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 MSL-17063, an unpublished study conducted by Monsanto Company.

Study Title

Molecular Analysis to Determine the Genetic Stability of Corn Rootworm Event MON 863 Across Additional Generations

Authors

Jeanna R. Hillyard
Ming Y. Deng
Tracey A. Cavato
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Study Completed On

December 21, 2000

Performing Laboratory

Monsanto Company
Product Characterization Center
Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
St. Louis, MO 63198

Laboratory Project ID

Study 00-01-39-28
MSL-17063

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA.10(d)(l)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to 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 EPA."

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

This study meets the requirements under GLP as specified in 40 CFR Part 160.

Submitter — Date

Ravinder S Sidhu — Dec 21, 2000
Sponsor Representative — Date

Tracey A. Cavato — December 21, 2000
Study Director — Date

QUALITY ASSURANCE UNIT STATEMENT

Study Title: Molecular Analysis to Determine the Genetic Stability of Corn Rootworm Event MON 863 Across Additional Generations

Study Number: 00-01-39-28

Reviews conducted by the QAU confirm that the final report reflects the raw data.

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

Dates Of Inspection / Audit	Phase	Date Reported To: Study Director	Management
May 12, 2000	Southern Blot	May 16, 2000	May 16, 2000
November 30, 2000	Restriction Enzyme Digestion	December 8, 2000	December 8, 2000
December 14, 2000	Raw Data Audit	December 21, 2000	December 21, 2000
December 19, 2000	Draft Report Audit	December 21, 2000	December 21, 2000

Joan M. Rejda-Heath

Joan Rejda-Heath, Ph.D
Quality Assurance
Monsanto Regulatory, Monsanto Company

Dec. 21, 2000
Date

Signatures of Approval

Study Number:	00-01-39-28
Title:	Molecular Analysis to Determine the Genetic Stability of Corn Rootworm Event MON 863 Across Additional Generations
Facility:	Monsanto Company 700 Chesterfield Parkway North St. Louis, MO 63198
Sponsor:	Ravinder S. Sidhu
Study Director:	Tracey A. Cavato
Contributors:	Jeanna R. Hillyard, Ming Y. Deng
Study Initiation Date:	May 5, 2000
Study Completion Date:	December 21, 2000
Records Retention:	All study specific raw data, protocols, final reports 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.

Signatures of Approval:

Tracey A. Cavato — December 21, 2000
Study Director — Date

Ravinder S Sidhu — Dec 21, 2000
Sponsor Representative — Date

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Abbreviations

~	approximately
35S	cauliflower mosaic virus (CaMV) 35S promoter
4-AS1	promoter containing 4 tandem copies of the AS1 element and the 35S promoter
ble	gene for bleomycin resistance
cry3Bb1	class III (Coleoptera-specific) crystal protein gene
Cry3Bb1	class III (Coleoptera-specific) crystal protein
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dCTP	deoxycytidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
HCl	hydrochloric acid
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
Na_2HPO_4	sodium phosphate
NOS 3'	nopaline synthase 3' polyadenylation sequence
nptII	gene for neomycin phosphotransferase II
PCR	polymerase chain reaction
ract1	intron from the rice actin gene
SDS	sodium dodecyl sulfate
SSC	20X is 3 M sodium chloride, 0.3 M sodium citrate
Tris	tris(hydroxymethyl)-aminomethane
tRNA	transfer RNA
tahsp17	3' nontranslated region of the wheat heat shock protein 17.3 containing the polyadenylation sequence
TE buffer	Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
UV	ultraviolet
wtCAB	5' untranslated leader of major wheat chlorophyll a/b binding protein

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

I. SUMMARY

The molecular characterization of corn rootworm event MON 863 has been previously described in detail (Cavato *et al.*, 2000). This characterization demonstrated that one copy of the DNA fragment used for transformation was present in corn event MON 863 and that the insert in corn event MON 863 was stable across three generations. The purpose of this study was to perform Southern blot fingerprint analysis by digesting genomic DNA from nine generations containing corn rootworm event MON 863 with the restriction enzyme *Nco* I and probing with the *nptII* coding region to assess the genetic stability of the integrated DNA. The data show that all of the generations analyzed yielded the expected size bands and no differences in banding pattern were observed among DNA extracted from any of the nine generations. These results, taken together with stability data developed previously (Cavato *et al.*, 2000), demonstrate the stability of the insert DNA in corn rootworm event MON 863 across the two major branches (A1 and A634) of the breeding tree.

II. INTRODUCTION

A. Background. Corn rootworm event MON 863 was generated through particle acceleration using a 4.7-Kb *Mlu I* restriction fragment from the plasmid vector PV-ZMIR13 (Figure 2). The DNA fragment used for transformation, PV-ZMIR13L (Figure 3), contained two gene expression cassettes: an *nptII* selectable marker cassette containing the *nptII* coding sequence under the regulation of the cauliflower mosaic virus (CaMV) 35S plant promoter, and a nopaline synthase (NOS) 3' polyadenylation sequence; and a *cry3Bb1* cassette containing the *cry3Bb1* coding sequence under the regulation of the 4-AS1 plant promoter, and the wtCAB leader, rice actin intron, and tahsp17 3' polyadenylation sequence. A description of the elements in the linear DNA fragment PV-ZMIR13L is given in Table 1. Previous molecular characterization of the insert in corn event MON 863 (Cavato *et al.*, 2000) demonstrated that one copy of the DNA fragment used for transformation is present in corn rootworm event MON 863. A schematic of the insert in corn event MON 863 is shown in Figure 1.

B. Purpose. The purpose of this study was to assess the genetic stability of the integrated DNA in corn event MON 863 using Southern blot fingerprint analysis in nine generations: MON863/A1BC2F3x23CDC1, A1F1, A1BC1F1, A1F2, A1BC2F1, A1BC2F2, A634F2a, A634F3 and LH82xA634F3 (Figure 4).

III. MATERIALS AND METHODS

A. Test substances. The test substance for this study was the corn rootworm event MON 863. The nine generations analyzed in this study containing the MON 863 event were: MON863/A1BC2F3x23CDC1 (Lot # TIO-0005-10294-I), A1F1 (Lot # TIO-0011-10742-S), A1BC1F1 (Lot # TIO-0011-10743-S), A1F2 (Lot # TIO-0011-10744-S), A1BC2F1 (#99-04-39-01, ID 00ZMLYO00030), A1BC2F2 (# 99-04-39-01, ID 00ZMGRO01028), A634F2a (# 99-01-39-08, ID 99ZMGRO00948), A634F3 (Lot # TIO-0011-10737-S), and LH82xA634F3 (Lot # TIO-0011-10748-S) (Figure 4).

B. Control substances. The control substances for this study were the non-transgenic corn lines A1x23CDC1 (Lot # TIO-0005-10295-I), A1 (Lot # TPC-0011-10741-S), A634 (Lot # TPC-0011-10740-S), and LH82xMON863-/A634F3 (Lot # TPC-0011-10752-S).

C. Reference substances. The reference substances include the plasmid PV-ZMIR13 from which the DNA fragment used in the transformation of the corn event was purified. DNA from the A1 non-transgenic control corn line was mixed with the plasmid, then digested and separated by electrophoresis on agarose gels. The mixed DNA samples provided an accurate size marker for the expected fragments of the plasmid. The plasmid DNA also served as a positive hybridization control and was spiked into control line DNA at concentrations representing approximately 1 copy of the plasmid DNA per copy of the genomic DNA to demonstrate the sensitivity of the Southern blotting method. The DNA from the A634F2a generation of corn rootworm event MON 863 was extracted as part of this study to also use as a reference substance in addition to using DNA extracted from this same generation in Study # 99-01-39-27 (Cavato *et al.*, 2000). Additionally, molecular size markers from Boehringer Mannheim [molecular size markers II (23.1 kb-0.6 kb) and IX (1.4 kb-0.072 kb), catalog #236 250 and #1449 460, respectively] were used for size estimations on Southern blots.

D. Test system. There was no test system. This study used analytical methods to characterize the corn event.

E. Identity of Test and Control Substances. The presence or absence of corn event MON 863 was confirmed by MON 863 event specific PCR for all substances. The confirmation of the identity of the test and control substances by verification of the chain of custody documentation occurred after the start of the study.

F. DNA isolation. DNA was extracted from leaf tissue for the A1F1, A1BC1F1, A1F2, A1BC2F1 and LH82xA634F3 generations. DNA was extracted from grain for the MON863/A1BC2F3x23CDC1, A1BC2F2, A634F2a and A634F3 generations in addition to the A1, A634, A1x23CDC1 and LH82xMON863-/A634F3 control lines. The Qiagen

DNeasy Plant Maxi Kit (Valencia, CA, Cat # 68163) was used to extract DNA from the A1F1, A1BC1F1, A1F2, A1BC2F1, A1BC2F2, LH82xA634F3, A1, A634, A634F3, A634F2a and LH82xMON863-/A634F3 samples as per the manufacturer's instructions. The MON863/A1BC2F3x23CDC1 and A1x23CDC1 samples were extracted using the following method. Corn grain tissue (4-5 g) was ground to a fine powder using a blender and transferred to a 50-ml centrifuge tube. Twenty milliliters of CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, and 1.4 M NaCl] were added to each tube and the tubes were incubated at 55°C for 60 min and then allowed to cool at room temperature for approximately 10 min. An equal volume (~20 ml) of chloroform:isoamyl alcohol (24:1) was added to each sample. The suspension was mixed by inversion of the tube several times and centrifuged for 10 min at approximately 16,139 x *g* at room temperature. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube and the chloroform:isoamyl alcohol extraction was repeated two additional times as above. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube and 2/3 volume (~13 ml) of isopropanol was added. The tubes were inverted several times to mix. The DNA was precipitated at approximately -20°C overnight. The tubes were then centrifuged at 16,139 x *g* at 4°C for 20 min. The pellet was re-dissolved in 4 ml of TE buffer, pH 8.0 and transferred to a 13 ml tube. Approximately 40 µl of 10 mg/ml RNase (preboiled) were added and the tubes incubated at 37°C for 30 min. An equal volume (~ 4 ml) of chloroform:isoamyl alcohol (24:1) was added to each sample. The suspension was mixed several times and centrifuged for 10 min. at approximately 16,139 x *g* at room temperature. The upper aqueous phase was transferred to a clean 50-ml centrifuge tube and the chloroform:isoamyl alcohol extraction was repeated once more as above. Approximately one half volume (2 ml) of 7.5 M ammonium acetate was added and the tubes were gently mixed. Two to two and one half volumes of 100% ethanol (~8ml) were added and the tubes were mixed and placed in a -20°C freezer for 2 hours. The DNA was pelleted by centrifugation at approximately 16,139 x *g* at 4°C for 20 min. The DNA was washed with 70% ethanol, vacuum dried, re-dissolved in 1 ml TE, pH 8.0, and stored in a 4°C refrigerator.

G. DNA quantitation and restriction enzyme digestion. Quantitation of the DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer (San Francisco, CA)(SOP BR-EQ-0065-01) using Boehringer Mannheim molecular size marker IX as a calibration standard when quantitating genomic DNA. Approximately 10 µg of genomic DNA from the test and control lines were used for the restriction enzyme digests. Overnight digests were performed at 37°C according to SOP GEN-PRO-010-01 in a total volume of 500 µl using 100 units of restriction enzyme. All restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). After digestion, the samples were precipitated by adding 1/10 volume (~50 µl) of 3M NaOAc and 2 volumes (~1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a

-20°C freezer for at least one hour. The digested DNA was pelleted by centrifugation at maximum speed, washed with 70% ethanol, vacuum dried for approximately 4-10 min. and re-dissolved at room temperature in TE, pH 8.0. One-tenth volume of 10X loading dye was added to each sample.

H. DNA probe preparation. The probe template was prepared by PCR using plasmid PV-ZMBK28 as a template to amplify the *nptII* coding sequence. PV-ZMBK28 contains the same *nptII* coding sequence as PV-ZMIR13. Approximately 25 ng of probe template were labeled with ^{32}P-dCTP (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Gibco BRL, Gaithersburg MD).

I. Southern blot analysis. Southern blot analysis (Southern, 1975) was performed according to SOP GEN-PRO-025-02 to assess the stability of the DNA that was integrated into the corn genome creating event MON 863. The samples of DNA digested with the restriction enzyme *Nco* I were separated, based on size, using 0.6% agarose gel electrophoresis according to SOP GEN-PRO-003-01. The samples were loaded onto the gel and typically electrophoresed for approximately 5.5-24 hours at 25 -85 volts. After photographing the gel, it was placed in a depurination solution (0.125 N HCl) for approximately 10 minutes followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for ~30 minutes and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for ~30 minutes. The DNA from the agarose gels was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) using a Turboblotter (Schleicher & Schuell, Keene, NH). The DNA was allowed to transfer for 12-18.5 hours (using 20X SSC as the transfer buffer) and covalently cross-linked to the membrane with a UV Stratalinker 1800 (Stratagene, La Jolla, CA) using the auto crosslink setting. The blots were prehybridized for 4-6.5 hours in an aqueous solution of 500 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 17-19 hours at approximately 65°C. Membranes were washed in an aqueous solution of 0.1% SDS and 0.1X SSC for two ~15 minute periods followed by two ~20 minute periods at approximately 65°C using fresh solution for each of the four washes. Multiple exposures of blots were generated at approximately -80°C using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen.

IV. RESULTS AND DISCUSSION

A. Southern blot strategy.

Genomic DNA from corn rootworm event MON 863 was digested with the *Nco* I restriction enzyme and subjected to Southern blot hybridization analysis to demonstrate the stability of the inserted DNA across nine generations. The previous molecular characterization of corn rootworm event MON 863 (Cavato *et al.*, 2000)

showed that when genomic DNA from corn event MON 863 is digested with *Nco* I and probed with the full-length *nptII* coding sequence, two unique bands at 0.4 and 8.0 kb are generated. The stability analysis using this restriction enzyme digest and probe combination was conducted in two separate Southern blot experiments since the generations tested were available at different times during the study. A map of the linear DNA fragment, PV-ZMIR13L, that was used to generate the MON 863 transgenic corn event, along with the locations of the restriction sites utilized for Southern analysis, is shown in Figure 3. The Southern blot figures presented in this report are representative of the data generated in the study.

B. Stability of inserted DNA in corn event MON 863.

1. Stability of the inserted DNA in the MON863/A1BC2F3x23CDC1 generation. Control DNA, control DNA mixed with PV-ZMIR13, and test DNA from the MON863/A1BC2F3x23CDC1 generation was digested with *Nco* I. The blot was probed with the full-length *nptII* coding region (Figure 5). The control DNA (lane 3) showed no hybridization signal as expected. Plasmid PV-ZMIR13 DNA mixed with the control DNA (lane 2) produced the expected 3.8, 2.0 and 0.4 kb bands in addition to faint bands at approximately 2.3, 6.0 and 6.6 kb most likely due to partial digestion (Figure 5). The MON 863 reference substance, the A634F2a generation, characterized in Cavato *et al.* (2000) (lane 4) produced the expected 0.4 kb band and the 8.0 kb border fragment from the 5' end of the insert. The test substance, MON863/A1BC2F3x23CDC1 DNA (lane 5), also produced the expected 0.4 kb band and the 8.0 kb border fragment from the 5' end of the insert.

2. Stability of the inserted DNA in the A1F1, A1BC1F1, A1BC2F2, A634F2a, A634F3, A1F2, A1BC2F1 and LH82xA634F3 generations. Control DNA, control DNA mixed with PV-ZMIR13 DNA, and test DNA from the A1F1, A1BC1F1, A1BC2F2, A634F2a, A634F3, A1F2, A1BC2F1 and LH82xA634F3 generations were digested with *Nco* I. The blot was probed with the entire *nptII* coding region (Figure 6). The control DNA samples, A634 and LH82xMON863-/A634F3 (lanes 3 and 4) showed no hybridization signals as expected. Plasmid PV-ZMIR13 DNA mixed with the A1 non-transgenic control DNA (lane 2) produced the expected size bands at 0.4, 2.0, and 3.8 kb, as well as multiple faint additional bands, most likely due to partial digestion. All eight generations tested, A1F1 (lane 5), A1BC1F1 (lane 6), A1F2 (lane 7), A1BC2F1 (lane 8), A1BC2F2 (lane 9), A634F3 (lane 10), A634F2a (lane 11) and LH82xA634F3 (lane 12) produced the expected 0.4 kb band and the 8.0 kb border fragment from the 5' end of the insert. Included in these generations was the A634F2a generations which was originally characterized in Cavato *et al.* (2000). There is a faint band at ~ 1.6 kb in the A634F3 generation (lane 10) which is likely due to incomplete digestion of the *Nco* I site at bp 1501 of PV-ZMIR13 (Figure 1). There are also some faint areas of hybridization which appear band-like in nature in a few of the generations on the blot which are most likely

due to non-specific hybridization and variations in DNA quality since they are not present in all of the corn event MON 863 generations analyzed. No other differences in banding pattern were observed in any of the nine generations included in these analyses. These results demonstrate the stability of the inserted DNA.

V. Conclusions

Previous molecular characterization of corn rootworm event MON 863 demonstrated that there is one complete copy of the DNA fragment used for transformation in corn rootworm event MON 863 (Cavato *et al.*, 2000). The genetic stability of the inserted DNA in corn event MON 863 was analyzed by performing Southern blot analysis on genomic DNA extracted from nine generations, including the generation used in the original molecular characterization of corn rootworm event MON 863 (Cavato *et al.*, 2000). No differences in banding pattern were observed between the DNA extracted from any of the generations demonstrating the stability of the inserted DNA in the nine generations.

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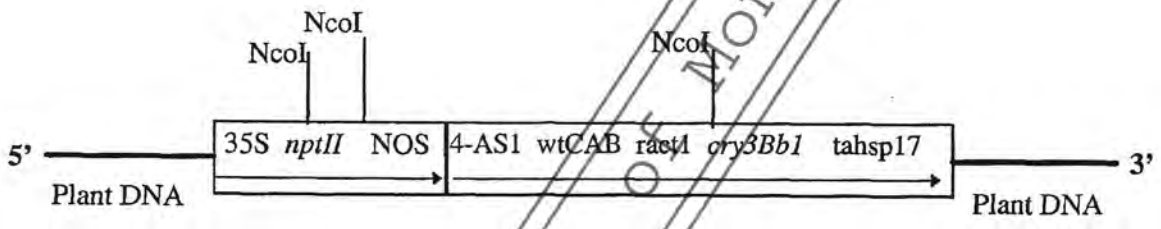

Figure 1. Schematic representation of the MON 863 insert. This figure depicts the predicted insert in corn event MON 863 based on data from Southern blot analyses and PCR confirmation of the sequences at the 5' and 3' ends of the insert. There is one copy of the PV-ZMIR13L fragment that was used to generate corn rootworm event MON 863. Genomic flanking sequence on the 3' end indicates that approximately 10 bp, including the *Hind* III site and *Mlu* I half site, are missing. However, the tahsp17 3' polyadenylation sequence is intact (Cavato *et al.*, 2000).

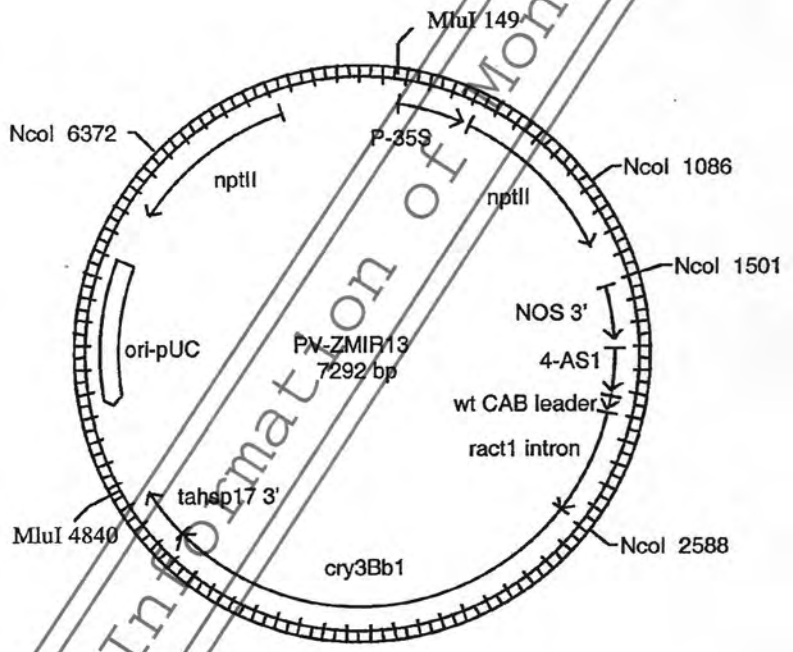

Figure 2. Plasmid map of PV-ZMIR13. The *Mlu* I fragment (bp 149-4840) of PV-ZMIR13 plasmid was used to generate corn rootworm event MON 863.

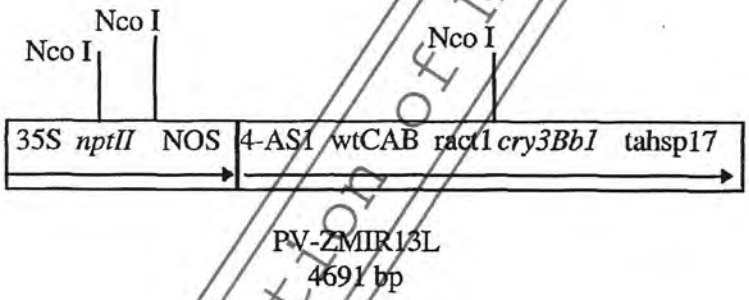

Figure 3. Map of DNA fragment PV-ZMIR13L. The DNA fragment PV-ZMIR13L was used to generate corn rootworm event MON 863 by particle acceleration technology.

Table 1. Summary of genetic elements in linear DNA fragment PV-ZMIR13L used for transformation of event MON 863

Genetic Element	Size Kb	Function
***Cry3Bb1* cassette:**		
4-AS1	0.22	Promoter containing four tandem copies of the AS-1 element (Lam, 1990) and a portion of the 35S promoter of cauliflower mosaic virus (Odell et al., 1985).
wt CAB	0.06	5' untranslated leader of the wheat chlorophyll a/b-binding protein (Lamppa et al., 1985)
ract1 intron	0.49	Intron from the rice actin gene (McElroy et al., 1990)
cry3Bb1	1.96	Gene encoding a synthetic variant of the Cry3Bb1 protein of *Bacillus thuringiensis* (Donovan et al., 1992)
MCS	0.017	Polylinker in the lacZ gene for alpha complementation
tahsp 17 3'	0.23	A 3' nontranslated region of the wheat heat shock protein 17.3 which terminates transcription and directs polyadenylation (McElwain and Spiker, 1989)
Selectable marker elements:		
35S	0.35	The cauliflower mosaic virus (CaMV) promoter (Odell et al., 1985)
nptII	0.97	The gene for the enzyme neomycin phosphotransferase type II from Tn5, a transposon isolated from *Escherichia coli* (Beck et al., 1982). The DNA derived from *E. coli* also includes a 153 bp segment of the gene (*ble*) encoding bleomycin binding protein.
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene of *Agrobacterium tumefaciens* T-DNA which terminates transcription and directs polyadenylation (Bevan et al., 1983)

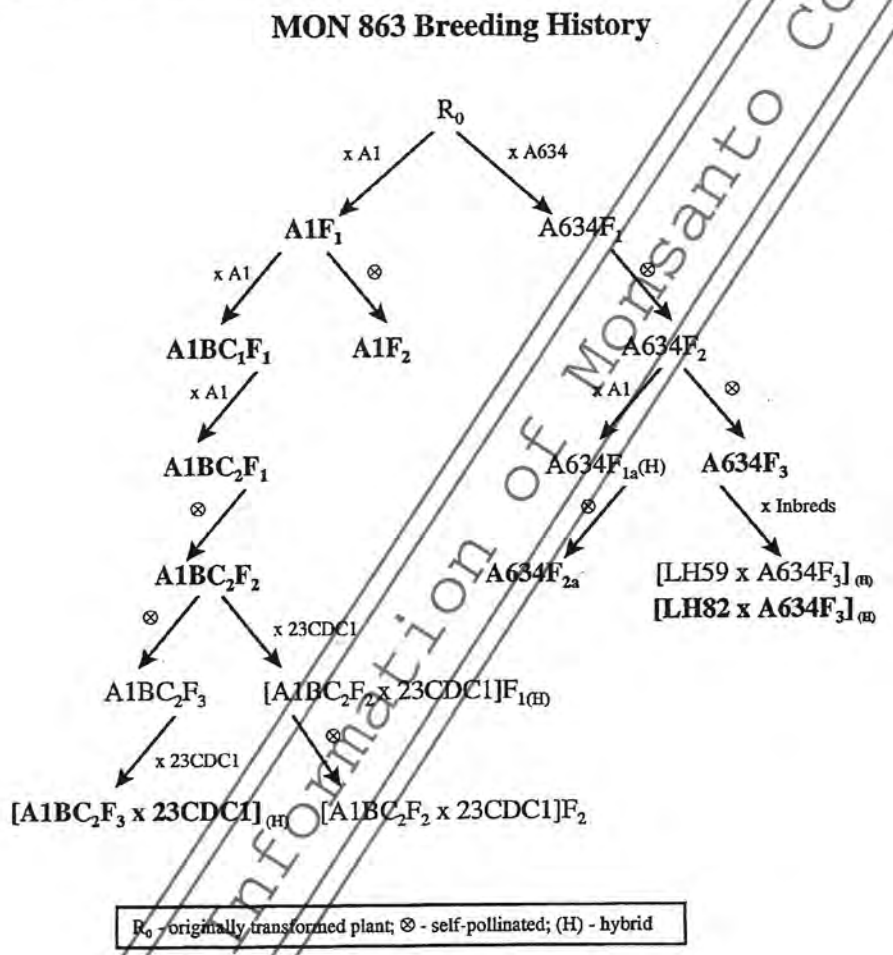

Figure 4. Corn event MON 863 breeding history. The A1BC2F3x23CDC1, A1F1, A1BC1F1, A1F2, A1BC2F1, A1BC2F2, A634F2a, A634F3 and LH82xA634F3 generations (shown in bold) were subjected to Southern blot analysis to assess the molecular genetic stability of the insert in corn rootworm event MON 863. [The previous molecular characterization of corn event MON 863 (Cavato *et al.*, 2000) provided stability data for the A634F2 and A634F2a generations].

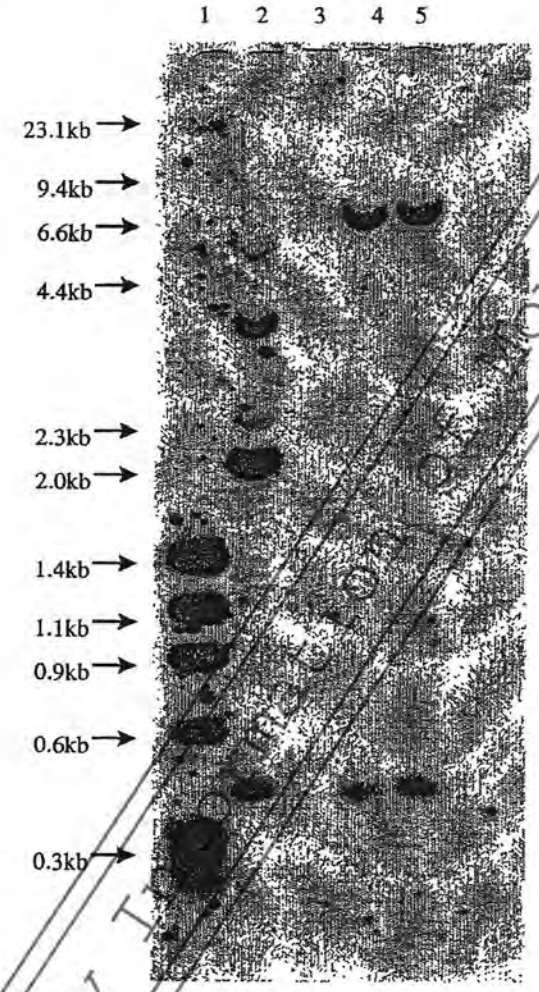

Figure 5. Corn event MON 863 event specific Southern blot fingerprint. Ten micrograms of genomic DNA extracted from corn grain were digested with *Nco* I. The DNA samples were then blotted and probed with the full length ^{32}P-labeled *nptII* coding sequence. Lane designations are as follows:

Lane 1: MW Marker II and MW Marker IX (Boehringer Mannheim)
2: A1x23CDC1 non-transgenic control spiked with 18 pg of plasmid PV-ZMIR13
3: A1x23CDC1 non-transgenic control
4: A634F2a
5: MON863/A1BC2F3x23CDC1
→ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

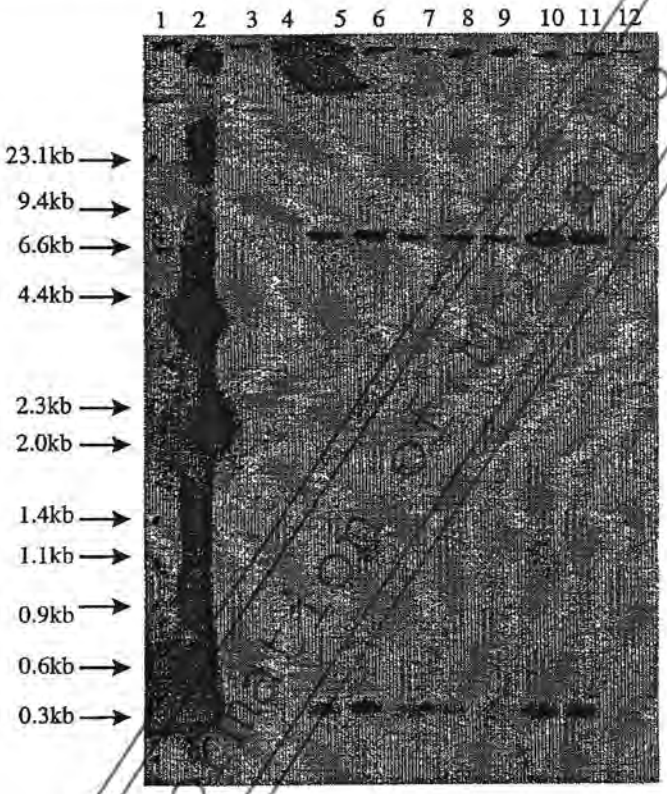

Figure 6. Corn event MON 863 event specific Southern blot fingerprint of additional generations. Ten micrograms of genomic DNA extracted from leaf or grain tissues were digested with *Nco* I. The DNA samples were then blotted and probed with the full-length ^{32}P-labeled *nptII* coding sequence. Lane designations are as follows:

Lane 1: MW Marker II and MW Marker IX (Boehringer Mannheim)
2: A1 non-transgenic control spiked with 22.8 pg of plasmid PV-ZMIR13
3: A634 non-transgenic control
4: LH82xMON863-/A634F3 non-transgenic control
5: A1F1
6: A1BC1F1
7: A1F2
8: A1BC2F1
9: A1BC2F2
10: A634F3
11: A634F2a
12: LH82xA634F3
→ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

Appendix 1

Standard Operating Procedures

BR-EQ-0065-01	DyNA Quant 200 Fluorometer
GEN-PRO-010-01	Procedure for Restriction Enzyme Digestion of DNA
GEN-PRO-003-01	Procedure for Agarose Gel Electrophoresis
GEN-PRO-025-02	Procedure for Southern Blot Analysis

Appendix 2

Study Protocol and Protocol Amendment

Monsanto Study #: **00-01-39-28**

Study Title: Additional molecular analysis of the genetic stability of corn event MON 863

Sponsor:
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May 5, 2000
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1.0 Regulatory Compliance

1.1 GLP Compliance

This is a product characterization study as defined by section §160.135(b) of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards (40 CFR Part 160) intended to characterize the physical and/or chemical properties of a potential commercial product. This study will be conducted in compliance with all requirements of section §160.135(b).

2.0 Purpose

The purpose of this study is to analyze the genetic stability of the inserted DNA in corn event MON 863 by Southern blot. Event MON 863 was created by modifying a corn line to express a protein from *Bacillus thuringiensis* strain EG11098 which has insecticidal activity against corn rootworm. In addition to the *cry3Bb1* coding region, the neomycin phosphostransferase II (*nptII*) coding region is present as the selectable marker, enabling selection of cells that contain the *cry3Bb1* coding region in tissue culture. The control line has background genetics representative of the test event, but has not been genetically modified and therefore, does not contain the *cry3Bb1* or *nptII* coding regions. The control line provides a background matrix used in the analysis of banding patterns on Southern blots. An event specific Southern blot fingerprint was developed for MON 863 and will be used in this study.

3.0 Timelines

3.1	Proposed Experimental Start Date:	May 5, 2000
3.2	Proposed Experimental Termination Date:	October 31, 2000

4.0 Test, Control and Reference Substances

4.1 Test Substance(s)

The test substance is corn event MON 863. Lot number TIO-0005-10294-I representing an A1BC2F3 Hybrid (A1BC2F3 x 23CDC1) of event MON 863 will be tested. Additional generations may be tested and will be added by amending the protocol.

[redacted]

4.2 Control Substance(s)

The control substance is the non-transgenic parental control corn line A1 x 23CDC1, Lot number TIO-0005-10295-I. Additional control substances may be used and will be added by amending the protocol.

4.3 Reference Substance(s)

The reference substances include the plasmid (PV-ZMIR13) from which the DNA fragment used in the transformation of the corn line was obtained. The plasmid DNA will be used as a size marker and positive hybridization control by digesting with *Nco*I. MON 863 DNA extracted as part of Study 99-01-39-27 will also be used as a reference substance. Additionally, molecular size markers from Boehringer Mannheim (molecular size markers II and IX, catalog #236 250 and #1449 460, respectively) will also be used for band size estimations.

4.4 Characterization of Test, Control and Reference Substances

The Study Director determined the identity of the test, control, and reference substances by verifying the chain-of-custody documentation supplied with the samples. The Southern blot analysis performed in this study will confirm the identity of the test and control substances.

5.0 Description of Experimental Design

Genomic DNA from the test substance will be analyzed by Southern blot for the stability of the inserted DNA. The genomic DNA will be digested with the restriction enzyme *Nco*I and the blot probed with the *nptII* coding region. Chain of custody for the reference substance originating in previous studies will be documented.

5.1 Analytical Methods

5.1.1 DNA Extraction

DNA was extracted from seed prior to the start of the study. A CTAB-based method was used and the raw data detailing the extraction will be archived with this study. All previously extracted DNAs have been stored at 2-8 °C. If necessary, additional DNA will be extracted under this protocol from seed using methods approved by the study director. Following extraction, the isolated DNA will be stored at 2-8 °C.

5.1.2 Restriction Enzyme Digestion

Ten micrograms of extracted genomic DNA and approximately 1 copy number equivalent of plasmid DNA (spiked into 10 ug of non-transgenic genomic DNA) will be digested with *Nco*I following SOP # GEN-PRO-010-01.

[redacted]

5.1.3 DNA Quantitation
Any DNA extracted in this study will be quantitated using Hoefer's DyNA Quant 200 Fluorometer according to SOP # BR-EQ-0065-01.

5.1.4 Agarose Gel Electrophoresis:
The digested DNA will be electrophoresed on agarose gels according to SOP # GEN-PRO-003-01.

5.1.5 Southern Blot Analysis:
The agarose gels containing digested DNA will be blotted to nylon membranes and probed with the full-length radiolabeled *nptII* coding region according to SOP # GEN-PRO-025-02.

6.0 Control of Bias
Proper positive and negative controls will be included on all agarose gels and Southern blots.

7.0 Records to be Maintained
Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters memoranda and other correspondence related to this study. These documents may include: photocopies, computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived in the Biotech Regulatory Science archives.

8.0 Changes to the Protocol
Planned changes to the protocol will be documented in the form of written protocol amendments and signed by the Study Director. Amendments become part of the protocol and will be archived with the protocol. All other changes will be in the form of written protocol deviations and will be filed with the raw data. All changes to the protocol will be addressed in the final report.

[redacted]

Protocol Amendment Form **Amendment #: 1**

Monsanto Study #: 00-01-39-28

Date changes implemented: November 30, 2000

Page number(s) and section(s): Page 4, Section 5.1.1 "DNA Extraction"

Protocol originally stated: DNA was extracted from seed prior to the start of the study. A CTAB-based method was used and the raw data detailing the extraction will be archived with this study. All previously extracted DNAs have been stored at 2-8°C. If necessary, additional DNA will be extracted under this protocol from seed using methods approved by the study director. Following extraction, the isolated DNA will be stored at 2-8°C.

Protocol amended as follows: DNA was extracted from seed from lot number TIO-0005-10294-I representing an A1BC2F3 Hybrid (A1BC2F3x23CDC1) of event MON 863 and the non-transgenic parental control corn line A1 x 23CDC1, lot number TIO-0005-10295-I prior to the start of the study using a CTAB based method. The raw data detailing the extraction will be archived with this study. DNA will be extracted in this study from leaf and/or seed tissue for all other generations tested using the Qiagen DNeasy Plant Maxi Kit (see Test Substances section of Amendment 1). All extracted DNA was/will be stored at 2-8°C.

Reason for the amendment and what impact will result from this change:
Additional generations needed to be tested by Southern blot analysis to analyze the genetic stability of the inserted DNA in corn event MON 863. There was little seed available for some of these generations, therefore the DNA will be extracted from leaf tissue where necessary. Since additional generations will be analyzed resulting in the generation of additional data supporting the stability of the insert, there will be a positive impact on the study.

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Protocol Amendment Form **Amendment #: 1**

Page number(s) and section(s): Page 3, Section 4.1. "Test Substance(s)"

Protocol originally stated: The test substance is corn event MON 863. Lot number TIO-0005-10294-I representing an A1BC2F3 Hybrid (A1BC2F3x23CDC1) of event MON 863 will be tested. Additional generations may be tested and will be added by amending the protocol.

Protocol amended as follows: The test substance is corn event MON 863. Additional generations may be tested and will be added by amending the protocol. The generations of MON 863 with their corresponding lot numbers which will be analyzed in this study are in the table below.

MON 863 Generation	Lot Number
A1BC2F3x23CDC1 (MON 863+)	TIO-0005-10294-I
A1F1	TIO-0011-10742-S
A1BC1F1	TIO-0011-10743-S
A1F2	TIO-0011-10744-S
A634F3	TIO-0011-10737-S
LH82xA634F3 (MON 863+)	TIO-0011-10748-S
A1BC2F1	# 99-04-39-01, LIMS ID 00ZMLYO00030
A634F2a	#99-01-39-08, LIMS ID 99ZMGRO00948
A1BC2F2	# 99-04-39-01, LIMS ID 00ZMGRO01028

Reason for the amendment and what impact will result from this change:
Additional generations of MON 863 need to be tested by Southern blot to analyze the genetic stability of the inserted DNA. Since additional generations will be analyzed resulting in the generation of additional data supporting the stability of the insert, there will be a positive impact on the study.

[redacted]

Protocol Amendment Form **Amendment #: 1**

Page number(s) and section(s): Page 4, Section 4.2 "Control Substance(s)"

Protocol originally stated: The control substance is the non-transgenic parental control corn line A1 x 23CDC1, Lot number TIO-0005-10295-I. Additional control substances may be used and will be added by amending the protocol.

Protocol amended as follows: The non-transgenic corn lines which will serve as control substances are shown in the table below along with their corresponding generation representing test substances. Additional control substances may be used and will be added by amending the protocol.

Control Substance	Lot Number	Corresponding Test Substance(s)
A1 x 23CDC1	TIO-0005-10295-I	A1BC2F3 x 23CDC1 (MON 863+)
A1	TPC-0011-10741-S	A1F1, A1BC1F1, A1F2, A1BC2F1, A1BC2F2
A634	TPC-0011-10740-S	A634F3, A634F2a
LH82xMON863-/A634F3	TPC-0011-10752-S	LH82xA634F3 (MON 863+)

Reason for the amendment and what impact will result from this change:
Additional generations of MON 863 will be tested by Southern blot analysis to analyze the genetic stability of the inserted DNA and the appropriate non-transgenic controls are needed for these generations. There is no impact on the study.

[redacted]

Protocol Amendment Form

Amendment #: 1

Approved By:

Patrick T. Weston
Testing Facility Management Representative

29, 2000
Date

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November 29, 2000
Date

Dennis P. Ward
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29 Nov 2000
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Nov. 29, 2000
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