H1} generation was used for the molecular analyses reported in Figures 5-16 and is referred to as MON 89034 in all Southern Blot figures. Generations used for stability analysis are bolded in the breeding tree.
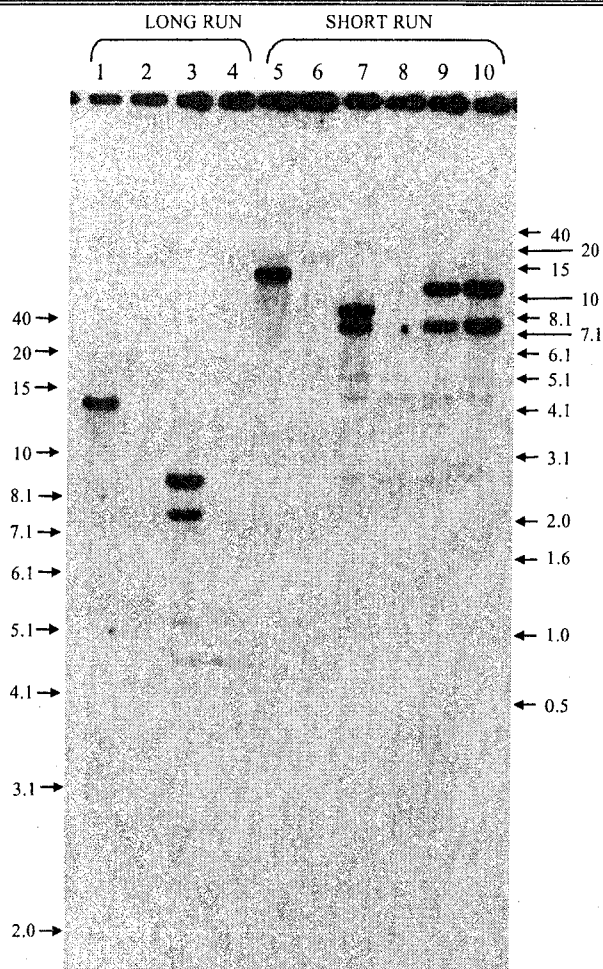
Figure 5. Southern blot analysis of MON 89034: Insert and copy number analysis

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 - 23, Figure 2). 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]
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
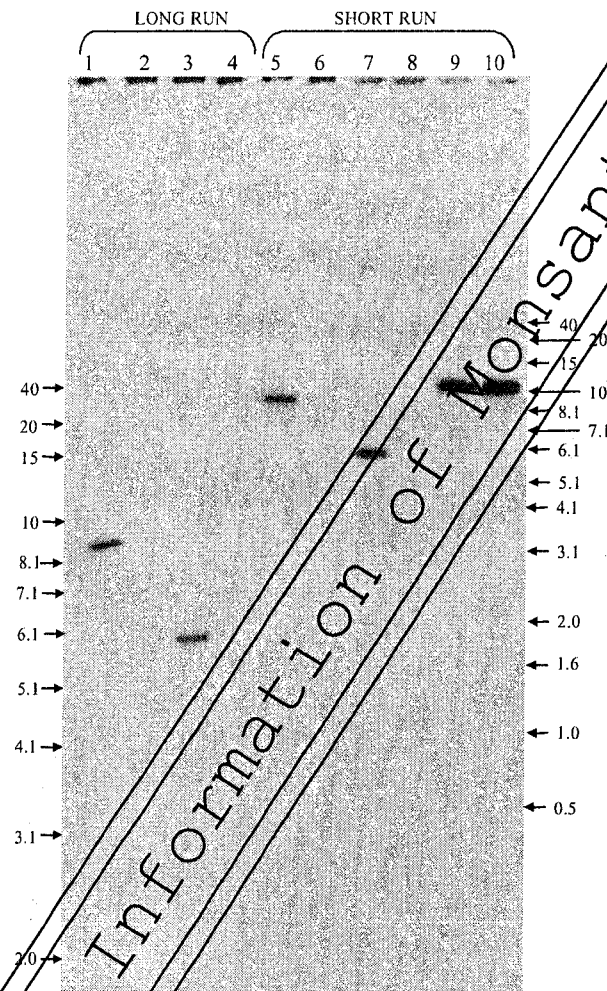
Figure 6. Southern blot analysis of MON 89034: *e35S* **promoter/***Cab* **leader**
The blot was examined with a ^{32}P-labeled probe that spanned the *e35S* promoter and *Cab* leader region (probe 1, Figure 1). Each lane contains approximately 10 µg of digested genomic DNA isolated from grain. Lane 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]
       Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
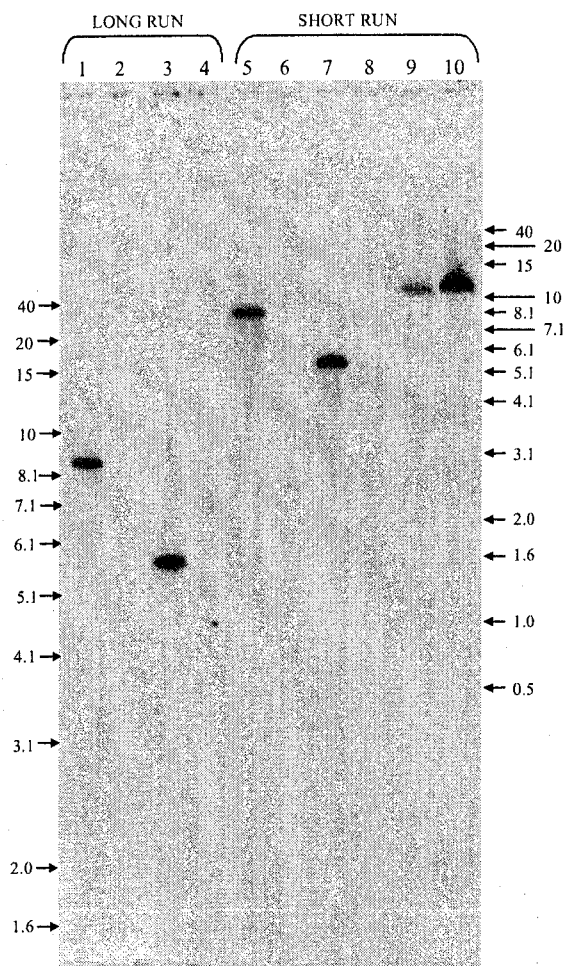
Figure 7. Southern blot analysis of MON 89034: _Ract1_ intron

The blot was examined with a ^{32}P-labeled probe that spanned the _Ract1_ intron (probe 2, Figure 1). 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]
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
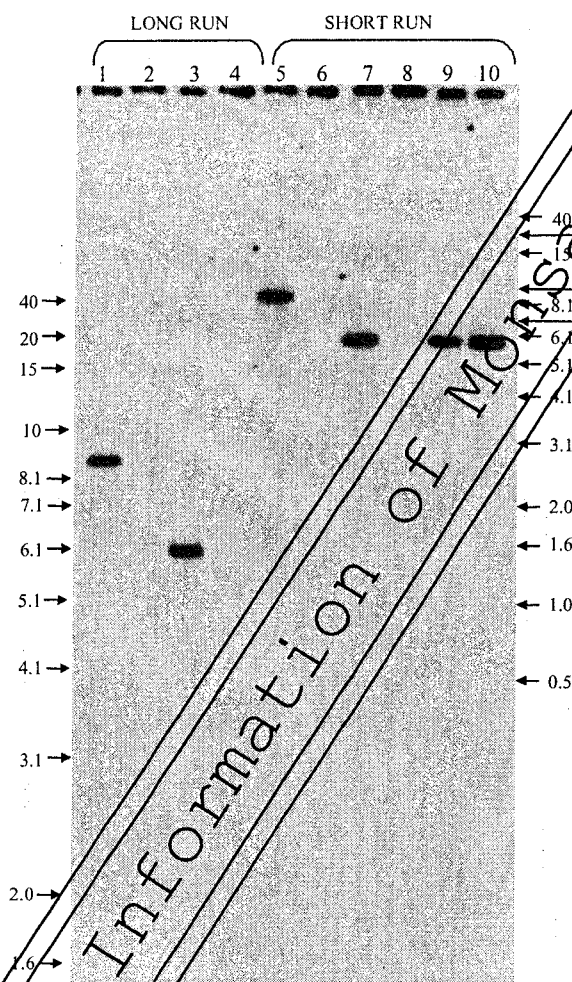
LONG RUN   SHORT RUN

1   2   3   4   5   6   7   8   9   10

40 →
20 →
15 →

10 →

8.1 →
7.1 →

6.1 →

5.1 →

4.1 →

3.1 →

2.0 →

1.6 →

← 40
← 20
← 15

← 10
← 8.1
← 7.1
← 6.1
← 5.1
← 4.1

← 3.1

← 2.0
← 1.6

← 1.0

← 0.5

Figure 8. Southern blot analysis of MON 89034: *cryIA.105* coding sequence
The blot was examined with overlapping ^{32}P-labeled probes that spanned the *cryIA.105* coding
sequence (probes 3 and 4, Figure 1). 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]
Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
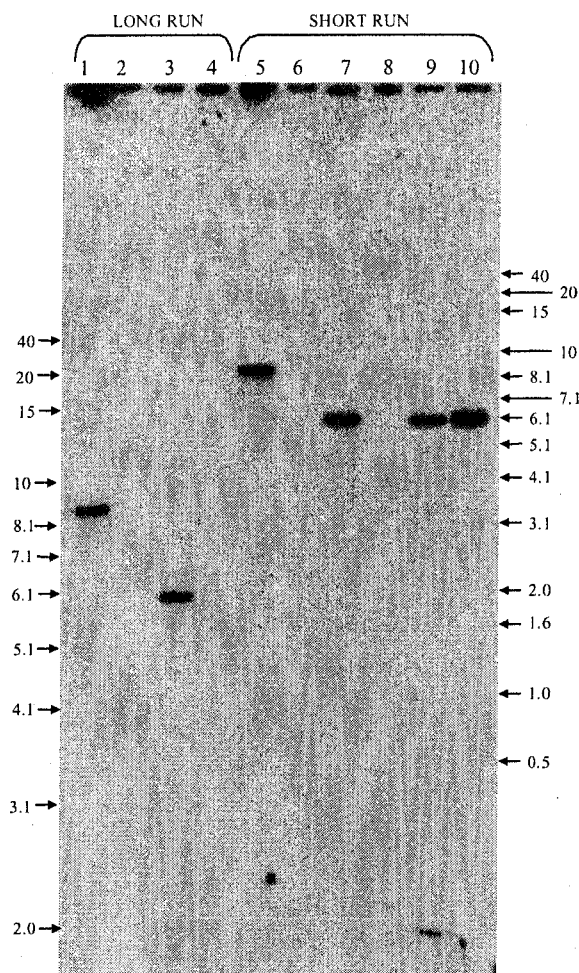
Figure 9. Southern blot analysis of MON 89034: _Hsp17_ 3' end sequence The blot was examined with a ^{32}P-labeled probe that spanned the _Hsp17_ 3' end sequence (probe 5, Figure 1). Each lane contains approximately 10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

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]
  → Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
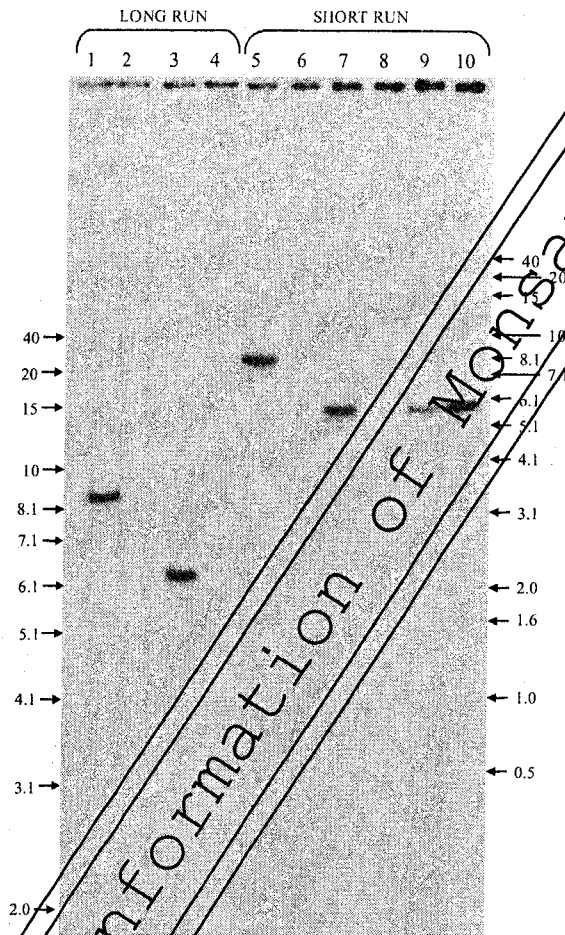
LONG RUN   SHORT RUN

1   2   3   4   5   6   7   8   9   10

40 →
20 →
15 →

10 →
8.1 →
7.1 →

6.1 →

5.1 →

4.1 →

3.1 →

2.0 →

← 40
← 20
← 15
← 8.1
← 7.1
← 6.1
← 5.1
← 4.1
← 3.1
← 2.0
← 1.6
← 1.0
← 0.5

Figure 10. Southern blot analysis of MON 89034: **FMV promoter** The blot was examined with a ^{32}P-labeled probe that spanned the FMV promoter (probe 6, Figure 1). 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]
→   Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
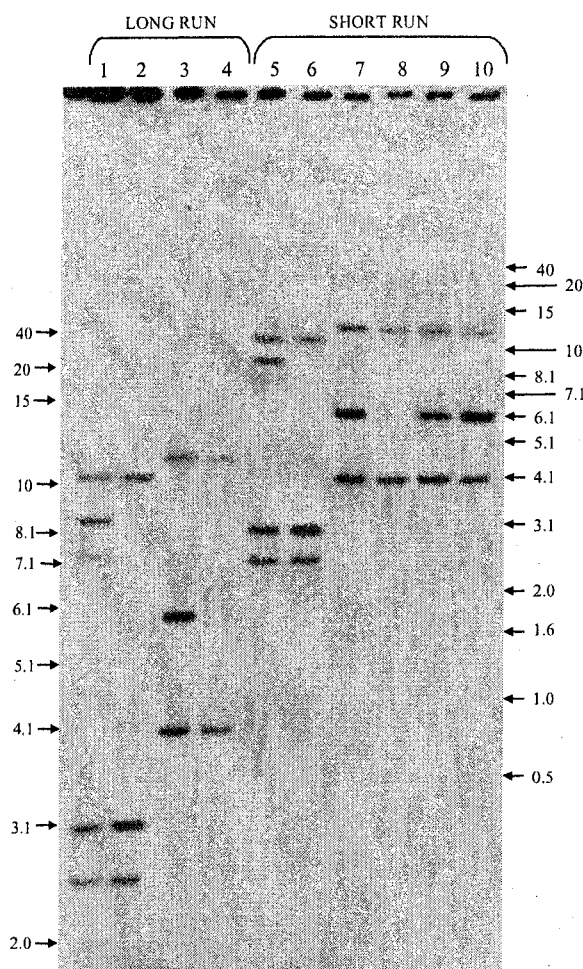
Figure 11. Southern blot analysis of MON 89034: *Hsp70* intron

The blot was examined with a ^{32}P-labeled probe that spanned the *Hsp70* intron (probe 7, Figure 1).
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]
→   Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
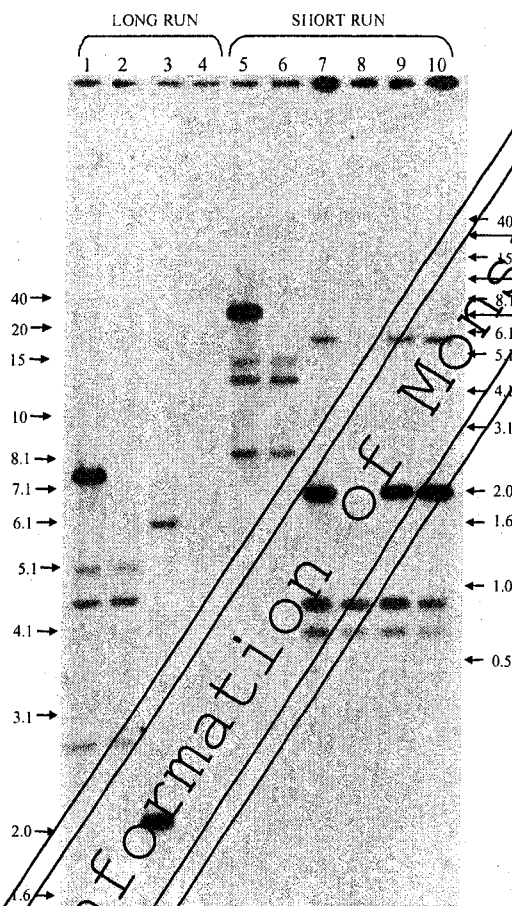
LONG RUN | SHORT RUN

1 2 3 4 | 5 6 7 8 9 10

40 →
20 →
15 →

10 →

8.1 →
7.1 →
6.1 →

5.1 →

4.1 →

3.1 →

2.0 →

1.6 →

← 40
← 15
← 10
← 8.1
← 7
← 6.1
← 5
← 4
← 3.1

← 2.0
← 1.6

← 1.0

← 0.5

Figure 12. Southern blot analysis of MON 89034: *SSU-CTP* **targeting sequence/***cry2Ab2*
coding sequence The blot was examined with overlapping ^{32}P-labeled probes that spanned the *SSU-
CTP* targeting sequence and *cry2Ab2* coding sequence (probes 8 and 9, Figure 1). 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]
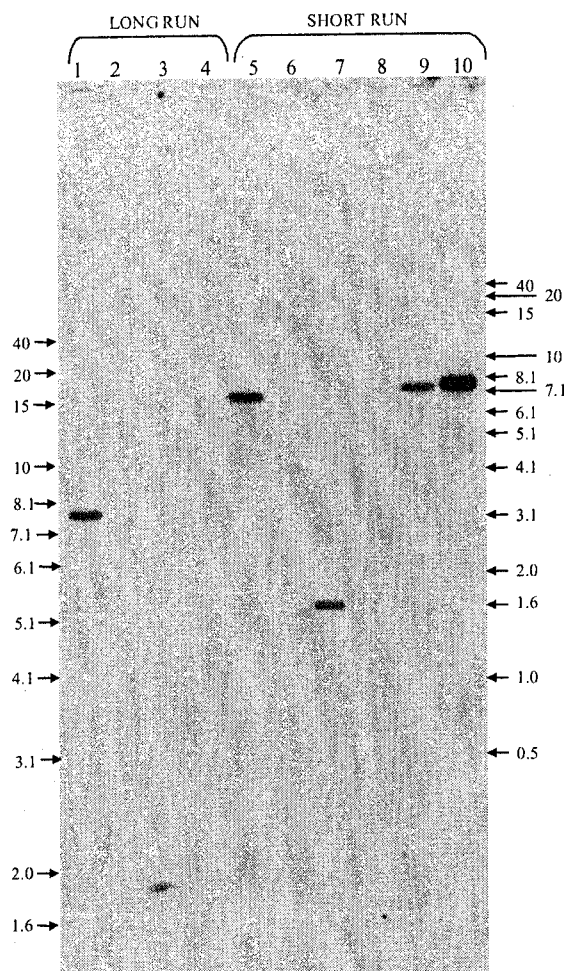   → Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Figure 13. Southern blot analysis of MON 89034: *nos* 3' end sequence
The blot was examined with a ^{32}P-labeled probe that spanned the *nos* 3' end sequence (probe10, Figure 1). 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]
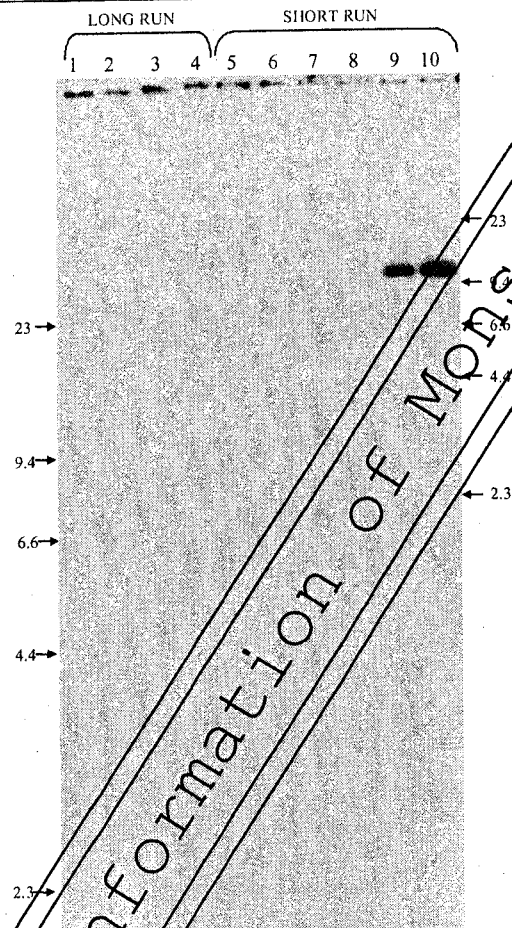→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Figure 14. Southern blot analysis of MON 89034: PV-ZMIR245 backbone sequence
The blot was examined with ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence
(probes 14-17, Figure 1). 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]
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
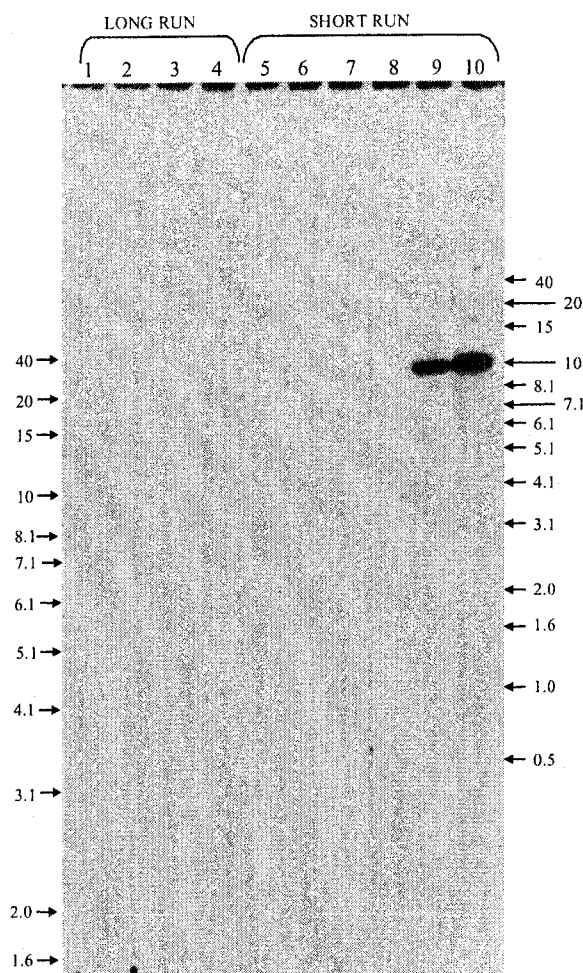
Figure 15. Southern blot analysis of MON 89034: *npt II* coding sequence The blot was examined with a ^{32}P-labeled probe that spanned the *npt II* coding sequence (probe 12, Figure 1). Each lane contains approximately 10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

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]

—➤ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
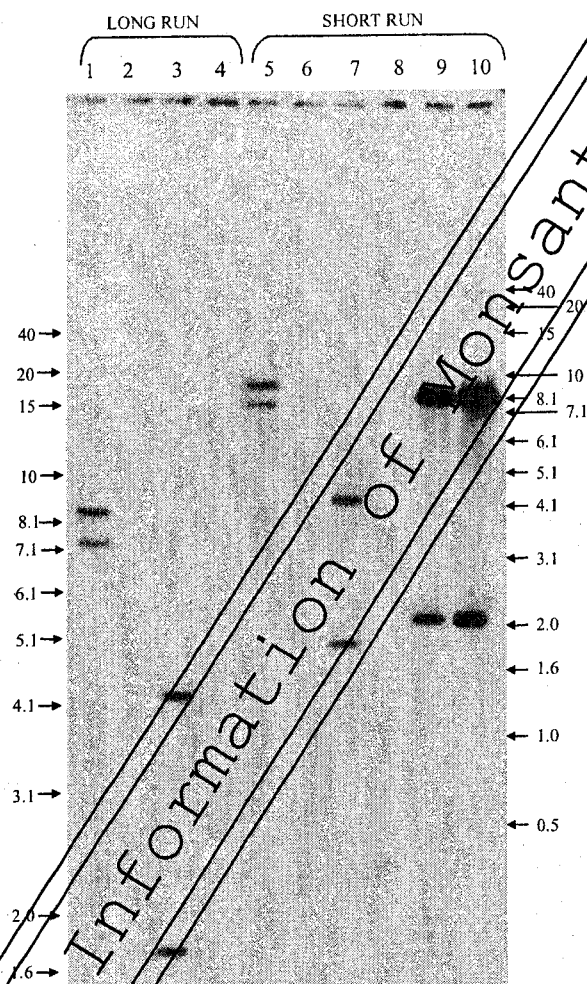
Figure 16. Southern blot analysis of MON 89034: T-DNA II
The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13, Figure 1). Each lane contains approximately 10 μg of digested genomic DNA isolated from grain. Lane designations are as follows:

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]
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
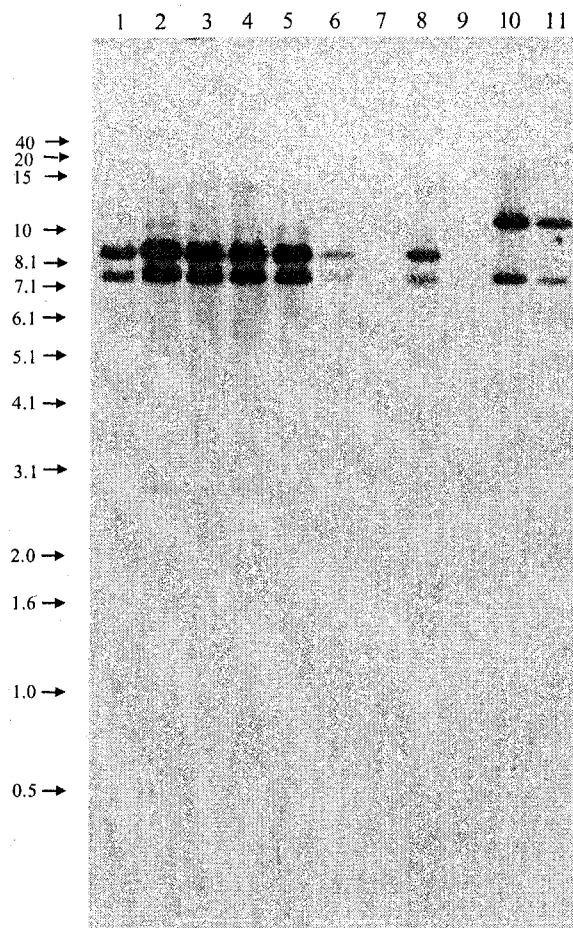
Figure 17. Insert stability of MON 89034: T-DNA I

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 18 - 23, Figure 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: 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 ((T1: BC1: F$_1$ x RP, *Ssp* I)
      9: Conventional corn A (*Ssp* I)
     10: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
     11: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→   Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
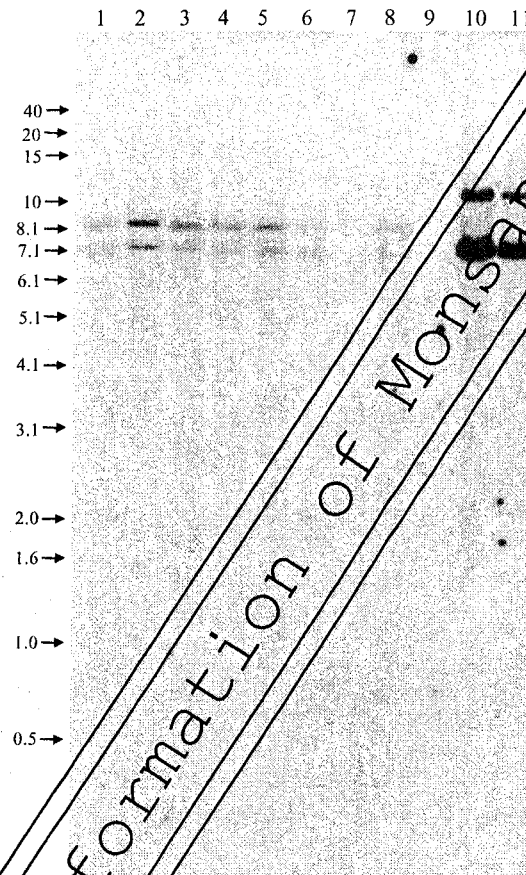
Figure 18.   T-DNA II analysis in multiple generations of MON 89034: T-DNA II

The blot was examined with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (probes 11-13, Figure 1). 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: 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 ((T1: BC1: F$_1$ x RP, Ssp I)

9: Conventional corn A (Ssp I)

10: Conventional corn spiked with PV-ZMIR245 (Ssp I) [1.0 copy]

11: Conventional corn spiked with PV-ZMIR245 (Ssp I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
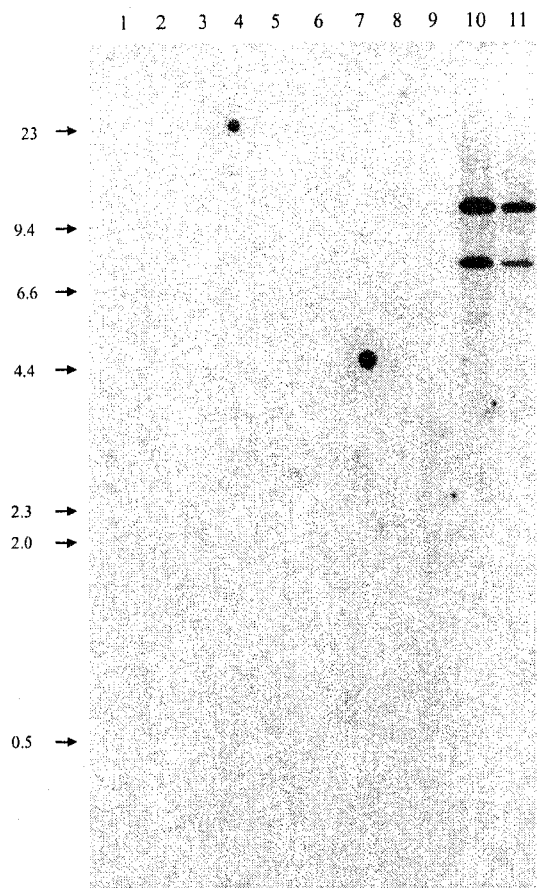
Figure 19. Backbone analysis in multiple generations of MON 89034: PV-ZMIR245 backbone sequence

The blot was examined with overlapping ^{32}P-labeled probes that spanned the PV-ZMIR245 backbone sequence (probes 14-17, Figure 1). 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: 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 ((T1: BC1: F$_1$ x RP, *Ssp* I)
        9: Conventional corn A (*Ssp* I)
     10: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [1.0 copy]
     11: Conventional corn spiked with PV-ZMIR245 (*Ssp* I) [0.5 copy]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
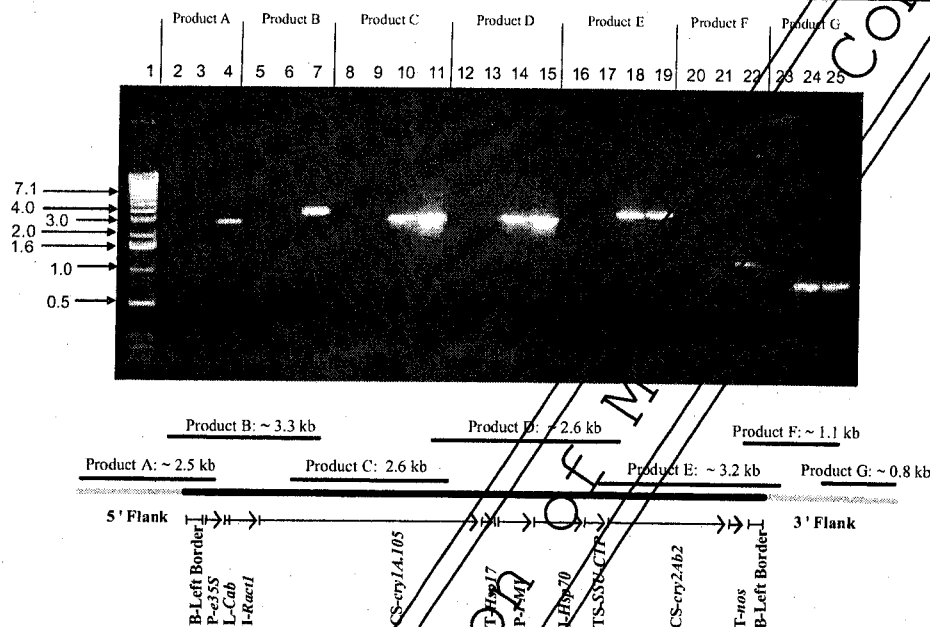
——▶ Symbol denotes sizes, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

Figure 20. 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 expected product size for each amplicon is provided in the illustration of the insert in MON 89034 that appears at the bottom of the figure. Ten to twenty-five microliters of each of the PCR products were loaded on the gel. This figure is representative of the data generated in the study; however the specific bands from this gel were not excised and sequenced.

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

[CBI CROSS REFERENCE 1]

[CBI CROSS REFERENCE 1]

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[CBI CROSS REFERENCE 1]

[CBI CROSS REFERENCE 1]

APPENDIX 1

Standard Operating Procedures

BR-EQ-0065-02	DyNA Quant 200 Fluorometer
BR-ME-0094-01	DNA Extraction from Plant Tissues (Based on Procedure of Rogers and Bendich)
BR-ME-0315-01	Procedure for Agarose Gel Electrophoresis
BR-ME-0315-02	Procedure for Agarose Gel Electrophoresis
BR-ME-0316-01	Procedure for Restriction Enzyme Digestion of DNA
BR-ME-0317-02	Procedure for Southern Blot Analysis
BR-ME-0486-01	Polymerase Chain Reaction
BR-ME-0611-01	Radiolabeling of Nucleic Acids
BR-ME-0626-01	Sarkosyl Method for DNA Isolation from Leaf Tissue Based on Fulton T. M. et al
BR-ME-0878-01	Harbil Paint Shaker
BR-ME-0920-01	Purification of Plasmid DNA (Qiagen QIA filter Plasmid Mini Kit)

APPENDIX 2

Notes for Reviewer

This report was amended to correct minor errors in references and nomenclature, and to improve clarity. These revisions are outlined in Appendix 2 as "Amendment 1". These changes do not impact the conclusions of this study.

The study title (MSL 20311 Pgs 1, 4, 6, and 'in the confidential attachment page 1') was revised to include the wording "Amended Report for MSL-20072:"

An Amended Report Completion Date (MSL 20311 Pgs 1, 6, and 'in the confidential attachment page 1') was added, and the Quality Assurance Statement (MSL 20311 Pg 4) was revised to add the new phase "Amended Report Audit."

The Table of Contents (MSL 20311 Pg 7) was amended to include "Appendix 2: Notes for Reviewer." and updated pagination (MSL 20311 Pg 7-9).

In Sections 1.0 and 2.1 (MSL 20311 Pgs 13 and 14), the first sentence was clarified by removing the word 'corn'.

In Section 2.1 (MSL 20311 Pg 14), the abbreviation, *e.g.*, was italicized.

In Section 3.13 (MSL 20311 Pg 18), the description of the protocol amendments and/or deviations was simplified.

In the first paragraph of Section 4.2 (MSL 20311 Pg 29), the text was corrected by deleting lane 24 from the list of conventional control reactions that did not generate a PCR product, and the reference to the PV-ZMIR245 control reaction (MSL 20311 Pg 30) in lane 19 being overloaded was removed, since only lanes 11 and 15 appear overloaded in this experiment.

In Table 1 (MSL 20311 Pg 36), the B-Right Border reference to "Barker et al., 1983" was corrected to "Depicker et al., 1982."

In Table 2 (MSL 20311 Pg 38), the B-Left Border reference to "Depicker et al., 1982" was corrected to "Barker et al., 1983."

In Figure 1 (MSL 20311 Pg 40), the PV-ZMIR245 diagram was corrected to illustrate that probes 6 and 7 overlap, and probes 9 and 10 do not overlap.

In Figure 4 (MSL 20311 Pg 43), the self pollination symbols, "⊗", were added as appropriate, and the period after "backcross generation" was removed.

In Figure 13 (MSL 20311 Pg 52), the text, *Spe* I, in the lane 5 description was corrected to *Ssp* I.

In Figure 20 (MSL 20311 Pg 59), ", in kilobase pairs," was added to the Figure legend

An Appendix 2 (MSL 20311 Pg 67), "Notes for Reviewer" section, was added detailing the contents of Amendment 1.

Title: Alignment of the MON 89034 Insert DNA
Sequence to the PV-ZMIR245
Transformation Vector DNA Sequence

Facility: Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, Missouri 63167

Date: August 31, 2006

Product Characterization
Center Molecular Team Lead: James D. Masucci, Ph.D.

Signatures of Approval:

————————————————————   ——————
James. F. Rice, Ph.D.   Date
Molecular Analyst

————————————————————   ——————
James D. Masucci, Ph.D.   Date
Molecular Team Lead

————————————————————   ——————
Yong Gao, Ph.D.   Date
Sponsor Representative

SUMMARY

The reported sequence of the insert in MON 89034[1] was aligned to the sequence of the transformation vector PV-ZMIR245 using the BestFit function in SeqLab[2]. Previously it was determined that the insert in MON 89034 matches the sequence of PV-ZMIR245 in two places (Rice et al., 2006). The 5' end of the insert, beginning at base 2061 and ending at base 2384 matches the sequence of PV-ZMIR245 between bases 14696 and 14373 (Figure 1). The remainder of the insert, beginning at base 2385 and ending at base 11377, matches PV-ZMIR245 between bases 2083 and 11075 (Figure 2). The sequence of the insert in MON 89034 is 100% identical to the corresponding sequences in PV-ZMIR245.

These results indicate that the P-*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. It is likely that this modification is the result of a crossover recombination event that occurred prior to the DNA being inserted into the genome between *35S* promoters in T-DNA I and T-DNA II (Figure 3). Therefore a modified *e35S* promoter regulates expression of the *cry1A.105* gene in MON 89034. The remaining MON 89034 insert sequence was not altered during the transformation process.

[1] Rice, J.F., Wolff, B.J., Scanlon, N.K., Groat, J.R., Scanlon, N.K., Jennings, J.C., and J.D. Masucci, 2006. Molecular Analysis of Corn MON 89034. Monsanto Technical Report MSL-20072, an unpublished study conducted for Monsanto Company, St. Louis, Missouri.

[2] SeqLab, Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA.

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Product Characterization Center

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Study Title

Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of the Inserted DNA in Corn MON 89034: Assessment of Putative Polypeptides

Authors

J. Scott McClain, Ph.D.
Andre Silvanovich, Ph.D.

Study Completed On

August 10, 2006

Sponsor and Performing Laboratory

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

Laboratory Project ID

MSL Number: 20306

Study Number: 06-01-39-11

The text below applies only to use of the data by the United States Environmental Protection Agency (US EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

STATEMENT OF DATA CONFIDENTIALITY CLAIM

A claim of data confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C).

We submit this material to the U.S. EPA specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by the EPA strictly in accordance with FIFRA. By submitting this material to the EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to the EPA.

Company: _____ Monsanto Company _____

Company Agent: _____

Title: _____

Signature: _____ Date:_____

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Statement of Compliance

This project does not meet the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.
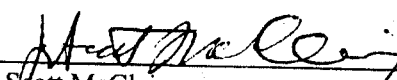
_____   Date: _____
Submitter

_____   Date: 9-Aug-2006
Yong Gao
Sponsor Representative

_____   Date: 10-Aug-2006
J. Scott McClain
Author

Summary of Quality Control Review

This report was checked to ensure that it accurately reflects the raw data of the study. The raw data was audited for compliance with the Monsanto Company Guidelines for Keeping Research Records (GRR 10/1/99), and where applicable, to Monsanto SOPs.

Valerie A. Zuk

Date: _Aug 10, 2006_

Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

Monsanto Company
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Study Certification Page

This report is an accurate and complete representation of the study/project activities.

Signatures of Final Report Approval:

_____ Date: 10-Aug-2006

J. Scott McClain
Author

_____ Date: 08-10-2006

Gary A. Bannon
Lead, Protein Sciences Team

Monsanto Company
Final Report
Product Characterization Center

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MSL No. 20306
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Study Information

Study Number: 06-01-39-11

Title: Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of the Inserted DNA in Corn MON 89034: Assessment of Putative Polypeptides

Facility: Monsanto Company
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800 North Lindbergh Blvd
St. Louis, Missouri 63167

Sponsor Representative: Yong Gao, Ph.D.

Authors: J. Scott McClain, Ph.D.
Andre Silvanovich, Ph.D.

Study Start Date: May 1, 2006

Study Completion Date: August 10, 2006

Records Retention: All study specific raw data and final report will be retained at Monsanto-St. Louis.

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Abbreviations and Definitions

aa	Amino acid
AD6	Allergen, gliadin, and glutenin protein sequence database
ALLPEPTIDES	Protein sequence database comprised of GenBank and SwissProt
Bt	*Bacillus thuringiensis* bacterium
BLOCKS	A database of amino acid motifs found in protein families
BLOSUM	BLOcks SUbstitution Matrix, used to score similarities between pairs of distantly related protein or nucleotide sequences
DATASET	Command used to create a GCG data library from a set of sequences in GCG format
E. coli	*Escherichia coli*
E-score	Expectation score
EMBL	A public genetic database maintained by the European Molecular Biology Laboratory at the European Bioinformatics Institute, Hinxton, England
Entrez	The main database searching system of the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA.
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
GCG	Genetics Computer Group, part of Accelrys and called the Wisconsin Package
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA.
NCBI	National Center of Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA.
ORF	Open reading frame
PubMed	A MEDLINE journal citation database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA.
STRINGSEARCH	Algorithm used to identify sequence entries by searching for character patterns, such as "toxin", in the annotation section of database flatfiles
SeqLab	The graphical X Windows-based interface for the GCG Wisconsin Package
SwissProt	A public protein database maintained by the Swiss Institute of Bioinformatics, Geneva, Switzerland, and the European Molecular Biology Laboratory at the European Bioinformatics Institute, Hinxton, England
TOXIN5	Toxin protein sequence database

UNIX   A computer operating system originally termed "UNiplexed Information and Computing Service"

1.0 Summary

Monsanto has developed 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 with 93.6% overall amino acid sequence identity to the Cry1Ac protein. Cry2Ab2 is a *Bt* (subsp. *kurstaki*) protein. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant allows better insect control and offers an additional insect-resistance management tool.

As part of a comprehensive safety assessment, bioinformatic analyses were performed to assess the potential for allergenicity, toxicity, or bioactivity of putative polypeptides encoded by the 5' and 3' inserted DNA-corn genomic DNA junctions. Sequences spanning the 5' corn genomic DNA-inserted DNA junction and the 3' inserted DNA-corn genomic DNA junction were translated from stop codon to stop codon in all six reading frames. Putative polypeptides from each reading frame were compared to allergen (AD6), toxin (TOXIN5), and public domain (ALLPEPTIDES) database sequences using bioinformatic tools.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD6, TOXIN5, and ALLPEPTIDES databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity, and the E score. In addition to structural congruence, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope.

No biologically relevant structural congruence to allergens, toxins, or bioactive proteins was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. These data demonstrate the lack of both structurally and immunologically relevant congruence to allergens for all of the putative polypeptides analyzed. These data also demonstrate the lack of structurally relevant correlates to toxins or other bioactive proteins for all of the putative polypeptides analyzed.

This bioinformatics analysis is theoretical. No empirical evidence exists to suggest that transcription of DNA sequence at the 5' or 3' junctions of the DNA inserted in MON 89034 occurs. Rather, the results of these bioinformatic analyses indicate that in the highly unlikely event that any of the junction sequences were to be transcribed and that a

transcript were to be translated, the translation product would not share a sufficient degree of sequence similarity or identity to indicate that it would be potentially allergenic, toxic, or have other health implications.

2.0 Introduction

Monsanto has developed 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 with 93.6% overall amino acid sequence identity to the Cry1Ac protein. Cry2Ab2 is a *Bt* (subsp. *kurstaki*) protein. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant allows better insect control and offers an additional insect resistance management tool.

As part of a comprehensive safety assessment, bioinformatic analyses were performed to assess the potential for allergenicity, toxicity, or bioactivity of putative polypeptides encoded by the 5' and 3' inserted DNA-corn genomic DNA junctions. Sequences spanning the 5' corn genomic DNA-inserted DNA junction and the 3' inserted DNA-corn genomic DNA junction were translated from stop codon to stop codon in all six reading frames. Putative peptides from each reading frame were compared to allergen (AD6), toxin (TOXIN5), and public domain (ALLPEPTIDES) database sequences using bioinformatic tools.

Exposure to allergens in foods may cause sudden, medically significant reactions in susceptible individuals. Gliadins and glutenins are suspected to cause celiac disease, a non-IgE mediated disorder (gluten-sensitive enteropathy), and are also considered important immunologically active proteins. Screening the amino acid sequences of proteins introduced into plants by modern biotechnology for similarity to sequences of known allergens, gliadins, and glutenins is one of many assessments performed to support product safety. Similarly, the amino acid sequences of introduced proteins are also screened against known toxins as well as all known proteins in publicly available genetic databases.

The FASTA algorithm can be used to evaluate the extent of sequence alignment between a query protein sequence and a database sequence. In principle, if two proteins share sufficient linear sequence similarity and identity, they will likely share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino

acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al, 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

A second bioinformatics tool, an eight amino acid sliding window search, is used to specifically identify short linear polypeptide matches to known or suspected allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain smaller immunologically significant epitopes. A query sequence may be considered allergenic if it has an exact sequence identity of at least eight contiguous amino acids with a potential allergen epitope (Metcalfe et al., 1996; Hileman et al., 2002; Goodman et al., 2002). However, most allergen epitopes have not been confirmed and the amino acid length for those that have been identified can vary widely, thus the relevance of an exact match of eight amino acids may have limited immunological relevance (Thomas et al., 2005). The eight amino acid bioinformatic strategy is currently an *in silico* search that can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006).

This report describes the bioinformatics assessment of putative polypeptides encoded at the corn genomic DNA-inserted DNA 5' junction and the inserted DNA-corn genomic DNA 3' junction of MON 89034. Inspection of the bioinformatic analysis data can be used to indicate whether the putative polypeptides have biologically relevant sequence congruence to known allergens, toxins, or other bioactive proteins.

3.0 Purpose

The purpose of this study was to evaluate the amino acid sequences of putative polypeptides obtained from all reading frames that span the corn genomic DNA-inserted DNA 5' junction and the inserted DNA-corn genomic DNA 3' junction in MON 89034 to sequences in established databases. Sequences spanning these two junctions were translated from stop codon to stop codon in all reading frames. Structural relatedness between the putative polypeptides and known allergens, toxins, and bioactive proteins was assessed using the FASTA sequence alignment tool. Using each putative polypeptide as a query sequence, FASTA searches were performed on allergen (AD6), toxin (TOXIN5), and public domain (ALLPEPTIDES) sequence databases. Immunologically relevant correlates were assessed using the pairwise comparison

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algorithm ALLERGENSEARCH, using the putative polypeptide as a query sequence to search against the AD6 database.

4.0 Methods

4.1 *Sequence Database Preparation.* The allergen, gliadin, and glutenin sequence database (AD6) was assembled from sequences found on the FARRP allergen database dated January 2006 located at www.allergenonline.com (FARRP, 2006). Gene identification (GI) numbers for the 1537 each sequences found on the FARRP database were used to assemble a list, and this list was used to batch query of the NCBI protein sequence database located at http://www.ncbi.nlm.nih.gov/entrez/batchentrez.cgi. For obsolete GI numbers found using the batch search, the GenPept format flat file associated with each obsolete GI number was examined and an up to date GI number was used to replace the obsolete GI number. Due to the collation of short peptide sequences into a single protein sequence, a total of 1,511 GI numbers were found to be valid as of February 13, 2006. These 1511 GI numbers were used to assemble a searchable database AD6 (Appendix 1, release date, March 6, 2006).

The toxin sequence database (TOXIN5) was assembled from public sequence databases, including GenBank and EMBL release 124 and SwissProt release 1. Protein sequences were retrieved using the STRINGSEARCH function (keyword = toxin) of the Wisconsin Package (version 10). This search was used to identify and retrieve 12,771 separate entries containing the word toxin within the flatfile annotation section. The list.data file was loaded into the editor window of SeqLab, selected, and compiled into a sub-database using the DATASET database utility. The actual number of unique toxin sequences is less than 12,771 because of the redundancy of these public databases and because some entries may contain the word toxin, but are not relevant protein toxins.

The ALLPEPTIDES sequence database was used to represent all currently known publicly available protein sequences and consisted of SwissProt release 40.0 (May 4, 2003) and NRAA release 65.0 (October 24, 2005).

4.2 *Translation of Putative Polypeptides.* DNA sequence spanning the 5' and 3' junctions of the MON 89034 insertion site (Rice et al., 2006) was analyzed for translational stop codons (TGA, TAG, TAA). All six possible reading frames originating or terminating within the MON 89034 insertion were translated using the standard genetic code from stop codon to stop codon.

4.3 *Sequence Database Searches.* All analyses were performed using the UNIX-based Wisconsin Package software, Genetics Computer Group (GCG, version 10.3, Madison, WI) on a personal computer supported with Reflection X Client Manager network software (version 7.20, WRQ, Inc. Seattle, WA). The DNA sequence was supplied in MSL 20072 and translated to the amino acid sequence with DNAStar, SeqBuilder software, version 5.08 or 7.00 (Table 1). The structural similarity of the translated protein sequences to sequences in each database (AD6, TOXIN5, and ALLPEPTIDES) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Specific FASTA comparison parameters used in this study included a wordsize (*k-tuple*) of two, a gap creation penalty of 12, a gap extension penalty of two, and an expectation threshold (*E* score) of ten. The *E* score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E* score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E* score of less than 1×10^{-5} if it is considered to have significant homology.

FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and, thus, recognizes distant evolutionary relationships (Pearson, 2000).

The extent of structural relatedness was evaluated by visual inspection of the aligned sequences, the calculated percent identity, and *E* score. The *E* score reflects the degree of similarity between a pair of sequences and can be used to evaluate the significance of an alignment. Similar amino acids are structurally related and share polar, hydrophobic, or charged states. Such substitutions are referred to as "conservative" since they are unlikely to change the structure of the protein and, by inference, the function of homologous proteins. The calculated *E* score depends on the overall length of joined (gapped) local sequence alignments,

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the quality (percent identity, similarity) of the overlap, and the size of the database (Pearson and Lipman, 1988; Baxevanis and Ouellette, 1998). For a pair of sequences, a very small E score may indicate a structurally relevant similarity. Conversely, large E scores are typically associated with alignments that do not represent a biologically relevant structural correlation.

In addition to the FASTA comparisons of each putative polypeptide to allergens (to assess overall structural similarity), an eight-mer search was performed. An algorithm (ALLERGENSEARCH) was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (ADB). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Hileman et al., 2002; Goodman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six-mer or seven-mer sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six-mer or seven-mer sliding-window searches in a comprehensive analysis of short-peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

4.4 *Significance of the Alignment.* An E score of 1 e-5 (1×10^{-5}) was set as an initial high cut-off value for alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an E score less than 1×10^{-5} was analyzed further to determine if such an alignment represented sequence homology.

5.0   Results and Discussion

Bioinformatics analyses were performed on putative polypeptides deduced from DNA sequence spanning the 5' and 3' inserted DNA-genomic DNA junctions of MON 89034 to assess the potential for similarity towards allergens, toxins, or other bioactive proteins. DNA sequence flanking the 5' (Figure 1) and 3' (Figure 2) junctions of the insertion site in MON 89034 (Rice et al., 2006) were translated from stop codon to stop codon in all possible reading frames. Polypeptide sequence from each reading frame was then inspected to confirm that the sequence was both encoded by DNA spanning the inserted DNA-genomic DNA junctions and was greater than or equal to eight amino acids in length. At the 5' flank, five deduced putative polypeptides spanned the genomic DNA-inserted DNA junction, while at the 3' flank, five putative polypeptides spanned the inserted DNA-genomic DNA junction (see Figure 3 and Table 1). Each putative polypeptide was designated as 5 or 3 (representing the 5' or 3' end, respectively), separated with an underscore by a numerical value 1 to 6 representing the respective reading frame (see Figures 1 and 2 for reading frame assignment). The putative polypeptides 5_2 and 3_4 were not analyzed because they were less than eight amino acids in length. Supporting dataset output files for each putative 5' polypeptide are contained in Appendices 2-6, while dataset output files for each putative 3' polypeptide are contained in Appendices 7-11.

5.1   *Assessment of Potential Allergenicity*: The results of the allergenicity assessment are shown in Tables 2 and 5. Potential allergenicity of the ten junction sequence-encoded putative polypeptides was assessed using the FASTA and ALLERGENSEARCH algorithms. Using the FASTA algorithm to search the AD6 database, no alignments with any of the ten query sequences generated an E score of less than 1×10^{-5}. Among all FASTA alignments between the ten query sequences and the AD6 database, the most significant alignment was between putative polypeptide 5_3 and allergen M-177 precursor (GI number 6492307), yielding an E score of 0.014 that corresponds to 50.0% identity in a window of 24 amino acids. This E-score of 0.014 is not reflective of homology between the putative flanking sequence 5_3 protein of the insert in MON89034 and the protein from the allergen M-177 precursor, as E-scores of ~1 or greater are expected to occur for alignments between random, non-homologous sequences (Pearson, 2000). Therefore, this low quality alignment is considered not relevant from an allergenic assessment perspective. The length of the overlap is relatively short (24 amino acids) when compared to the full length (1668 amino acids) allergen M-177 precursor protein and 2 gaps were required to optimize the alignment. Consequently, no structural and/or functional homology between the 5_3 putative protein and the allergen M-177 precursor protein can be inferred. This alignment neither met nor exceeded the threshold of 35% identity over 80 amino acids (Codex

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Alimentarius, 2003). Furthermore, a minimum similarity required for a allergenic cross-reactivity is likely $\geq$ 50% identity across the entire length of the protein (Aalberse, 2000). Thus, it is extremely unlikely that cross-reactivity exists between the putative protein sequences generated from putative flanking sequence 5_3 of the insert in MON 89034 protein and the protein from the M-177 precursor protein. Inspection of the remaining alignments did not show any significant similarities between the flanking sequence 5_3 protein and allergens. Based on the small alignment window relative to the lengths of the aligned AD6 database proteins, the aforementioned FASTA alignment does not represent a homologous match (Doolittle, 1990) and the hypothetical flanking sequence 5_3 protein, therefore, is not likely to be significant in its structural relatedness to known allergens.

When using the ALLERGENSEARCH algorithm to search the AD6 database, no alignments of eight or more identical amino acids with any of the ten query sequences were demonstrated. As a result, these ten putative polypeptides are unlikely to contain any cross-reactive IgE binding epitopes.

5.2 *Assessment of Potential Toxicity:* The results of the toxicity assessment are shown in Tables 3 and 6. Potential toxicity of the ten junction sequence-encoded putative polypeptides was assessed using the FASTA algorithm. Using the FASTA algorithm to search the TOXIN5 database, no alignments with any of the ten query sequences generated an E score of less than 1×10^{-5}. Among all FASTA alignments between the ten query sequences and the TOXIN5 database, the most significant alignment was between putative polypeptide 3_5 and *E. coli* putative integral transmembrane protein (accession number BAB37562), yielding an E score of 0.056 that corresponds to 57.1% identity in a window of 14 amino acids. Based on the small alignment window relative to the lengths of the aligned TOXIN5 database proteins, the aforementioned FASTA alignment does not represent a homologous match (Doolittle, 1990) and putative polypeptide 3_5 is, therefore, not likely to be significant in its structural relatedness to known toxin proteins.

5.3 *Assessment of Potential Adverse Biological Activity:* The results of this assessment are shown in Tables 4 and 7. Potential untoward biological activity of the ten junction sequence-encoded putative polypeptides was assessed using the FASTA algorithm. Using the FASTA algorithm to search the ALLPEPTIDES database, no alignment among the ten query sequences generated an E score of less than 1×10^{-5}. Among all FASTA alignments between the ten query sequences and the ALLPEPTIDES database, the top alignment was between putative polypeptide 3_2 and Putative CD9/CD37/CD63 antigens (GI number 29840953), yielding an E score of 1.3 that corresponds to 34.6% identity in a window of 26 amino acids. Based on the small alignment windows relative to the lengths of the aligned

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ALLPEPTIDES database proteins, the aforementioned FASTA alignment does not represent a homologous match (Doolittle, 1990) and putative polypeptide 3_2 is, therefore, not likely to be significant in its structural relatedness to proteins of adverse activity.

6.0 Conclusions

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' junctions of the MON 89034 inserted DNA were performed using bioinformatic tools. Results of the FASTA sequence alignments demonstrated a lack of structurally relevant congruence between any known allergenic, toxic, or bioactive proteins and the ten putative polypeptides. Moreover, results from the epitope matching algorithm (ALLERGENSEARCH) demonstrated the lack of potential immunologically relevant sequence matches between any of the putative polypeptides and the AD6 database. The results of these bioinformatic analyses demonstrate that even in the highly unlikely event that any of the junction polypeptides were translated; they would not share a sufficient degree of sequence similarity with other proteins to indicate that they would be potentially allergenic, toxic, or have other health implications.

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Cross Reference Number 1

This cross reference number is used in the report in place of the following page(s) that are found in of the **Confidential Attachment**.

Page   MSL-20306 Page 23 of 182

Page Title   **Figure 1**. Reading frame assignment and DNA
                                  sequence at the 5′ junction of the MON 89034
                                  insert.

Reason for Deletion   Discloses commercial information.

FIFRA Reference   10(d)(1)(A)

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Cross Reference Number 2

This cross reference number is used in the report in place of the following page(s) that are found in of the **Confidential Attachment.**

Page   MSL-20306 Page 24 of 182

Page Title   **Figure 2.** Reading frame assignment and DNA sequence at the 3' junction of the MON 89034 insert.

Reason for Deletion   Discloses commercial information.

FIFRA Reference   10(d)(1)(A)

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Cross Reference Number 3

This cross reference number is used in the report in place of the following page(s) that are found in of the **Confidential Attachment**.

Page   MSL-20306 Page 25 of 182

Page Title   **Figure 3**. Graphic mapping of the flanking DNA sequences and putative polypeptides encoded by each reading frame at the 5' and 3' junctions of the MON 89034 insert.

Reason for Deletion   Discloses commercial information.

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Cross Reference Number 4

This cross reference number is used in the report in place of the following page(s) that are found in of the **Confidential Attachment.**

Page

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Page Title

Table 1. The predicted sequence of putative polypeptides encoded by each reading frame at the 5' and 3' junctions of the MON 89034 insert.

Reason for Deletion

Discloses commercial information.

FIFRA Reference

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