Title

Amended Report for MSL0021960: Molecular Analysis of Insect-Protected Soybean MON 87701

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Study Completed

Amendment 2

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Sponsor/ Testing Facility

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Laboratory Project ID

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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 exception:

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
Eddie Zl	July 31, 2009
Sponsor Representative	Date
Jeanna R Great	7/31/09
Study Director	Date

QUALITY ASSURANCE STATEMENT

Study Title: Amended Report for MSL0021960: Molecular Analysis of Insect-Protected Soybean MON 87701

Study Number: REG-07-317

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
05/29/2008	Southern Blot	06/03/2008	06/03/2008
07/15/2008	PCR/Sequence	07/28/2008	07/28/2008
11/07/2008	Raw Data Audit	11/12/2008	11/12/2008
12/04/2008	Raw Data Audit	12/05/2008	12/05/2008
12/04/2008	Draft Report Review	12/05/2008	12/05/2008
04/08/2009	Draft Report Amendment Review	04/08/2009	04/08/2009
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Quality Assurance Specialist
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Date

STUDY INFORMATION PAGE

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Primary Facility:	Monsanto Company 800 North Lindbergh Blvd. St. Louis, Missouri 63167
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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 Company, St. Louis, Missouri.
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto Company, St. Louis, Missouri.

STUDY CERTIFICATION PAGE

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

Jeanna R. Groat 7/31/2009

Jeanna R. Groat Date
Study Director
Regulatory Product Characterization Team

Qing Tian 7/31/09

Qing Tian, Ph.D. Date
Molecular Team Lead
Regulatory Product Characterization Team

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ABBREVIATIONS AND DEFINITIONS [1]

aadA	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3' (9)-O-nucleotidyltransferase from the transposon Tn7 that confers spectinomycin and streptomycin resistance
~	Approximately
2-ME	2-Mercaptoethanol
2T-DNA	Plasmid vector containing two separate T-DNA regions each delineated by left and right borders of the Ti plasmid
B-Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
bp	Base pair
B-Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
B.t.	*Bacillus thuringiensis*
Ci	Curie
COA	Certificate of Analysis
CPM	Counts per minute
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4
Cry1Ac	Class I (lepidopteran-specific) crystal protein
CS–*cp4 epsps*	Codon modified coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein
CS-*cry1Ac*	Codon-modified coding sequence of the Cry1Ac protein of *Bacillus thuringiensis*
CS-*rop*	Coding sequence for repressor of primer protein derived from ColE1 plasmid for maintenance of plasmid copy number in *E. coli*
CTAB	Cetyltrimethylammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
kb	Kilobase
L-*ShkG*	5' non-translated leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS that helps regulate gene expression
ml	Milliliter
mmol	Millimole
MW	Molecular weight

[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 (CONT.)

OR–*ori V*	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium*
OR–*ori-pBR322*	Origin of replication from *pBR322* for maintenance of plasmid in *Escherichia coli*
PCI	25:24:1 Phenol: chloroform: isoamyl alcohol mixture
PCR	Polymerase chain reaction
PEG	Polyethylene glycol (MW 8000)
P-*FMV*	Promoter for the 35S RNA from figwort mosaic virus (FMV) that directs transcription in most plant cells
P-*RbcS4*	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1, 5-bisphosphate carboxylase small subunit 1A, and promoter expresses in above ground tissues
SOP	Standard operating procedure
sp.	Species
subsp.	Subspecies
T-*7Sα*'	3' region of the *Sphas1* gene of *Glycine max* encoding the 7*Sα'* seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence. The element functions to terminate transcription and direct polyadenylation of the mRNA.
T-DNA	Transfer(ed) DNA
TE	Tris EDTA, pH 8.0
T-*E9*	3' non-translated sequence from *RbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA
TS-*CTP1*	Targeting sequence encoding the transit peptide of the *Arabidopsis RbcS4* encoding small subunit 1A transit peptide, from *Arabidopsis thaliana*, present to direct the *cry1Ac* protein to the chloroplast
TS–*CTP2*	Targeting sequence encoding the chloroplast transit peptide from the *ShkG* gene of *Arabidopsis thaliana* encoding EPSPS
µg	Microgram
µl	Microliter
% w/v	Percentage weight of a substance of the total volume

1.0 SUMMARY

Monsanto Company has developed insect-protected soybean MON 87701 that produces the Cry1Ac insecticidal crystal (Cry) protein (δ-endotoxin) derived from *Bacillus thuringiensis* (*B.t.*) subsp. *kurstaki*. The Cry1Ac protein provides protection from feeding damage caused by targeted lepidopteran insect pests.

MON 87701 was produced by *Agrobacterium*-mediated transformation of soybean tissue using the 2T-DNA transformation plasmid PV-GMIR9 (Figure 1) that contains two separate T-DNAs (transfer DNAs), which are delineated by right and left border sequences. The first T-DNA, designated as T-DNA I, contains the expression cassette containing the *cry1Ac* coding sequence regulated by the *RbcS4* promoter and the *7Sα*' 3' non-translated region sequence. The second T-DNA, designated as T-DNA II, contains the expression cassette containing the *cp4 epsps* coding sequence regulated by the FMV promoter and the *E9* polyadenylation sequence. During transformation, both T-DNAs were inserted into the genome at independent, unlinked loci. Subsequently, traditional breeding and segregation was used to isolate those plants that contain the *cry1Ac* expression cassette (T-DNA I) but do not contain the *cp4 epsps* expression cassette (T-DNA II), resulting in the production of marker-free, insect-protected soybean MON 87701.

In this study, Southern blot analyses confirmed the copy number of T-DNA I sequences in the genome and the absence of transformation vector backbone and selectable marker (T-DNA II) sequences. The data indicate that MON 87701 contains one copy of the insert at a single integration locus and all expression elements are present in the T-DNA I. These data also demonstrate that MON 87701 does not contain detectable backbone or selectable marker sequence from plasmid PV-GMIR9. The complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 87701 confirmed the integrity of the inserted *cry1Ac* expression cassette within the inserted sequences and identified the 5' and 3' insert-to-genomic DNA junctions. Additional characterization of the insertion site in conventional soybean confirmed the flanking genomic DNA is native to the soybean genome and it also indicates that 32 bases of genomic DNA were deleted and 14 bases inserted during the *Agrobacterium*-mediated transformation. Furthermore, Southern blot fingerprint analysis demonstrated that the insert in MON 87701 has been maintained through five generations of breeding, thereby confirming the stability of the insert over multiple generations.

2.0 INTRODUCTION

2.1 Background

Monsanto Company has developed insect-protected soybean MON 87701 that produces the Cry1Ac protein to provide protection from feeding damage caused by targeted lepidopteran insect pests. MON 87701 was produced through *Agrobacterium*-mediated transformation using 2T-DNA vector technology to generate marker-free plants.

2.2 Purpose

The purpose of this study was to characterize the integrated DNA in MON 87701. Genomic DNA was analyzed using Southern blot analyses and the complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 87701 was determined. The copy number of T-DNA I, the integrity of the inserted *cry1Ac* gene cassette (T-DNA I), and the absence of plasmid backbone and selectable marker sequences (T-DNA II) were evaluated. Insert stability analysis was performed to confirm the stability of the transgene insertion in multiple generations of MON 87701. In addition, PCR and DNA sequence analysis were performed to confirm that the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87701 are native to the soybean genome, thereby characterizing the insertion site in conventional soybean.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance was MON 87701. Genomic DNA was extracted from leaf tissue harvested from Production Plan 07-01-59-05.

Production Plan	Seed Lot Number	Virgo Number	Sample IDs
07-01-59-05	GLP-0705-18705-S	60080819037	07015905-00006
			07015905-00007

Additional samples from the conventional breeding of MON 87701 (Figure 2) were used to assess the stability of the test substance across generations. Genomic DNA was extracted from the following leaf tissues listed below.

Generation	Production Plan	ORION[2] ID	Source ID	Container ID
R4	REG-08-152	10002639	10002140	10002639-002
R5	REG-08-152	10002640	10002141	10002640-002
R6	REG-08-152	10002641	10002142	10002641-002
R8	REG-08-152	10002642	10002143	10002642-002
R9	REG-08-152	10002643	10002144	10002643-002

3.2 Control Substance

The control substance is conventional soybean variety A5547 which has the same genetic background as the test substance. Genomic DNA was extracted from leaf

[2] ORION is a proprietary database used at Monsanto Company to track Regulatory plant samples.

tissue harvested from Production Plan 07-01-59-05 and used for Southern blot and PCR analyses in this study.

Production Plan	Seed Lot Number	Virgo Number	Sample ID
07-01-59-05	GLP-0612-17895-S	60077863314	07015905-00002
			07015905-00003

3.3 Reference Substances

The reference substance, plasmid PV-GMIR9, was used in the transformation process to develop MON 87701. Digested whole plasmid and probe templates generated from this plasmid served as positive hybridization controls. The plasmid was isolated prior to the study and its identity confirmed by restriction enzyme digestion. The 1 kb DNA extension ladder and λ DNA/*Hin*d III fragments from Invitrogen (Carlsbad, CA) were used for size estimations on agarose gels for Southern analyses. Additionally, the 500 bp ladder from Invitrogen and GeneRuler 1kb DNA ladder Plus from Fermentas were used for size estimations on agarose gels.

3.4 Characterization of Test, Control and Reference Substances

The identity of the control substance was determined by event-specific PCR prior to use in the study per SOP BR-PO-0573-02. The Study Director reviewed the chain-of-custody documentation for the control starting seed to confirm its identity prior to use of the leaf tissue in the study. Additional identity confirmation of the leaf tissue for both the test and control substance samples was performed by event-specific PCR prior to use in the study. Data from the event-specific PCR analysis of the leaf samples as well as a copy of the COA for the control substance were archived with the study in the Monsanto Regulatory archives. Because of the qualitative nature of the Southern blot and PCR analyses being performed, the test substance was considered stable during storage because it yielded interpretable signals on the Southern blot analyses and produced specific PCR products. The control substance was considered stable during storage because the sample did not appear visibly degraded on ethidium-stained gels.

3.5 Genomic DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA from the test and control substances was isolated from soybean leaf tissue. The leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted from the processed leaf tissue using the following method. Approximately 5-6 grams of soybean leaf tissue was processed in liquid nitrogen using a mortar and pestle on dry ice. To each sample, 25 milliliters (ml) of a pre-warmed lysis solution was added [24.25 ml pre-warmed (50-60°C) CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl) pH 8.0, 0.5 ml 2-mercaptoethanol (2-ME), and 0.25 ml of 10 mg/ml proteinase K for a final

concentration of 2% 2-ME and 100 µg/ml proteinase K]. The tube was incubated for at least 60 minutes at 50-60°C, with periodic shaking. Twenty ml of a phenol: chloroform: isoamyl alcohol (PCI 25:24:1) mixture was added to each tube and vigorously mixed by hand. The tubes were centrifuged for 10 minutes at 13,000 x g at 15-25°C and the supernatant was transferred to a pre-spun 50 ml MaXtract High Density conical tube (Qiagen, Carlsbad, CA). Twenty ml of PCI 25:24:1 was added to each tube and vigorously mixed by hand. The tubes were centrifuged for 10 minutes at 1500 x g at 15-25°C. This was repeated for a total of two MaXtract High Density extractions. After the last extraction, the upper aqueous phase was transferred to a clean 50 ml conical tube and approximately two times the volume of -20°C 100% ethanol was added. The tube was gently inverted by hand several times to mix. To precipitate the DNA, the tubes were placed in a -20°C freezer for at least 30 minutes. To pellet the DNA, the tubes were centrifuged at 13,000 x g for 20 minutes at 1-9°C. The DNA was rinsed at least twice with 70% ethanol and residual ethanol was removed by heating at 37-65°C. The pellets were redissolved in 3 ml of TE (10 mM Tris HCl, 1 mM EDTA), pH 8.0. The tubes were incubated at 60-70°C for at least 1 hour to resuspend the pellets completely. The tubes were then centrifuged at 15,000 x g for 10 minutes at 15-25°C to remove undissolved material. The supernatants were transferred to a 13 ml Sarstedt tube and approximately 4 µl of 100 mg/ml RNase A was added to each tube. The tubes were then incubated at 60°C for 15 minutes. To remove residual polysaccharide compounds, the DNA was PEG precipitated according to SOP BR-ME-1157-01 with the exception of using a smaller volume of TE buffer to resuspend the pellet, which created a more concentrated DNA solution for use in the Southern analyses.

Genomic DNA from the test substance samples used in the insert stability analyses was isolated according to SOP BR-ME-1153-01. Some of the genomic DNA from the test substance used in the T-DNA I copy number analyses was also isolated according to this SOP, except that the amount of processed leaf tissue was increased and the other volumes of material were increased accordingly. This was acknowledged in the raw data as a protocol deviation. All extracted DNA was stored in a 4°C refrigerator and/or -20°C freezer.

3.6 Genomic DNA Quantification

Genomic DNA was quantified using a Hoefer 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

Ten micrograms (µg) of genomic DNA extracted from the test and control substances was digested with the appropriate restriction enzymes according to SOP BR-ME-0316-01 in a total volume of ~500 µl using ~100 units of the restriction enzyme with the exception of the reactions presented in Figure 5, which used ~50 units of restriction enzyme. For the purpose of running positive hybridization controls, ~10 µg of

genomic DNA extracted from the control substance was digested and the appropriate positive hybridization control(s) were added to these digests and loaded.

3.8 Agarose Gel Electrophoresis

Digested DNA was resolved on 0.8% (% w/v) agarose gels according to SOP BR-ME-0315-02. Individual digests of ~10 µg each of test and control DNA were loaded on the same gel in a long run/short run format. For the insert stability analysis, individual digests of ~10 µg of genomic DNA extracted from leaf tissue across multiple generations were loaded on the agarose gel in a single short run format. The longer run allows for greater resolution of large molecular weight DNA, whereas the short run allows for the detection of small molecular weight DNA. The positive hybridization controls were only run in the short run format.

3.9 Probe Preparation

Probe template DNA containing sequences of plasmid PV-GMIR9 was prepared by PCR amplification according to SOP BR-ME-0486-01. The probes were designed based on the nucleotide content (% GC) so that the entire probe would hybridize under the conditions used. Approximately 25 ng of each probe template were radiolabeled with either ^{32}P-deoxycytidine triphosphate (dCTP) or ^{32}P-deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Invitrogen) according to SOP BR-ME-0611-01. Probe locations relative to the genetic elements in plasmid PV-GMIR9 are depicted in Figure 1.

3.10 Southern Blot Analyses

Digested genomic DNA isolated from test and control materials was evaluated using Southern blot analyses, according to SOP BR-ME-0317-02. When multiple probes were used for the analysis, the appropriate probe templates were used as positive hybridization controls (Figure 1). The plasmid DNA was digested with *Bgl* II / *Nco* I and added to conventional soybean genomic DNA as an additional positive hybridization control. The DNA was then separated by agarose gel electrophoresis. Southern blots were hybridized and washed at 50°C, 55°C, 60°C, or 65°C depending on the melting temperature of the probe. The table below lists the temperature and radiolabeling conditions of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer.

Probe	DNA Probe	Labeling Method	Probe labeled with dNTP (^{32}P)	Hybridization/ Wash Temperature (°C)
1	Backbone Probe 1	RadPrime	dATP	60
2	Backbone Probe 2	RadPrime	dATP	60
3	Backbone Probe 3	RadPrime	dATP	60
4	Backbone Probe 4	RadPrime	dCTP	65
5	T-DNA II Probe 5	RadPrime	dATP	55
6	T-DNA II Probe 6	RadPrime	dATP	55

7	T-DNA I Probe 7	RadPrime	dATP	50
8	T-DNA I Probe 8	RadPrime	dATP	60
9	T-DNA I Probe 9	RadPrime	dATP	55
10	T-DNA I Probe 10	RadPrime	dATP	60
11	T-DNA I Probe 11	RadPrime	dATP	55

3.11 PCR and Sequence Analyses

Overlapping PCR products were generated that span the insert and adjacent 5' and 3' flanking genomic DNA sequence in MON 87701 (Figure 10). These products were sequenced to determine the nucleotide sequence of the insert in MON 87701, as well as determining the nucleotide sequence of the genomic DNA flanking the 5' and 3' ends of the insert. The PCR analysis was performed according to SOP BR-ME-0486-01. The table below lists the PCR reaction and cycling conditions used in this study.

Product Name	Product A		Product B		Product C		Product D	Product E		Product F	Product G		Product H	Product I
DNA template (ng) in Reaction Volume (µl)	30 ng in 50µl		48 ng in 25µl		96 ng in 25µl		48 ng in 25µl	96 ng in 25µl		48 ng in 25µl	48 ng in 25µl		96 ng in 25µl	30 ng in 50µl
Reaction Conditions	1♦		2♣		2		2	2		2	2		2	1
Cycling Conditions	Cycles	Temp Time	Cycles	Temp Time	Cycles	Temp Time	** Same cycling conditions as Product C	Cycles	Temp Time	** Same cycling conditions as Product C	Cycles	Temp Time	** Same cycling conditions as Product E	** Same cycling conditions as Product A
	1 cycle	95˚C 2 min	1 cycle	94˚C 2 min	1 cycle	94˚C 2 min		1 cycle	94˚C 2 min		1 cycle	94˚C 2 min		
		94°C 30 sec		94°C 45 sec		94°C 45 sec			94°C 45 sec		10 cycles	94°C 45 sec		
	40 cycles	65°C 30 sec	35 cycles	69°C 45 sec	35 cycles	68°C 45 sec		35 cycles	65°C 45 sec		Decrease 1°C per cycle	70°C 45 sec		
		72°C 1.5 min		72°C 5 min		72°C 5 min			72°C 5 min			72°C 5 min		
	1 cycle	72°C 5 min	1 cycle	72°C 10 min	1 cycle	72°C 10 min		1 cycle	72°C 10 min			94°C 45 sec		
											35 cycles	60°C 45 sec		
												72°C 5 min		
											1 cycle	72°C 10 min		

1♦ 2 mM $MgSO_4$,0.2 µM of each primer,0.1 mM each dNTP,1M Betaine, and 0.2 U KOD Hot Start DNA Polymerase
2♣ 1 mM $MgSO_4$,0.8 µM of each primer,0.2 mM each dNTP,1M Betaine, and 0.02 U KOD Hot Start DNA polymerase

Aliquots of each PCR product were separated on 0.8 % (w/v) agarose E-gels (Invitrogen) and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. To remove residual excess primer following PCR amplification, Products B, C, and G were treated with a 2 µl mixture of Exonuclease I (EXO) (USB Cleveland, OH) and Shrimp Alkaline Phosphatase (SAP) (USB) (0.1 Units (U)/ µl each) per 5µl of PCR product and cycled as follows: one cycle at 37°C for 15 minutes and one cycle at 80°C for 15 minutes. Not all products were treated with EXO-SAP prior to sequencing as documented in the raw

data. The PCR products were sequenced using multiple primers, including some of the primers used for PCR amplification. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry (ABI, Foster City, CA).

3.12 PCR and DNA Sequence Analysis to Examine the MON 87701 Insertion Site

To demonstrate that the DNA sequences flanking the insert in MON 87701 are native to the soybean genome and to characterize the insertion site in conventional soybean, PCR analyses were performed on genomic DNA from both MON 87701 and the conventional soybean control, variety A5547 (Figure 12). The product resulting from the PCR analysis on conventional soybean control, variety A5547 was sequenced. Genomic DNA from MON 87701 did not yield a product. The primers used in this analysis were designed from the genomic DNA sequences flanking the insert in MON 87701. One primer designed from the genomic DNA sequence flanking the 5' end of the insert was paired with a second primer located in the genomic DNA sequence flanking the 3' end of the insert. The PCR analysis was performed according to SOP BR-ME-0486-01. The table below lists the PCR reaction and cycling conditions used in this study.

Product Name	Product
DNA template (ng) in Reaction volume (µl)	96 ng in 25 µl
Reaction Conditions	1 mM $MgSO_4$ 0.2 µM of each primer 0.1 mM each dNTP 1M Betaine 0.02 U KOD Hot Start DNA Polymerase
Cycling Conditions	94°C 2 minutes — 1 cycle 94°C 45 seconds 65°C 45 seconds 72°C 5 minutes — 35 cycles 72°C 10 minutes — 1 cycle

Aliquots of each PCR product were separated on 0.8 % (w/v) agarose E-gels (Invitrogen) and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. To remove residual excess primer following PCR amplification, products B, C, and G were treated with a 2 µl mixture of EXO and SAP (0.1 U / µl each) per 5µl of PCR product and cycled as follows: one cycle at 37°C for 15 minutes and one cycle at 80°C for 15 minutes. The PCR products were sequenced using multiple primers, including primers used for PCR amplification and primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry (ABI).

3.13 Data Rejected or Not Reported

Some Southern blot analyses conducted as part of this study were not reported due to weak hybridization signals, restriction fragments that were not retained on the agarose gel, or duplicate blots being generated. Southern blots were rejected if high levels of background signal hindered the ability to draw accurate conclusions or in cases of restriction enzyme digestion issues, such as partial digestion or star activity. Some PCR analyses were rejected if expected amplification products were not produced. Other PCR analyses were not reported if the amplification products were too weak for use as a sequencing template. 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.

3.14 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. None of these changes had any negative impact on the conclusions of this study.

1. The protocol was amended to correct a typographical error in the study number listed in the text.

2. The protocol was amended to clarify that the Study Director reviewed the certificate of analysis (COA) for the control starting seed to confirm its identity prior to use of the leaf tissue in the study.

3. The test substance samples for the insert stability analyses were finalized after the protocol was signed. The protocol was amended to include additional test substances used for the insert stability analyses, and to clarify the study procedures used for this analysis.

4.0 RESULTS AND DISCUSSION

The R5 generation (Figure 2) was used for the molecular characterization analyses in this study, because it is the same generation provided to initiate commercial breeding. A description of the genetic elements and their location within PV-GMIR9 is shown in Table 1. Characterization of the R5 generation confirms that all generations derived from this generation would not contain any unintended elements other than those associated with the insert in MON 87701. Genomic DNA was analyzed using Southern blot analyses and sequencing of the insert and adjacent genomic DNA sequence in MON 87701. The Southern blots examined the genome for the presence of all sequences contained in PV-GMIR9, which consisted of T-DNA-I, T-DNA II, and backbone sequences. Additionally, the stability of integrated DNA was examined by confirming that the expected Southern blot fingerprint of MON 87701 has been maintained in multiple generations produced by conventional breeding. PCR and DNA sequence analysis confirmed the intactness of all genetic elements within the cassette, confirmed

the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87701 are native to the soybean genome, and determined the integration site in conventional soybean.

4.1 Southern Blot Analyses of MON 87701

Southern blot analysis was used to characterize the insert present in MON 87701 and determine whether the insert reported in Figures 3 and 12 represents the only detectable insert in MON 87701. Genomic DNA from MON 87701 was digested using various restriction enzymes and subjected to Southern blot analyses utilizing probes that cover the entire transformation vector (Figure 1). The selection and design of the probes used allow for a determination of the copy number of all sequences from PV-GMIR9. Therefore, it can be determined if any elements within the insert are present at multiple loci or if any backbone or T-DNA II sequences are present in MON 87701. A map of plasmid vector PV-GMIR9 annotated with the probes used in the Southern analysis is presented in Figure 1. A linear map depicting the restriction sites within the insert DNA sequence, as well as within the soybean genomic DNA immediately flanking the insert in MON 87701, is shown in Figure 3. Based on the insert linear map and the plasmid map, a table summarizing the expected DNA fragments for Southern analyses is presented in Table 3. For estimating the sizes of bands present in the long-run lanes of Southern blots, the molecular weight markers on the left side of the figures were used. For estimating the band sizes present in the short-run lanes, the molecular weight markers on the right side of the figures were used. In most of the Southern blots, the migration of the genomic DNA is slightly different when compared to the migration of the molecular weight markers and, in some instances, there are slight migration differences between different DNA preparations. These altered migrations are likely the result of a difference in salt concentrations between the genomic DNA samples and the molecular weight marker (Sambrook and Russell, 2001). The Southern blots (Figures 4 – 9) presented in this report are representative of the data generated in the study.

4.1.1 Southern Blot Analyses to Confirm the Copy Number of T-DNA I

The number of copies and insertion sites of T-DNA I sequences in the soybean genome were evaluated by digesting the test and control genomic DNA samples with the two enzyme sets *Nco* I / *Vsp* I and *Xho* I / *Nde* I, which cleave within the insert and known flanking sequences. The enzymes used generate a restriction fragment containing T-DNA I and adjacent plant genomic DNA so that each insert would generate a unique banding pattern. If T-DNA I sequences are present at a single integration site in MON 87701, then probing with the sequence from T-DNA I should result in the restriction fragments described in Figure 3 and Table 3. Any additional integration sites would be detected as additional bands. The blots were hybridized with overlapping T-DNA I probes spanning the entire inserted DNA sequence (Probes 7-11 in Figure 1). Each Southern blot contained several controls. Genomic DNA isolated from the conventional soybean control, A5547, was used as a negative control to determine if the probes hybridized to any endogenous sequences. Conventional soybean spiked with either plasmid DNA or probe template was used as

a positive hybridization control and to demonstrate the sensitivity of the Southern blot. The results of these analyses are shown in Figures 4, 5, and 6.

4.1.1.1 Probe 7

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure 4, lanes 1 and 8) or *Xho* I / *Nde* I (Figure 4, lanes 3 and 10) and hybridized with Probe 7 (Figure 1) showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA, previously digested with *Bgl* II/ *Nco* I, (Figure 1, Probe 7) produced the expected bands at ~6.0 kb and ~9.5 kb as shown in Figure 4, lane 7 with the ~1.0 genome equivalent spike. In Figure 4, lane 6, the ~0.1 genome equivalent spike produced the expected band at ~6.0 kb, but the ~9.5 kb band is too faint to identify, since only a small portion of Probe 7, which spans the Right Border region, has homology to the ~9.5 kb portion of the vector. The ability to detect the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I (Figure 4, lanes 2 and 9) and hybridized to Probe 7 is expected to produce one band at ~ 4.0 kb. The long run (Figure 4, lane 2) produced a single band at ~4.1 kb (at or above the 4.1 kb marker) and the short run (Figure 4, lane 9) also produced a single band of the correct size. MON 87701 DNA digested with *Xho* I / *Nde* I (Figure 4, lanes 4 and 11) and hybridized with Probe 7 is expected to produce a single band of ~5.7 kb. The long run (Figure 4, lane 4) produced a single band at ~6.2 kb (at or above the 6.1 kb marker) and the short run (Figure 4, lane 11) produced a single band at ~ 5.7 kb. The apparent shift in migrations of the bands in the long run versus the short run can be attributed to the method used to record the molecular weight markers on the agarose gel and on the autoradiograph. The slight discrepancy in the bands does not alter the conclusion that a single band was detected of the correct size, thus there is a single detectable insert containing Probe 7 sequences. The results presented in Figure 4 indicate that the sequence covered by Probe 7 resides at a single detectable locus of integration in MON 87701.

4.1.1.2 Probe 8 and Probe 10

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure 5, lanes 1 and 8) or *Xho* I / *Nde* I (Figure 5, lanes 3 and 10) and hybridized with Probes 8 and 10 (Figure 1) showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template (Figure 1, Probes 8 and 10) generated from plasmid PV-GMIR9 produced the expected bands at ~1.5 kb, and ~1.8 kb (Figure 5, lanes 5 and 6). In lane 6, there is a faint band at ~3.6 kb, which likely represents a minor PCR artifact that was generated during probe template preparation. Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II /*Nco* I produced the two expected bands at ~6.0 kb and ~9.5 kb (Figure 5, lane 7). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I and hybridized with Probes 8 and 10 (Figure 5, lanes 2 and 9) produced two bands. The ~4.0 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) and the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~6.3 kb band represents the 3' border fragment containing the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert. MON 87701 DNA digested with *Xho* I / *Nde* I (lanes 4 and 11) produced two bands. The ~5.7 kb band observed in Figure 5 (lanes 4 and 11) is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) and the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~1.9 kb band observed in Figure 5 (lanes 4 and 11) represents an internal fragment contained in the inserted T-DNA. The results presented in Figure 5 indicate that sequence covered by Probes 8 and 10, resides at a single detectable locus of integration in MON 87701.

4.1.1.3 Probe 9 and Probe 11

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure 6, lanes 2 and 11) or digested with *Xho* I / *Nde* I (Figure 6, lanes 4 and 13) and hybridized with Probes 9 and 11 (Figure 1) produced several hybridization signals. This was expected as the *7Sα*' 3'non-translated region genetic element within T-DNA I was originally isolated from soybean. These hybridization signals result from the probes hybridizing to endogenous targets residing in the soybean genome and are not specific to the inserted DNA. These signals were produced in all lanes, including those containing the conventional soybean DNA material and, therefore, are considered to be endogenous background hybridization. Pre-digested conventional soybean DNA spiked with probe template (Figure 1, Probes 9 and 11) generated from plasmid PV-GMIR9 produced the expected bands at ~ 1.2 kb for probe template 9 (Figure 6, lanes 5 and 6) and ~1.1 kb for probe template 11 (Figure 6, lanes 7 and 8). Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II/ *Nco* I produced the two bands at ~6.0 kb and ~9.5 kb (Figure 6, lane 9). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I and hybridized with Probes 9 and 11 (Figure 6, lanes 1 and 10) produced one unique band in addition to the endogenous background hybridization. The ~6.3 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I) and the adjacent genomic DNA flanking the 3' end of the insert (Figure 3). MON 87701 DNA digested with *Xho* I/ *Nde* I (Figure 6, lanes 3 and 12) produced three unique bands, as expected, in addition to the endogenous background hybridization. The expected band at ~5.7 kb migrated together with an endogenous hybridization signal observed in Figure 6, lanes 3 and 12. The ~5.7 kb band represents the 5' border fragment containing the 5' end of the inserted DNA along with the adjacent genomic DNA flanking the 5' end of the insert. The ~1.9 kb band represents a portion of the *cry1Ac* expression cassette. The ~2.7 kb band represents the 3' border fragment containing

the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert. The results presented in Figure 6 indicate that sequences covered by Probes 9 and 11 reside at a single detectable locus of integration in MON 87701. Together, the data presented in Figures 4, 5, and 6 indicate that MON 87701 contains a single copy of T-DNA I at a single insertion site.

4.1.2 Southern Blot Analysis to Determine the Presence or Absence of Plasmid PV-GMIR9 Backbone Sequence

The presence or absence of plasmid backbone sequences in the soybean genome was evaluated by digesting the test and control genomic DNA samples with *Nco* I / *Vsp* I or *Xho* I / *Nde* I and hybridizing with backbone probes spanning the entire backbone sequence of PV-GMIR9 (Figure 1, Probes 1, 2, 3, and 4). If backbone sequences are present in MON 87701, then probing with overlapping backbone sequences should result in hybridizing bands. The results of this analysis are shown in Figures 7 and 8. Each Southern blot contains the same controls as described in Section 4.1.1.

4.1.2.1 Probe 1, Probe 2, and Probe 3

Conventional soybean control DNA digested with *Nco* I / *Vsp* I (Figure 7, lanes 1 and 8) or *Xho* I / *Nde* I (Figure 7, lanes 3 and 10) and hybridized simultaneously with overlapping probes spanning most of the vector backbone of PV-GMIR9 (Figure 1, Probes 1, 2, and 3) showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template (Figure 1, Probes 1, 2, and 3) generated from plasmid PV-GMIR9 produced the expected bands at ~1.5 kb, ~1.8 kb, and ~1.1 kb, respectively (Figure 7, lanes 5 and 6). Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II / *Nco* I produced the expected size band of ~9.5 kb (Figure 7, lane 7). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I (Figure 7, lanes 2 and 9) or *Xho* I / *Nde* I (Figure 7, lanes 4 and 11) and hybridized with Probes 1, 2, and 3 produced no detectable bands. There is a diffuse area of hybridization that overlaps with lane 4. Because this hybridization is not a distinct band, and it is not present in lane 11, which contains the same enzyme set, this area of hybridization is considered non-specific binding. The data indicate that MON 87701 contains no backbone elements from PV-GMIR9 that overlaps Probes 1, 2, and 3.

4.1.2.2 Probe 4

Conventional soybean control DNA digested with *Nco* I / *Vsp* I (Figure 8, lanes 1 and 7) or *Xho* I / *Nde* I (Figure 8, lanes 3 and 9) and hybridized with Probe 4 from the vector backbone of PV-GMIR9 (Figure 1, Probe 4) showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II / *Nco* I produced the expected band at ~6.0 kb (Figure 8, lanes 5 and 6). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I (Figure 8, lanes 2 and 8) or *Xho* I / *Nde* I (Figure 8, lanes 4 and 10) and hybridized with Probe 4 produced no detectable hybridization bands, indicating that MON 87701 contains no detectable PV-GMIR9 backbone elements that are contained within Probe 4. These data, in combination with the data presented in 4.1.2.1, indicate that MON 87701 contains no detectable PV-GMIR9 backbone elements.

4.1.3 Southern Blot Analysis to Determine the Presence or Absence of T-DNA II

The presence or absence of T-DNA II sequences in the soybean genome was evaluated by digesting the test and control genomic DNA samples with the *Nco* I / *Vsp* I or *Xho* I / *Nde* I enzyme sets and hybridizing with overlapping T-DNA II probes spanning the entire T-DNA II sequence of PV-GMIR9 (Figure 1, Probes 5 and 6). The left and right border regions of T-DNA II share 100% homology with those of T-DNA I and thus were not included in the T-DNA II analysis. If T-DNA II sequences are present in MON 87701, then probing with the T-DNA II sequences should result in hybridizing bands. The results of this analysis are shown in Figure 9. The Southern blot contained the same controls as described in Section 4.1.1.

4.1.3.1 Probe 5 and Probe 6

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure 9, lanes 1 and 9) or *Xho* I / *Nde* I (Figure 9, lanes 3 and 11) and hybridized with Probes 5 and 6 (Figure 1) showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template generated from plasmid PV-GMIR9 produced the expected bands at ~2.0 kb and ~1.2 kb (Figure 9, lanes 6 and 7). Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II/ *Nco* I produced the expected size bands of ~6.0 kb and ~9.5 kb (Figure 9, lane 8). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I/ *Vsp* I (Figure 9, lanes 2 and 10) or *Xho* I / *Nde* I (Figure 9, lanes 4 and 12) produced no hybridization bands. These results indicate that MON 87701 contains no detectable T-DNA II elements.

4.2 Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87701

The organization of the elements within the MON 87701 insert was confirmed using PCR analysis by amplifying nine overlapping regions of DNA that span the entire length of the insert and the associated flanking genomic DNA. The positions of the PCR products relative to the insert, as well as the results of the PCR analyses, are shown in Figure 10.

In Figure 10, the control reactions containing no template DNA (lanes 4, 8, 11, 16, 20, 25, 28, 32, and 36), and all but one of the conventional control reactions (lanes 2, 6, 9, 13, 17, 22, 26, and 34) did not generate PCR products with any of the primer sets, as

expected. The conventional control reaction in lane 30 produced a product of equal size to MON 87701 (lane 31) because the primer sequences for this product are both located in the flanking genomic sequence adjacent to the 3' end of the insert in MON 87701. Plasmid PV-GMIR9 was used as a positive control template (lanes 15, 19, and 24) and produced the expected size PCR products of ~1.2 kb, 2.0 kb, and 2.0 kb, respectively, for each reaction. PCR reactions using genomic DNA from MON 87701 produced the expected size products: ~2.2 kb for Product A (lane 3); ~1.6 kb for Product B (lane 7); ~1.7 kb for Product C (lane 10); ~1.2 kb for Product D (lane 14); ~2.0 kb for Product E (lane 18); ~2.0 kb for Product F (lane 23); ~2.0 kb for Product G (lane 27); ~1.4 kb for Product H (lane 31); and ~2.9 kb for Product I (lane 35). These overlapping PCR products confirm the organization of the insert is as expected.

To determine the sequence of the insert in MON 87701 and genomic DNA flanking the insert, the PCR products A-I (Figure 10) were subjected to DNA sequencing. The consensus sequence representing the insert in MON 87701, including the genomic DNA flanking the ends of the insert, is shown in Figure 11 and is described in Table 2. This consensus sequence was generated by compiling numerous sequencing reactions using PCR products spanning the length of the insert and the 5' and 3' junctions with the flanking soybean genomic DNA. The amplification and sequencing of the insert and flanking DNA from MON 87701 establishes that the arrangement and linkage of elements in the insert are consistent with those in plasmid PV-GMIR9 and are as depicted in Figure 3.

The DNA sequence of the MON 87701 insert is 6426 base pairs long, beginning at base 3908 of PV-GMIR9 located in the Right Border region and ending at base 10333 in the Left Border region of PV-GMIR9. The sequence of 2000 base pairs of soybean genomic DNA flanking the 5' end of the insert and 2109 base pairs of soybean genomic DNA flanking the 3' end of the insert were also determined (Figure 11). The sequence confirmed the presence and organization of the integrated genetic elements as described in Table 2.

4.3 PCR and DNA Sequence Analysis to Examine the MON 87701 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from MON 87701 and conventional soybean control, A5547, to demonstrate that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87701 are native to the soybean genome and to characterize the insertion site in conventional soybean. The PCR was performed with one primer designed from the genomic DNA sequence flanking the 5' end of the insert (Primer A, Figure 13) paired with a second primer designed from the genomic DNA sequence flanking the 3' end of the insert (Primer B, Figure 13). The results of the PCR analysis are shown in Figure 12. The control PCR containing no template DNA (Figure 12, lane 4) did not generate a PCR product, as expected. The reaction using the conventional genomic DNA template (Figure 12, lane 2) generated a PCR product of a ~3 kb band. As expected, a PCR product across the ~ 9.5 kb span of DNA between Primer A and Primer B in MON 87701 (Figure 12, lane 3) was not

generated in this analysis since the PCR conditions necessary to generate a product of this size were not used.

The ~3 kb PCR product generated from the conventional genomic DNA was sequenced and the resulting data are shown in Figure 13. A sequence comparison between the PCR product generated from the conventional soybean and the sequence generated from the 5' and 3' flanking sequences of MON 87701 indicated that there was a 32 bp deletion (bases 1441-1472 in Figure 13) and a 14 bp insertion (Bases 1987-2000 in Figure 11) just 5' to the MON 87701 insertion site. These likely occurred in MON 87701 upon insertion of the T-DNA. Bases 1-1440 and 1473-2968 in Figure 13, which are from conventional soybean, are identical to the 5' and 3' genomic DNA sequence flanking the insert in MON 87701, respectively (547-1986 bp, 8427-9922 bp, Figure 11). This analysis confirms that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87701 are native to the soybean genome and that no major unexpected rearrangements occurred during the formation of MON 87701.

4.4 Southern Blot Analyses to Examine Insert Stability in Multiple Generations of MON 87701

In order to demonstrate the stability of the T-DNA in MON 87701, Southern blot analysis was performed using DNA obtained from multiple generations of MON 87701. For reference, the breeding history of MON 87701 is presented in Figure 2. The specific generations tested are indicated in the legend of Figure 14. The R5 generation was used for the molecular characterization analyses shown in Figures 4-9. To analyze stability, four additional generations were evaluated by Southern analysis and compared to the R5 generation. Genomic DNA, isolated from each of the selected generations of MON 87701, were digested with the restriction enzymes *Nco* I / *Vsp* I (Figure 3) and hybridized with Probe 8 (Figure 1). Probe 8 is designed to detect both fragments generated by the *Nco* I / *Vsp* I digest. Any instability associated with the insert would be detected as novel bands within the fingerprint on the Southern blot. The results are shown in Figure 14. The Southern blot has the same controls as described in Section 4.1.1.

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure 14, lane 4) and hybridized with Probe 8 showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template (Figure 1, Probe 8) generated from plasmid PV-GMIR9 produced the expected band at ~1.5 kb (Figure 14, lanes 1 and 2). Pre-digested conventional soybean DNA spiked with plasmid PV-GMIR9 DNA, previously digested with *Bgl* II / *Nco* I, produced the expected size bands of ~6.0 kb and ~9.5 kb (Figure 14, lane 3). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

DNA extracted from MON 87701 generations R4, R5, R6, R8, and R9 digested with *Nco* I / *Vsp* I (lanes 5-9) and hybridized with Probe 8 each produced two bands of ~6.3 kb and ~4.0 kb. The ~4.0 kb band is the expected size for the 5' border fragment and the ~6.3 kb band is consistent with the expected size of the 3' border fragment. These bands

are consistent with the bands detected in Figure 5 (lanes 2 and 9) indicating that the single copy of T-DNA I in MON 87701 is stably maintained across the selected generations.

5.0 CONCLUSIONS

Molecular characterization of MON 87701 by Southern blot analyses confirmed that the introduced DNA was inserted into the soybean genome at a single locus and contains one copy of the *cry1Ac* expression cassette. There were no other elements detected other than those associated with the reported insert. No backbone DNA from plasmid PV-GMIR9 or selectable marker sequences, or T-DNA II sequences were detected.

PCR and DNA sequence analyses confirmed the predicted organization and intactness of the genetic elements within the insert, determined the 5' and 3' insert-to-plant junctions, determined the complete DNA sequence of the MON 87701 insert and adjacent genomic DNA sequence, determined the insertion site sequences in conventional soybean, and confirmed that the genomic DNA sequences flanking the 5' and 3' ends of the MON 87701 insert are native to the soybean genome.

Analysis of multiple MON 87701 generations via Southern blot demonstrated that the introduced DNA has been stably maintained across the generations tested.

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Table 1. Summary of Genetic Elements in Plasmid Vector PV-GMIR9

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA II (Continued from bp 15532)		
Intervening Sequence	1-14	Sequences used in DNA cloning
L[1]-*ShkG*	15-81	5' non-translated leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS (Klee et al., 1987) that is involved in regulating gene expression
TS[2]-*CTP2*	82-309	Targeting sequence encoding the chloroplast transit peptide from the *ShkG* gene of *Arabidopsis thaliana* encoding EPSPS (Klee et al., 1987)
CS[3]-*cp4-epsps*	310-1677	Codon modified coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Padgette et al., 1996; Barry et al., 1997)
Intervening Sequence	1678-1719	Sequences used in DNA cloning
T[4]-*E9*	1720-2362	3' non-translated sequence from *RbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	2363-2409	Sequences used in DNA cloning
B[5]-Left Border	2410-2851	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	2852-2937	Sequences used in DNA cloning
OR[6]-*ori V*	2938-3334	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	3335-3595	Sequences used in DNA cloning

L[1] –Leader; **TS[2]** – Targeting Sequence; **CS[3]** – Coding Sequence; **T[4]** – Transcription Termination Sequence; **B[5]** – Border; **OR[6] –** Origin of Replication

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA I		
B-Right Border	3596-3952	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	3953-4061	Sequences used in DNA cloning
P[7]-*RbcS4*	4062-5784	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1, 5-bisphosphate carboxylase small subunit 1A, (Krebbers et al., 1988) and promoter expresses in above ground tissues
TS-*CTP1*	5785-6048	Targeting sequence encoding the transit peptide of the *Arabidopsis RbcS4* encoding small subunit 1A transit peptide, from *Arabidopsis thaliana*, present to direct the *cry1Ac* protein to the chloroplast (Krebbers et al., 1988).
CS-cry1Ac	6049-9585	Codon-modified coding sequence of the Cry1Ac protein of *Bacillus thuringiensis* (Fischhoff and Perlak, 1995)
Intervening Sequence	9586-9594	Sequences used in DNA cloning
T-7Sα'	9595-10033	3' region of the *Sphas1* gene of *Glycine max* encoding the 7*Sα'* seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler et al., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA.
Intervening Sequence	10034-10069	Sequences used in DNA cloning
B-Left Border	10070-10511	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Vector Backbone (Continued from bp 3595)		
Intervening Sequence	10512-11786	Sequences used in DNA cloning

P[7]- Promoter

CS-*rop*	11787-11978	Coding sequence for repressor of primer protein derived from ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	11979-12405	Sequences used in DNA cloning
Genetic Element	**Location in Plasmid**	**Function (Reference)**
OR-*ori-pBR322*	12406-12994	Origin of replication from pBR322 for maintenance of plasmid in *Escherichia coli* (Sutcliffe, 1978)
Intervening Sequence	12995-13524	Sequences used in DNA cloning
aadA	13525-14413	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) that confers spectinomycin and streptomycin resistance
Intervening Sequence	14414-14549	Sequences used in DNA cloning
T-DNA II (Continued from bp 2851)		
B-Right Border	14550-14906	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	14907-14939	Sequences used in DNA cloning
P-*FMV*	14940-15503	Promoter for the 35S RNA from figwort mosaic virus (FMV) (Rogers, 2000) that directs transcription in most plant cells
Intervening Sequence	15504-15532	Sequences used in DNA cloning

Table 2. Summary of Genetic Elements in MON 87701

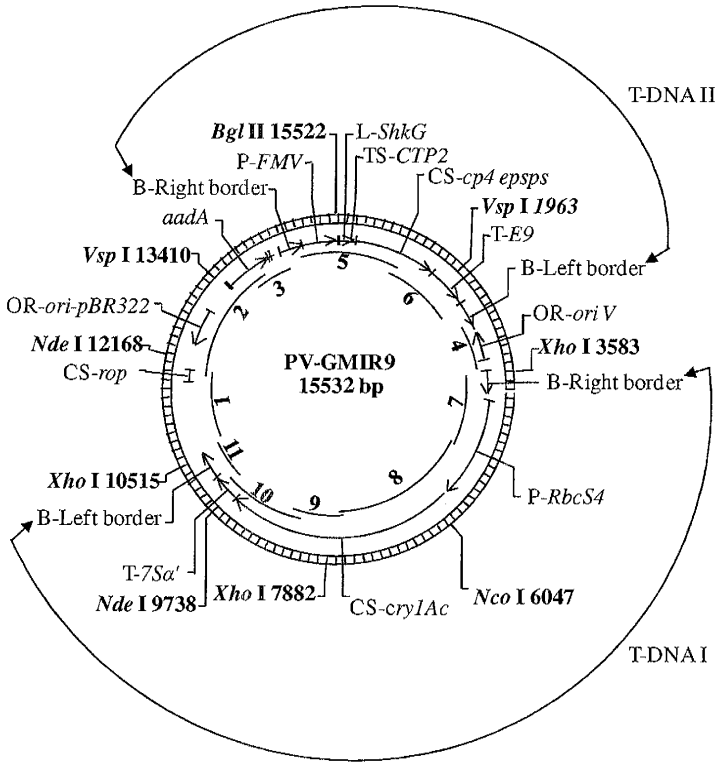
Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Sequence flanking 5' end of the insert	1-2000	Soybean genomic DNA
B[3]-Right Border[r1]	2001-2045	45 bp DNA region from the Right Border region remaining after integration (Depicker *et al.*, 1982)
Intervening Sequence	2046-2154	Sequence used in DNA cloning
P[4]- *RbcS4*	2155-3877	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1, 5-bisphosphate carboxylase small subunit 1A, (Krebbers et al., 1988) and promoter expresses in above ground tissues
TS[5]-*CTP1*	3878-4141	Targeting sequence encoding the transit peptide of the *Arabidopsis RbcS4* encoding small subunit 1A transit peptide, from *Arabidopsis thaliana*, present to direct the *cry1Ac* protein to the chloroplast (Krebbers et al., 1988).
CS*[6]*-Cry1Ac	4142-7678	Codon-modified coding sequence of the Cry1Ac protein of *Bacillus thuringiensis* (Fischhoff and Perlak, 1995)
Intervening Sequence	7679-7687	Sequences used in DNA cloning
T[7]-*7Sα'*	7688-8126	3' region of the *Sphas1* gene of *Glycine max* encoding the 7*Sα'* seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler et al., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA.
Intervening Sequence	8127-8162	Sequence used in DNA cloning
B-Left Border [r1]	8163-8426	264 bp DNA region from the Left Border region remaining after integration (Barker *et al.*, 1983)
Sequence flanking 3' end of the insert	8427-10535	Soybean genomic DNA

[1] Although flanking sequences and intervening sequences are not functional genetic elements, they comprise a portion of the sequence reported in Figure 11.
[2] Numbering refers to the sequence from Figure 11 that includes the insert in MON 87701 and adjacent genomic DNA.
[3]B – Border; [4]P – Promoter; [5]TS – Targeting Sequence; [6]CS – Coding Sequence
[7]T –3' non-translated transcriptional termination sequence and polyadenylation signal sequences.
[r1] Superscripts in Left and Right Borders indicate that the sequences in MON 87701 were truncated compared to the sequences in the PV-GMIR9 plasmid.

Table 3. Summary Chart of the Expected DNA Fragments Based on Restriction Enzymes and Probes

Probes Used	**7**	**8, 10**	**9, 11**	**1, 2, 3**	**4**	**5, 6**	**8**
Southern Blot Figure	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Plasmid PV-GMIR9							
Bgl II/*Nco* I	**~6.0 kb ~9.5 kb**	**~6.0 kb ~9.5 kb**	**~6.0 kb ~9.5 kb**	**~9.5 kb**	**~6.0 kb**	**~6.0 kb ~9.5 kb**	**~6.0 kb ~9.5 kb**
Probe Templates [1]	**~~**[2]	**~1.5 kb ~1.8 kb**	**~1.2 kb ~1.1 kb**	**~1.8 kb ~1.5 kb ~1.1 kb**	**~~**[2]	**~2.0 kb ~1.2 kb**	**~1.5 kb**
MON 87701							
Nco I / *Vsp* I	**~4.0 kb**	**~4.0 kb ~6.3 kb**	**~6.3 kb**	**No band**	**No band**	**No band**	**~6.3 kb ~4.0 kb**
Xho I / Nde I	**~5.7 kb**	**~5.7 kb ~1.9 kb**	**~5.7 kb ~2.7 kb ~1.9 kb**	**No band**	**No band**	**No band**	**--**[3]

[1] probe templates were spiked when multiple probes are used in Southern blot analysis.
[2] '~~' indicates that the only plasmid template was used since the Southern blot was hybridized with one probe.
[3] '--' indicates that the particular restriction enzyme or the combination of the enzymes was not used in the analysis.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	Backbone Probe 1	10513	12013	1.5
2	Backbone Probe 2	11813	13640	1.8
3	Backbone Probe 3	13440	14549	1.1
4	Backbone Probe 4	2852	3595	0.74
5	T-DNA II Probe 5	14907	1375	2.0
6	T-DNA II Probe 6	1225	2409	1.2
7	T-DNA I Probe 7	3596	5596	2.0
8	T-DNA I Probe 8	5471	6971	1.5
9	T-DNA I Probe 9	6846	8046	1.2
10	T-DNA I Probe 10	7846	9650	1.8
11	T-DNA I Probe 11	9450	10512♦	1.1

Figure 1. Plasmid Map of Vector PV-GMIR9 Showing Probes 1-11
A circular map of the plasmid vector PV-GMIR9 used to develop soybean MON 87701 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map. PV-GMIR9 contains two T-DNA regions designated as T-DNA I and T-DNA II. The left and right border regions of T-DNA II share 100% homology with those of T-DNA I and thus were not included in the T-DNA II analysis.

♦ Nucleotide 10512 is vector backbone sequence

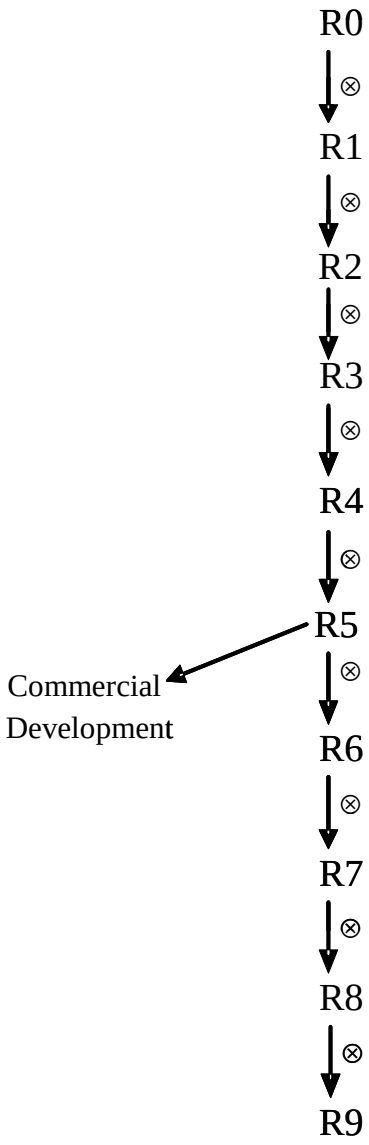


Figure 2. Breeding History for MON 87701
All generations were self-pollinated (⊗). The R5 generation seed material was used for regulatory molecular characterization and commercial development. Seed lots from the R4, R5, R6, R8, and R9 generations were used in the molecular generational stability analysis.

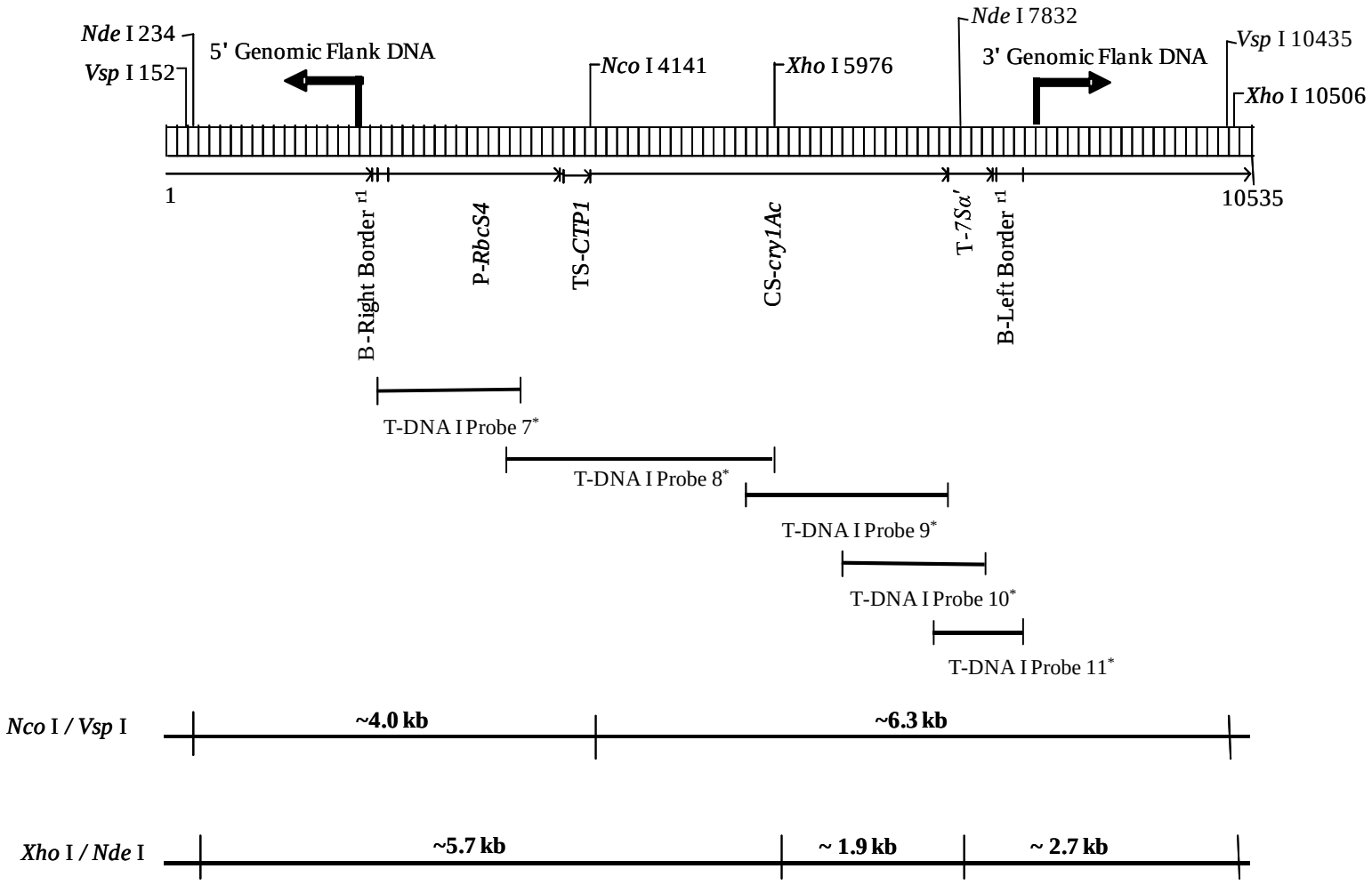


Figure 3. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87701

Linear DNA derived from T-DNA I of vector PV-GMIR9 incorporated into MON 87701. Arrows indicate the end of the insert and the beginning of soybean genomic flanking sequence. Identified on the map are genetic elements within the insert, including restriction sites with positions relative to the size of the genomic flanking sequences and the insert sequence reported in Figure 11 for enzymes used in the Southern analyses.

* These probes are not drawn to scale and are the estimated locations of the T-DNA I probes. Probes are described in Figure 1.

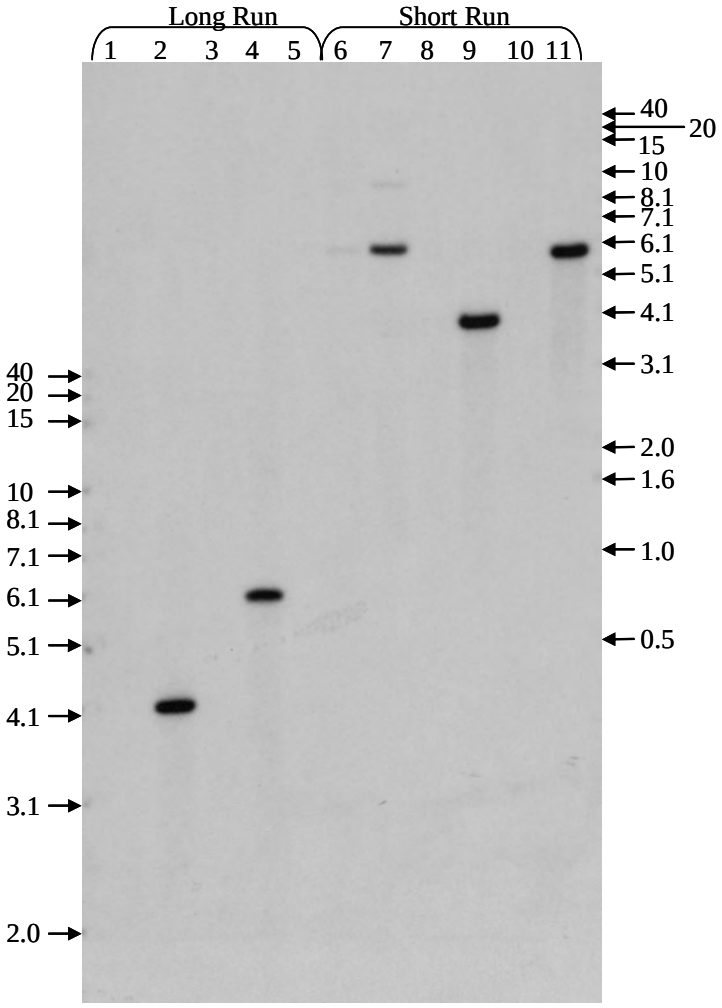


Figure 4. Southern Blot Analysis of MON 87701: T-DNA I Copy Number Analysis (Probe 7).

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA I sequence (Figure 1, Probe 7). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Blank
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~0.1 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I)
9: MON 87701 (*Nco* I / *Vsp* I)
10: Conventional soybean (*Xho* I / *Nde* I)
11: MON 87701 (*Xho* I / *Nde* I)

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

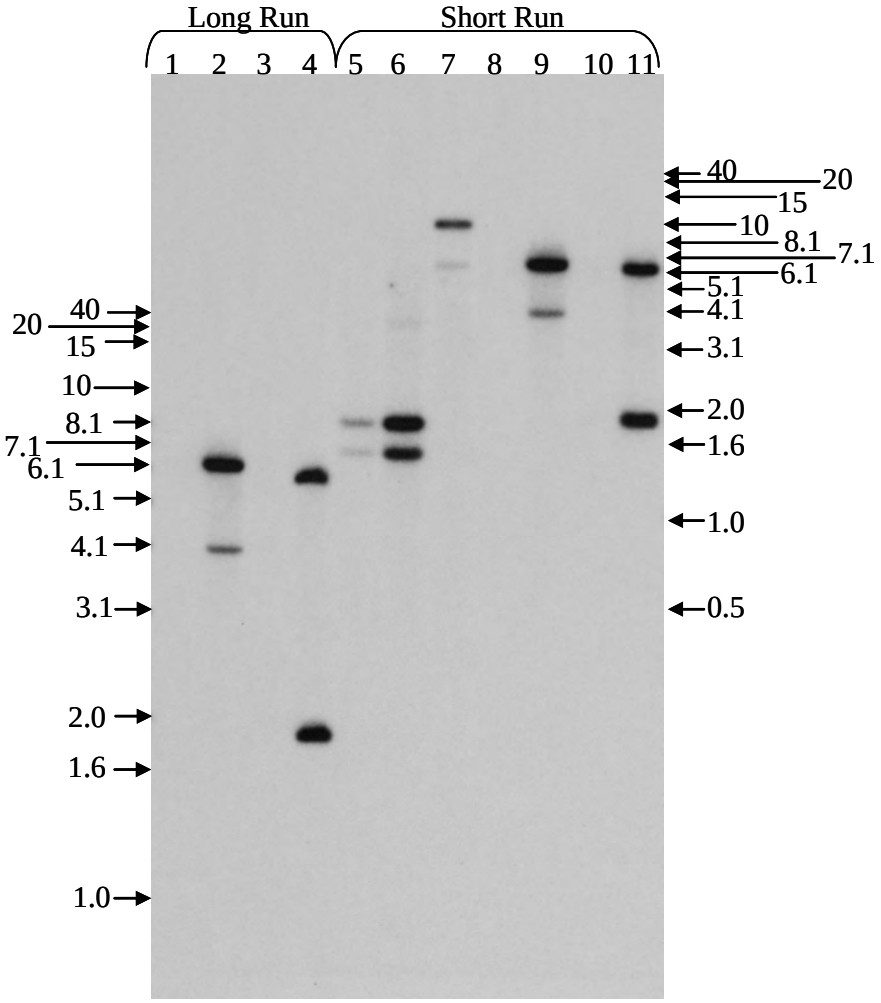


Figure 5. Southern Blot Analysis of MON 87701: T-DNA I Copy Number Analysis (Probe 8 and Probe 10).

The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure 1, Probes 8 and 10). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~0.1 genome equivalent]
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I)
9: MON 87701 (*Nco* I / *Vsp* I)
10: Conventional soybean (*Xho* I / *Nde* I)
11: MON 87701 (*Xho* I / *Nde* I)

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

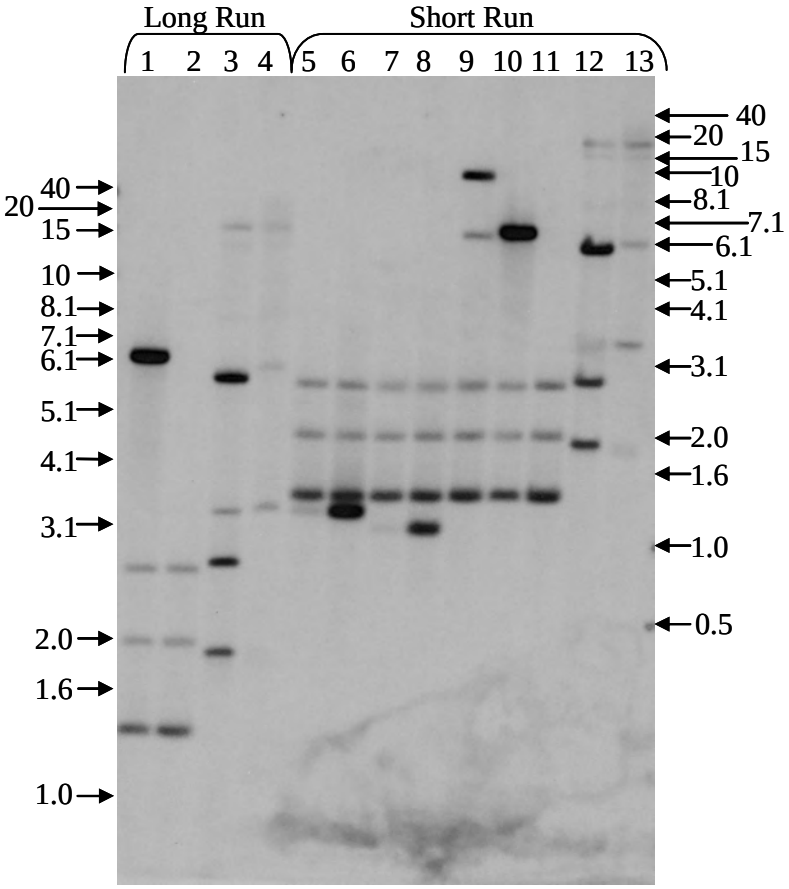


Figure 6. Southern Blot Analysis of MON 87701: T-DNA I Copy Number Analysis (Probe 9 and Probe 11).

The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure 1, Probes 9 and 11). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: MON 87701 (*Nco* I / *Vsp* I)
2: Conventional soybean (*Nco* I / *Vsp* I)
3: MON 87701 (*Xho* I / *Nde* I)
4: Conventional soybean (*Xho* I / *Nde* I)
5: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe 9 template [~0.1 genome equivalent]
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe 9 template [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe 11 template [~0.1 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe 11 template [~1.0 genome equivalent]
9: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl II*) [~1.0 genome equivalent]
10: MON 87701 (*Nco* I / *Vsp* I)
11: Conventional soybean (*Nco* I / *Vsp* I)
12: MON 87701 (*Xho* I / *Nde* I)
13: Conventional soybean (*Xho* I / *Nde* I)

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

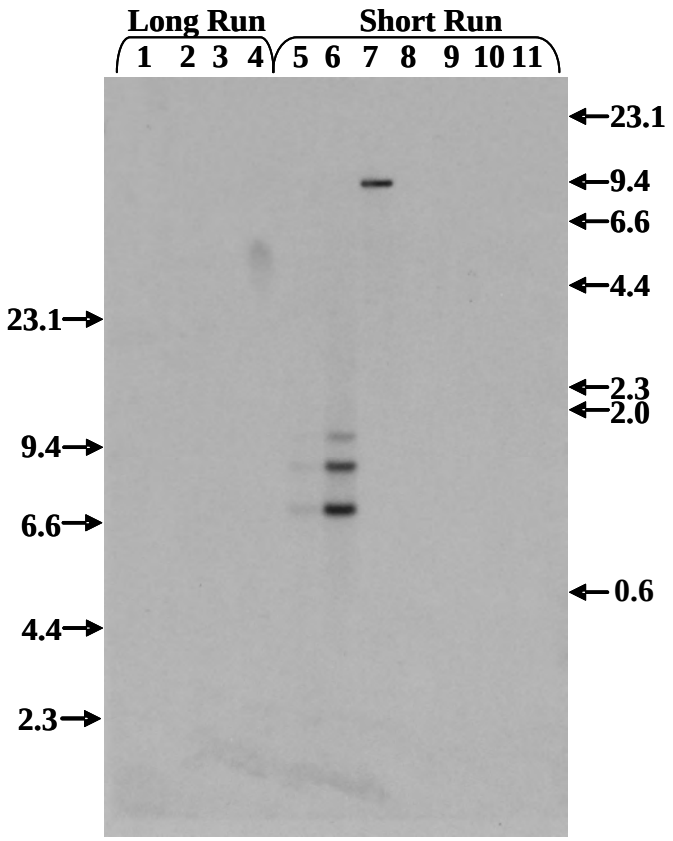


Figure 7. Southern Blot Analysis of MON 87701: PV-GMIR9 Backbone Sequence (Probe 1, Probe 2, and Probe 3).

The blot was hybridized with three ^{32}P-labeled probes that spanned a portion of the PV-GMIR9 backbone sequence (Figure 1, Probes 1, 2, and 3). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~0.1 genome equivalent]
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I)
9: MON 87701 (*Nco* I / *Vsp* I)
10: Conventional soybean (*Xho* I / *Nde* I)
11: MON 87701 (*Xho* I / *Nde* I)

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

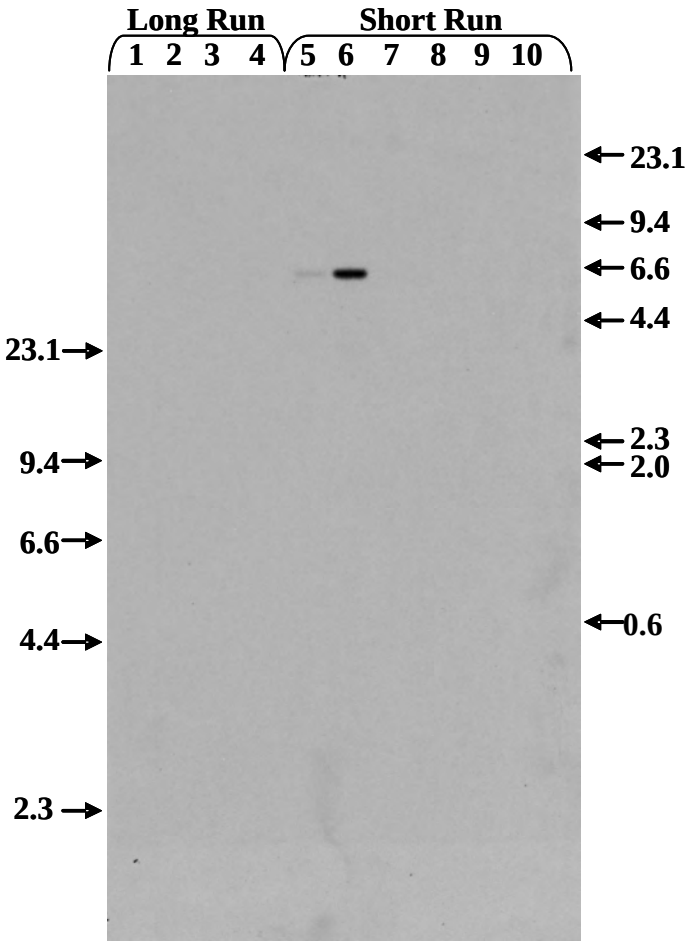


Figure 8. Southern Blot Analysis of MON 87701: PV-GMIR9 Backbone Sequence (Probe 4).

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the PV-GMIR9 backbone sequence (Figure 1, Probe 4). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~0.1 genome equivalent]
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I)
8: MON 87701 (*Nco* I / *Vsp* I)
9: Conventional soybean (*Xho* I / *Nde* I)
10: MON 87701 (*Xho* I / *Nde* I)

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

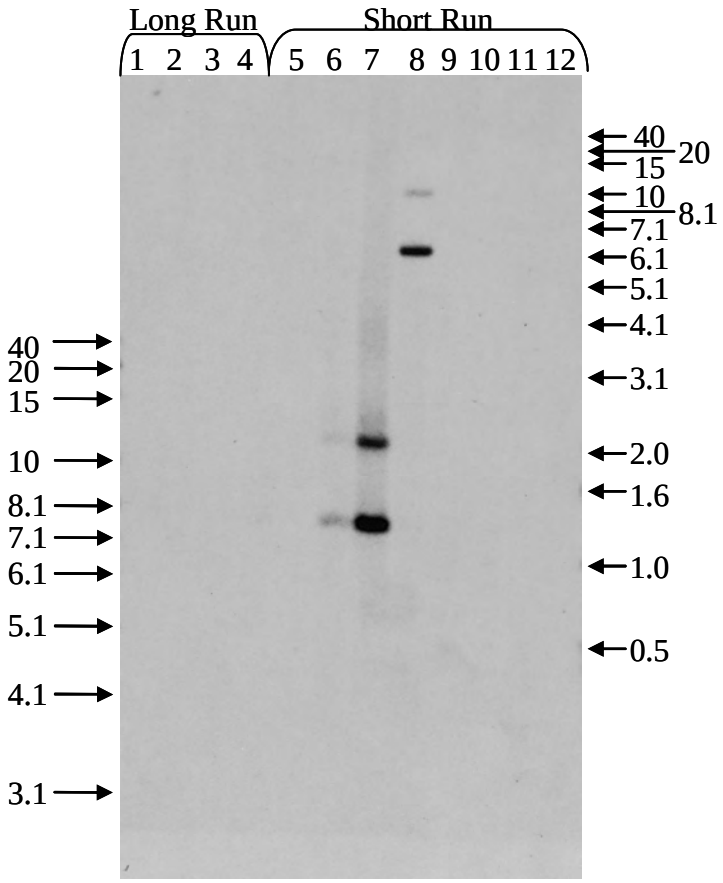


Figure 9. Southern Blot Analysis of MON 87701: T-DNA II (Probe 5 and Probe 6).
The blot was hybridized with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (Figure 1, Probes 5 and 6). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Blank
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~0.1 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl II*) [~1.0 genome equivalent]
9: Conventional soybean (*Nco* I / *Vsp* I)
10: MON 87701 (*Nco* I / *Vsp* I)
11: Conventional soybean (*Xho* I / *Nde* I)
12: MON 87701 (*Xho* I / *Nde* I)

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

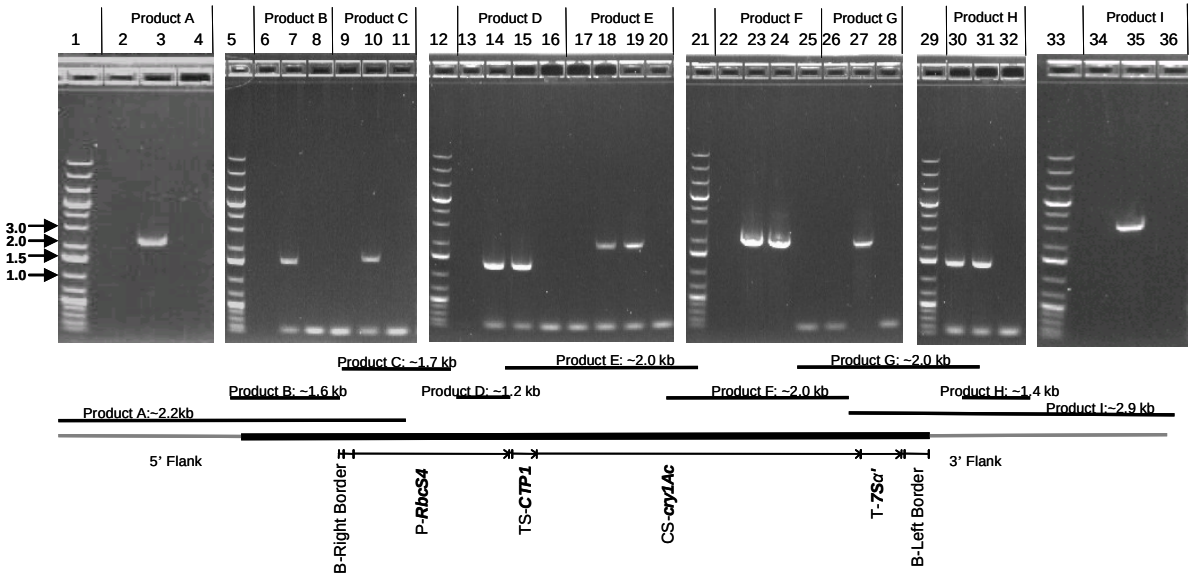


Figure 10. Overlapping PCR Analysis Across the Insert in MON 87701

PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 87701 were performed on MON 87701 genomic DNA extracted from leaf tissue (Lanes 3, 7, 10, 14, 18, 23, 27, 31 and 35). Lanes 2, 6, 9, 13, 17, 22, 26, 30, and 34 contain reactions with conventional soybean control DNA extracted from leaf tissue. Lanes 4, 8, 11, 16, 20, 25, 28, 32, and 36 are reactions containing no template DNA. Lanes 15, 19, and 24 contain reactions with PV-GMIR9 control DNA. Lanes 1, 5, 12, 21, 29, and 33 contain Fermentas GeneRuler™ 1 kb Plus DNA Ladder. Only lanes containing PCR reactions are shown in the figure. Lanes are marked to show which product has been loaded and is visualized on the agarose E-gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87701 that appears at the bottom of the figure. Three to six µl of each of the PCR reactions were loaded on the gel. PCR amplicons reported in this figure were not necessarily used in sequencing, but are representative of the study data.

Lanes	Lanes	Lanes	Lanes
1.) GeneRuler™ 1 kb Plus DNA Ladder	10.) MON 87701 genomic DNA	19.) PV-GMIR9 control DNA	28.) No template DNA control
2.) Conventional soybean control DNA	11.) No template DNA control	20.) No template DNA control	29.) GeneRuler™ 1 kb Plus DNA Ladder
3.) MON 87701 genomic DNA	12.) GeneRuler™ 1 kb Plus DNA Ladder	21.) GeneRuler™ 1 kb Plus DNA Ladder	30.) Conventional soybean control DNA
4.) No template DNA control	13.) Conventional soybean control DNA	22.) Conventional soybean control DNA	31.) MON 87701 genomic DNA
5.) GeneRuler™ 1 kb Plus DNA Ladder	14.) MON 87701 genomic DNA	23.) MON 87701 genomic DNA	32.) No template DNA control
6.) Conventional soybean control DNA	15.) PV-GMIR9 control DNA	24.) PV-GMIR9 control DNA	33.) GeneRuler™ 1 kb Plus DNA Ladder
7.) MON 87701 genomic DNA	16.) No template DNA control	25.) No template DNA control	34.) Conventional soybean control DNA
8.) No template DNA control	17.) Conventional soybean control DNA	26.) Conventional soybean control DNA	35.) MON 87701 genomic DNA
9.) Conventional soybean control DNA	18.) MON 87701 genomic DNA	27.) MON 87701 genomic DNA	36.) No template DNA control

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

[CBI CROSS REFERENCE 1]

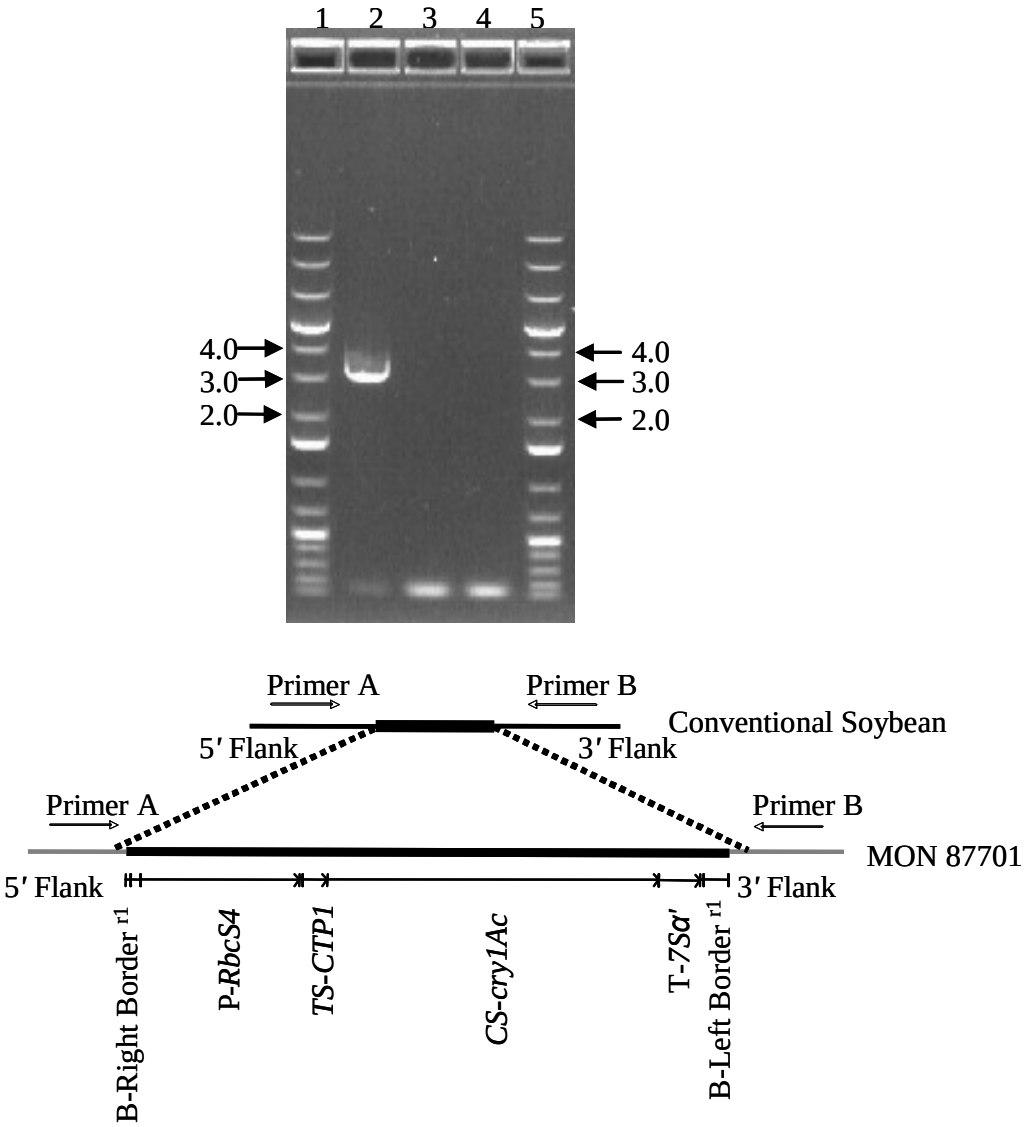


Figure 12. PCR Amplification of the MON 87701 Insertion Site in Conventional Soybean. Depiction of the MON 87701 insertion site in conventional soybean (upper panel) and the MON 87701 insert (lower panel). PCR amplification was performed using Primer A in the 5' flanking sequence and Primer B in the 3' flanking sequence of the insert in MON 87701 to verify that the flanking sequences are native to the soybean genome. Three to five µl of each of the PCR reactions were loaded on the gel. Lane designations are as follows:

Lane 1: GeneRuler™ 1 kb Plus DNA Ladder
2: Conventional soybean control DNA
3: MON 87701 genomic DNA
4: No template DNA control
5: GeneRuler™ 1 kb Plus DNA Ladder

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained E-gel.

[CBI CROSS REFERENCE 2]

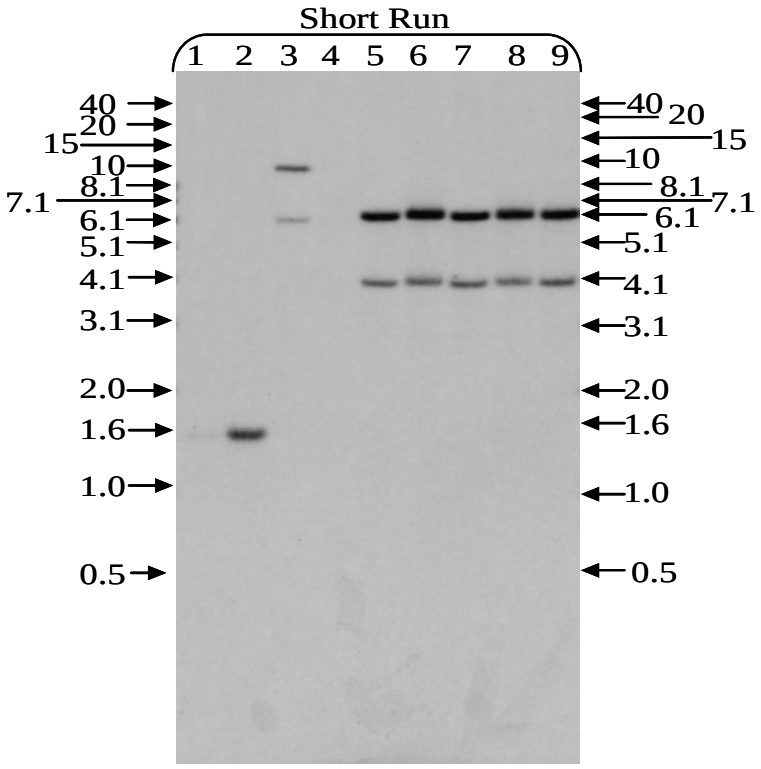


Figure 14. Insert Stability of MON 87701: T-DNA I (Probe 8)
The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA I sequence (Figure 1, Probe 8). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue from the designated generation. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~0.1 genome equivalent]
2: Conventional soybean (*Nco* I / *Vsp* I) spiked with probe templates [~1.0 genome equivalent]
3: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
4: Conventional soybean (*Nco* I / *Vsp* I)
5: MON 87701 (R4) (*Nco* I / *Vsp* I)
6: MON 87701 (R5) (*Nco* I / *Vsp* I)
7: MON 87701 (R6) (*Nco* I / *Vsp* I)
8: MON 87701 (R8) (*Nco* I / *Vsp* I)
9: MON 87701 (R9) (*Nco* I / *Vsp* I)

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

APPENDIX 1

Standard Operating Procedures

BR-EQ-0065-02	DyNA Quant 200 Fluorometer
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-PO-0573-02	TCR Material Confirmation of Identity
BR-ME-0611-01	Radiolabeling of Nucleic Acids
BR-ME-0889-01	Purification of DNA from an Agarose Gel (Adaptation of Qiagen Gel Extraction Kit)
BR-ME-1153-01	Quick CTAB DNA Extraction from Leaf Tissue
BR-ME-1157-01	PEG Precipitation of Genomic Plant DNA

APPENDIX 2

Notes for Reviewer

Appendix 2 contains amendments for the original final report. These changes do not impact the conclusions of this study.

Page Number in MSL0022176	Change
1	Updated report title, MSL number, and report completion date, added "Amendment 2" after Study Completed and added new Molecular Team Lead to list of Authors.
2	Added the statement "The inclusion of this page in all studies is for quality assurance purposes and does not necessarily indicate that this study has been submitted to the U.S. EPA." to the section of the Statement of Data Confidentiality Claim.
4	Added "Amended Report Audit" to list of phases, and updated study title.
5	Updated the study title, MSL number, and added "Amended Report Completion Date," added new Molecular Team Lead, and added "Original" to Study Completion Date.
6	Updated new Molecular Team Lead.
9	Updated "Appendix 2: Notes for Reviewer" in the Table of Contents.
11	In the Abbreviation Section, changed the sentence "3' non-translated region of the *Sphas1* gene of *Glycine max* from soybean 7*Sα'* seed storage protein, which terminates transcription and directs polyadenylation of the mRNA." to "3' region of the *Sphas1* gene of *Glycine max* encoding the 7*S*α' seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence. The element functions to terminate transcription and direct polyadenylation of the mRNA."
11	Deleted the abbreviation and definition " var." from the Abbreviations and Definitions
11	Added the abbreviation and definition " subsp." to the Abbreviations and Definitions
12	Replaced the abbreviation "var." with "subsp."

Page Number in MSL0022176	Change
18	Added Figure number to the sentence, To demonstrate that the DNA sequences flanking the insert in MON 87701 are native to the soybean genome and to characterize the insertion site in conventional soybean, PCR analyses were performed on genomic DNA from both MON 87701 and the conventional soybean control, variety A5547 (Figure 12).
22	Changed the sentence "The ~1.9 kb band observed in Figure 5 (lanes 4 and 11) 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." to "The ~1.9 kb band observed in Figure 5 (lanes 4 and 11) represents an internal fragment contained in the inserted T-DNA."
23	Changed the sentence "The presence or absence of plasmid backbone sequences in the soybean genome was evaluated by digesting the test and control genomic DNA samples with *Nco* I / *Vsp* I or *Xho* I / *Nde* I and hybridizing with overlapping backbone probes spanning the entire backbone sequence of PV-GMIR9 (Figure 1, Probes 1, 2, 3, and 4)." to "The presence or absence of plasmid backbone sequences in the soybean genome was evaluated by digesting the test and control genomic DNA samples with *Nco* I / *Vsp* I or *Xho* I / *Nde* I and hybridizing with backbone probes spanning the entire backbone sequence of PV-GMIR9 (Figure 1, Probes 1, 2, 3, and 4)."
25	Changed the sentence "The DNA sequence of the MON 87701 insert is 6427 base pairs long, beginning at base 3908 of PV-GMIR9 located in the Right Border region and ending at base 10334 in the Left Border region of PV-GMIR9." to "The DNA sequence of the MON 87701 insert is 6426 base pairs long, beginning at base 3908 of PV-GMIR9 located in the Right Border region and ending at base 10333 in the Left Border region of PV-GMIR9."
26	Changed the sentence "In order to demonstrate the stability of MON 87701, Southern blot analysis was performed using DNA obtained from multiple generations of MON 87701." to "In order to demonstrate the stability of the T-DNA in MON 87701, Southern blot analysis was performed using DNA obtained from multiple generations of MON 87701."
26	Changed the sentence "The R5 generation was used for the molecular characterization analyses shown in Figures 4-9, 12 and 14." to "The R5 generation was used for the molecular characterization analyses shown in Figures 4-9."
30	Added a hyphen to "T-DNA II" in Table 1.

Page Number in MSL0022176	Change
31	Added a hyphen to "T-DNA I" in Table 1.
31	In Table 1, changed the sentence "3' non-translated region of the *Sphas1* gene of *Glycine max* from soybean 7*Sα'* seed storage protein, which terminates transcription and directs polyadenylation of the mRNA (Schuler et al., 1982)." to "3' region of the *Sphas1* gene of *Glycine max* encoding the 7*S*α' seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler et al., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA."
32	Added a hyphen to "T-DNA II" in Table 1.
33	Added the footnote to define the "r1" superscript.
33	In Table 2, changed the sentence "3' non-translated region of the *Sphas1* gene of *Glycine max* from soybean 7*Sα*' seed storage protein, which terminates transcription and directs polyadenylation of the mRNA (Schuler et al., 1982)." to "3' region of the *Sphas1* gene of *Glycine max* encoding the 7*S*α' seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler et al., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA."
37	Added a hyphen to "T-DNA I" to the probes in the figure and in the Figure 3 legend.
37	Changed the sentence in the legend of Figure 3 from "Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analyses." to "Identified on the map are genetic elements within the insert, including restriction sites with positions relative to the size of the genomic flanking sequences and the insert sequence reported in Figure 11 for enzymes used in the Southern analyses."
39	Changed the sentence in the legend of Figure 5 from "The blot was hybridized with two overlapping ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure 1, Probes 8 and 10)." to "The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure 1, Probes 8 and 10)."

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40	Changed the sentence in the legend of Figure 6 from "The blot was hybridized with two overlapping ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure 1, Probes 9 and 11)." to "The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure 1, Probes 9 and 11)."
44	In the legend of Figure 10, corrected the typographical errors of the lane numbers used to represent the MON 87701 genomic DNA from " Lanes 3, 6, 11, 15, 21, 25, and 30" to "Lanes 3, 7, 10, 14, 18, 23, 27, 31, and 35."
45	In the legend of Figure 11, base pair position"10334" was changed to "10333."
54-57	Updated "Appendix 2: Notes for Reviewer."
Confidential Attachment Title Page	Updated report title, MSL number, and report completion date, added "Amendment 2" after Study Completed and added new Molecular Team Lead to list of Authors.