Title

Amended Report for MSL0022130: Molecular Analysis of Soybean MON 87705

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Amendment 2

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Sponsor/ Testing Facility

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Study # REG-08-060
MSL0022384

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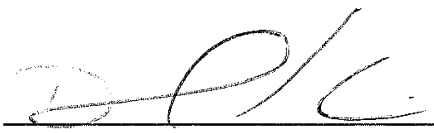
Statement of Compliance

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

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

Submitter	Date
	19 NOV 09
Sponsor Representative	Date
Xingtian	11/19/2009
Study Director	Date

Quality Assurance Statement

Study Title: Amended Report for MSL0022130: Molecular Analysis of Soybean MON 87705

Study Number: REG-08-060

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
05/15/2008	Southern Blot	05/16/2008	05/16/2008
05/22/2008	PCR/Sequence	05/22/2008	05/22/2008
08/20/2008	Raw Data Audit	08/26/2008	08/26/2008
03/13/2009	Draft Report and Data Audit	03/19/2009	03/19/2009
07/10/2009	Draft Report Amendment Review	07/10/2009	07/10/2009
11/18/2009	Draft Report Amendment Review	11/18/2009	11/18/2009

Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

Nov 19th, 2009
Date

Study Certification Page

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

Qing Tian 11/19/2009

Qing Tian, Ph.D. Date
Study Director / Team Lead
Regulatory Product Characterization Center

Study Information Page

Study Number:	REG-08-060
MSL Number:	0022384
Title:	Amended Report for MSL0022130: Molecular Analysis of Soybean MON 87705
Primary Facility:	Monsanto Company 800 North Lindbergh Blvd. St. Louis, Missouri 63167
Additional Testing Facility:	Monsanto Company Genomics Sequencing Center 800 North Lindbergh Blvd. St. Louis, Missouri 63167
Sponsor Representative:	Daniel J. Jenkins, M.S., J.D.
Team Lead:	Qing Tian, Ph.D.
Study Director:	Qing Tian, Ph.D.
Contributors:	Aurelia Skipwith and Kim R. Lawry
Study Initiation Date:	April 11, 2008
Original Study Completion Date:	March 26, 2009
Amended Report Completion Date:	November 19, 2009
Records Retention:	All study-specific raw data (including data rejected and data not reported), protocol, final report and facility records will be retained at Monsanto St. Louis.
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto, St. Louis.

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ABBREVIATIONS AND DEFINITIONS [1]

~	Approximately
aadA	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3" (9)-O-nucleotidyltransferase from the transposon Tn7 that confers spectinomycin and streptomycin resistance
B-Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
B-Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
bp	Base pair
CS-*cp4 epsps*	Codon modified coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein
CS-*rop*	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
CTAB	Hexadecyltrimethylammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
FAD2-1A	Sequence from the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase
FAD2-1A^p	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase which forms part of the suppression cassette
FATB1A	Sequence from the *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase
FATB1A^p	Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase which forms part of the suppression
I-*Tsf1*	Intron with flanking exon sequence from the *Tsf1* gene of *Arabidopsis thaliana* encoding elongation factor EF-1 alpha
L-*Tsf1*	5' nontranslated leader (exon 1) from the *Tsf1* gene of *Arabidopsis thaliana* encoding elongation factor EF-1 alpha
kb	Kilobase pair

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

ABBREVIATIONS AND DEFINITIONS (CONTINUED)

OR-*ori-pBR322*	Origin of replication from pBR322 for maintenance of plasmid in *E. coli*
OR-*ori V*	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium*
P-*7Sα'*	Promoter and leader from the *Sphas1* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'-bcsp) that directs transcription in seed
PCR	Polymerase chain reaction
P-*FMV/Tsf1*	Chimeric promoter consisting of enhancer sequences from the promoter of Figwort Mosaic virus (FMV) 35S RNA combined with promoter from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1 alpha
PV-GMPQ/HT4404	Plasmid vector used to develop MON 87705
T-DNA	Transferred DNA
T-*E9*	3' nontranslated region of the pea *RbcS2* gene which functions to direct polyadenylation of the mRNA
T-*H6*	3' UTR sequence of the *H6* gene from *Gossypium barbadense* encoding a protein involved in secondary cell wall assembly
TS-*CTP2*	Targeting sequence from the gene *shkG* encoding the transit peptide region of *Arabidopsis thaliana* EPSPS that functions to direct transport of the CP4 EPSPS protein to the chloroplasts

1.0 SUMMARY

Monsanto Company has developed biotechnology-derived soybean, MON 87705, to generate nutrionally-improved soybean oil with decreased levels of saturated fats (16:0 palmitic acid and 18:0 stearic acid) and increased levels of oleic acid (18:1). MON 87705 was produced using transformation plasmid PV-GMPQ/HT4404, which contains two separate T-DNAs that are delineated by left and right border sequences. Both T-DNAs integrated into the same locus creating a *cp4 epsps* expression cassette and a *FAD2-1A* and *FATB1A* suppression cassette *in planta*. The expression from the *FAD2-1A* and *FATB1A* suppression cassette results in the formation of an inverted repeat transcript which is expected to lead to suppression of the endogenous *FAD2-1A* and *FATB1A* RNA levels.

In this study, Southern blot analyses confirmed the copy number of the two T-DNA (T-DNA I and T-DNA II) sequences in the genome and the absence of transformation vector backbone. The data indicate that MON 87705 contains one copy of the insert at a single integration locus. These data also demonstrate that MON 87705 does not contain detectable backbone sequence from plasmid PV-GMPQ/HT4404. The complete DNA sequence of the insert and adjacent genomic DNA in MON 87705 confirmed the integrity of the inserted *cp4 epsps*, *FAD2-1A*, and *FATB1A* expression cassettes within the inserted sequences and identified the 5' and 3' insert-to-genomic DNA junctions. Additional DNA sequence analyses demonstrated that the genomic DNA sequence flanking the 5' and 3' ends of the insert in MON 87705 is native to the soybean genome and the analyses characterized the insertion site in conventional soybean. Furthermore, Southern blot analysis demonstrated that the Southern blot fingerprint of the insert in MON 87705 has been maintained through three generations of breeding, thereby confirming the stability of the insert in multiple generations.

2.0 INTRODUCTION

2.1 Background

Monsanto Company has developed MON 87705 using gene suppression technology to suppress FAD2-1 and FATB1 oil biosynthetic enzymes, in order to improve soybean oil composition. Using *Agrobacterium*-mediated transformation, a conventional soybean variety, A3525, was transformed using plasmid PV-GMPQ/HT4404 to generate MON 87705. MON 87705 contains an insert that encodes for a *FAD2-1A* and *FATB1A* inverted repeat transcript and the CP4 EPSPS protein. PV-GMPQ/HT4404 contains two separate T-DNAs. T-DNA I contains a *cp4 epsps* expression cassette and a partial suppression cassette. The *cp4 epsps* expression cassette is under the regulation of *FMV/Tsf1* chimeric promoter and *E9* polyadenylation sequence. The partial suppression cassette in T-DNA I contains fragments of the *FAD2-1A* intron and *FATB1A* 5' nontranslated region including the

chloroplast targeting sequence (referred to as *FAD2-1A* and *FATB1A* fragments), which are under the regulation of the *7Sα*' promoter. T-DNA II contains a partial suppression cassette, which consists of the *FAD2-1A* and *FATB1A* fragments, which are under the regulation of the *H6* 3' UTR sequence. During plant transformation, the two T-DNAs co-integrated at one locus in the soybean genome creating a DNA insert that contains a *cp4 epsps* cassette and a *FAD2-1A* and *FATB1A* suppression cassette. The composition of these cassettes was confirmed by Southern blot analyses.

2.2 Purpose

The purpose of this study was to characterize the integrated DNA in MON 87705. Genomic DNA was analyzed using Southern blot methods to determine the number of copies and insertion sites of the integrated DNA, as well as the presence or absence of plasmid backbone sequence. PCR and DNA sequence analyses were performed to determine the complete DNA sequence of the insert and the adjacent 5' and 3' flanking genomic DNA sequence in MON 87705, the 5' and 3' insert-to-genomic DNA junctions, and that the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87705 are native to the soybean genome. In addition, the stability of the inserted DNA in MON 87705 was assessed by Southern blot fingerprint analysis on genomic DNA from generations R4, R5, and R6.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance was MON 87705. Genomic DNA was extracted from the leaf tissue, as indicated in the table below, and was used for analyses in this study:

Production Plan	Seed Lot Number	Virgo Number	Tissue Type	Sample ID
07-01-83-30	GLP-0704-18620-S	60080299540	Leaf Tissue	07018330-00025
				07018330-00026

In order to assess the stability of the transgene insertion in MON 87705 across multiple generations, genomic DNA extracted from the following leaf tissues, as indicated in the table below, was used in this study:

Generation	Production Plan	Orion[2] ID	Source ID	Container ID
R4	PPN-08-178	10005232	10002230	10005232-001
R5	PPN-08-178	10005233	10002231	10005233-001
R6	PPN-08-178	10005234	10002232	10005234-001

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

3.2 Control Substance

The control substance was conventional soybean variety A3525 which has the same genetic background as the test substance. Genomic DNA was extracted from the leaf tissue indicated in the table below and was used for analyses in this study:

Production Plan	Seed Lot Number	Virgo Number	Tissue Type	Sample ID
				07018330-00028
				07018330-00029
07-01-83-30	GLP-0704-18621-S	60080299487	Leaf Tissue	07018330-00030

3.3 Reference Substance

The reference substance, plasmid PV-GMPQ/HT4404 (Figures 1 and 2), was used as the transformation vector to develop MON 87705. The plasmid was digested and used as a positive hybridization control in Southern analyses. Probe templates generated from this plasmid also served as positive hybridization controls. As additional reference standards, appropriate molecular size markers from commercial vendors were used for size estimations on Southern blots, agarose gels, and size estimation for PCR analyses.

3.4 Characterization of Test, Control, and Reference Substances

The identity of the pooled leaf tissue for the test and control substances was confirmed by event-specific PCR prior to use in the study. DNA was extracted using the method outlined in Section 3.5, and analyzed following SOP BR-ME-1188-01. The positive hybridization controls were generated from plasmid PV-GMPQ/HT4404, which was isolated prior to the study, and its identity confirmed by restriction enzyme digestion. The raw data was archived in the Regulatory Archives. The Study Director reviewed the chain of custody documentation to confirm the identity of the test, control, and reference substances prior to use of the materials in the study. The test and reference substances were considered stable during storage if they yielded interpretable signals on the Southern blot or produced specific PCR products. The control substance was considered stable during storage if the sample did not appear visibly degraded on ethidium bromide-stained gels or if it produced a specific PCR product in the analysis of the wild type insertion site.

3.5 Genomic DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA from the test and control substances was extracted using a hexadecyltrimethylammonium bromide (CTAB)-based method according to SOP BR-ME-1153-01. DNA extractions were stored in a 4°C refrigerator or a -20°C freezer.

3.6 Quantification of DNA

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

3.7 Restriction Enzyme Digestion of Genomic DNA

Approximately 10 or 20 μg of genomic DNA extracted from the test and control substances were used for restriction enzyme digestions. When digesting genomic DNA with *Nco* I (New England BioLabs, Beverly, MA), 10X NE buffer 3 (New England BioLabs) was used. When digesting genomic DNA with the *Spe* I (Roche, Indianapolis, IN), 10X Tango buffer (Fermentas) was used. All digestions were performed at 37°C according to SOP BR-ME-0316-01 in a total volume of ~500 μl using ~25-100 units of the appropriate restriction enzyme.

3.8 Agarose Gel Electrophoresis

Digested DNA were separated based on size using 0.8% (w/v) agarose gels according to SOP BR-ME-0315-02. Approximately 20 μg of digested test and control genomic DNA were divided in half to load approximately an equal amount on the long and short runs of the agarose gel. For copy number determination of T-DNA I and T-DNA II, and backbone analyses, a 'long run' and 'short run' were performed during the gel electrophoresis. The long run enabled greater resolution of the higher molecular weight restriction fragments, while the short run allowed the smaller molecular weight restriction fragments to be retained on the gel.

3.9 DNA Probe Preparation for Southern Blot Analyses

Probe template DNA containing sequences of plasmid PV-GMPQ/HT4404 was prepared by PCR amplification according to SOP BR-ME-0486-01 and gel purified according to SOP BR-ME-0889-01. The probes were designed based on the nucleotide content (%GC) so that the entire probe would be hybridized under the conditions appropriate for the sequence. Approximately 25 ng of each probe template were radiolabeled with ^{32}P-deoxyadenosine triphosphate (dATP) or ^{32}P-deoxycytosine triphosphate (dCTP) (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Invitrogen) according to SOP BR-ME-0611-01. Approximately 1×10^6 cpm of labeled probe per ml of hybridization solution was hybridized to the Southern blot. Probe locations relative to the genetic elements in plasmid PV-GMPQ/HT4404 are depicted in Figures 1 and 2.

3.10 Southern Blot Analyses of Genomic DNA

Digested genomic DNA isolated from test and control material, in addition to pre-digested control material mixed with appropriate positive hybridization controls were evaluated using Southern blot analyses according to SOP BR-ME-0317-02. The

plasmid DNA was digested and then added to the pre-digested conventional soybean genomic DNA to serve as a positive hybridization control. Probe templates were added to pre-digested control material to serve as an additional positive hybridization control. The DNA was then separated by agarose gel electrophoresis. Southern blots were hybridized and washed at 55°C or 60°C depending on the calculated melting temperature (T_m) of the probes. The table below lists the hybridization 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	Element Sequence Spanned by DNA Probe	Hybridization/Wash Temperature (°C)
1	T-DNA I Probe 1A	Left Border + P-*FMV/Tsf1* + L-*Tsf1* + I-*Tsf1* (portion)	55
2	T-DNA I Probe 2B	I-*Tsf1* (portion) + TS-*CTP2* (portion)	55
3	T-DNA I Probe 3C	TS-*CTP2* (portion) + CS-*cp4 epsps* (portion)	60
4	T-DNA I Probe 4D	CS-*cp4 epsps* (portion) + T-*E9* + P-*7Sα'* + *FAD2-1A*^{p} (portion)	55
5	T-DNA I Probe 5E	*FAD2-1A*^{p} (portion) + *FATB1A*^{p} + Right Border	55
6	T-DNA II Probe 1A	T-*H6* + *FAD2-1A*^{p} + *FATB1A*^{p}	55
7	Backbone Probe 1	Backbone Sequence	60
8	Backbone Probe 2	Backbone Sequence	60
9	Backbone Probe 3	Backbone Sequence	60
10	Backbone Probe 4	Backbone Sequence	60

3.11 PCR and Sequence Analyses

Overlapping PCR products were generated that span the insert in MON 87705. These products were sequenced to determine the nucleotide sequence of the insert in MON 87705, 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 PCR analyses were conducted using ~96-100 ng of genomic DNA template or ~12 ng of plasmid DNA in a 50 µl reaction volume or ~48-50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 1 mM $MgSO_4$, 1 M Betaine, 0.8 µM of each primer, 0.2 mM each dNTP, and 0.02 units of KOD Hot Start DNA polymerase from Novagen (Gibbstown, NJ). The amplification of Products A, D, and E was performed under the following cycling conditions: 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 65°C for 45 seconds, 72°C for 5 minutes; one cycle at 72°C for 10 minutes. The amplification of Products B, C, and F

was performed under the following cycling conditions: 94°C for 2 minutes; 35 cycles at 94°C for 55 seconds, 68°C for 60 seconds, 72°C for 5 minutes; and one cycle at 72°C for 10 minutes.

Aliquots of each PCR product were separated on 0.8% (w/v) agarose E-gel® (Invitrogen) or separated on a 0.8% agarose gel according to SOP BR-ME-0315-02. Prior to sequencing, the PCR products were visualized by ethidium staining to verify the products were of the expected size prior to sequencing. To remove excess primers following PCR amplification, products were treated with a mixture of 0.1 unit Exonuclease I, designated as EXO, from USB (Cleveland, OH) and 0.1 unit Shrimp Alkaline Phosphatase, designated as SAP (USB) per 5 µl of PCR product and cycled at the following conditions: one cycle at 37°C for 15 minutes, one cycle at 80°C for 15 minutes. As documented in the raw data, not all products were treated with EXO-SAP prior to sequencing. The PCR products were sequenced using multiple primers, including primers used for PCR amplification. In addition, primers internal to the amplified PCR sequences were used to sequence the regions of the amplicon. 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 87705 Insertion Site

To demonstrate that the DNA sequences flanking the insert in MON 87705 are native to the soybean genome and to examine the MON 87705 insertion site in conventional soybean, PCR and sequence analyses were performed on genomic DNA from both MON 87705 and conventional soybean. The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 87705. 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 PCR analyses were conducted using ~96-100 ng of genomic DNA template in a 50 µl reaction volume or ~50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 1 mM $MgSO_4$, 1 M Betaine, 0.8 µM of each primer, 0.2 mM each dNTP, and 0.02 units of KOD Hot Start DNA polymerase (Novagen). The amplification of the product was performed under the following cycling conditions: 94°C for 2 minutes; 35 cycles at 94°C for 55 seconds, 68°C for 60 seconds, 72°C for 5 minutes; one cycle at 72°C for 10 minutes.
Aliquots of each PCR product were separated on 0.8% (w/v) agarose E-gel® (Invitrogen) and visualized using the UV transilluminator to verify that the products were of the expected size prior to sequencing. To remove excess primer following

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PCR amplification, the PCR product containing the conventional soybean template was treated with a mixture of 0.1 unit EXO and 0.1 unit SAP per 5µl of PCR product and cycled as follows: 1 cycle at 37°C for 15 minutes, one cycle at 80°C for 15 minutes. The PCR products were sequenced using multiple primers, including primers used for PCR amplification. In addition, primers internal to the amplified PCR sequences were used to sequence the regions of the amplicon. 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 duplicate blots generated, high levels of background, or partial digestion or star activity caused by restriction enzyme issues. Southern blot analyses were rejected if there was mis-loading of the agarose gel, unexpected positive results in the negative DNA control, or inaccurate amounts of positive hybridization control.

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 poor 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, changes to the original protocol were required. These changes were documented as protocol amendments and are summarized below. None of these changes had any negative impact on the conclusions of this study.

1. The protocol was amended to provide a unique Sample ID for each test and sample to further clarify the samples used in the study.

2. The test substances for generational stability were added to the study by protocol amendment. Additionally, the protocol was amended to correct a typographical error in the Production Plan Number of the generational stability samples.

3. The protocol originally stated that the event-specific PCR would be performed according to SOP BR-PO-0573-02. The protocol was amended to clarify that this SOP was not applicable for the purpose of the identity confirmation of the samples used in this study.

4. The protocol was amended to perform additional Southern analyses, which provided confirmation of incomplete digestion and star activity when digesting

MON 87705 DNA with *Spe I*. However, the original Southern analyses using *Spe I* were optimized and the data generated from the additional analyses were not reported.

5. The protocol was amended to allow the report quality photograph to be generated using a 0.8-1.0% agarose gel according to the current version of SOP BR-ME-0315 for the gel showing the products for the MON 87705 insert and flanking sequence. The originally protocol stated that all report quality photographs would be generated using the 0.8% agarose Invitrogen E-gel®.

6. The protocol was amended to reflect change in study directorship.

4.0 RESULTS AND DISCUSSION

The R3 generation (Figure 4) was used for the molecular characterization analyses of MON 87705, because this generation is prior to the initiation of commercial breeding. Genomic DNA from MON 87705 was subjected to Southern blot analysis to characterize the DNA that was integrated into the soybean genome and to confirm the presence or absence of PV-GMPQ/HT4404 backbone sequence in MON 87705. Additionally, the complete DNA sequence of the insert and adjacent flanking genomic DNA in MON 87705 was confirmed. The PCR and DNA sequence analysis confirmed that the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87705 are native to the soybean genome. Furthermore, the stability of integrated DNA in MON 87705 was examined by confirming that the expected Southern blot fingerprint of MON 87705 had been maintained in multiple generations.

4.1 Southern Blot Analyses of MON 87705

Southern blot analysis was performed to characterize the insert present in MON 87705 and determine whether the insert reported in Figures 3 and 11 represent the only detectable insert in MON 87705. Genomic DNA from MON 87705 was digested using a variety of restriction enzymes and subjected to Southern blot analysis utilizing probes that cover the entire sequence of the transformation vector. A description of the genetic elements and their location within PV-GMPQ/HT4404 are shown in Table 1. The genetic elements integrated in MON 87705 are summarized in Table 2. Maps of plasmid vector PV-GMPQ/HT4404, used in the transformation to produce MON 87705 and annotated with the probes used in the Southern analysis are presented in Figures 1 and 2. A linear map depicting restriction sites within the insert as well as within the known soybean genomic DNA immediately flanking the insert in MON 87705 is shown in Figure 3. The relative positions of the restriction sites in Figure 3 are based on the sequence of the insert and adjacent genomic DNA discussed below in Section 4.2. Based on the linear map of the insert and the plasmid map, a table summarizing the expected DNA fragments for Southern analyses is presented in

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Table 3. The generations used in this study are depicted in the breeding history shown in Figure 4. 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 sizes of bands present in the short run lanes, the molecular weight markers on the right side of the figures were used. In some of the Southern blots, the migration of the genomic DNA is slightly different when compared to the migration of the molecular weight markers. 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).

4.1.1 Southern Blot Analyses to Confirm the Copy Number and the Integrity of the Inserted T-DNA I and T-DNA II in MON 87705

The copy number and insertion sites of T-DNA I and T-DNA II were assessed by digesting test DNA with restriction enzymes *Nco* I or *Spe* I and hybridizing Southern blots with probes that span T-DNA I and T-DNA II (Figure 1). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table 3). Since each detected fragment contains flanking genomic DNA, any additional integrated sites would produce a different banding pattern with additional bands.

The restriction enzyme *Nco* I cuts once in the MON 87705 insert and once in each of the known 5' and 3' flanking sequences of MON 87705. Therefore, if T-DNA I and T-DNA II sequences are present at a single integration site in MON 87705, the digestion with *Nco* I was expected to generate two border fragments with expected sizes of ~4.0 kb and ~5.7 kb (Figure 3). The ~4.0 kb restriction fragment contains genomic DNA flanking the 5' end of the insert, Left Border^{r1}, *FMV/Tsf1* promoter, *Tsf1* leader, and *Tsf1* intron. The ~5.7 kb restriction fragment contains the *CTP2* targeting sequence, *cp4 epsps* coding sequence, *E9* 3' nontranslated sequence, *7Sα'* promoter, *FAD2-1A*^{p} and *FATB1A*^{p} sequences, Right Border^{r1}, Left Border^{r2}, *FATB1A*^{p1} and *FAD2-1A*^{p} sequences, *H6* 3' UTR sequence, Left Border^{r3} and genomic DNA flanking the 3' end of the insert.

The restriction enzyme *Spe* I cuts once in the MON 87705 insert and once in the known 3' flanking sequence of MON 87705. Therefore, if T-DNA I and T-DNA II sequences are present at a single integration site in MON 87705 digestion with *Spe* I was expected to release two border fragments with expected sizes of ~5.0 kb and greater than 8.1 kb (Figure 3). Since the *Spe* I site in the soybean genome flanking the 5' end of the insert lies outside of the known sequence, it was not possible to predict a precise fragment size. However, the fragment size was determined by Southern blot analyses to be ~11 kb (Figures 5, 6, and 7). The ~11 kb contains genomic DNA flanking the 5' end of the insert, Left Border^{r1}, *FMV/Tsf1* promoter, *Tsf1* leader, *Tsf1* intron, *CTP2* targeting sequence, *cp4 epsps* coding sequence, *E9* 3' nontranslated

sequence, and a portion of *7Sα'* promoter. The ~5.0 kb restriction fragment contains the remaining portion of the *7Sα'* promoter, *FAD2-1A*^{p} and *FATB1A*^{p} sequences, Right Border^{r1}, Left Border^{r2}, *FATB1A*^{p1} and *FAD2-1A*^{p} sequences, *H6* 3' UTR sequence, Left Border^{r3} and genomic DNA flanking the 3' end of the insert.

In the Southern blot analyses performed, each Southern blot contained a negative and several positive controls. Conventional soybean DNA digested with *Nco* I or *Spe* I was used as a negative control to determine if the probes hybridized to any endogenous soybean sequences. As a positive control on the Southern blots, digested plasmid and probe templates were used. Plasmid PV-GMPQ/HT4404 digested with a combination of *Xho* I and *Nco* I was mixed with pre-digested conventional soybean genomic DNA and loaded on the gel to serve as a positive hybridization control. For a Southern blot hybridized with multiple probes, each probe template was mixed with pre-digested conventional soybean DNA as a positive hybridization control for each probe. The positive hybridization control was spiked at 0.1 and 1 genome equivalent to demonstrate sufficient sensitivity of the Southern blot. Individual Southern blots were hybridized with the following probe sets: Probes 1, 4, and 6; Probes 2 and 5; and Probe 3 (refer to Section 3.10 and Figure 1). The results of this analysis are shown in Figures 5 -7. As described above, the blots differ in hybridization and wash temperatures based on the sequence composition of the probes.

4.1.1.1 Copy Number Analysis of T-DNA I and II: Probes 1, 4, and 6

Conventional soybean DNA digested with *Nco* I (Figure 5, lanes 1 and 8) or *Spe* I (Figure 5, lanes 3 and 10) and hybridized with the probes 1, 4, and 6 (Figure 1) produced several hybridization signals. These hybridization signals result from the probes (Probes 1, 4, and 6, Figure 1) which contain some soybean sequence, hybridizing to endogenous sequences residing in the soybean genome and are not specific to the inserted DNA. These signals, as expected, were produced in both test and conventional soybean lanes, and therefore the bands are considered to be endogenous background hybridization.

Probe template spikes (Probes 1, 4 and 6, Figure 1) generated from plasmid PV-GMPQ/HT4404 were mixed with the conventional soybean DNA pre-digested with *Spe* I and produced the expected bands at ~1.8, ~2.1, and ~1.1 kb, respectively, (Figure 5, lanes 5-6) in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 5, lane 10). Plasmid PV-GMPQ/HT4404 digested with a combination of *Xho* I and *Nco* I and mixed with conventional soybean DNA pre-digested with *Spe* I (Figure 5, lane 7) produced the expected size bands of ~3.2 and ~9.9 kb (refer to Figure 1 plasmid map for digested fragment sizes) in addition to the endogenous background

hybridization observed in the conventional soybean DNA (Figure 5, lane 10). These results indicate that the probes are hybridizing to their target control sequences.

MON 87705 DNA digested with *Nco* I (Figure 5, lanes 2 and 9) produced two unique bands of ~4.0 and ~5.7 kb in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 5, lanes 1 and 8). The ~4.0 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~5.7 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I and T-DNA II) along with the adjacent genomic DNA flanking the 3' end of the insert (Figure 3).

MON 87705 DNA digested with *Spe* I (Figure 5, lanes 4 and 11) produced two unique bands of ~5.0 and ~11 kb, in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 5, lanes 3 and 10). The ~11 kb fragment is consistent with the expected band being greater than 8.1 kb. This band in the short run appears slightly larger, at ~13 kb, than the corresponding band in the long run. This border fragment contains the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~5.0 kb band (Figure 5, lane 4) is consistent with the expected band of 5.0 kb; however, the migration of the fragment is slightly higher at ~5.2 kb in the short run (Figure 5, lane 11) than indicated by the molecular weight marker. The ~5.0 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I and T-DNA II) along with the adjacent genomic DNA flanking the 3' end of the insert (Figure 3).
There were no additional bands detected using the probes 1, 4, and 6. Based on the results presented in Figure 5, it was concluded that T-DNA sequences covered by probes 1, 4, and 6 reside at a single detectable locus of integration in MON 87705.

4.1.1.2 Copy Number Analysis of T-DNA I and II: Probes 2 and 5

Conventional soybean DNA digested with *Nco* I (Figure 6, lanes 1 and 8) or *Spe* I (Figure 6, lanes 3 and 10) and hybridized with the probes 2 and 5 (Figure 1) produced several hybridization signals. These hybridization signals result from the probes (Probes 2 and 5, Figure 1), which contain some soybean sequence, hybridizing to endogenous sequences residing in the soybean genome and are not specific to the inserted DNA. These signals, as expected, were produced in both test and conventional soybean lanes, and therefore the bands are considered to be endogenous background.

Probe template spikes (Probes 2 and 5, Figure 1) generated from plasmid PV-GMPQ/HT4404 mixed with the conventional soybean DNA pre-digested with *Spe* I produced the expected bands at ~0.8 and ~1.0 kb (Figure 6, lanes 5-6) in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 6, lane 10). Plasmid PV-GMPQ/HT4404 digested with a combination of *Xho* I and *Nco* I and mixed with conventional soybean DNA pre-digested with *Spe* I (Figure 6, lane 7) produced the expected size bands of ~3.2 and ~9.9 kb (refer to Figure 1 plasmid map) in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 6, lane 10). These results indicate that the probes are hybridizing to their target control sequences.

MON 87705 DNA digested with *Nco* I (Figure 6, lanes 2 and 9) produced two unique bands of ~4.0 and ~5.7 kb in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 6, lanes 1 and 8). The ~4.0 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~5.7 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I and T-DNA II) along with the adjacent genomic DNA flanking the 3' end of the insert (Figure 3).

MON 87705 DNA digested with *Spe* I (Figure 6, lanes 4 and 11) produced two unique bands of ~5.0 and ~11 kb, in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 6, lanes 3 and 10). The ~11 kb fragment is consistent with the expected band being greater than 8.1 kb and because this band is seen with probes 1, 4, and 6 (Figure 5). The ~11 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~5.0 kb border fragment contains the 3' end of the inserted DNA (T-DNA I and T-DNA II) along with the adjacent genomic DNA flanking the 3' end of the insert (Figure 3).

There were no additional bands detected using probes 2 and 5. Based on the results presented in Figure 6, it was concluded that sequence covered by probes 2 and 5 resides at a single detectable locus of integration in soybean MON 87705.

4.1.1.3 Copy Number Analysis of T-DNA I and II: Probe 3

Conventional soybean DNA digested with *Nco* I (Figure 7, lanes 1 and 7) or *Spe* I (Figure 7, lanes 3 and 9) and hybridized with the probe 3 (Figure 1) showed no detectable hybridization bands, as expected for the negative control.

Plasmid PV-GMPQ/HT4404 digested with a combination of *Xho* I and *Nco* I and mixed with conventional soybean DNA pre-digested with *Spe* I (Figure 7, lanes 5-6) produced the expected size band of ~3.2 kb (refer to Figure 1 plasmid map). This hybridization indicates that the probe is hybridizing to its target control sequence.

MON 87705 DNA digested with *Nco* I (Figure 7, lanes 2 and 8) produced the expected band of ~5.7 kb. The ~5.7 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I and T-DNA II) along with the adjacent genomic DNA flanking the 3' end of the insert (Figure 3).

MON 87705 DNA digested with *Spe* I (Figure 7, lanes 4 and 10) produced the expected band of ~11 kb. The ~11 kb band is consistent with expected band being greater than 8.1 kb and this band is also seen with probes 1, 4, and 6 (Figure 5) and probes 2 and 5 (Figure 6). The ~11 kb band represents the border fragment containing the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure 3).

There were no additional bands detected using probe 3. Based on the results presented in Figure 7, it was concluded that sequence covered by probe 3 resides at a single detectable locus of integration in soybean MON 87705.

Taken together, the data presented in Figures 5, 6, and 7 indicate that a single copy of the T-DNA I and T-DNA II sequences integrated into the soybean genome at a single detectable site in MON 87705.

4.1.2 Southern Blot Analyses to Determine the Presence or Absence of Plasmid PV-GMPQ/HT4404 Backbone Sequence

Test and conventional soybean DNA were digested with the restriction enzymes *Nco* I or *Spe* I. Probe template spikes (probes 7-10, Figure 2) generated from plasmid PV-GMPQ/HT4404 were mixed with the pre-digested conventional soybean genomic DNA to serve as positive hybridization controls. Additionally, plasmid PV-GMPQ/HT4404 DNA previously digested with the combination of *Xho* I and *Nco* I was mixed with conventional soybean DNA digested with *Spe* I and loaded on

the gel to serve as a positive hybridization control. The blots were hybridized with probes 7-10 (Figure 2) that covered the entire backbone sequence of PV-GMPQ/HT4404. If backbone sequences are present in MON 87705, then probing with backbone sequence should result in unique hybridizing bands. The results are shown in Figures 8 and 9.

4.1.2.1 Plasmid Backbone Sequence Analysis: Probes 7 and 9

Conventional soybean DNA digested with the restriction enzyme *Nco* I (Figure 8, lanes 1 and 8) or *Spe* I (Figure 8, lanes 3 and 10) and hybridized with probes 7 and 9 (Figure 2) showed no detectable hybridization bands, as expected for the negative control.

Probe template spikes (Probes 7 and 9, Figure 2) generated from plasmid PV-GMPQ/HT4404 and mixed with conventional soybean DNA pre-digested with *Spe* I produced the expected bands at ~1.3 and ~1.5 kb (Figure 8, lanes 5 and 6). In addition, there is an unexpected faint band at ~3.0 kb (Figure 8, lane 6). Based on size, this band is likely derived from dimers of the probe template. Since this extra band is only present in the positive control and is not present in the conventional or test soybean DNA, it was concluded that the presence of this extra band did not impact or alter the final results for MON 87705. Plasmid PV-GMPQ/HT4404 *Xho* I/*Nco* I restriction fragments mixed with conventional soybean DNA digested with *Spe* I (Figure 8, lane 7) produced a band at ~9.4 kb. This band is consistent with the expected band at ~9.9 kb; however, the migration of the ~9.4 kb fragments is slightly lower than indicated by molecular weight marker most likely due to differences in salt concentrations between sample and marker. Overall, these results indicate that these probes are hybridizing to their target control sequences.

MON 87705 DNA digested with either *Nco* I (Figure 8, lanes 2 and 9) or *Spe* I (Figure 8, lanes 4 and 11) showed no detectable hybridization signal, indicating that MON 87705 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ/HT4404.

4.1.2.2 Plasmid Backbone Sequence Analysis: Probes 8 and 10

Conventional soybean DNA digested with the restriction enzyme *Nco* I (Figure 9, lanes 1 and 8) or *Spe* I (Figure 9, lanes 3 and 10) and hybridized with probes 8 and 10 (Figure 2) showed no detectable hybridization bands, as expected for the negative control.

Probe template spikes (Probes 8 and 10, Figure 2) generated from plasmid PV-GMPQ/HT4404 and mixed with conventional soybean DNA pre-digested

with *Spe* I produced two expected bands that migrated at ~2.1 and~0.7 kb (Figure 9, lanes 5 and 6). Plasmid PV-GMPQ/HT4404 *Xho* I/*Nco* I restriction fragments mixed with conventional soybean DNA digested with *Spe* I (Figure 9, lane 7) produced a band at ~9.4 kb, which is consistent the expected band at ~9.9 kb. The migration of the positive hybridization controls (Figure 9, lanes 5-7) is slightly different than indicated by the molecular weight marker, most likely due to differences in salt concentrations between samples and marker. Overall, these results indicate that these probes are hybridizing to their target control sequences.

MON 87705 DNA digested with either *Nco* I (Figure 9, lanes 2 and 9) or *Spe* I (Figure 9, lanes 4 and 11) showed no detectable hybridization signal. These results indicate that MON 87705 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ/HT4404.

4.2 Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87705

The organization of the elements within the insert in MON 87705 was confirmed using PCR analysis to amplify six 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, 12, 15, 18, and 21), and the conventional soybean control reactions (lanes 2, 5, 9, 13, 16, and 19) did not generate PCR products with any of the primer sets, as expected for the negative control. Plasmid PV-GMPQ/HT4404 was used as a positive control template (Figure 10, lanes 7 and 11) and produced the expected size PCR products of ~1.9 kb and ~2.0 kb respectively for each reaction. There is an unexpected faint band at ~4.0 kb (Figure 10, lanes 7 and 11), which is likely derived from dimers of the PCR products. PCR reactions using genomic DNA from MON 87705 produced the expected size products of ~4.0 kb for Product A (Figure 10, lane 3), ~1.9 kb for Product B (Figure 10, lane 6), ~2.0 kb for Product C (Figure 10, lane 10), ~2.3 kb for Product D (Figure 10, lane 14), ~1.0 kb for Product E (Figure 10, lane 17), and ~3.5 kb for Product F (Figure 10, lane 20).

To determine the sequence of the insert in MON 87705 and the adjacent genomic DNA flanking the insert, the PCR products A-F (Figure 10) were sequenced. The consensus sequence representing the insert in MON 87705, including the genomic DNA flanking the insert, is shown in Figure 12 (CBI Cross Reference 1) and is described in Table 2. This consensus sequence was generated by compiling multiple

sequencing reactions performed on the overlapping PCR products which spanned the length of the insert and the 5' and 3' flanking soybean genomic DNA. The sequence of the insert establishes that the arrangement and linkage of elements within each individual T-DNA in the insert are consistent with those in plasmid PV-GMPQ/HT4404 and are as depicted in Figures 3, 11, and 12. The sequence between T-DNA I and T-DNA II in MON 87705 are consistent with the border sequences in PV-GMPQ/HT4404. The sequence confirms that T-DNA I and T-DNA II are tandem to one another such that an inverted repeat of *FAD2-1A*^{p} and *FATB1A*^{p} is formed.

The arrangement of T-DNA I and T-DNA II in the insert in MON 87705 is compared to the arrangement of the two T-DNAs in PV-GMPQ/HT4404 in Figure 11. The insert in MON 87705 is 7251 base pairs and matches the sequence of PV-GMPQ/HT4404 in four distinct locations as described below (see Figure 11).

1. The 5' portion of the insert, beginning at base 3280 and ending at base 9114 (Figures 11, 12, and Table 2), matches the T-DNA I sequence of PV-GMPQ/HT4404 between bases 7840-13088 and 1-586 (Table 1).
2. The sequence between the coding regions of T-DNA I and T-DNA II in MON 87705 aligns to border sequences in PV-GMPQ/HT4404, as indicated below.
    a. There are 20 base pairs of the insert sequence, beginning at base 9115 and ending at base 9134 (Figures 11, 12 and Table 2) that matches the Right Border sequence of PV-GMPQ/HT4404 at base location 640-659 (Table 1).
    b. There are 38 base pairs, beginning at 9135 and ending at 9172 (Figures 11, 12, and Table 2) in MON 87705 that match the Left Border sequence of PV-GMPQ/HT4404 from both T-DNA I and T-DNA II. The 38 base pairs in MON 87705 matches bases 7833-7870 in T-DNA I and between 5304-5341 in T-DNA II (Table 1).
3. The remainder of the insert, beginning at 9173 and ending at base 10530 (Figures 11, 12, and Table 2), represents T-DNA II which contains the *FAD2-1A*^{p} and *FATB1A*^{p} sequences. However, there was a 30 base pair truncation at the 3' end of *FATB1A*^{p} sequence detected in MON 87705. This region of the MON 87705 insert matches the sequence of PV-GMPQ/HT4404 beginning at base 6652 and ending at bases 5295 (Table 1). The T-DNA II sequence of PV-GMPQ/HT4404 was inverted, relative to the T-DNA I sequence, upon integration into the soybean genome.

In addition to the insert sequence, 3279 base pairs of soybean genomic DNA flanking the 5' end of the insert and 2713 base pairs of soybean genomic DNA flanking the 3'

end of the insert were reported in Figure 12. 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 87705 Insertion Site

PCR and DNA sequence analysis were performed on genomic DNA extracted from MON 87705 and conventional soybean to demonstrate that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87705 are native to the soybean genome and to examine the MON 87705 insertion site in conventional soybean. The PCR was performed with one primer designed to the genomic DNA sequence flanking the 5' end of the insert (Figure 14, Primer A) paired with a second primer designed to the genomic DNA sequence flanking the 3' end of the insert (Figure 14, Primer B). The results of the PCR analyses are shown in Figure 13. The control PCR containing no template DNA (Figure 13, lane 4) did not generate a PCR product, as expected. The reaction using the conventional soybean genomic DNA template (Figure 13, lane 2) generated a PCR product of ~3.7 kb band across the insertion site of MON 87705. As expected, a PCR product across the insert in MON 87705 (Figure 13, lane 3) was not generated in this analysis since the PCR conditions to generate a product of this size (13,349 bp) were not used.

The ~3.7 kb PCR product generated from the conventional soybean genomic DNA was sequenced and the resulting data are shown in Figure 14 (CBI Cross Reference 2). Comparison of this sequence to the 5' and 3' genomic sequences flanking the insert (Figure 12) indicate that a small deletion and a duplication of the genomic DNA occurred at the MON 87705 insertion site. Nucleotides 1-895 in Figure 14 match bases 11-905 of the 5' genomic flanking sequence shown in Figure 12. Bases 896-931 of conventional soybean DNA sequence (Figure 14) are not found in the 5' flanking sequence (Figure 12), indicating that the 36 bases were most likely deleted upon the insertion of T-DNA I and T-DNA II. Bases 932-3309 from the conventional soybean sequence (Figure 14) is identical to 3' genomic DNA sequence flanking the MON 87705 insert (Figure 12). Of the 2378 bases, bases 936-3309 match genomic DNA sequence in both the 5' and 3' flanking sequence of MON 87705 (bases 906-3279 and 10535-12908 in Figure 12). These 2374 bases are most likely from the 3' end of the flanking genomic DNA and were duplicated at the 5' end of the insertion site when T-DNA I and T-DNA II integrated into the genome, because of the following reasons: 1) there is a single nucleotide change detected in the duplicated 2374 base pairs at the 5' flanking sequence; and 2) there are 4 bases located at the 3' junction of the insert (10531-10534, Figure 12) that match conventional soybean (932-935 bp, Figure 14; see Figure 13), but these 4 bases are not present at the 5' end duplicated sequence. Bases 3310-3635 (Figure 14) match bases 12909 – 13234 of the 3' end of the 3' genomic flanking sequence reported in Figure 12. This analysis confirms that the genomic sequences flanking the insert in

MON 87705 are native to the soybean genome and that a 36 base pair deletion and a 2374 base pair duplication which contains a single base change occurred at the insertion site during integration of the T-DNA sequences. These molecular rearrangements presumably resulted from double-stranded break repair mechanisms in the plant during *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998).

4.4 Southern Blot Analyses of MON 87705 in Multiple Generations

In order to assess the stability of the T-DNA I and T-DNA II in MON 87705 in multiple generations, Southern blot analyses were performed using DNA obtained from multiple generations of the MON 87705 breeding history. For reference, the breeding history of MON 87705 is presented in Figure 4. The specific generations tested are indicated in the legends of Figures 4 and 15. DNA samples from R3, R4, R5, and R6 generations of MON 87705 (refer to Figure 4) were digested with *Nco* I and were expected to release two border fragments with the expected sizes of 4.0 and 5.7 kb (Figure 3, refer to Section 4.1). The detected hybridization bands in R4, R5, and R6 generations are compared to the fully characterized MON 87705 R3 generation to evaluate stability. Any instability associated with the insert would be detected as faint novel bands within the fingerprint on the Southern blot. The blot was hybridized simultaneously with two radiolabeled probes that cover both border fragments generated by the digest (probes 1 and 6, Figure 1). This blot has two of the same positive hybridization controls (probes 1 and 6, Figure 1) as described in Section 4.1.1.1. The results of this analysis are shown in Figure 15.

4.4.1 Southern Blot Analysis to Examine Generational Stability of the Insert

Conventional soybean DNA digested with *Nco* I and hybridized with probes 1 and 6 (Figure 15, lane 4) showed hybridization bands. These hybridization signals result from the probes hybridizing to endogenous targets residing in the soybean genome and are not specific to the inserted DNA.

Probe templates spikes (Probes 1and 6, Figure 1), generated from plasmid PV-GMPQ/HT4404 and mixed with conventional soybean DNA pre-digested with *Nco* I (Figure 15, lanes 1 and 2) produced the expected size bands at ~1.8 and ~1.1 kb. The detection of the probe template positive hybridization controls demonstrates that both probes were hybridizing to the target DNA. Plasmid PV-GMPQ/HT4404 digested with a combination of *Xho* I and *Nco* I and mixed with conventional soybean DNA pre-digested with *Nco* I (Figure 15, lane 3) produced the expected size band at ~9.9 kb, which indicates that the probes are hybridizing to their corresponding sequence in the transformation vector. This

expected band at ~9.9 kb migrated together with an endogenous hybridization signal observed in Figure 15, lane 4.

Digestion of MON 87705 from multiple generations (refer to Breeding History of MON 87705, Figure 4) with restriction enzyme *Nco* I produced two bands at ~4.0 and ~5.7 kb (Figure 15, lanes 5-8) in addition to the endogenous background hybridization observed in the conventional soybean DNA (Figure 15, lane 4). The ~4.0 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) along with the adjacent genomic DNA flanking the 5' end of the insert (Figure 3). The ~5.7 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I and T-DNA II) along with the adjacent genomic DNA flanking the 3' end of the insert (Figure 3). However, the migration of this fragment appears slightly lower than indicated by the molecular weight marker most likely due to differences in salt concentrations between the samples and marker. This restriction pattern is the same as the restriction pattern observed in the Southern blot analysis of the R3 generation shown in Figure 5 (lanes 2 and 9).

There were no additional unexpected bands detected, indicating that the single copy of T-DNA I and T-DNA II in MON 87705 is stably maintained in the selected generations.

5.0 CONCLUSIONS

Molecular characterization of MON 87705 by Southern blot analyses demonstrated that a single copy of the T-DNA sequences from the transformation vector PV-GMPQ/HT4404 was integrated into the soybean genome at a single locus. The data show that MON 87705 contains a single copy of T-DNA I and T-DNA II. There was no additional sequence from the transformation vector PV-GMPQ/HT4404 detected other than those associated with the reported insert. Backbone sequence from plasmid PV-GMPQ/HT4404 was not detected.

PCR and DNA sequence analyses were performed on MON 87705, which confirmed the organization of the elements within the insert, determined the 5' and 3' insert-to-plant junctions, determined the complete DNA sequence of the insert and adjacent soybean genomic DNA in MON 87705, and confirmed that the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87705 are native to the soybean genome. The PCR and DNA sequence analysis identified 36 bp of conventional soybean DNA sequence deleted at the insertion site in MON 87705. Additionally, a 2374 bp duplication was

identified in the 5' genomic flanking sequence of MON 87705. This duplicated sequence is most likely from the 3' flanking sequence of MON 87705 and it contains a single nucleotide change.

Generational stability analysis demonstrated that an expected Southern blot fingerprint of MON 87705 has been maintained through several generations of the breeding history, thereby confirming the stability of the insert.

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Table 1. Summary of Genetic Elements in Plasmid Vector PV-GMPQ/HT4404

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA I		
B[1]-Left Border	7657 – 8098	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	8099 – 8134	Sequence used in DNA cloning
P[2]-*FMV/Tsf1*	8135 – 9174	Chimeric promoter consisting of enhancer sequences from the promoter of the Figwort Mosaic virus 35S RNA (Richins et al., 1987) combined with the promoter from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1alpha (Axelos et al., 1989)
L[3]-*Tsf1*	9175 – 9220	5' nontranslated leader (exon 1) from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1 alpha (Axelos et al., 1989)
I[4]-*Tsf1*	9221 – 9842	Intron with flanking exon sequence from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1alpha (Axelos et al., 1989)
Intervening Sequence	9843 – 9851	Sequence used in DNA cloning
TS[5]-*CTP2*	9852 – 10079	Targeting sequence from the *ShkG* gene encoding the transit peptide region of *Arabidopsis thaliana* EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS[6]-*cp4 epsps*	10080 –11447	Codon modified coding sequence of the *aroA* gene from the *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Padgette et al., 1996; Barry et al., 1997)
Intervening Sequence	11448 – 11505	Sequence used in DNA cloning
T[7]-*E9*	11506 – 12148	3′ nontranslated region of the pea *RbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	12149 – 12236	Sequence used in DNA cloning
P-7Sα′	12237 – 13077	Promoter and leader from the *Sphas1* gene of Glycine max encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle et al., 1986) that directs transcription in seed

B[1] –Border; **P[2]** – Promoter; **L[3]**– Leader; **I[4]** – Intron; **TS[5]** – Targeting Sequence; **CS[6]** – Coding Sequence; **T[7]** – 3' nontranslated transcriptional termination sequence and polyadenylation signal sequences;

Table 1 (cont.) Summary of Genetic Elements in Plasmid Vector PV-GMPQ/HT4404

Intervening Sequence	13078 – 11	Sequence used in DNA cloning
FAD2-1A^{p}	12 – 277	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which forms part of the suppression cassette
FATB1A^{p}	278 – 578	Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette
Intervening Sequence	579 – 616	Sequence used in DNA cloning
B-Right Border	617 – 973	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Zambryski et al., 1982)
Vector Backbone		
Intervening Sequence	974 – 1109	Sequence used in DNA cloning
aadA	1110 – 1998	Promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9)-O-nucleotidyltransferase from the transposon Tn7 (Flung et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	1999 – 2528	Sequence used in DNA cloning
OR^{8}-*ori-pBR322*	2529 – 3117	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	3118 – 3544	Sequence used in DNA cloning
CS-*rop*	3545 – 3736	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	3737 – 5127	Sequence used in DNA cloning
T-DNA II		
B-Left Border	5128 – 5569	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)

P **–** Partial sequence; **OR^{8} –** Origin of Replication.

Table 1 (cont.) Summary of Genetic Elements in Plasmid Vector PV-GMPQ/HT4404

Intervening Sequence	5570 – 5667	Sequence used in DNA cloning
T-*H6*	5668 – 6103	3' UTR sequence of the *H6* gene from *Gossypium barbadense* encoding a fiber protein involved in secondary cell wall assembly (John and Keller, 1995)
Intervening Sequence	6104 – 6115	Sequence used in DNA cloning
***FAD2-1A*^{p}**	6116 – 6381	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which forms part of the suppression cassette
***FATB1A*^{p}**	6382 – 6682	Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette
Intervening Sequence	6683 – 6693	Sequence used in DNA cloning
B-Right Border	6694 – 7024	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Zambryski et al., 1982)
Vector Backbone		
Intervening Sequence	7025 – 7173	Sequence used in DNA cloning
OR-*ori V*	7174 – 7570	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	7571 – 7656	Sequence used in DNA cloning

Table 2. Summary of Genetic Elements in MON 87705

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Unique 5' flanking sequence of the insert	1 – 905	Soybean genomic DNA
Sequence flanking 5' end of the insert	906-3279	2374 bp of soybean genomic DNA duplicated from the 3' end of the flanking sequence of the insert
B[3]-Left Border[r1]	3280 – 3538	259 bp sequence from the B-Left Border region remaining after integration (Barker et al., 1983)
Intervening Sequence	3539 – 3574	Sequence used in DNA cloning
P[4]-*FMV/Tsf1*	3575 – 4614	Chimeric promoter consisting of enhancer sequences from the promoter of the Figwort Mosaic virus 35S RNA (Richins et al., 1987) combined with the promoter from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1 alpha (Axelos et al., 1989)
L[5]-*Tsf1*	4615 – 4660	5' nontranslated leader (exon 1) from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1 alpha (Axelos et al., 1989)
I[6]-*Tsf1*	4661 – 5282	Intron with flanking exon sequence from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1alpha (Axelos et al., 1989)
Intervening Sequence	5283 – 5291	Sequence used in DNA cloning
TS[7]-*CTP2*	5292 – 5519	Targeting sequence from the *ShkG* gene encoding the transit peptide region of *Arabidopsis thaliana* EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS[8]-*cp4 epsps*	5520 – 6887	Codon modified coding sequence of the *aroA* gene from the *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Padgette et al., 1996; Barry et al., 1997)
Intervening Sequence	6888 – 6945	Sequence used in DNA cloning

[1] Although flanking sequences and intervening sequences are not functional genetic elements, they comprise a portion of the sequence reported in Figure 12.
[2] Numbering refers to the sequences from Figure 12 that includes the insert in MON 87705 and adjacent genomic DNA.

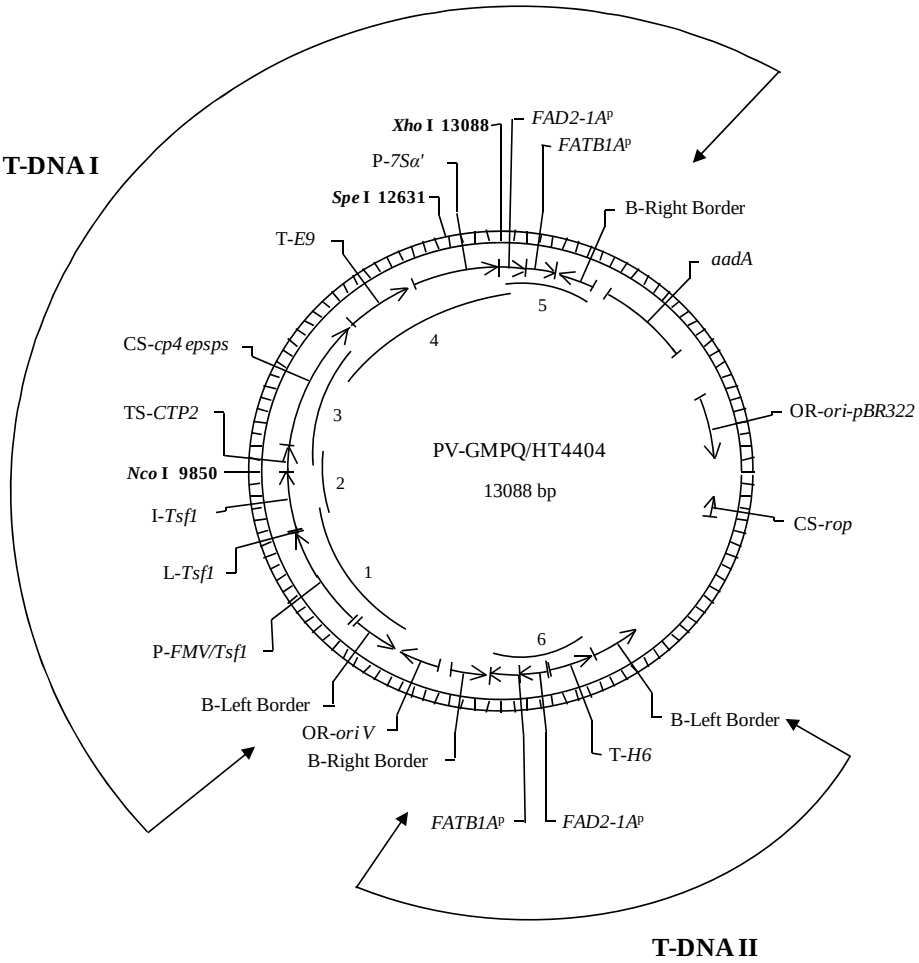
Table 2 (cont.) Summary of Genetic Elements in MON 87705

Genetic Element	Location in Sequence	Function (Reference)
T^{9}-*E9*	6946 – 7588	3' nontranslated region of the pea *RbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	7589 – 7676	Sequence used in DNA cloning
P-*7Sα'*	7677 – 8517	Promoter and leader from the *Sphas1* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle et al., 1986) that directs transcription in seed
Intervening Sequence	8518 – 8539	Sequence used in DNA cloning
FAD2-1A^{p}	8540 – 8805	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which forms part of the suppression cassette
FATB1A^{p}	8806 - 9106	Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette
Intervening Sequence	9107 - 9114	Sequence used in DNA cloning
B-Right Border^{r1}	9115 – 9134	20 bp sequence from the B-Right Border region remaining after integration (Zambryski et al., 1982)
B-Left Border^{r2}	9135 - 9172	38 bp sequence from the B-Left Border region remaining after integration (Barker et al., 1983)
FATB1A^{p1}	9173 – 9443	Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette
FAD2-1A^{p}	9444 – 9709	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which forms part of the suppression cassette

T^{9} – 3' nontranslated transcriptional termination sequence and polyadenylation signal sequences; ***P*** – Partial sequence; ***P1*** – Truncated partial sequence of *FATB1A*.

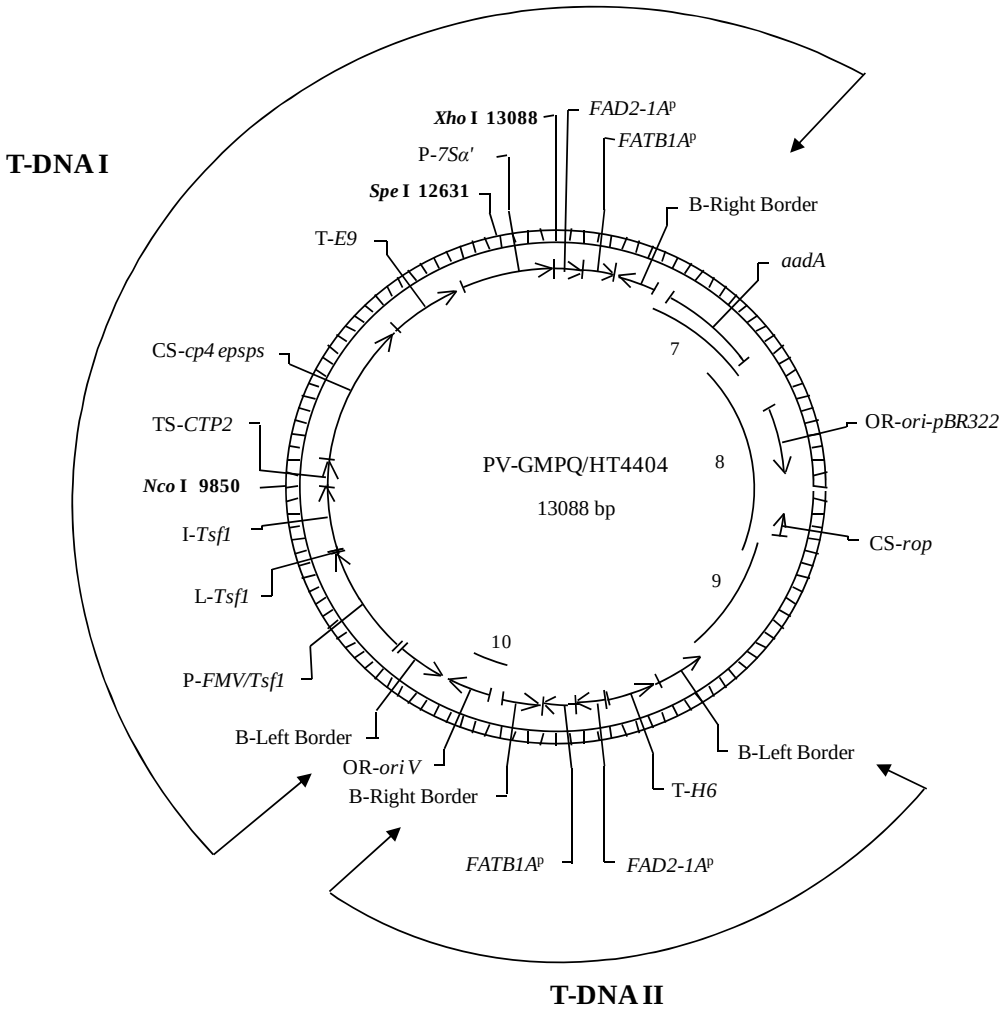
Table 2 (cont.) Summary of Genetic Elements in MON 87705

Genetic Element	Location in Sequence	Function (Reference)
Intervening Sequence	9710 – 9721	Sequence used in DNA cloning
T-*H6*	9722 – 10157	3' UTR sequence of the *H6* gene from *Gossypium barbadense* encoding a fiber protein involved in secondary cell wall assembly (John and Keller, 1995)
Intervening Sequence	10158 –10255	Sequence used in DNA cloning
B-Left Border^{r3}	10256 - 10530	275 bp sequence from the B-Left Border region remaining after integration (Barker et al., 1983)
Sequence flanking 3' end of the insert	10531 – 12908	Soybean genomic DNA including the 2374 bases duplicated at the 5' end of the flanking sequence of the insert
Unique 3' flanking sequence of the insert	12909 – 13243	Soybean genomic DNA



Probe	DNA Probe	Start Position	Stop Position	Total Length (~kb)
1	T-DNA I Probe 1A	7657	9406	1.8
2	T-DNA I Probe 2B	9270	10042	0.8
3	T-DNA I Probe 3C	9943	11325	1.4
4	T-DNA I Probe 4D	11151	160	2.1
5	T-DNA I Probe 5E	13080	973	1.0
6	T-DNA II Probe 1A	5570	6693	1.1

Figure 1. Circular Map of Plasmid PV-GMPQ/HT4404 showing probes 1-6
Plasmid PV-GMPQ/HT4404 containing the T-DNAs used in *Agrobacterium*-mediated transformation to produce MON 87705. Genetic elements (depicted in the exterior of the map) and restriction sites for enzymes used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The T-DNA I and T-DNA II probes used in the Southern analyses (labeled 1-6 within the interior of the map) are detailed in the accompanying table.



Probe	DNA Probe	Start Position	Stop Position	Total Length (~kb)
7	Backbone Probe 1	974	2280	1.3
8	Backbone Probe 2	2140	4080	1.9
9	Backbone Probe 3	3631	5127	1.5
10	Backbone Probe 4	7025	7656	0.6

Figure 2. Circular Map of Plasmid PV-GMPQ/HT4404 showing probes 7-10
Plasmid PV-GMPQ/HT4404 containing the T-DNAs used in *Agrobacterium*-mediated transformation to produce MON 87705. Genetic elements (depicted in the exterior of the map) and restriction sites for enzymes used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The backbone probes used in the Southern analyses (labeled 7-10 within the interior of the map) are detailed in the accompanying table.

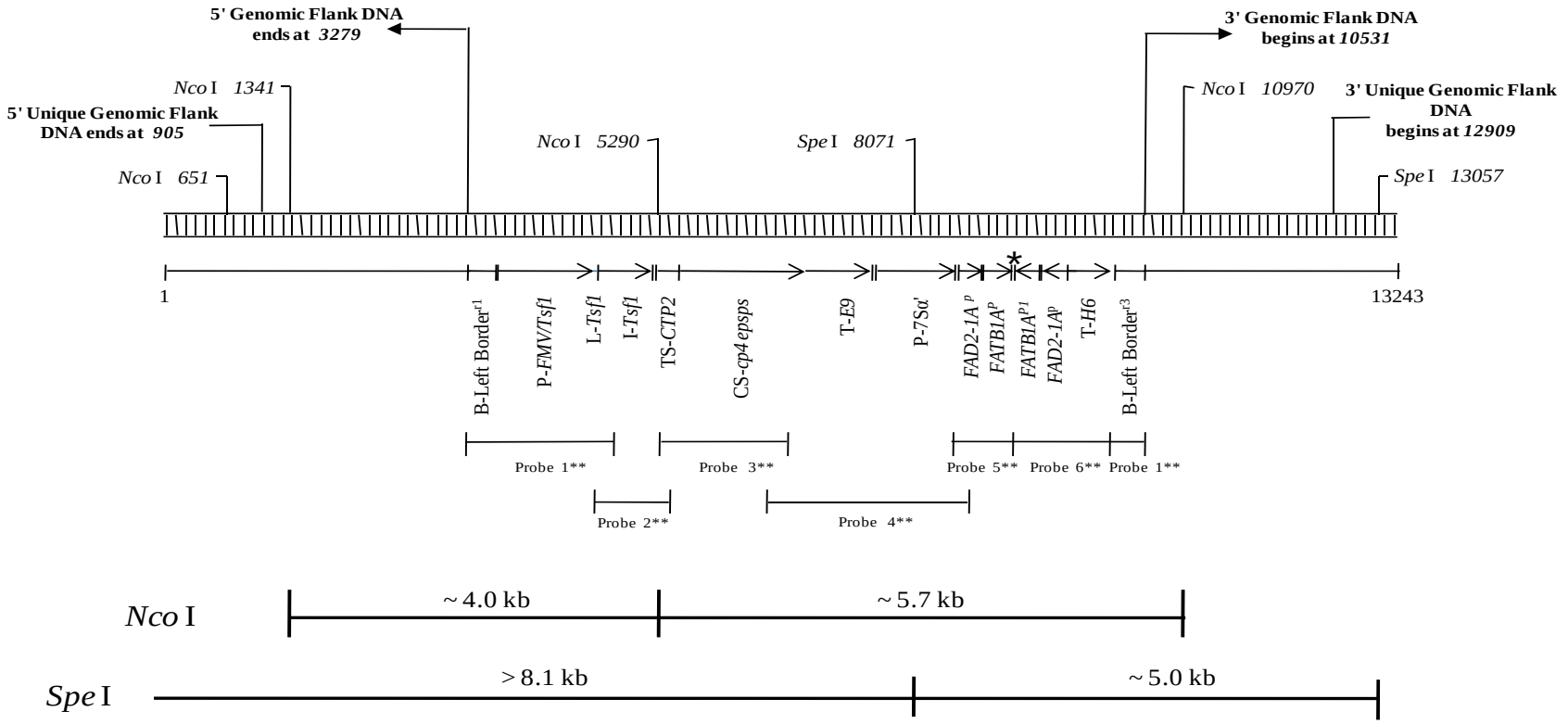


Figure 3. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87705

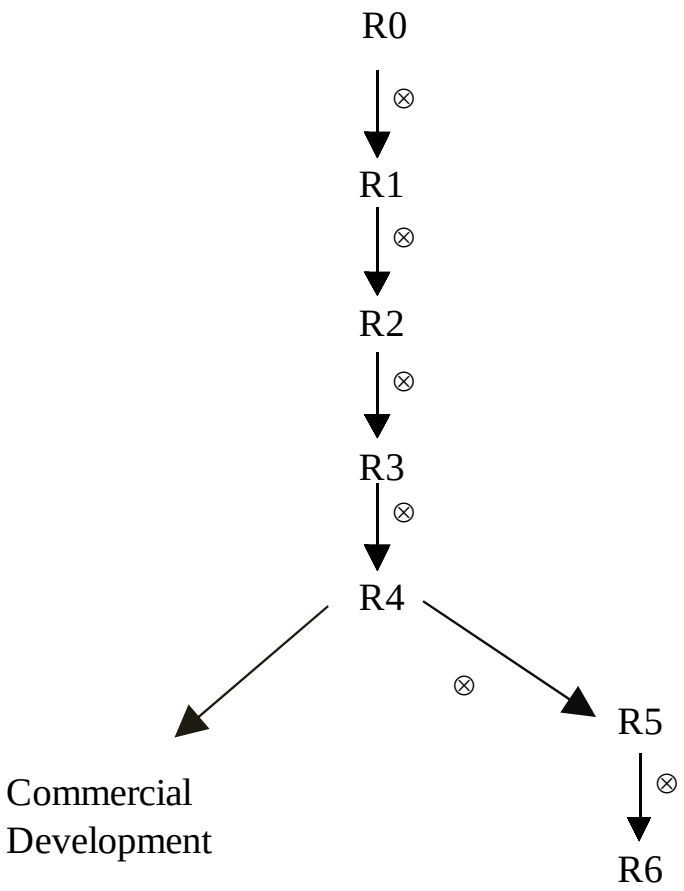
A linear map of the insert and genomic DNA flanking the insert in MON 87705 is shown. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analyses. Shown on the lower portion of the map are the expected sizes of the DNA fragments after digestion with respective restriction enzymes. Arrowheads (→) indicate the end of the insert and the beginning of soybean genomic flanking sequence. The arrows (→) indicated the sequence direction of the elements in MON 87705. The * denotes sequences from Right Border^{r1} and Left Border^{r2} after integration into MON 87705 (Table 2).

**These probes are not drawn to scale and are the estimated locations of the T-DNA I and T-DNA II probes in MON 87705. Refer to the Figure 1 for the description of the probes.

Table 3. Summary Chart of the Expected DNA Fragments Based on Hybridizing Probes and Restriction Enzymes Used in MON 87705 Analysis

Southern blot Figure	5	6	7	8	9	10
Probes Used in Analysis	**1, 4, 6**	**2, 5**	**3**	**7, 9**	**8, 10**	**1, 6**
Positive Hybridization Controls						
***Xho* I + *Nco* I Digested Plasmid**	**~3.2, ~9.9 kb**	**~3.2, ~9.9 kb**	**~3.2 kb**	**~9.9 kb**	**~9.9 kb**	**~9.9 kb**
Probe Templates [1]	**~1.8, ~2.1, and ~1.1 kb**	**~0.8 and ~1.0 kb**	**~~**[2]	**~1.3 and ~1.5 kb**	**~1.9 and ~0.6 kb**	**~1.8 and ~1.1 kb**
MON 87705 DNA Digestion						
***Nco* I**	**~4.0 and ~5.7 kb**	**~4.0 and ~5.7 kb**	**~5.7 kb**	**No band**	**No band**	**~4.0 and ~5.7 kb**
***Spe* I**	**> 8.1*** **and ~5.0kb**	**> 8.1*** **and ~5.0kb**	**> 8.1*** **kb**	**No band**	**No band**	**--**[3]

[1] probe templates were added to pre-digested conventional soybean DNA when multiple probes are used in Southern blot analysis.
[2] '~~' indicates that the plasmid template was the only positive control used, because the Southern blot was hybridized with one probe.
[3] '--' indicates that the particular restriction enzyme was not used in the analysis.
*Southern analysis indicates this fragment to be ~ 11 kb.



R0 – originally transformed plant; ⊗ – self pollinated

Figure 4. Breeding History of MON 87705
All generations were self pollinated (⊗). The R3 generation was used for the molecular analyses of MON 87705 reported in Figures 5-9 and is referred to as MON 87705 in all Southern blot figures. The R4, R5, and R6 generations were used for analyzing the stability of the insert in multiple generations.

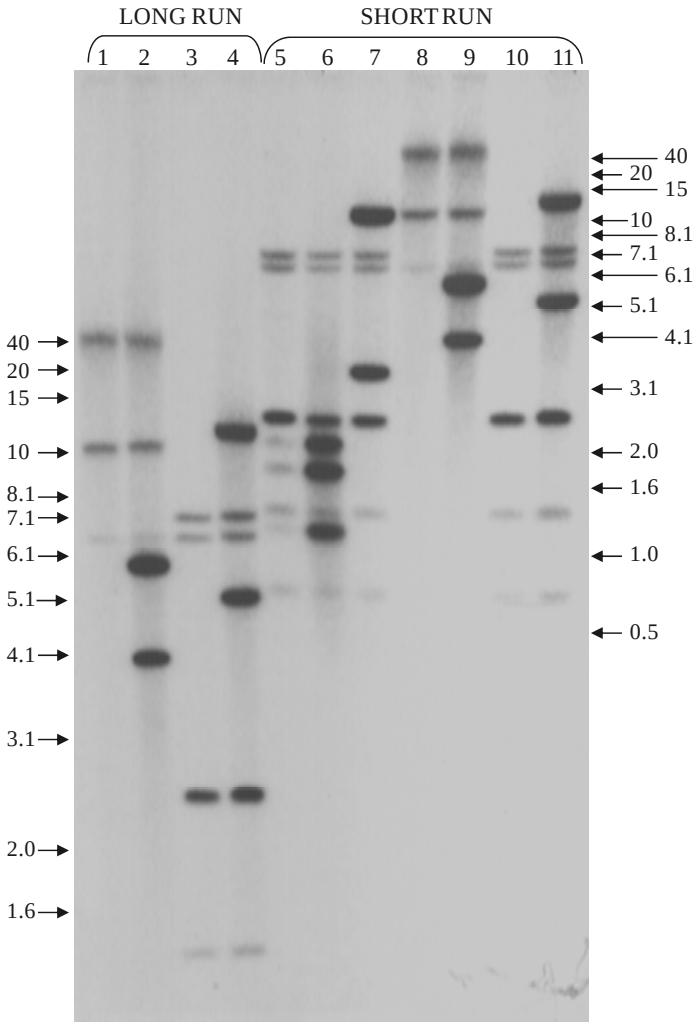


Figure 5. Southern Blot Analysis of MON 87705: Probes 1, 4, and 6

The blot was hybridized with ^{32}P-labeled probes that span a portion of T-DNA I and T-DNA II sequences (probes 1, 4, and 6, Figure 1). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I)
2: MON 87705 (*Nco* I)
3: Conventional soybean (*Spe* I)
4: MON 87705 (*Spe* I)
5: Conventional soybean (*Spe* I) spiked with probe templates [~0.1 genomic equivalent]
6: Conventional soybean (*Spe* I) spiked with probe templates [~1 genomic equivalent]
7: Conventional soybean (*Spe* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~1 genomic equivalent]
8: Conventional soybean (*Nco* I)
9: MON 87705 (*Nco* I)
10: Conventional soybean (*Spe* I)
11: MON 87705 (*Spe* I)

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

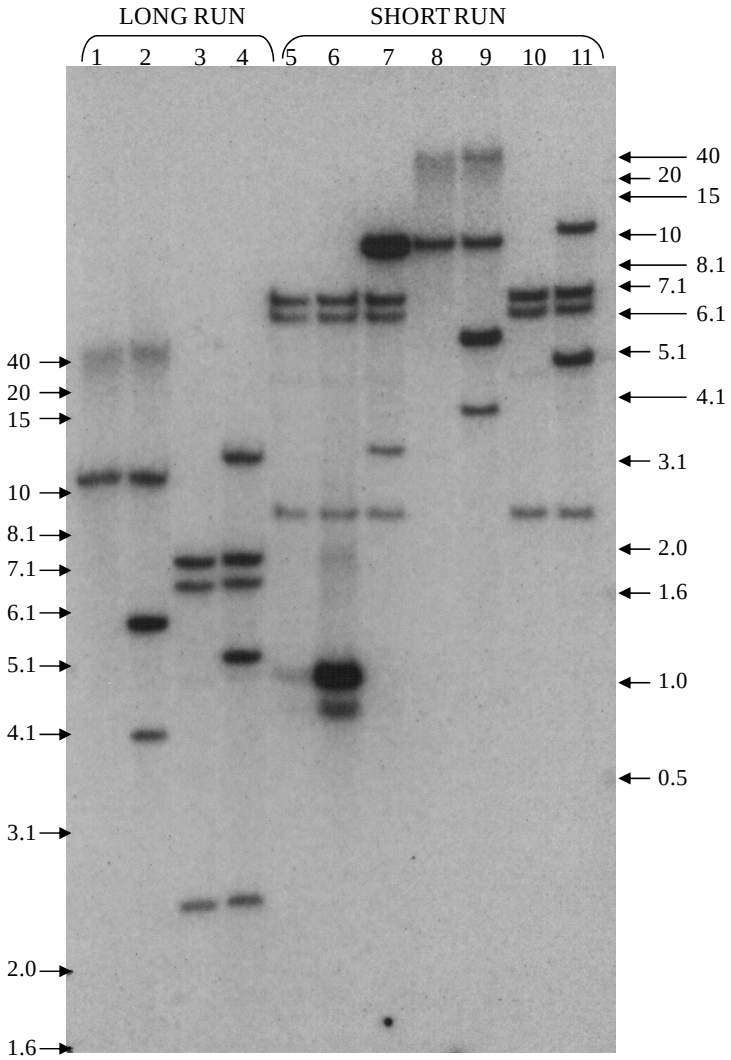


Figure 6. Southern Blot Analysis of MON 87705: Probes 2 and 5

The blot was hybridized with ^{32}P-labeled probes that span a portion of T-DNA I sequences (probes 2 and 5, Figure 1). Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I)
2: MON 87705 (*Nco* I)
3: Conventional soybean (*Spe* I)
4: MON 87705 (*Spe* I)
5: Conventional soybean (*Spe* I) spiked with probe templates [~0.1 genomic equivalent]
6: Conventional soybean (*Spe* I) spiked with probe templates [~1 genomic equivalent]
7: Conventional soybean (*Spe* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~1 genomic equivalent]
8: Conventional soybean (*Nco* I)
9: MON 87705 (*Nco* I)
10: Conventional soybean (*Spe* I)
11: MON 87705 (*Spe* I)

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

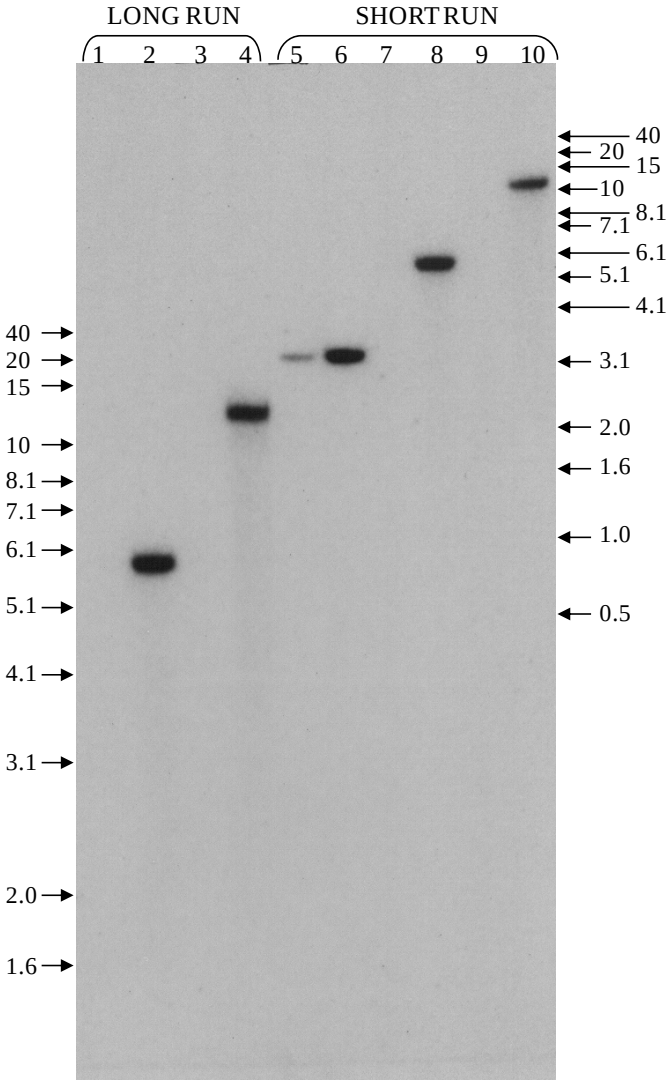


Figure 7. Southern Blot Analysis of MON 87705: Probe 3

The blot was hybridized with a ^{32}P-labeled probe that span a portion T-DNA I sequences (probe 3, Figure 1). Each lane contains ~10 μg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I)
2: MON 87705 (*Nco* I)
3: Conventional soybean (*Spe* I)
4: MON 87705 (*Spe* I)
5: Conventional soybean (*Spe* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~0.1 genomic equivalent]
6: Conventional soybean (*Spe* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~1 genomic equivalent]
7: Conventional soybean (*Nco* I)
8: MON 87705 (*Nco* I)
9: Conventional soybean (*Spe* I)
10: MON 87705 (*Spe* I)

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

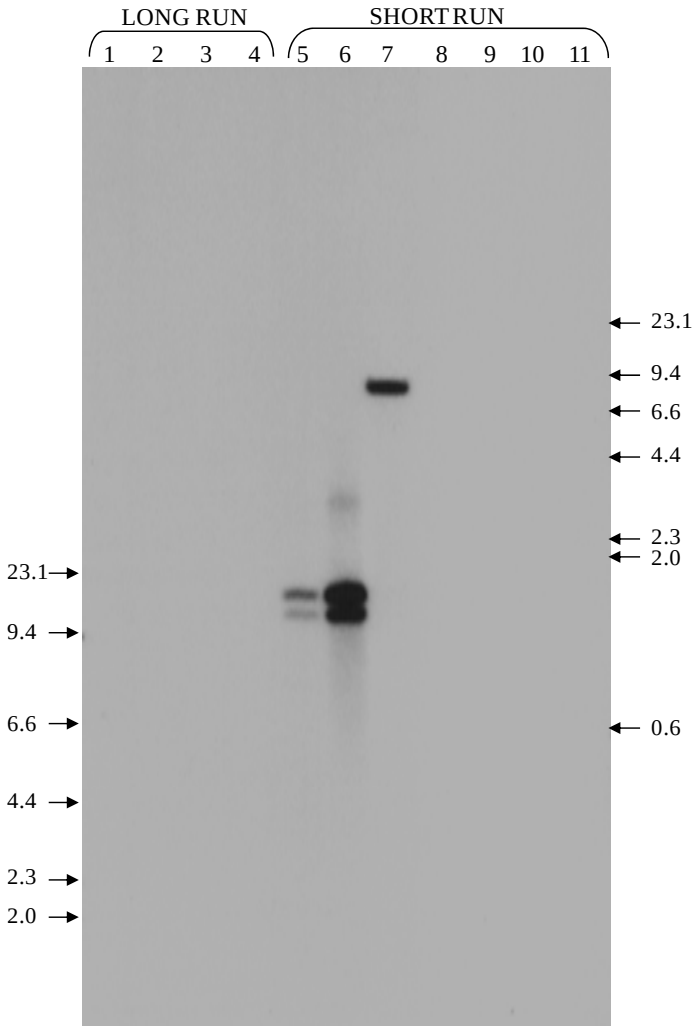


Figure 8. Southern Blot Analysis of MON 87705:PV-GMPQ/HT4404 Backbone Probes 7& 9

The blot was hybridized with ^{32}P-labeled probes that span a portion of backbone sequences (probes 7 and 9, Figure 2) of plasmid PV-GMPQ/HT4404. Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I)
2: MON 87705 (*Nco* I)
3: Conventional soybean (*Spe* I)
4: MON 87705 (*Spe* I)
5: Conventional soybean (*Spe* I) spiked with probe templates [~0.1 genomic equivalent]
6: Conventional soybean (*Spe* I) spiked with probe templates [~1 genomic equivalent]
7: Conventional soybean (*Spe* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~1 genomic equivalent]
8: Conventional soybean (*Nco* I)
9: MON 87705 (*Nco* I)
10: Conventional soybean (*Spe* I)
11: MON 87705 (*Spe* I)

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

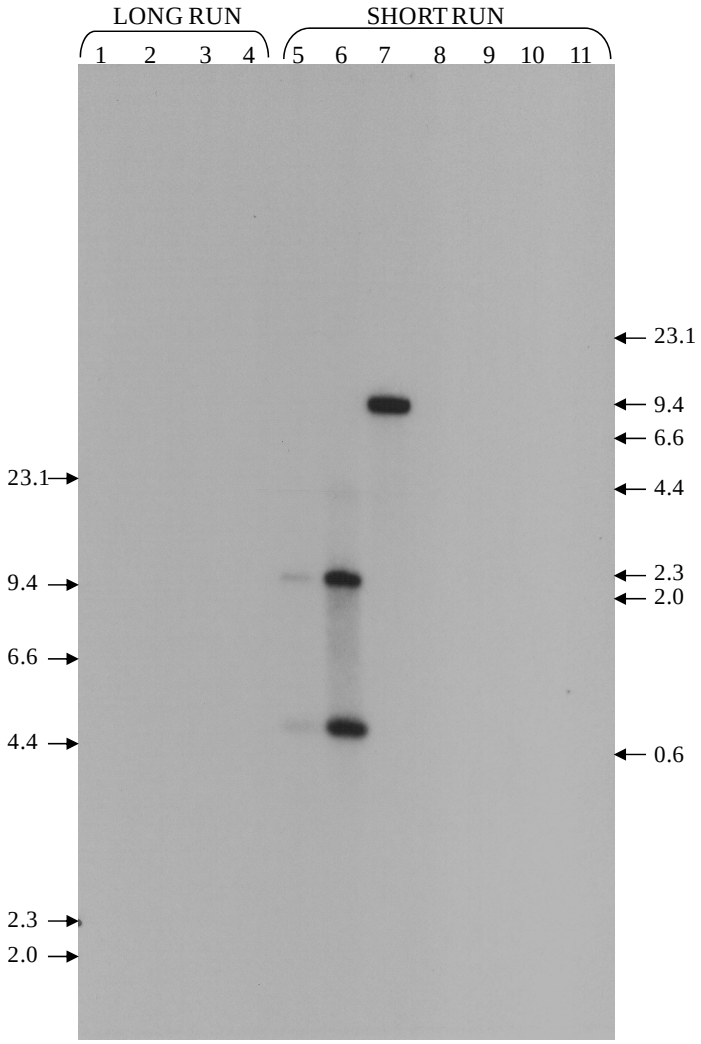


Figure 9. Southern Blot Analysis of MON 87705: PV-GMPQ/HT4404 Backbone Probes 8 & 10
The blot was hybridized with ^{32}P-labeled probes that span a portion of backbone sequences (probes 8 and 10, Figure 2) of plasmid PV-GMPQ/HT4404. Each lane contains ~10 μg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I)
2: MON 87705 (*Nco* I)
3: Conventional soybean (*Spe* I)
4: MON 87705 (*Spe* I)
5: Conventional soybean (*Spe* I) spiked with probe templates [~0.1 genomic equivalent]
6: Conventional soybean (*Spe* I) spiked with probe templates [~1 genomic equivalent]
7: Conventional soybean (*Spe* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~1 genomic equivalent]
8: Conventional soybean (*Nco* I)
9: MON 87705 (*Nco* I)
10: Conventional soybean (*Spe* I)
11: MON 87705 (*Spe* I)

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

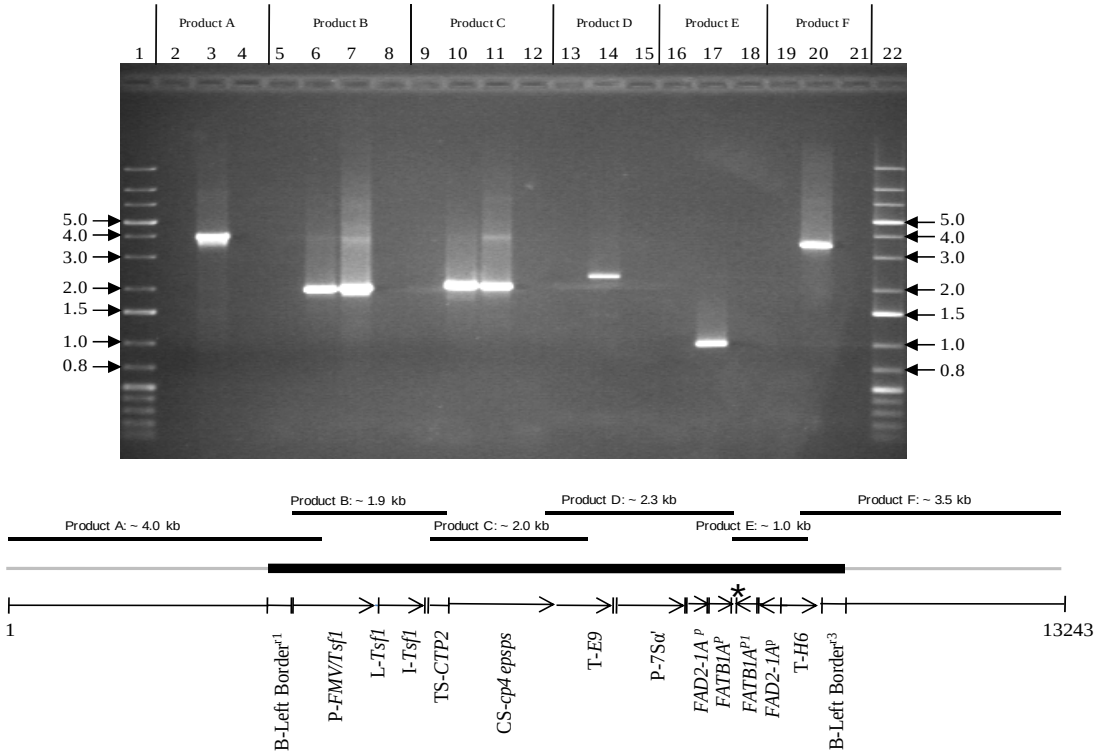
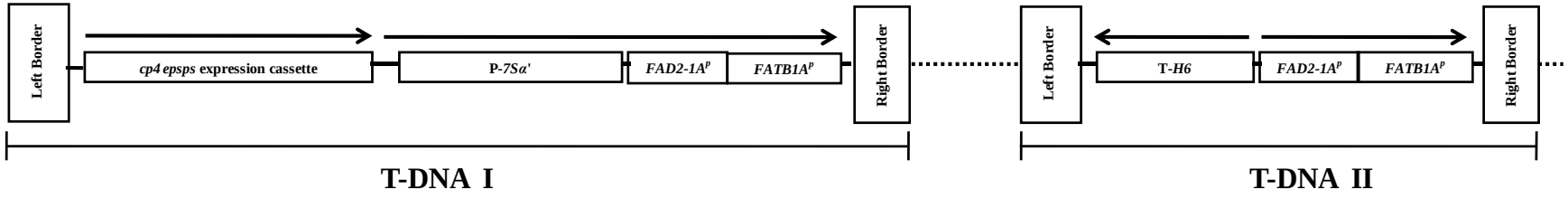


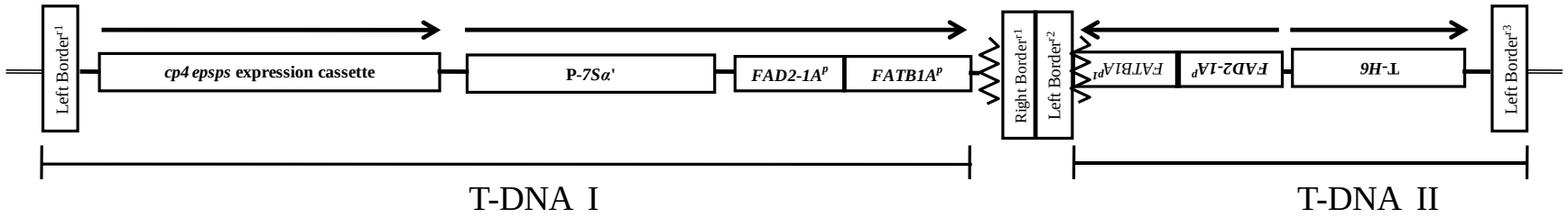
Figure 10. Overlapping PCR Analysis Across the Insert in MON 87705
PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 87705 were performed on MON 87705 genomic DNA extracted from leaf (Lanes 3, 6, 10, 14, 17, and 20). Lanes 2, 5, 9, 13, 16, and 19 contain reactions with conventional soybean DNA while lanes 4, 8, 12, 15, 18, and 21 are reactions containing no template DNA. Lanes 7 and 11 contain reactions with PV-GMPQ/HT4404 plasmid control DNA. Lanes 1and 22 contain Fermentas GeneRuler™ 1 kb Plus DNA Ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87705 that appears near the bottom of the figure. Three to fifteen microliters of each of the PCR products was loaded on the gel. This figure is representative of the data generated in the study; however the PCR amplicons reported in this figure were not necessarily used in sequencing.

Lane
1: GeneRuler™ 1 kb Plus DNA Ladder
2: Conventional soybean DNA
3: MON 87705 genomic DNA
4: No template DNA control
5: Conventional soybean DNA
6: MON 87705 genomic DNA
7: PV-GMPQ/HT4404 control DNA
8: No template DNA control
9: Conventional soybean DNA
10: MON 87705 genomic DNA
11: PV-GMPQ/HT4404 control DNA
12: No template DNA control
13: Conventional soybean DNA
14: MON 87705 genomic DNA
15: No template DNA control
16: Conventional soybean DNA
17: MON 87705 genomic DNA
18: No template DNA control
19: Conventional soybean DNA
20: MON 87705 genomic DNA
21: No template DNA control
22: GeneRuler™ 1 kb Plus DNA Ladder

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.
***** Symbol denotes sequences from Right Border^{r1} and Left Border^{r2} after integration into MON 87705 (Table 2).



Panel A. PV-GMPQ/HT4404 Sequence



Panel B. MON 87705 Sequence

	T-DNA I		Right Border^{r1}		Left Border^{r2}		T-DNA II	
	Starting Base	Ending Base	Starting Base	Ending Base	Starting Base	Ending Base	Starting Base	Ending Base
Sequence location in PV-GMPQ/HT4404	7840	586	640	659	7833 or (5304)[1]	7870 or (5341)[1]	6652	5295
Sequence location in MON 87705	3280	9114	9115	9134	9135	9172	9173	10530

Figure 11. Schematic Representation of the Insert DNA in Soybean MON 87705
Panel A represents a linear map of the T-DNA I and T-DNA II arrangement in the transformation plasmid, PV-GMPQ/HT4404. Panel B represents a linear map of the arrangement of the two T-DNAs as they exist in MON 87705. In MON 87705, the *FAD2-1A*^{p} and *FATB1A*^{p} sequences are tandem to each other as inverted repeats. The chart below the diagrams displays the corresponding base location of the T-DNA I and T-DNA II regions in the transformation plasmid PV-GMPQ/HT4404 and the base location in the MON 87705 insert sequence. The arrows indicate the sequence direction of the elements in the plasmid and MON 87705.

[1] Bases 9135 to 9172 in MON 87705 match sequence from each Left Border region in PV-GMPQ/HT4404.

[CBI CROSS REFERENCE 1]

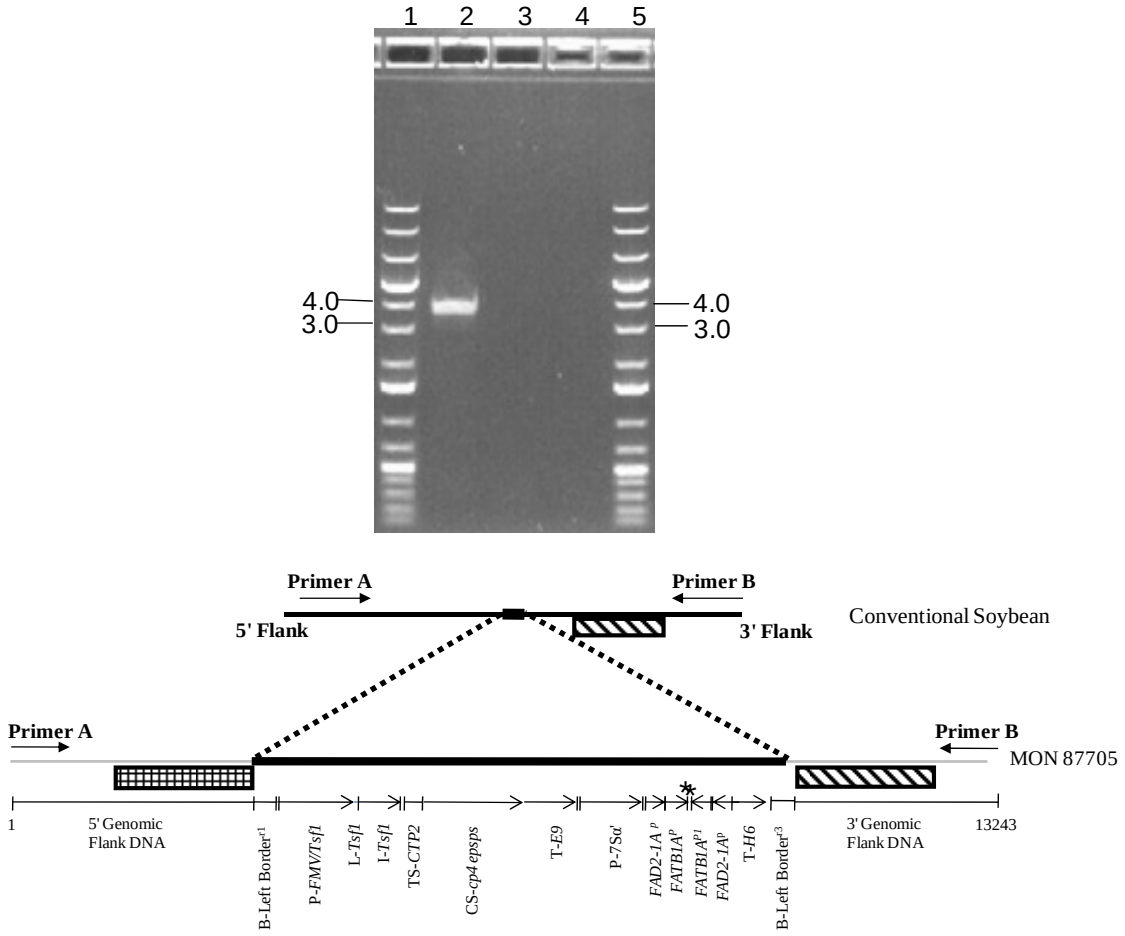


Figure 13. PCR Amplification of the MON 87705 Insertion Site in Conventional Soybean
The above figure is a depiction of the MON 87705 insertion site in conventional (upper panel) and MON 87705 soybean (lower panel). PCR amplification was performed using Primer A located in the 5' flanking sequence and Primer B located in the 3' flanking sequence of the insert in MON 87705 to verify that the flanking sequences are native to the soybean genome. Three to fifteen microliters of each of the PCR products were loaded on the gel. The ▦ box represents the 2374 bp direct repeat that was identified when the amplicon generated from MON 87705 was sequenced (Figure 14). The ▨ box represents that the 2374 bp repeat originated from the 3' flanking sequence and was duplicated at the 5' end of the insertion. Lane designations are as follows:

Lane 1: GeneRuler™ 1 kb Plus DNA Ladder
2: Conventional soybean
3: MON 87705
4: No template DNA control
5: GeneRuler™ 1 kb Plus DNA Ladder

— Symbol denotes size of DNA, in base pairs, obtained from MW markers on gel.
***** Symbol denotes sequences from Right Border^{r1} and Left Border^{r2} after integration into MON 87705 (Table 2).

[CBI CROSS REFERENCE 2]

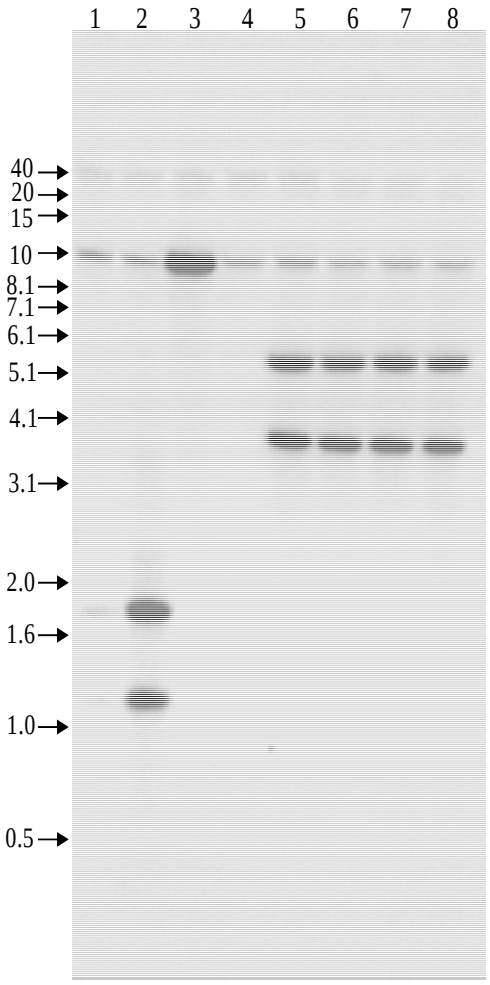


Figure 15. Generational Stability of MON 87705: Probes 1 and 6

The blot was hybridized with ^{32}P-labeled probes that spanned a portion of T-DNA I and T-DNA II sequences (probes 1, and 6, Figure 1). Each lane contains ~ 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87705 is illustrated in Figure 4. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I) spiked with probe templates [~0.1 genomic equivalent]
2: Conventional soybean (*Nco* I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Nco* I) spiked with PV-GMPQ/HT4404 (*Xho* I/*Nco* I) [~1 genomic equivalent]
4: Conventional soybean (*Nco* I)
5: MON 87705 [R3, *Nco* I]
6: MON 87705 [R4, *Nco* I]
7: MON 87705 [R5, *Nco* I]
8: MON 87705 [R6, *Nco* 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-1188-01	OMNISOY Soybean MON 87705 Qualitative Event-specific TaqMan® PCR
BR-ME-1153-01	Quick CTAB DNA Extraction from Leaf Tissues
BR-ME-0315-02	Agarose Gel Electrophoresis
BR-ME-0316-01	Restriction Enzyme Digestion of DNA
BR-ME-0317-02	Southern Blot Analysis
BR-ME-0486-01	Polymerase Chain Reaction
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)

APPENDIX 2
Notes for Reviewer

The original final report was amended to ensure clarity and correct typographical errors. These revisions are outlined in Appendix 2. These changes do not impact the conclusions of this study.

Page Number in MSL0022384	Change
1	Updated report title, MSL number, added "Amendment 2" after Study Completed, and revised report completion date.
4	Added "Draft Report Amendment Review" to list of phases and updated study title, and changed the name of the Quality Assurance Specialist.
6	Updated the study title, MSL number, added "Original" to Study Completion Date, and added "Amended Report Completion Date."
9	Added "Appendix 2: Notes to Reviewer" to the Table of Contents.
10	Updated the *FAD2-1A*^{p} and *FATB1A*^{p} definitions and added dCTP definition.
15	Added "or ^{32}P-deoxycytosine triphosphate (dCTP)".
16	Added superscript "p" to *FAD2-1A* and added "(portion)" to *FAD2-1A*^{p}.
17	Added E-gel® registered trademark information in footnote.
19	Changed Section number '4.3' to '4.2' and added E-gel® registered trademark information in footnote.
20	Changed "partial sequences of Right Border and Left Border" to "Right Border^{r1}, Left Border^{r2}" and added the superscript "r1" or "r3" to Left Border.
21	Changed "partial sequences of Right Border and Left Border" to "Right Border^{r1}, Left Border^{r2}" and added the superscript "r3" to Left Border.
32	Changed Sutcliffe, 1978 reference to "Sutcliffe, J.G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harb Symp Quant Biol. 43:77-90."
34	Changed the year on Sutcliffe reference from "1978" to "1979", updated the *FAD2-1A*^{p} function to "Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which forms part of the suppression cassette," and updated the *FATB1A*^{p} function to "Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette."
35	Updated the *FAD2-1A*^{p} function to "Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which forms part of the suppression cassette," and updated the *FATB1A*^{p} function to "Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette."

Page Number in MSL0022384	Change
37	Updated the *FAD2-1A*^{p} function to "Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti et al., 2003) which which forms part of the suppression cassette" and updated the *FATB1A*^{p} and *FATB1A*^{p1} functions to "Partial sequence from the 5' nontranslated region and the chloroplast targeting sequence from *Glycine max FATB1A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti et al., 2003) which forms part of the suppression cassette."
41	Changed the explanation of the * to "denotes sequences from Right Border^{r1} and Left Border^{r2} after integration into MON 87705 (Table 2)." Updated Figure 3 to include the estimated location of probes in the T-DNA insert and added the footnote,"**These probes are not drawn to scale and are the estimated locations of the T-DNA I and T-DNA II probes in MON 87705. Refer to the Figure 1 for the description of the probes."
49, 56	Changed the explanation of the * to "denotes sequences from Right Border^{r1} and Left Border^{r2} after integration into MON 87705 (Table 2)."
50	Added superscript "r1" to Left Border in Panel B illustration.
58	Added base "A" at location 3635 in conventional soybean DNA sequence that was present in MSL0021487, but was absent in the amended MSL report, MSL0022130.
61	Updated "Appendix 2: Notes to Reviewer"
Confidential Attachment Title Page	Updated report title, MSL number, report completion date, and added "Amendment 2" after Completed On.