Title

Amended Report for MSL0021074: Molecular Analysis of Stearidonic Acid Producing Soybean MON 87769

Authors
Renee Girault
Zihong Song
Aihong Pan
Dongyan Feng
James F. Rice, Ph.D.
Qing Tian, Ph.D.
James D. Masucci, Ph.D.

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Sponsor/Testing Facility

Monsanto Company
Regulatory Product Characterization Team
Biotechnology Regulatory Sciences
800 North Lindbergh Blvd.
St. Louis, MO 63167

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Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice (GLP) 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
Cheriaw Georr	3-2-2009
Sponsor Representative	Date
James F. Thomas	3-2-2009
Study Director	Date

Quality Assurance Statement

Study Title: Amended Report for MSL0021074: Molecular Analysis of Stearidonic Acid Producing Soybean MON 87769

Study Number: 06-01-83-23

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
03/01/2007	PCR/Sequence	03/01/2007	03/01/2007
03/05/2007	Southern Blot	03/19/2007	03/19/2007
07/03/2007	Southern Blot	07/03/2007	07/03/2007
08/20/2007	Raw Data Audit	08/31/2007	08/31/2007
01/25/2008	Southern Blot	01/25/2008	01/25/2008
07/03/2008	Raw Data Audit	07/08/2008	07/08/2008
07/03/2008	Raw Data Audit	07/08/2008	07/08/2008
08/07/2008	Draft Report Review	08/07/2008	08/07/2008
02/26/2009	Draft Report Amendment Review	02/26/2009	02/26/2009

IL. [illegible]
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

MAR 2nd, 2009
Date

Study Certification Page

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

James F. Rice, Ph.D. Date
Study Director
Regulatory Product Characterization Team

3/2/09

James D. Masucci, Ph.D. Date
Team Lead
Regulatory Product Characterization Team

Study Information Page

Study Number:	06-01-83-23
MSL Number:	0021926
Title:	Amended Report for MSL0021074: Molecular Analysis of Stearidonic Acid Producing Soybean MON 87769
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:	Cherian George
Team Lead:	James D. Masucci, Ph.D.
Study Director:	James F. Rice, Ph.D.
Contributors:	Renee Girault, Zihong Song, Aihong Pan, Dongyan Feng, and Qing Tian, Ph.D.
Study Initiation Date:	January 31, 2007
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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 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]

35S	gene from the cauliflower mosaic virus (CaMV) encoding the 35S RNA
aadA	bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
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
BSA	bovine serum albumin
CS-*cp4 epsps*	codon modified coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein
CS-*Nc.Fad3*	codon modified coding sequence for the gene from *Neurospora crassa* encoding delta-15 desaturase
CS-*Pj.D6D*	coding region for the fatty acid delta-6 desaturase from *Primula juliae*
CS-*rop*	coding sequence for repressor of primer protein used for maintenance of plasmid copy number in *E. coli*
CTAB	hexadecyltrimethylammonium bromide
dCTP	deoxycytidine triphosphate
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
GLP	Good Laboratory Practice
L-*ShkG*	5' UTR from the Arabidopsis *ShkG* gene encoding EPSPS
MW	Molecular Weight
OR-*ori-PBR322*	origin of replication from pBR322 necessary for maintenance of plasmid in *E. coli*
OR-*ori V*	origin of replication from the broad host range plasmid RK2 necessary for maintenance of plasmid in *Agrobacterium*
P-*7Sα'*	promoter and leader from the *Sphas1* gene of *Glycine max*
P-*7Sα*	promoter and leader from the *Sphas2* gene from soybean
P-*FMV*	promoter for the 35S RNA from figwort mosaic virus (FMV)
PCR	polymerase chain reaction
PV-GMPQ1972	plasmid vector used to develop MON 87769
SOP	Standard Operating Procedure
T-DNA	transferred DNA

[1] Standard abbreviations, e.g., units of measure, will be used according to the format described in 'Instructions for Authors' in the *Journal of Biological Chemistry* (http://www.jbc.org/).

ABBREVIATIONS AND DEFINITIONS (CONTINUED)

T-*E9*	3′ non-translated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA
T-*tml*	3′ non-translated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid which functions to direct polyadenylation of the mRNA
TS-*CTP2*	Transit peptide region of *Arabidopsis thaliana* EPSPS used to direct proteins into chloroplasts
w/v	weight per volume

1.0 SUMMARY

Monsanto has developed soybean MON 87769 that produces stearidonic acid (SDA), an omega-3 fatty acid. In MON 87769 soybean, two proteins are expressed, delta-6 desaturase from *Primula julea* (PjΔ6D) and delta-15 desaturase from *Neurospora crassa* (NcΔ15D). MON 87769 was developed through *Agrobacterium*-mediated transformation of meristematic soybean tissue of the conventional soybean variety A3525 with the transformation plasmid PV-GMPQ1972. PV-GMPQ1972 is a 2T-DNA vector. The first T-DNA (T-DNA I) contains both the *Pj.D6D* and *Nc.Fad3* expression cassettes, and is responsible for the production of stearidonic acid. The *Pj.D6D* expression cassette consists of the *Pj.D6D* coding sequence under the regulation of the *7Sα'* promoter and the *tml* 3' non-translated region. The *Nc.Fad3* expression cassette consists of the *Nc.Fad3* coding sequence under the regulation of the *7Sα* promoter, and the *E9* 3' non-translated region. The second T-DNA (T-DNA II) contains the *cp4 epsps* expression cassette, encoding the CP4 EPSPS protein that was used as a selectable marker for transformation. The *cp4 epsps* expression cassette in T-DNA II consists of the *cp4 epsps* coding sequence region under the regulation of the *FMV* promoter and the *E9* 3′ non-translated region.

The DNA insert contained in the MON 87769 genome was characterized with respect to the number of integration sites within the soybean genome (insert number), the number of copies of the transferred DNA (T-DNA I) within one locus (copy number), the presence or absence of elements associated with T-DNA II or the transformation vector backbone sequences, the integrity of the inserted *Pj.D6D* and *Nc.Fad3* expression cassettes, the organization of the elements within the insert, and the 5′ and 3′ genomic DNA flanking the insert.

The data show that MON 87769 contains one copy of the insert at a single integration locus. No additional elements from the transformation vector PV-GMPQ1972, linked or unlinked to the intact *Pj.D6D* and *Nc.Fad3* expression cassettes, were detected. The MON 87769 genome does not contain detectable elements uniquely associated with T-DNA II or backbone sequences from plasmid PV-GMPQ1972.

Generational stability analysis demonstrated that the Southern blot fingerprint of MON 87769 has been maintained across the four generations analyzed; thereby confirming the stability of the insert over multiple generations. No elements uniquely associated with T-DNA II or backbone sequences from plasmid PV-GMPQ1972 were detected in any generation.

Finally, the complete DNA sequence of the insert and the adjacent genomic DNA was determined in MON 87769. These data characterized the 5' and 3' insert-to-genomic DNA junctions and confirmed the organization of the elements within the insert. Further, polymerase chain reaction (PCR) and DNA sequencing analyses determined the genomic

organization of the MON 87769 insertion site in the conventional soybean variety A3525, which confirms that the genomic DNA sequence flanking the 5' and 3' ends of the insert in MON 87769 is native to the soybean genome.

2.0 INTRODUCTION

2.1 Background

Monsanto Company has developed biotechnology derived soybean MON 87769 that produces stearidonic acid (SDA), an omega-3 fatty acid. Production of SDA in soybean seed was achieved through the introduction of genes encoding the production of *Neurospora crassa* delta-15 desaturase (NcΔ15D) and *Primula juliae* delta-6 desaturase (PjΔ6D). These two genes were driven by seed-specific promoters, resulting in the production of SDA only in soybean seeds.

MON 87769 was produced by *Agrobacterium*-mediated transformation of embryonic soybean cells of the conventional soybean variety A3525 with the transformation plasmid PV-GMPQ1972. PV-GMPQ1972 is a 2T-DNA vector. The first T-DNA (T-DNA I) contains both the *Pj.D6D* and *Nc.Fad3* expression cassettes, and is responsible for the production of stearidonic acid. The *Pj.D6D* expression cassette consists of the *Pj.D6D* coding sequence under the regulation of the *7Sα′* promoter and the *tml* 3' non-translated region. The *Nc.Fad3* expression cassette consists of the *Nc.Fad3* coding sequence under the regulation of the *7Sα* promoter, and the *E9* 3' non-translated region. The second T-DNA (T-DNA II) contains the *cp4 epsps* expression cassette encoding the CP4 EPSPS protein that was used as a selectable marker for transformation. The *cp4 epsps* expression cassette consists of the *cp4 epsps* coding region under the regulation of the *FMV* promoter and the *E9* 3′ non-translated region.

2.2 Purpose

The purpose of this study was to characterize the integrated DNA in MON 87769. MON 87769 genomic DNA was analyzed using Southern blot methods to determine the insert number and copy number of T-DNA I, the number of copies of each element within T-DNA I, the presence or absence of the *cp4 epsps* expression cassette in T-DNA II, and the presence or absence of plasmid backbone sequences. Multiple generations were analyzed to confirm the stability of the inserted DNA in MON 87769. PCR and DNA sequence analyses were performed to determine: the organization of the elements within the insert, the 5' and 3' insert-to-genomic DNA junctions, the complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 87769, and that the genomic DNA sequence flanking the 5' and 3' ends of the insert in MON 87769 is native to the soybean genome.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance is MON 87769. Leaf tissue from production plan 06-01-83-18 (Sample IDs 06018318-00002, 06018318-00004, 06018318-00006, and 06018318-00008) generated from seed lot number GLP-0509-16624-S was used in this study.

In addition, leaf tissue from seed lot numbers GLP-0703-18305-S (R3, Sample IDs 07018320-00001 and 07018320-00006), GLP-0703-16624-S (R4, Sample ID 07018320-00002), GLP-0604-17267-S (R5[a], Sample ID 07018320-00003), GLP-0703-18307-S (R6[c], Sample IDs 07018320-00004 and 07018320-00009), and GLP-0704-18475-S (R6[b], Sample IDs 07018320-00005 and 07018320-00010) from Production Plan 07-01-83-20 were used to assess the stability of the transgene insertion across generations.

3.2 Control Substance

The control substance is conventional soybean with the same genetic background as the test substance. Leaf tissue from production plan 06-01-83-18 (Sample IDs 06018318-00001, 06018318-00003, and 06018318-00005) generated from seed lot number GLP-0509-16625-S was used in this study.

3.3 Reference Substance

The reference substance was plasmid PV-GMPQ1972 (Figures 1 and 2) that was used as the transformation vector to produce MON 87769. This plasmid was 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, the 1 kb DNA extension ladder and λ DNA/*Hin*d III fragments from Invitrogen (Carlsbad, CA) were used for size estimations on Southern blots and agarose gels. The 100 bp, 250 bp, 500bp, and 1 kb DNA ladders (Invitrogen) were used for size estimations for PCR analyses**.**

3.4 Characterization of Test, Control and Reference Substances

The identity of the starting seed used to generate the test and control substances was confirmed by event-specific PCR prior to use in the study following SOP BR-PO-0573-02. The identity of the pooled leaf tissue for the test and control substances was confirmed by event-specific PCR prior to the use in the study following SOP BR-ME-0914-02, with the following exception; genomic DNA was extracted according to SOP BR-ME-1153-01 and was diluted to approximately 5 ng/µL. The identity of additional pools of leaf tissue and generational stability substances used in the study were confirmed by Southern blot analyses.

The Study Director reviewed the chain of custody documentation and the event-specific PCR to confirm the identity of the test and control substances prior to use of the materials in the study. The Certificates of Analysis are archived as facility records by their lot number, except for generational stability substances which do not have Certificates of Analysis. Documentation of the event-specific PCR on the leaf tissue test and control substances is archived with the raw data. 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 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 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 *Lgu* I (Fermentas, Hanover, MD) or the enzyme combination *Bsr*G I and *Psh*A I (New England BioLabs, Ipswich, MA), 1x and 2x Tango Buffer (Fermentas) was used, respectively. When digesting genomic DNA with the enzyme combination *Lgu* I and *Bst*X I (New England BioLabs), NEbuffer 2 (New England BioLabs) was used. All digests were performed at 37°C or 55°C according to SOP BR-ME-0316-01 in a total volume of approximately 500 µl using approximately 50-100 units of the appropriate restriction enzyme(s).

3.8 Agarose Gel Electrophoresis

Digested DNA was separated based on size using 0.8% (w/v) agarose gels according to SOP BR-ME-0315-02. For Southern blot analyses, except for generational stability, a 'long run' and 'short run' gel electrophoresis were performed. 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. For Southern blot analysis, approximately 10 µg of digested test and control genomic DNA was loaded into each lane of the agarose gel. For those

agarose gels containing both long and short runs, except for the *Pj.D6D* coding sequence analysis, 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.

3.9 DNA Probe Preparation for Southern Blot Analyses

Probe template DNA containing sequences of plasmid PV-GMPQ1972 was prepared by PCR amplification according to SOP BR-ME-0486-01. Approximately 18-25 ng of each probe template were radiolabeled with ^{32}P-deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Invitrogen). Probe locations relative to the genetic elements in plasmid PV-GMPQ1972 are depicted in Figures 1 and 2.

3.10 Southern Blot Analyses of Genomic DNA

Digested genomic DNA isolated from test material and control material mixed with appropriate positive hybridization controls were evaluated using Southern blot analyses, according to SOP BR-ME-0317-02. The positive hybridization controls were generated from PV-GMPQ1972 DNA, which was isolated prior to the study, and its identity confirmed by restriction enzyme digestion. The PV-GMPQ1972 DNA was digested and then added to digested conventional soybean genomic DNA to serve as the positive hybridization control. In the case where more than one probe was hybridized to the Southern blot, probe templates generated from PV-GMPQ1972 were used as additional positive hybridization controls and were added to digested conventional soybean genomic DNA. The DNA was then separated by agarose gel electrophoresis. Southern blots were hybridized at 65°C with the exception of the *E9* 3′ non-translated region/Left Border and the T-DNA II Southern blots which were hybridized at 60°C. 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.

3.11 PCR and Sequence Analyses

Overlapping PCR products that span the insert in MON 87769 were generated. These products were sequenced to determine the nucleotide sequence of the insert in MON 87769 as well as the nucleotide sequence of the genomic DNA flanking the 5' and 3' ends of the insert.

The PCR analyses were conducted according to SOP BR-ME-0486-01 using 60 ng of genomic DNA template or approximately 0.9 ng of PV-GMPQ1972 DNA in a 50 µl reaction volume utilizing manufacturer buffer containing a final concentration of 1.8 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM each dNTP, and 1 unit of DNA polymerase mix. The specific DNA polymerase mix used to generate the products was Platinum *Taq* (Invitrogen, Figure 17, product A) or Platinum High Fidelity *Taq*

(Invitrogen, Figure 17, products B, C, D, and E). To generate product A, 1/5x of PCR Enhancer (Invitrogen) was added to the reaction mix. The amplification of product A, B and E was performed under the following cycling conditions: 1 cycle at 97°C for 1 minute; 34 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 2 minutes; 1 cycle at 68°C for 5 minutes. The amplification of product C was performed under the following cycling conditions: 1 cycle at 97°C for 1 minute; 32 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 3 minutes and 20 seconds; 1 cycle at 68°C for 5 minutes. The amplification of product D was performed under the following cycling conditions: 1 cycle at 97°C for 1 minute; 36 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 2 minutes and 30 seconds; 1 cycle at 68°C for 5 minutes.

Aliquots of each PCR reaction were separated on a 1.0 % (w/v) agarose gel according to SOP BR-ME-0315-02 and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. Each PCR product was sequenced with the multiple primers used for PCR amplification. In addition, primers internal to the PCR primers were used to sequence other regions of the amplified product. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry.

3.12 PCR and DNA Sequence Analysis to Examine the MON 87769 Insertion Site

To demonstrate that the DNA sequences flanking the insert in MON 87769 are native to the soybean genome, PCR analysis was performed on genomic DNA from both MON 87769 and conventional soybean. The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 87769. 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 60 ng of genomic DNA template in a 50 µl reaction volume utilizing manufacturer buffer containing a final concentration of 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM each dNTP, and 1 unit of DNA polymerase mix. The specific DNA polymerase mix used to amplify the products was Platinum *Taq* (Invitrogen). The amplification of the product was performed under the following cycling conditions: 1 cycle at 97°C for 1 minute; 34 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 1 minute; 1 cycle at 68°C for 5 minutes.

Aliquots of each PCR reaction were separated on a 1.0% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the product was of the expected

size prior to sequencing. The PCR product was sequenced with the primers used for PCR amplification. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry.

3.13 Data Rejected or Not Reported

Some Southern blot analyses conducted as part of this study were not reported because the results were not of report quality or these Southern blots were repeated using a modified restriction enzyme strategy. Southern blot analyses were rejected if there were high levels of background signal that hindered the ability to draw accurate conclusions, if there was inconsistent loading of the positive hybridization controls, or if there were Southern blot artifacts due to poor DNA quality, partial restriction enzyme digestion, or star activity of the restriction enzyme.

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 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 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 originally outlined the DNA extraction method. It was amended to reflect DNA extraction performed according to SOP BR-ME-1153-01, which became effective on February 12, 2007.

2. The protocol was amended to clarify that only *Eco*N I and *Bss*S I digests were bulk digested, however the bulk digest was not used in the study.

3. The test substances for the generational stability were added to the study by protocol amendment.

4. The protocol was amended to change the restriction enzymes used for digestion of genomic DNA to obtain report quality Southern blots.

5. The protocol was amended to reopen the study to recalculate data to accurately reflect that there are 43 bp of Right Border sequence , and MSL0021074 was amended as outlined in Appendix 2.

6. Deviations to the protocol include: use of one enzyme set for the stability analysis; use of an isoschizomer of the restriction enzyme stated in the protocol;

pair-wise DNA sequence alignments were conducted; Southern blot analysis was used to confirm the identity of test, control, and generational stability substances which were not subjected to event specific PCR; a test substance Sample ID which was not listed in the protocol was used, however this material is from the same Lot Number of seed and is the same material as those listed in the protocol; the Right and Left Border sequences were contained in the probes used in Southern analysis. These deviations do not have an impact on the conclusions of the study.

4.0 RESULTS AND DISCUSSION

Genomic DNA from MON 87769 was digested with a variety of restriction enzymes and subjected to Southern blot analysis to characterize the DNA that was integrated into the soybean genome. A description of the genetic elements and their location within PV-GMPQ1972 is shown in Table 1. The genetic elements integrated in MON 87769 are summarized in Table 2. Maps of plasmid vector PV-GMPQ1972, used in the transformation to produce MON 87769 and annotated with the probes used in the Southern blot 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 87769 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 4.4. The generations used in this study are depicted in Figure 4. The sizes of bands present in the long run lanes of Southern blots were estimated using the molecular weight markers on the left side of the figures. The sizes of bands present in the short run lanes were estimated using the molecular weight markers on the right side of the figures.

4.1 Southern Blot Analyses to Determine MON 87769 Insert and Copy Number, and Presence or Absence of Plasmid PV-GMPQ1972 Backbone and T-DNA I Sequences

4.1.1 Southern Blot Analyses to Determine Insert and Copy Number

The insert number (the number of integration sites of T-DNA I in the soybean genome) was evaluated by digesting the test and control DNA with *Lgu* I, a restriction enzyme that does not cleave within T-DNA I. This enzyme generates a restriction fragment containing T-DNA I and adjacent, plant genomic DNA. The number of restriction fragments detected indicates the number of inserts present in MON 87769. The number of copies of the T-DNA I integrated at a single locus was determined by digesting test and control genomic DNA samples with *Lgu* I and *Bst*X I, which cleave once within the insert and within the DNA flanking the 3′ and 5′ end of the insert resulting in two restriction fragments. These blots were examined with six overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 13-18 in Figure 2). The six overlapping

probes were split between two blots to reduce background hybridization. If MON 87769 contains one copy of T-DNA I, then two restriction fragments would be present. The first set of overlapping T-DNA I probes, including the coding sequence for *Pj.D6D*, should hybridize to one band, representing the T-DNA I along with adjacent plant genomic DNA. The second set of overlapping T-DNA I probes, including the coding sequence for *Nc.Fad3*, should hybridize to both bands, each containing a portion of the T-DNA I along with adjacent plant genomic DNA. The results of this analysis are shown in Figure 5.

In Figure 5a, probes 13-15 were used and in Figure 5b, probes 16-18 were used (probes are found in Figure 2). Genomic DNA isolated from conventional soybean DNA digested with *Lgu* I (lanes 3 and 7) or *Lgu* I and *Bst*X I (lanes 1 and 5) produced several hybridization signals (Figure 5a and 5b). This was expected as several genetic elements within T-DNA I were originally derived 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.

In Figure 5a, plasmid PV-GMPQ1972 DNA digested with *Bam*H I and mixed with *Lgu* I and *Bst*X I digested conventional soybean DNA (lane 9) produced expected bands at approximately 1.3, 1.9, 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA. Conventional soybean DNA spiked with probe templates (lanes 10 and 11) produced expected bands at 1.6, 1.7, and 2.0 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA. The 0.1 genomic equivalent of the probe template spikes in lane 11 are faint but detectable in the reported exposure.

In Figure 5b, plasmid PV-GMPQ1972 DNA digested with *Bam*H I and mixed with *Lgu* I and *Bst*X I digested conventional soybean DNA (lane 9) produced expected bands at approximately 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA. Conventional soybean DNA spiked with probe templates (lanes 10 and 11) produced expected bands at 0.8, 1.0, and 1.3 kb, in addition to the endogenous background hybridization. The 0.1 genomic equivalent of the probe template spikes in lane 11 are faint but detectable in the reported exposure.

In Figure 5 (a and b), MON 87769 DNA digested with *Lgu* I (lanes 4 and 8) produced a single unique band of approximately 10.0 kb in addition to the endogenous background hybridization observed in the conventional soybean

DNA (lanes 3 and 7). This result confirms that MON 87769 contains one insert located on an approximately 10.0 kb *Lgu* I restriction fragment.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 5a, lanes 2 and 6) hybridized with probes 13-15 produced one single unique band of approximately 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean DNA (lanes 1 and 5). MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 5b, lanes 2 and 6) hybridized with probes 16-18 produced two bands of approximately 1.6 and 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean DNA (lanes 1 and 5). The results presented in Figure 5 indicate that MON 87769 contains one copy of T-DNA I that resides at a single locus of integration on an approximately 10.0 kb *Lgu* I restriction fragment.

4.1.2 Southern Blot Analysis to Determine the Presence or Absence of Plasmid PV-GMPQ1972 Backbone Sequence

Test and control DNA were digested with either a combination of the restriction enzymes *Lgu* I and *Bst*X I or the combination of restriction enzymes *Bsr*G I and *Psh*A I. Probe template spikes (probes 1, 2, 3, and 6, Figure 1) generated from the plasmid PV-GMPQ1972 were mixed with the pre-digested control genomic DNA to serve as a positive hybridization control. Additionally, plasmid PV-GMPQ1972 DNA previously digested with the restriction enzyme *Bam*H I was mixed with control genomic DNA digested with *Lgu* I and *Bst*X I to serve as a positive hybridization control. The blot was hybridized simultaneously with four overlapping probes (probes 1, 2, 3, and 6, Figure 1) that spanned the backbone sequence of PV-GMPQ1972. The results are shown in Figure 6.

Conventional soybean DNA digested with a combination of *Lgu* I and *Bst*X I (Figure 6, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 6, lanes 3 and 7) produced no hybridization signals.

Plasmid PV-GMPQ1972 *Bam*H I restriction fragments mixed with conventional soybean DNA digested with *Lgu* I and *Bst*X I produced the expected bands at 2.3 and 7.9 kb (Figure 6, lane 9). Probe template spikes (Probes 1, 2, 3, and 6, Figure 1) generated from plasmid PV-GMPQ1972 were mixed with the control DNA pre-digested with *Lgu* I and *Bst*X I. The expected bands at 0.7, 0.9, 1.5, and 1.8 kb (Figure 6, lanes 10 and 11) were observed. The results show that the four probes hybridized, as expected, to the target DNA. MON 87769 DNA digested with the combination of *Lgu* I and *Bst*X I (Figure 6, lanes 2 and 6) or *Bsr*G I and *Psh*A I (Figure 6, lanes 4 and 8) showed no detectable hybridization signal, indicating that MON 87769 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ1972.

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

Test and control DNA were digested with either a combination of the restriction enzymes *Lgu* I and *Bst*X I or the combination of restriction enzymes *Bsr*G I and *Psh*A I. Probe template spikes (probes 4 and 5, Figure 1) generated from the plasmid PV-GMPQ1972 were mixed with the pre-digested control genomic DNA to serve as a positive hybridization control. Additionally, plasmid PV-GMPQ1972 DNA previously digested with the restriction enzyme *Bam*H I was mixed with control genomic DNA digested with *Bsr*G I and *Psh*A I to serve as a positive hybridization control. The blot was hybridized simultaneously with two overlapping probes (probes 4 and 5, Figure 1) that spanned the T-DNA II sequence of PV-GMPQ1972. The results are shown in Figure 7.

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 7, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 7, lanes 3 and 7) examined with overlapping probes spanning T-DNA II (probes 4 and 5, Figure 1) showed no detectable hybridization bands as expected for the negative control.

Conventional soybean DNA (digested with *Bsr*G I and *Psh*A I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 7, lane 9) produced two expected size bands at approximately 7.9 and 2.3 kb. Conventional soybean DNA containing probe template spikes (Figure 7, lanes 10 and 11) produced two expected size bands at approximately 2.0 and 1.9 kb. The 0.1 genomic equivalent of the probe template spikes in lane 11 are faint but detectable in the reported exposure.

The overlapping probes spanning T-DNA II contain the Right Border sequence, the *E9* 3′ non-translated region sequence, and the Left Border sequence that are also contained on T-DNA I. Therefore, the T-DNA II probes are expected to hybridize to fragments derived from T-DNA I.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 7, lanes 2 and 6) hybridized with probes 4 and 5 (Figure 1) produced one unique band of approximately 1.6 kb. This band is consistent with the 1.6 kb band detected with the *E9* 3′ non-translated region and the Left Border sequence (Figure 13, lanes 2 and 6). A band at 6.8 kb is also expected that represents the Right Border sequence in the insert. This band was not detected because the probe only overlaps with 43 base pairs of the Right Border sequence within this fragment.

MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 7, lanes 4 and 8) hybridized with probes 4 and 5 (Figure 1) produced one unique band of approximately 4.2 kb. This band is consistent with the 4.2 kb expected band

detected with the *E9* 3′ non-translated region and the Left Border sequence (Figure 13, lanes 4 and 8). A band at 2.0 kb is also expected representing the Right Border sequence in the insert, however this band was not detected because the probe only overlaps with 43 base pairs of the Right Border sequence within this fragment. No additional fragments were seen in the over exposure. No unexpected bands were detected indicating that MON 87769 contains no additional detectable T-DNA II elements other than those associated with T-DNA I.

4.2 Southern Blot Analyses to Confirm the Cassette Copy Number of the Elements Comprising the *Pj.D6D* and *Nc.Fad3* Cassettes in MON 87769

The cassette copy number (the number of times each element of T-DNA I is integrated into the soybean genome of MON 87769) was evaluated by digesting the test and control DNA and hybridizing Southern blots with probes for each element of T-DNA I.

Digestion of the test substance with *Lgu* I and *Bst*X I releases two border fragments with the expected size of approximately 6.8 and 1.6 kb (Figure 3). The approximately 6.8 kb fragment contains genomic DNA flanking the 5′ end of the insert, Right Border sequence, *7Sα′* promoter sequence, *Pj.D6D* coding sequence, *tml* 3′ non-translated region, *7Sα* promoter sequence, and a portion of the *Nc.Fad3* coding sequence. The approximately 1.6 kb fragment contains the remaining portion of the *Nc.Fad3* coding sequence, *E9* 3′ non-translated region, Left Border sequence, and genomic DNA flanking the 3′ end of the insert (Figure 3).

Digestion of the test substance with *Bsr*G I and *Psh*A I releases one internal restriction fragment and two border fragments (Figure 3). The 5′ border fragment is expected to be approximately 2.0 kb and contains genomic DNA flanking the 5′ end of the insert, Right Border sequence, *7Sα′* promoter sequence, and a portion of the *Pj.D6D* coding sequence. The approximately 1.9 kb internal fragment contains the remaining portion of the *Pj.D6D* coding sequence, and a portion of the *tml* 3′ non-translated region. The 3′ border fragment is expected to be approximately 4.2 kb and contains the remaining portion of the *tml* 3′ non-translated region, *7Sα* promoter sequence, *Nc.Fad3* coding sequence, *E9* 3′ non-translated region, Left Border sequence, and genomic DNA flanking the 3′ end of the insert.

Plasmid PV-GMPQ1972 DNA was digested with *Bam*H I and combined with conventional soybean DNA (digested with *Lgu* I and *Bst*X I) to serve as a positive hybridization control. Individual Southern blots were examined with the following probes: Right Border/*7Sα′* promoter, *Pj.D6D* coding region, *tml* 3′ non-translated

region, *7Sα* promoter, *Nc.Fad3* coding sequence, and *E9* 3′ non-translated region /Left Border sequence. (probes 7-12, Figure 1).

4.2.1 Right Border/*7Sα′* Promoter Probe

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 8, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 8, lanes 3 and 7) and hybridized with the Right Border/*7Sα′* promoter probe (probe 7, Figure 1) each produced a single hybridization signal. This hybridization signal results from the probe hybridizing to an endogenous sequence residing in the soybean genome and is not specific to the inserted DNA. This signal was produced in both test and control lanes and, therefore, the band was considered to be endogenous background. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 8, lanes 10 and 11) produced the expected size bands at approximately 1.3, 2.3 and 7.9 kb in addition to the endogenous hybridization band.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 8, lanes 2 and 6) and hybridized with probe 7 (Figure 1) produced the expected single unique band of approximately 6.8 kb in addition to the endogenous hybridization band observed in the conventional soybean control (Figure 8, lanes 1 and 5). MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 8, lanes 4 and 8) produced a single unique band of approximately 2.0 kb in addition to the endogenous hybridization band observed in the conventional soybean control (Figure 8, lanes 3 and 7). No unexpected bands were detected, other than the endogenous bands, indicating that MON 87769 contains no additional detectable Right Border or *7Sα′* promoter elements other than those associated with the *Pj.D6D* expression cassette.

4.2.2 *Pj.D6D* Coding Sequence Probe

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 9, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 9, lanes 3 and 7) and hybridized with the *Pj.D6D* coding sequence (probe 8, Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 9, lanes 10 and 11) produced the expected size bands at approximately 1.3 and 1.9 kb.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 9, lanes 2 and 6) and hybridized with probe 8 (Figure 1) produced the expected single unique band of approximately 6.8 kb. MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 9, lanes 4 and 8) produced the expected unique bands of approximately 1.9 and 2.0 kb. In lane 8, these two bands at 1.9 and 2.0 kb appear as a doublet

in the short run, however the two bands are separated in the long run (lane 4). No unexpected bands were detected indicating that MON 87769 contains no additional detectable *Pj.D6D* coding sequence elements other than those associated with the *Pj.D6D* expression cassette.

4.2.3 *tml* 3′ Non-Translated Region Probe

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 10, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 10, lanes 3 and 7) and hybridized with the *tml* 3′ non-translated region probe (probe 9, Figure 1) showed no detectable hybridization as expected for the negative control. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 10, lanes 10 and 11) produced the expected size band at approximately 1.9 kb**.**

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 10, lanes 2 and 6) and hybridized with probe 9 (Figure 1) produced the expected single unique band of approximately 6.8 kb. MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 10, lanes 4 and 8) produced two expected unique bands of approximately 1.9 and 4.2 kb. No unexpected bands were detected, indicating that MON 87769 contains no additional, detectable *tml* 3′ non-translated region elements other than those associated with the *Pj.D6D* expression cassette.

4.2.4 *7Sα* Promoter Probe

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 11, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 11, lanes 3 and 7) and hybridized with the *7Sα* promoter probe (probe 10, Figure 1) produced two hybridization signals. Each of these hybridization signals result from the probe hybridizing to endogenous sequences residing in the soybean genome and are not specific to the inserted DNA. These signals were produced in both test and control lanes and, therefore, the bands are considered to be endogenous background. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 11, lanes 10 and 11) produced the expected size band at approximately 3.1 kb in addition to the endogenous hybridization bands observed in conventional soybean DNA (lanes 1 and 5).

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 11, lanes 2 and 6) and hybridized with probe 10 (Figure 1) produced the expected single unique band of approximately 6.8 kb in addition to the endogenous hybridization bands. MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 11, lanes 4 and 8) produced the expected single unique band of approximately 4.2 kb in addition to the endogenous hybridization bands. No unexpected bands were detected, other

than the endogenous bands, indicating that MON 87769 contains no additional, detectable *7Sα* promoter elements other than those associated with the *Nc.Fad3* expression cassette.

4.2.5 *Nc.Fad3* Coding Sequence Probe

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 12, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 12, lanes 3 and 7) and hybridized with the *Nc.Fad3* coding sequence probe (probe 11, Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional soybean DNA digested with *Lgu* I and *Bst*X I containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 12, lanes 10 and 11) produced the expected size band at approximately 3.1 kb.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 12, lanes 2 and 6) and hybridized with probe 11 (Figure 1) produced two expected unique bands of approximately 1.6 and 6.8 kb. The migration of the approximately 1.6 kb fragment is slightly lower than indicated by the molecular weight marker band sizes. The altered migrations may be due to the difference in salt concentrations between the DNA sample and the molecular weight marker (Sambrook and Russell, 2001). MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 12, lanes 4 and 8) produced the expected band of approximately 4.2 kb. No unexpected bands were detected indicating that MON 87769 contains no additional, detectable *Nc.Fad3* coding sequence elements other than those associated with the *Nc.Fad3* expression cassette.

4.2.6 *E9* 3′ Non-Translated Region/Left Border Probe

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 13, lanes 1 and 5) or *Bsr*G I and *Psh*A I (Figure 13, lanes 3 and 7) and hybridized with the *E9* 3′ non-translated region/Left Border probe (probe 12, Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 13, lanes 10 and 11) produced two expected size bands at approximately 2.3 and 7.9 kb.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (Figure 13, lanes 2 and 6) and hybridized with probe 12 (Figure 1) produced the expected single unique band of approximately 1.6 kb. MON 87769 DNA digested with *Bsr*G I and *Psh*A I (Figure 13, lanes 4 and 8) produced the expected single unique band of approximately 4.2 kb. No unexpected bands were detected indicating that MON 87769 contains no additional detectable *E9* 3′ non-translated region or Left Border elements other than those associated with the *Nc.Fad3* expression cassette

4.3 Southern Blot Analyses of MON 87769 In Multiple Generations

In order to assess the stability of the T-DNA in MON 87769 in multiple generations, five DNA samples from four generations, including two separate R6 generation samples, were analyzed by Southern blot analyses. For reference, the breeding history of MON 87769 is presented in Figure 4. The specific generations tested are indicated in the legends of Figures 4, 14, 15, 16, and 17.

4.3.1 Southern Blot Analysis to Examine Generational Stability of the Insert

DNA from four generations of MON 87769 soybean (refer to Figure 4) were isolated and subjected to digestion with *Lgu* I and *Bst*X I. Digestion of the test materials with *Lgu* I and *Bst*X I was expected to release two border fragments with the expected sizes of 6.8 kb and 1.6 kb**.** The detected band in each generation is compared to the fully characterized MON 87769 generation (Figure 4) to determine stability. The blot was examined with six overlapping T-DNA I probes spanning the entire inserted DNA sequence (probes 13-18, Figure 2) on two blots. The results of this analysis are shown in Figure 14.

In Figure 14a, probes 13-15 were used and in Figure 14b, probes 16-18 were used (Figure 2). Genomic DNA isolated from conventional soybean digested with *Lgu* I and *Bst*X I (lane 4) produced several hybridization signals (Figure 14a and 14b). This is expected as several genetic elements within T-DNA I were originally derived 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.

In Figure 14a, plasmid PV-GMPQ1972 DNA digested with *Bam*H I mixed with conventional soybean DNA (digested with *Lgu* I and *Bst*X I, lane 1) produced the expected bands at approximately 1.3, 1.9, 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA. Conventional soybean DNA spiked with probe templates (lanes 2 and 3) produced expected bands at 1.6, 1.7, and 2.0 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA.

In Figure 14b, plasmid PV-GMPQ1972 DNA digested with *Bam*H I mixed with conventional soybean DNA (digested with *Lgu* I and *Bst*X I, lane 1) produced the expected bands at approximately 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean

DNA. Conventional soybean DNA spiked with probe templates (lanes 2 and 3) produced expected bands at 0.8, 1.0, and 1.3 kb, in addition to the endogenous background hybridization.

MON 87769 DNA from generations R3, R4, R5[a], R6[c], and R6[b] (see Figure 4) digested with *Lgu* I and *Bst*X I (Figure 14a, lanes 5 - 9) and hybridized with probes 13-15 produced one single unique band of approximately 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean control DNA (lane 4). MON 87769 DNA from generations R3, R4, R5[a], R6[c], and R6[b] digested with *Lgu* I and *Bst*X I (Figure 14b, lanes 5 - 9) and hybridized with probes 16-18 produced two bands of approximately 1.6 and 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean control DNA (lane 4). The results presented in Figure 14 indicate that MON 87769 contains one copy of T-DNA I that is stable across the selected generations.

4.3.2 Southern Blot Analysis to Determine the Presence or Absence of Plasmid PV-GMPQ1972 Backbone Sequence

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 15, lane 4) examined with four overlapping probes spanning the PV-GMPQ1972 backbone sequence (probes 1-3, and 6, Figure 1) showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 15, lane 1) hybridized with probes 1-3, and 6 (Figure 1) produced the two expected size bands at approximately 2.3 and 7.9 kb. Conventional soybean DNA digested with *Lgu* I and *Bst*X I containing probe template spikes (Figure 15, lanes 2 and 3) produced four expected size bands at approximately 0.7, 0.9, 1.5, and 1.8 kb.

MON 87769 from generations R3, R4, R5[a], R6[c], and R6[b] digested with *Lgu* I and *Bst*X I (Figure 15, lanes 5 - 9) and hybridized with probes 1-3, and 6 (Figure 1) produced no detectable hybridization bands indicating that MON 87769 contains no detectable PV-GMPQ1972 backbone elements across the generations tested. No unexpected bands were detected, indicating that MON 87769 generations R3, R4, R5[a], R6[c], and R6[b] contain no detectable PV-GMPQ1972 backbone elements.

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

Conventional soybean DNA digested with *Lgu* I and *Bst*X I (Figure 16, lane 4) examined with two overlapping probes spanning T-DNA II (probes 4 and 5, Figure 1) showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA (digested with *Lgu* I and *Bst*X I) containing

plasmid PV-GMPQ1972 DNA digested with *Bam*H I (Figure 16, lane 1) produced the two expected size bands at approximately 2.3 and 7.9 kb. Conventional soybean DNA containing probe template spikes (Figure 16, lanes 2 and 3) produced two expected size bands at approximately 2.0 and 1.9 kb.

The overlapping probes spanning T-DNA II contain the Right Border sequence, *E9* 3′ non-translated region sequence, and the Left Border sequence, which are contained on T-DNA I. Therefore, the T-DNA II probes are expected to hybridize to fragments derived from T-DNA I. MON 87769 generations R3, R4, R5[a], R6[c], and R6[b] digested with *Lgu* I and *Bst*X I (Figure 16, lanes 5 - 9) and hybridized with probes 4 and 5 (Figure 1) each produced two expected bands of approximately 1.6 kb and 6.8kb. The 1.6 kb band is consistent with the 1.6 kb band detected with the *E9* 3′ non-translated region and the Left Border sequence (Figure 13, lanes 2 and 6). The band at approximately 6.8 kb is also expected representing the Right Border sequence in the insert, which is faint since there are only 43 base pairs of the Right Border in the MON87769 insert. No unexpected bands were detected indicating that MON 87769 generations R3, R4, R5[a], R6[c], and R6[b] contain no additional detectable T-DNA II elements other than those associated with T-DNA I.

4.4 Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87769

The organization of the elements within the insert in MON 87769 was confirmed using PCR analysis by amplifying five 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 17. As expected, the control reactions containing no template DNA (lanes 2, 5, 9, 13, and 17), and the conventional soybean control reactions (lanes 3, 6, 10, 14, and 18) did not generate PCR products with any of the primer sets. The plasmid control reactions in lanes 8, 12, and 16 produced products of equal size to those of MON 87769 (lanes 7, 11 and 15) because the primer sequences for each product are located within the sequence of the MON 87769 insert and within PV-GMPQ1972.

In Figure 17, PCR reactions using genomic DNA from MON 87769 produced the expected size products of approximately 1.9 kb for product A (lane 4); approximately 1.2 kb for product B (lane 7); approximately 3.1 kb for product C (lane 11); approximately 2.3 kb for product D (lane 15); and approximately 1.5 kb for product E (lane 19). Plasmid PV-GMPQ1972 was used as a positive control template (lanes 8, 12 and 16). As expected, these positive control reactions produced PCR products of 1.2, 3.0, and 2.3 kb, respectively. The amplification of the predicted size PCR products from MON 87769 establishes that the arrangement and linkage of elements

in the insert are consistent with those in plasmid PV-GMPQ1972 and are as depicted in Figure 3.

PCR products (Figure 17, A-E) generated from MON 87769 genomic DNA were subjected to DNA sequencing to further confirm the organization of the elements within the insert. The consensus sequence representing the insert in MON 87769, including the genomic DNA flanking the ends of the insert, is shown in Figure 18 (CBI Cross Reference 1) and is described in Table 2. This consensus sequence was generated by compiling numerous sequencing reactions performed on the PCR products A-E (Figure 17).

The DNA sequence of the MON 87769 insert is 7367 base pairs long, beginning at base 9387 of PV-GMPQ1972 located in the Right Border region, and ending at base 288 of PV-GMPQ1972 located in the Left Border region. There is sequence for 933 base pairs of soybean genomic DNA flanking the 5' end of the insert and 831 base pairs of soybean genomic DNA flanking the 3' end of the insert (Figure 18, CBI Cross Reference 1). The sequence confirmed the presence and organization of the integrated genetic elements as described in Table 2.

4.5 PCR and DNA Sequence Analysis to Examine the MON 87769 Insertion Site

PCR and sequence analyses were performed on genomic DNA extracted from MON 87769 and conventional soybean to demonstrate that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87769 are native to the soybean genome. The PCR was performed with one primer designed from the genomic DNA sequence flanking the 5' end of the insert (Figure 19, Primer A) paired with a second primer designed from the genomic DNA sequence flanking the 3' end of the insert (Figure 19, Primer B). The results of the PCR analysis are shown in Figure 19. The control PCR containing no template DNA (lane 4) did not generate a PCR product, as expected. The reaction using the conventional soybean genomic DNA template (lane 2) generated the expected PCR product of an approximately 600 bp across the insertion site of MON 87769. As expected, a PCR product across the 7367 bp DNA insert in MON 87769 (lane 3) was not generated in this analysis since the PCR conditions necessary to generate a product of this size were not used.

The approximately 600 bp PCR product generated from the conventional soybean genomic DNA was sequenced and the resulting data are shown in Figure 20 (CBI Cross Reference 2). The consensus sequence is the result of double stranded sequence reads and is shorter than the PCR product used to generate the sequence because it can be difficult to generate reliable sequence information immediately adjacent to the primers used for sequencing. A sequence comparison between the product generated from the conventional soybean and the sequence generated from

the 5' and 3' flanking sequence of MON 87769 indicated a 9 bp deletion (bases 334-342 in Figure 20), and two small insertions of 17 and 8 bp (bases 917-933 and 8301-8308 in Figure 18) had occurred. These likely occurred in MON 87769 upon insertion of the T-DNA. Bases 1-333 and 343-489 from conventional soybean represent genomic DNA sequences that are identical to 5' and 3' genomic DNA sequence flanking the insert in MON 87769, respectively (584-916 bp, 8309-8455 bp, Figure 20). This analysis confirms that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87769 is native to the soybean genome.

5.0 CONCLUSIONS

Molecular characterization of MON 87769 by Southern blot analyses demonstrated that the introduced DNA was inserted into the soybean genome at a single locus and contains one copy each of the *Pj.D6D* and *Nc.Fad3* expression cassettes. All expression elements are present in the inserted DNA and no additional elements were detectable other than those associated with the respective insert. No backbone plasmid DNA or T-DNA II sequences were detected other than those associated with T-DNA I.

Analysis of multiple MON 87769 generations demonstrated that the introduced DNA was stably maintained across the generations tested. No PV-GMPQ1972 backbone DNA or T-DNA II sequences were detected other than those associated with T-DNA I.

Finally, PCR and DNA sequence analyses were performed on MON 87769, 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 sequence in MON 87769, and confirmed that the genomic DNA sequence flanking the 5' and 3' ends of the insert in MON 87769 is native to the soybean genome.

6.0 REFERENCES

Barker, R.F., K. B. Idler, D. V. Thompson, and J. D. Kemp. 1983. Nucleotide Sequence of the T-DNA Region from the *Agrobacterium tumefaciens* Octopine Ti Plasmid pTi15955. *Plant Mol. Biol.* **2**: 335-350.

Barry, G.F., G.M. Kishore, S.R. Padgette, and W.C. Stallings. 1997. Glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. United States Patent #5,633,435.

Coruzzi, G., R. Broglie, C. Edwards, and N. H. Chua. 1984. Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. *EMBO J* **3**(8): 1671-9.

Depicker, A., S. Stachel, P. Dhaese, P. Zambryski, and H. M.Goodman. 1982. Nopaline Synthase: Transcript Mapping and DNA Sequence. *J. Molec. Appl. Genet*. **1**: 561-573.

Doyle, J. J., M. A. Schuler, W. D. Godette, V. Zenger, R. N. Beachy, and J. L. Slightom. 1986. The glycosylated seed storage proteins of Glycine max and Phaseolus vulgaris. Structural homologies of genes and proteins. *J Biol Chem* **261**(20): 9228-38.

Fling, M., J. Kopf, and C. Richards. 1985. Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase. *Nucleic Acids Res*. **13**: 7095-7106.

Giza, P.E. and R.C. Huang. 1989. A self-inducing runaway-replication plasmid expression system utilizing the Rop protein. *Gene* **78**: 73-84.

Kemp, J. D., R. F. Barker, and M. J. Adang. 2000. Octopine T-DNA structural genes. United States Patent No. 6090627.

Klee, H. J., Y. M. Muskopf, and C. S. Gasser. 1987. Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Mol. Gen. Genet.* **210**: 437-42.

Padgette, S.R., D.B. Re, G.F. Barry, D.E. Eichhlotz, X. Delannay, R.L. Fuchs, G.M. Kishore, and R.T. Fraley. 1996. New weed control opportunities: Development of soybeans with a Roundup Ready™ gene. In: Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory, and Technical Aspects. Ed. S. O. Duke. CRC Press, New York.

Rogers, S.G. 2000. Promoter for transgenic plants. United States Patent No. 6,018,100.

Sambrook, J., and D. Russell. 2001. Molecular Cloning third edition, Cold Spring Harbor Laboratory Press. Chapter 5, Protocol 1: Agarose gel electrophoresis.

Stalker, D. M., C. M. Thomas, and D. R. Helinski. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. *Mol. Gen. Genetics*. **181**: 8-12.

Sutcliffe, J.G. 1978. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Symposia on Quantitative Biology*. **43**: 77-103.

Ursin, V., B. Froman, J., Gonzales, S. Screen, D. Fenggao, T. La Rosa. 2005. Fatty Acid Desaturases from Primula. WO05021761A1

Ursin, V., T. Voelker, B. Froman. 2003. Fatty Acid Desaturase from Fungi. WO03099216A2

Wang, Q., and P. Dubois. 2004. Seed specific 7S.alpha. promoter for expressing genes in plants. United States Patent No. 6,825,398.

Table 1. Summary of Genetic Elements in Plasmid Vector PV-GMPQ1972

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA I (continued from bp 16465)		
Intervening Sequence	1–14	Sequence used in DNA cloning
B[1]-Left Border	15-456	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker et al., 1983)
Vector Backbone		
Intervening Sequence	457–1619	Sequence used in DNA cloning
CS[2]-*rop*	1620-2092	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	2093-2340	Sequence used in DNA cloning
OR[3]-*ori-PBR322*	2341-2969	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978)
Intervening Sequence	2970-3469	Sequence used in DNA cloning
aadA	3470-4358	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	4359-4494	Sequence used in DNA cloning
T-DNA II (not present in MON 87769)		
B-Right Border	4495-4851	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	4852-4884	Sequence used in DNA cloning
P[4]-*FMV*	4885-5448	Promoter for the 35S RNA from figwort mosaic virus (FMV) (Rogers, 2000) that directs transcription in plant cells
Intervening Sequence	5449-5491	Sequence used in DNA cloning
L[5]-*ShkG*	5492–5558	5' non-translated leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS (Klee et al., 1987) that helps regulate gene expression

[1]B – Border; [2]CS – Coding Sequence; [3]OR – Origin of Replication; [4]P – Promoter; [5]L-Leader;

Table 1 (cont.) Summary of Genetic Elements in Plasmid Vector PV-GMPQ1972

T-DNA II (cont.)		
TS[6]–*CTP2*	5559–5786	Transit peptide region of *Arabidopsis thaliana* EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS-*cp4 epsps*	5787-7154	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	7155-7196	Sequence used in DNA cloning
T[7]-*E9*	7197-7839	A 3' non-translated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	7840-7886	Sequence used in DNA cloning
B-Left Border	7887-8328	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker et al., 1983)
Vector Backbone		
Intervening Sequence	8329-8414	Sequence used in DNA cloning
OR-*ori V*	8415-8811	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	8812-9072	Sequence used in DNA cloning
T-DNA I		
B-Right Border	9073-9429	DNA area from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	9430-9480	Sequence used in DNA cloning
P-*7Sα'*	9481-10321	Promoter and leader from the *Sphas1* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle et al., 1986) that directs mRNA transcription in seed
Intervening Sequence	10322-10337	Sequence used in DNA cloning
CS-*Pj.D6D*	10338-11678	Coding region for the fatty acid delta-6 desaturase from *Primula juliae* (Ursin et al., 2005)
Intervening Sequence	11679-11686	Sequence used in DNA cloning

[6]TS – Targetting Sequence; [7]T – Transcript Termination Sequence

Table 1 (cont.) Summary of Genetic Elements in Plasmid Vector PV-GMPQ1972

T-*tml*	11687-12636	3' non-translated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid (Kemp et al., 2000) that directs polyadenylation of the mRNA
Intervening Sequence	12637-12737	Sequence used in DNA cloning
P-*7Sα*	12738-14417	Promoter and leader from the *Sphas2* gene from soybean encoding the alpha subunit of beta-conglycinin (Wang et al., 2004) that directs mRNA transcription in seed
Intervening Sequence	14418-14445	Sequence used in DNA cloning
CS-*Nc.Fad3*	14446-15735	Codon optimized coding sequence for the gene from *Neurospora crassa* encoding delta-15 desaturase (Ursin et al., 2003)
Intervening Sequence	15736-15787	Sequence used in DNA cloning
T-*E9*	15788-16430	3' non-translated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	16431-16465	Sequence used in DNA cloning

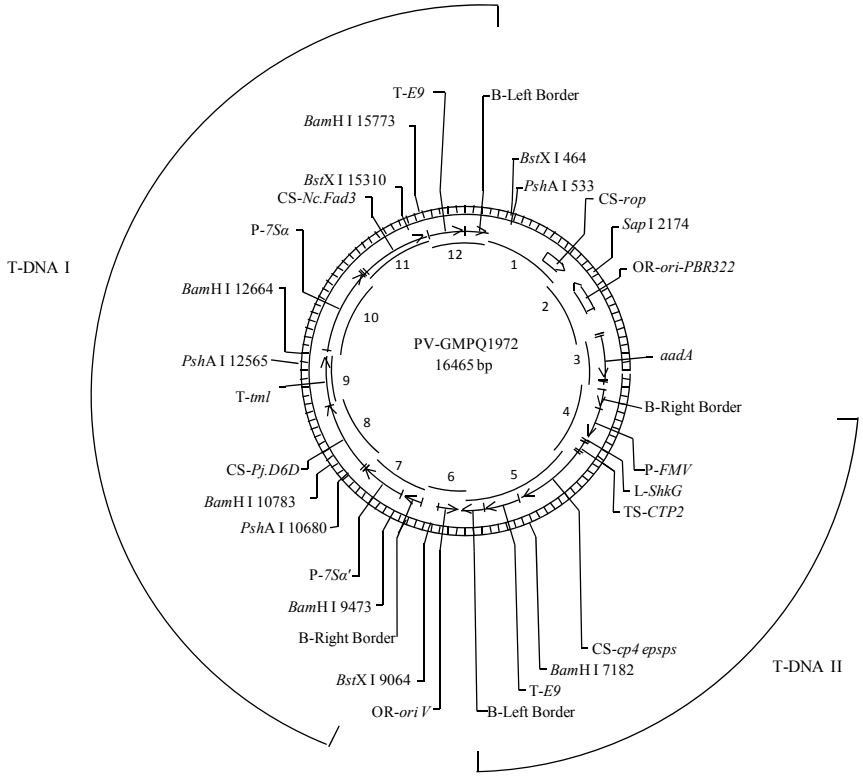
Table 2. Summary of Genetic Elements in MON 87769

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Sequence flanking 5' end of the insert	1-933	Soybean genomic DNA
B[3]-Right Border	934-976	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	977-1027	Sequence used in DNA cloning
P[4]-*7Sα'*	1028-1868	Promoter and leader from the *Sphas1* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle et al., 1986)
Intervening Sequence	1869-1884	Sequence used in DNA cloning
CS[5]-*Pj.D6D*	1885-3225	Coding region for the fatty acid delta-6 desaturase from *Primula juliae* (Ursin et al., 2005)
Intervening Sequence	3226-3233	Sequence used in DNA cloning
T[6]-*tml*	3234-4183	3′ non-translated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid (Kemp et al., 2000)
Intervening Sequence	4184-4284	Sequence used in DNA cloning
P-*7Sα*	4285-5964	Promoter and leader from the *Sphas2* gene from soybean encoding the alpha subunit of beta-conglycinin (Wang et al., 2004)
Intervening Sequence	5965-5992	Sequence used in DNA cloning
CS-*Nc.Fad3*	5993-7282	Codon optimized coding sequence for the gene from *Neurospora crassa* encoding delta-15 desaturase (Ursin et al., 2003)
Intervening Sequence	7283-7334	Sequence used in DNA cloning
T-*E9*	7335-7977	3′ non-translated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al, 1984)
Intervening Sequence	7978-8026	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 17.
[2] Numbering refers to the sequence from Figure 17 that includes the insert in MON 87769 and adjacent genomic DNA.
[3]B – Border; [4]P – Pomoter; [5]CS – Coding Sequence; [6]T – 3' non-translated transcriptional termination sequence and polyadenylation signal sequences.

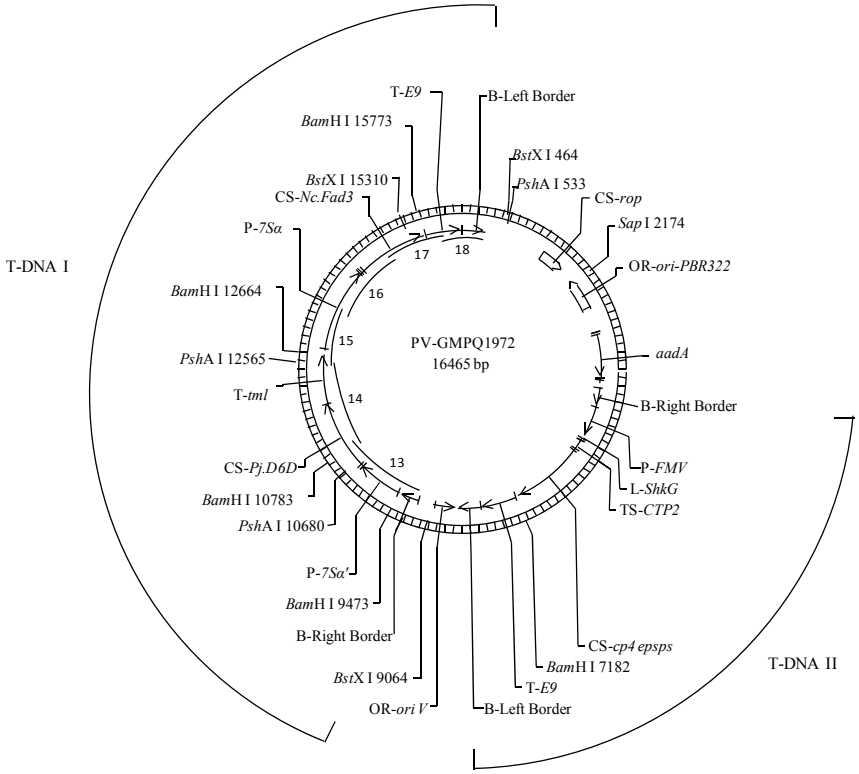
Table 2 (cont.) Summary of Genetic Elements in MON 87769

B-Left Border	8027-8300	DNA region from Agrobacterium tumefaciens containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Sequence flanking 3' end of the insert	8301-9131	Soybean genomic DNA



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	Backbone 1	457	2254	1.8
2	Backbone 2	2168	3656	1.5
3	Backbone 3	3553	4494	0.9
4	T-DNA II probe 1	4495	6512	2.0
5	T-DNA II probe 2	6436	8330	1.9
6	Backbone 4	8329	9072	0.7
7	B-Right Border/P-7*Sα'*	9073	10329	1.3
8	CS-*Pj.D6D*	10338	11680	1.3
9	T-*tml*	11687	12636	0.95
10	P-7*Sα*	12728	14431	1.7
11	CS-*Nc.Fad3*	14446	15736	1.3
12	T-*E9*/B-Left Border	15786	458	1.1

Figure 1. Plasmid Map of Vector PV - GMPQ1972 Showing Probes 1-12
A circular map of the plasmid vector PV- GMPQ1972 used to develop soybean MON 87769 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analysis are shown on the interior of the map. PV- GMPQ1972 contains two T-DNA regions designated as T-DNA I and T-DNA II above.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
13	T-DNA I probe 1	9073	11046	2.0
14	T-DNA I probe 2	10966	12710	1.7
15	T-DNA I probe 3	12545	14151	1.6
16	T-DNA I probe 4	14012	15300	1.3
17	T-DNA I probe 5	15168	16205	1.0
18	T-DNA I probe 6	16116	458	0.8

Figure 2. Plasmid Map of Vector PV- GMPQ1972 Showing Probes 13-18
A circular map of the plasmid vector PV- GMPQ1972 used to develop soybean MON 87769 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The overlapping T-DNA I probes used in the Southern analysis are shown on the interior of the map.

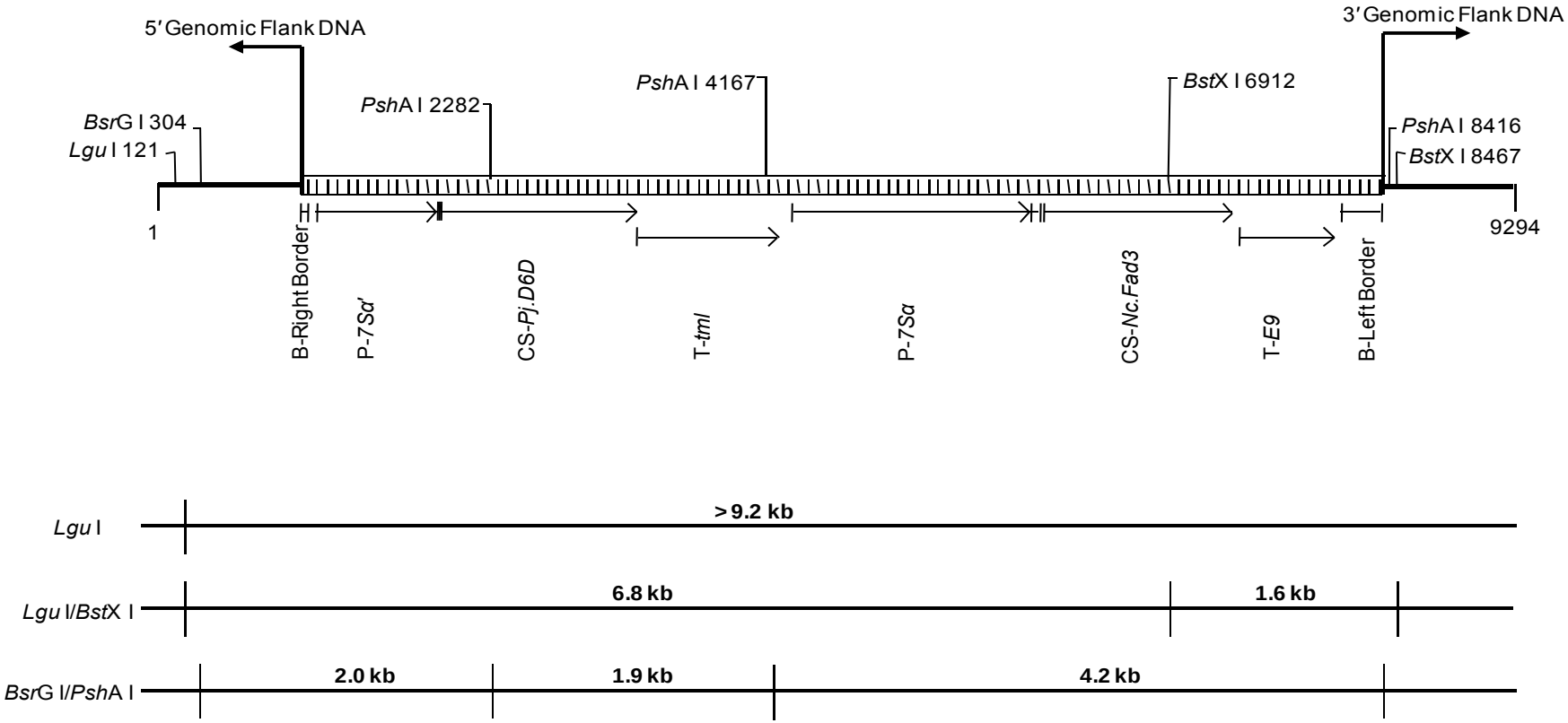
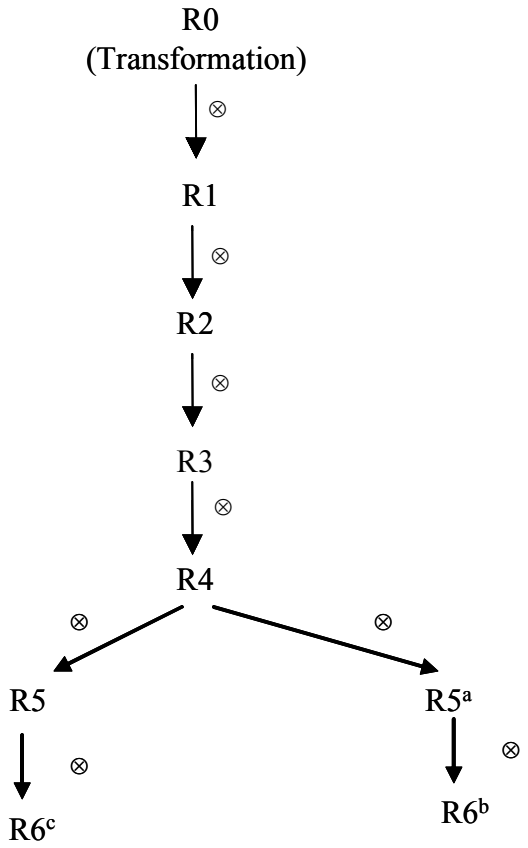


Figure 3. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87769
A linear map of the insert and genomic DNA flanking the insert in MON 87769 is shown. Identified on the map are genetic elements within the insert, as well as restriction sites that fall within the known sequence reported in Figure 18. Restriction site positions relative to the size of the linear map for enzymes used in the Southern analyses are shown. Shown on the lower portion of the map are the expected sizes of the DNA fragments after digestions with respective restriction enzyme or combination of enzymes. Arrows indicate the end of the insert and the beginning of soybean genomic flanking sequence.



R0 – originally transformed plant; ⊗ – self pollinated

Figure 4. Breeding History of MON 87769
The R4 generation was used for the molecular analyses reported in Figures 5-13 and is referred to as MON 87769 in all Southern blot figures. MON 87769 from generations R3, R4, R5[a], R6[b], and R6[c] are used for analyzing the stability of the insert and the absence of the T-DNA II and Backbone sequences across generations (Figures 14-16).

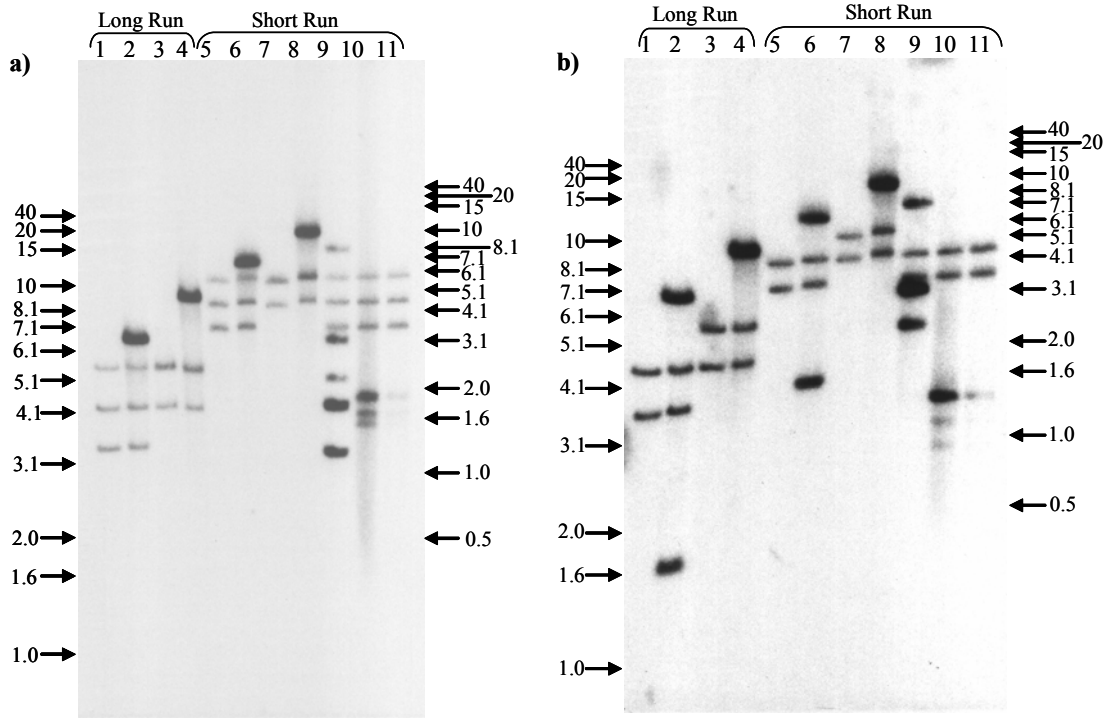


Figure 5. Southern Blot Analysis of MON 87769: Insert and Copy Number Probes
The blots were hybridized with overlapping ^{32}P-labeled probes that spanned the T-DNA I sequence (probes 13-18, Figure 2). Each blot was hybridized simultaneously with three of the overlapping probes. Figure 5a was hybridized with probes 13-15 and figure 5b was hybridized with probes 16-18. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Lgu* I)
4: MON 87769 (*Lgu* I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Lgu* I)
8: MON 87769 (*Lgu* I)
9: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]

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

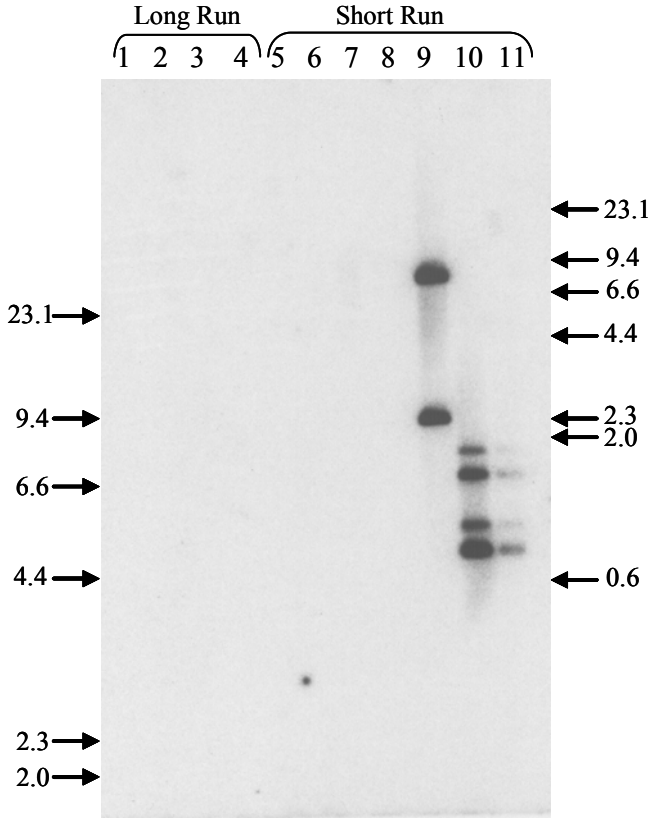


Figure 6. Southern Blot Analysis of MON 87769: PV-GMPQ1972 Backbone Probes
The blot was hybridized simultaneously with four overlapping ^{32}P-labeled probes that span the entire backbone sequence (probes 1, 2, 3, and 6, Figure 1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]

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

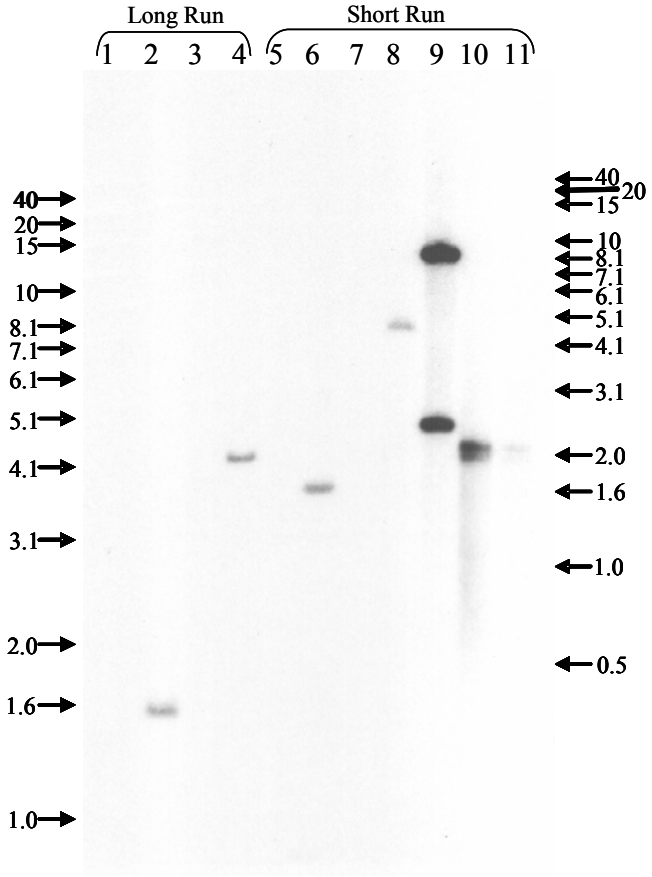


Figure 7. Southern Blot Analysis of MON 87769: T-DNA II Probes
The blot was hybridized simultaneously with two overlapping ^{32}P-labeled probes that span the entire T-DNA II sequence (probes 4 and 5, Figure 1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Conventional soybean (*Bsr*G I and *Psh*A I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
10: Conventional soybean (*Bsr*G I and *Psh*A I) spiked with probe templates [~1 genomic equivalent]
11: Conventional soybean (*Bsr*G I and *Psh*A I) spiked with probe templates [~0.1 genomic equivalent]

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

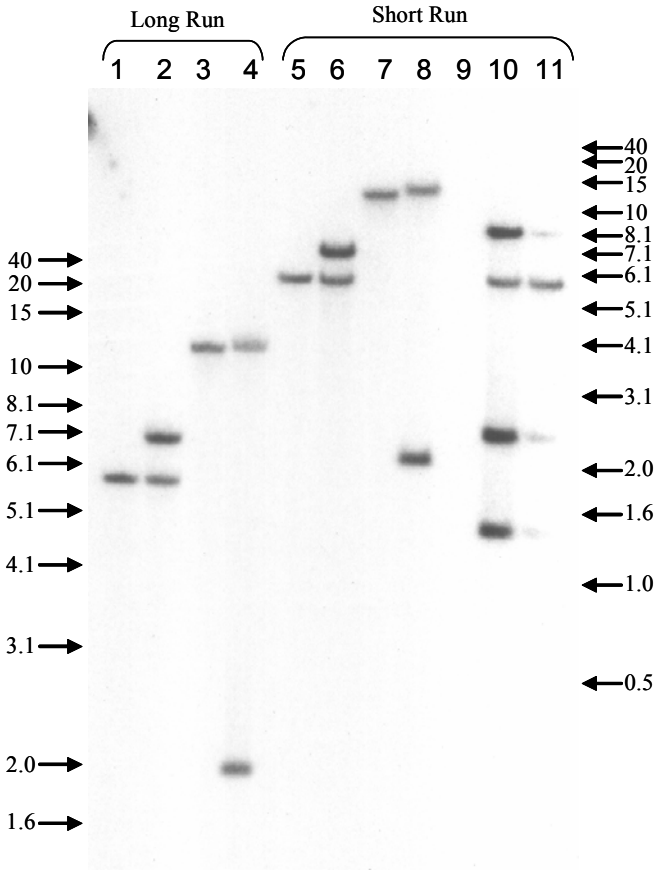


Figure 8. Southern Blot Analysis of MON 87769: Right Border/P-*7sα′* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the Right Border and *7sα′* promoter sequence (probe 7, Figure 1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

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

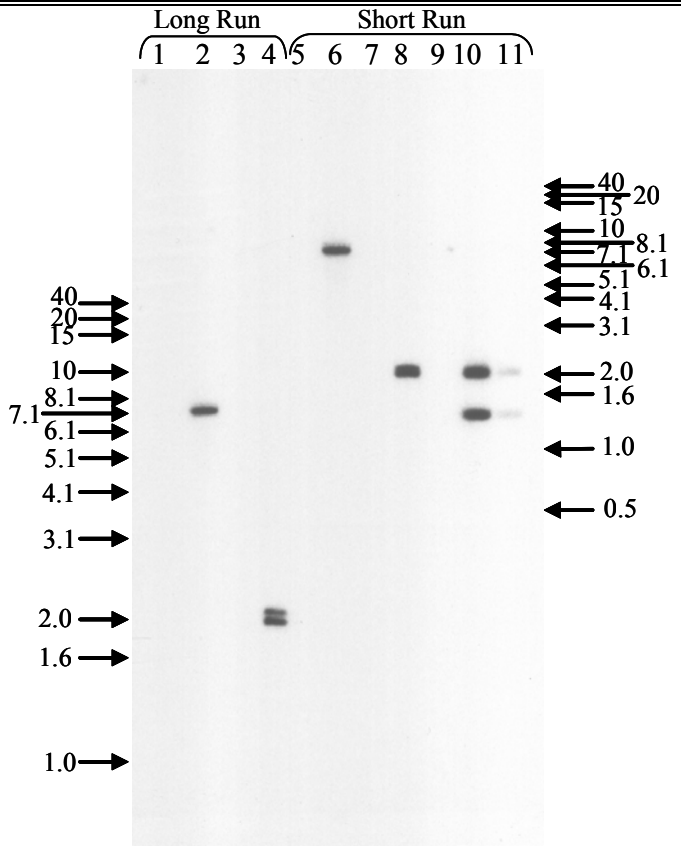


Figure 9. Southern Blot Analysis of MON 87769: CS-*Pj.D6D* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *Pj.D6D* coding sequence (probe 8, Figure 1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

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

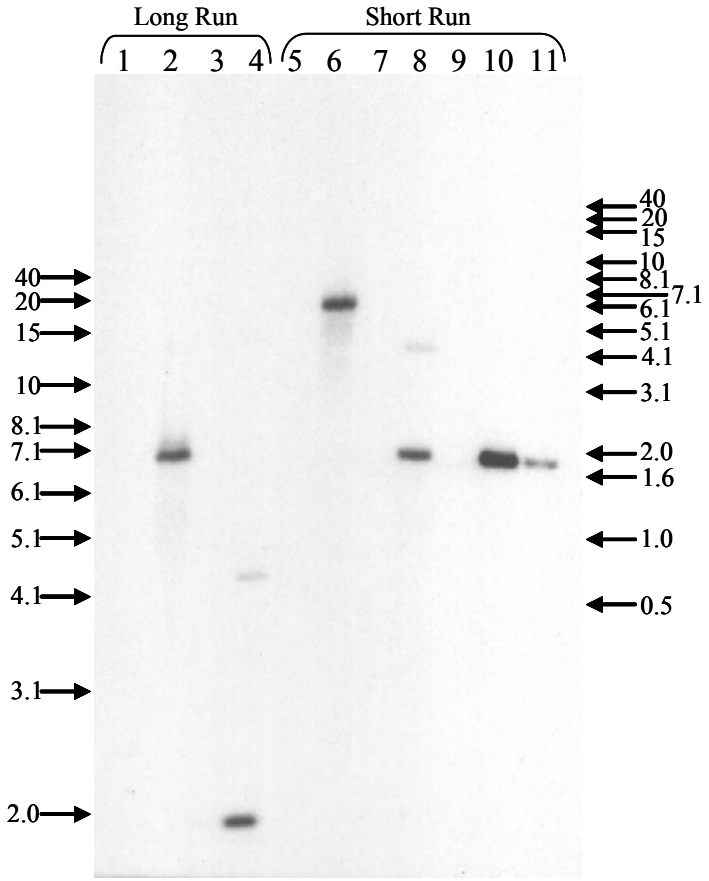


Figure 10. Southern Blot Analysis of MON 87769: T-*tml* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *tml* non-translated region (probe 9, Figure 1). Each lane contains approximately 10 μg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

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

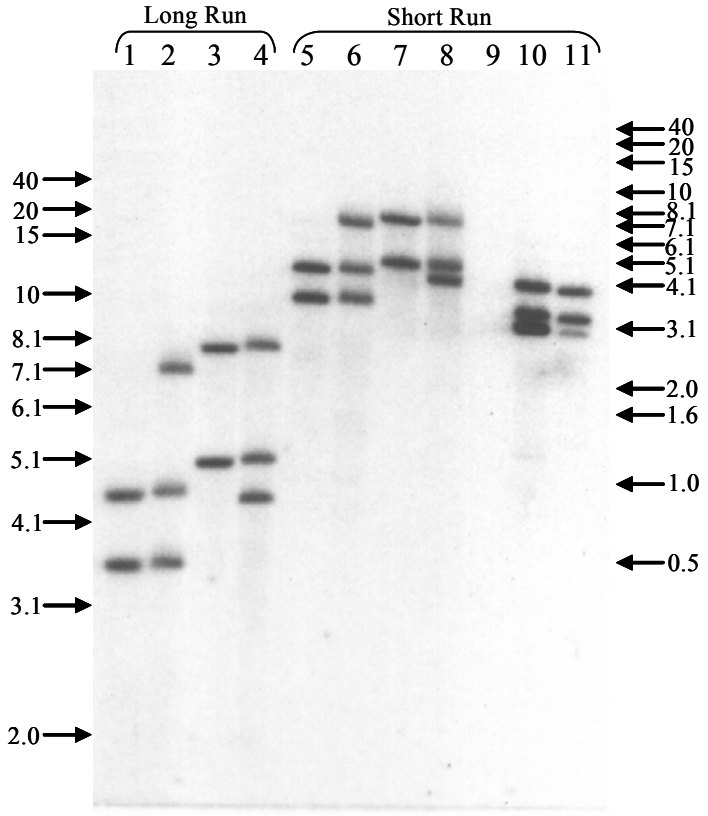


Figure 11. Southern Blot Analysis of MON 87769: P-*7sα* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *7sα* promoter sequence (probe 10, Figure 1). Each lane contains approximately 10 μg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

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

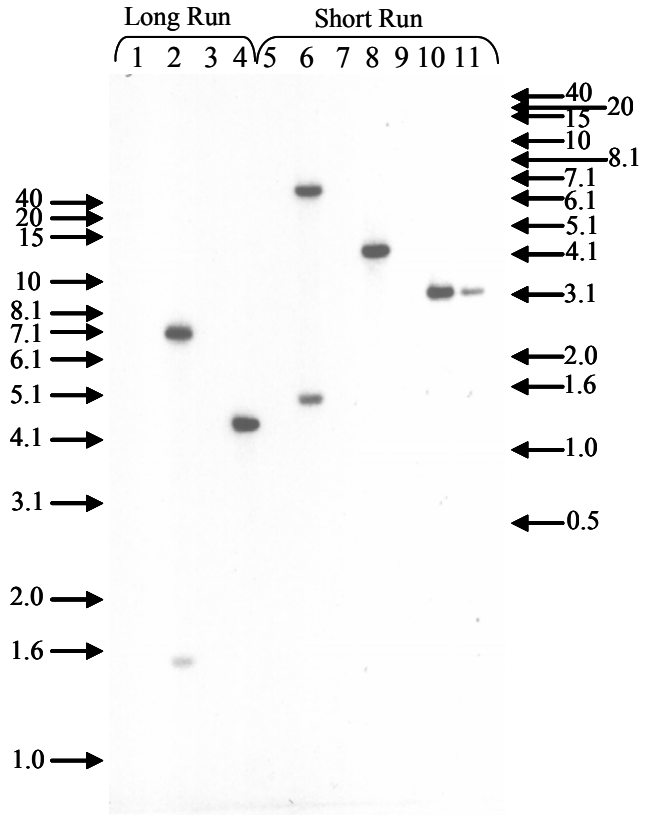


Figure 12. Southern Blot Analysis of MON 87769: CS-*Nc.Fad3* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *Nc.Fad3* coding sequence (probe 11, Figure 1). Each lane contains approximately 10 μg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

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

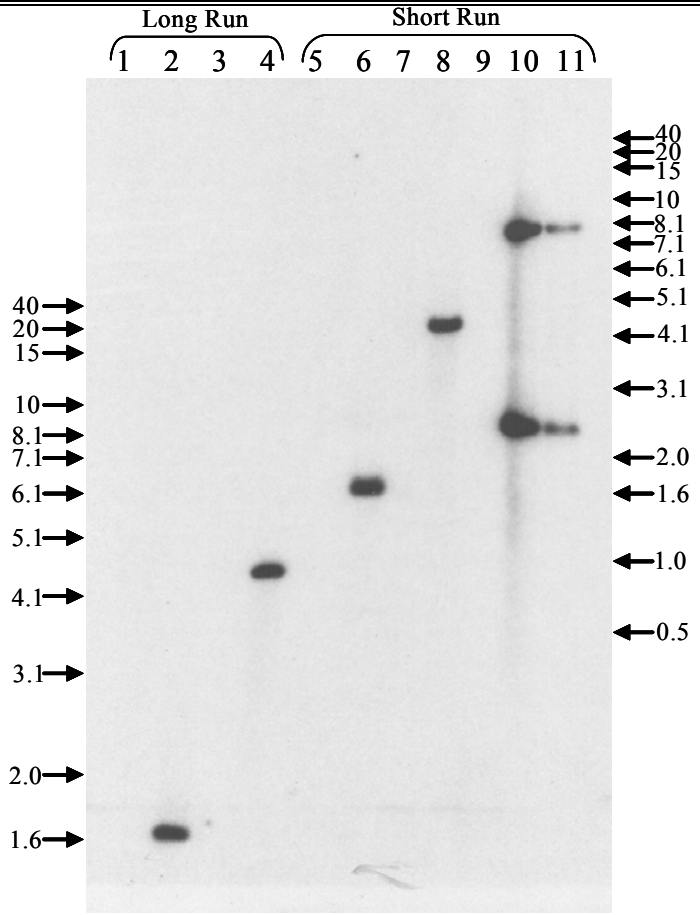


Figure 13. Southern Blot Analysis of MON 87769: T-*E9*/Left Border Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *E9* non-translated region and Left Border (probe 12, Figure 1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

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

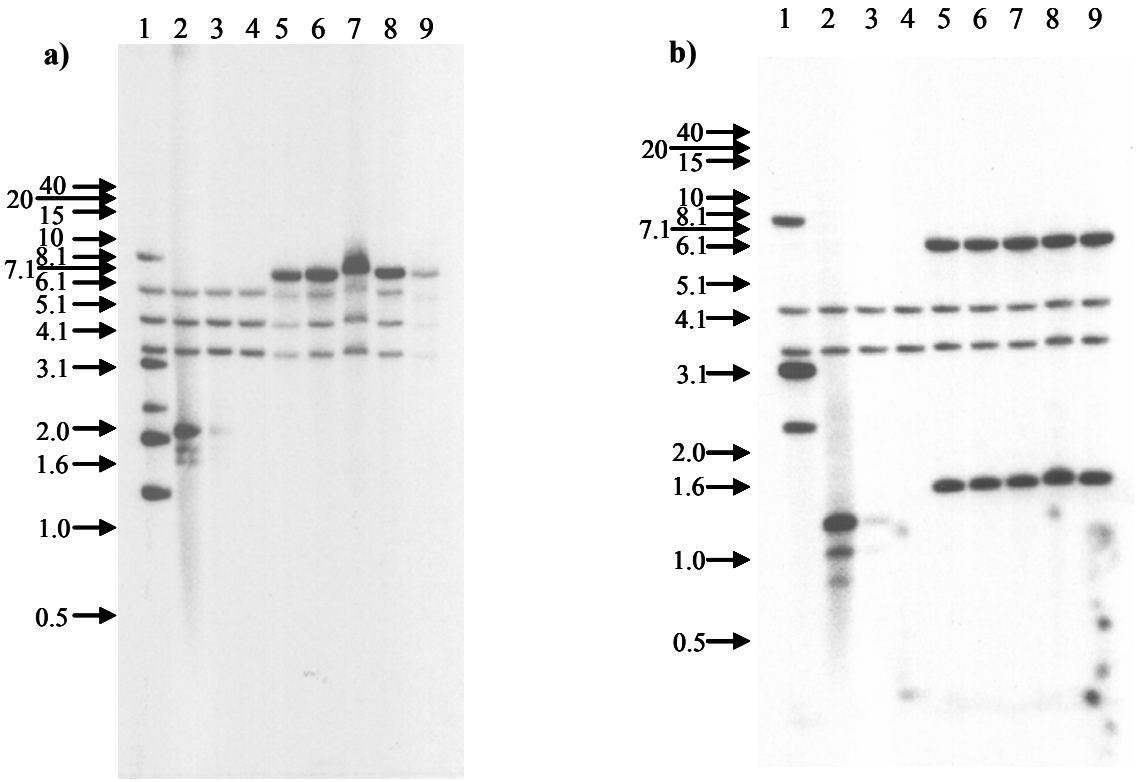


Figure 14. Generational Stability of MON 87769: T-DNA I Probes
The blot was hybridized with overlapping ^{32}P-labeled probes that spanned the T-DNA (probes 13-18, Figure 2). Each blot was hybridized simultaneously with three of the overlapping probes. Figure 14a was hybridized with probes 13-15 and figure 14b was hybridized with probes 16-18. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87769 is illustrated in Figure 4. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
2: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]
4: Conventional soybean (*Lgu* I and *Bst*X I)
5: R3: MON 87769 (*Lgu* I and *Bst*X I)
6: R4: MON 87769 (*Lgu* I and *Bst*X I)
7: R5[a]: MON 87769 *(Lgu* I and *Bst*X I)
8: R6[c]: MON 87769 *(Lgu* I and *Bst*X I)
9: R6[b]: MON 87769 *(Lgu* I and *Bst*X I)

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

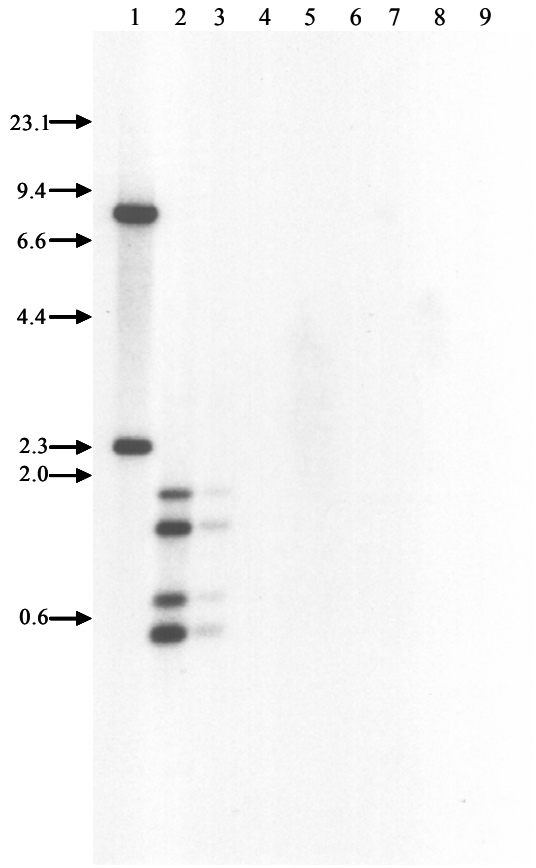


Figure 15. Generational Stability of MON 87769: PV-GMPQ1972 Backbone Probes
The blot was hybridized with four overlapping ^{32}P-labeled probes that span the entire backbone sequence (probes 1, 2, 3, and 6, Figure 1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87769 is illustrated in Figure 4. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
2: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]
4: Conventional soybean (*Lgu* I and *Bst*X I)
5: R3: MON 87769 (*Lgu* I and *Bst*X I)
6: R4: MON 87769 (*Lgu* I and *Bst*X I)
7: R5[a]: MON 87769 *(Lgu* I and *Bst*X I)
8: R6[c]: MON 87769 *(Lgu* I and *Bst*X I)
9: R6[b]: MON 87769 *(Lgu* I and *Bst*X I)

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

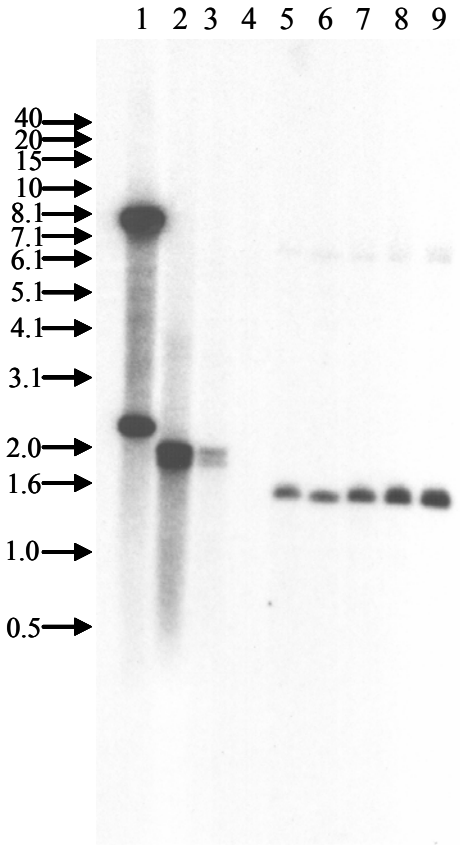


Figure 16. Generational Stability of MON 87769: T-DNA II Probes
The blot was hybridized with two overlapping ^{32}P-labeled probes that span the entire T-DNA II (probes 4 and 5, Figure 1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87769 is illustrated in Figure 4. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
2: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]
4: Conventional soybean (*Lgu* I and *Bst*X I)
5: R3: MON 87769 (*Lgu* I and *Bst*X I)
6: R4: MON 87769 (*Lgu* I and *Bst*X I)
7: R5[a]: MON 87769 *(Lgu* I and *Bst*X I)
8: R6[c]: MON 87769 *(Lgu* I and *Bst*X I)
9: R6[b]: MON 87769 *(Lgu* I and *Bst*X I)

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

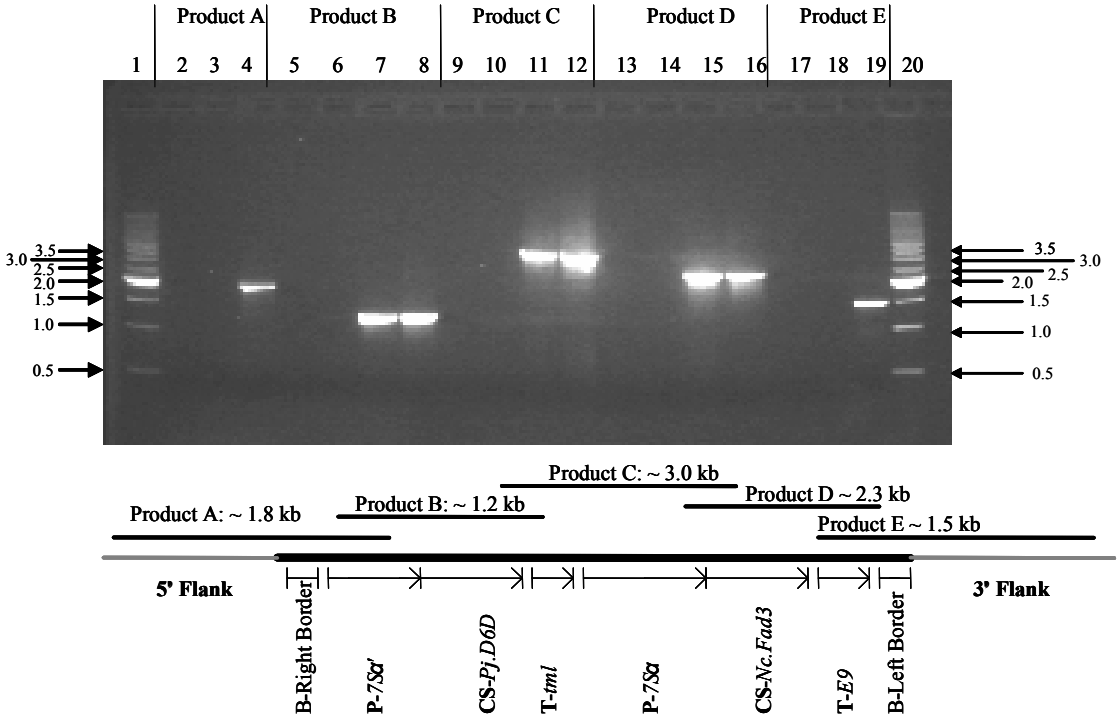


Figure 17. PCR Analysis Across the Insert in MON 87769
PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 87769 were performed on MON 87769 genomic DNA extracted from leaf (Lanes 4, 7, 11, 15, and 19). Lanes 3, 6, 10, 14, and 18 contain reactions with Conventional soybean control DNA while lanes 2, 5, 9, 13, and 17 are reactions containing no template DNA. Lanes 8, 12, and 16 contain reactions with PV-GMPQ1972 control DNA. Lane 1 and 20 contain Invitrogen 500 bp 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 87769 that appears at the bottom of the figure. Three to ten microliters of each of the PCR products were loaded on the gel.

Lane
1: Invitrogen 500 bp DNA ladder
2: No template
3: Conventional soybean DNA
4: MON 87769
5: No template
6: Conventional soybean DNA
7: MON 87769
8: PV-GMPQ1972
9: No template
10: Conventional soybean DNA
11: MON 87769
12: PV-GMPQ1972
13: No template
14: Conventional soybean DNA
15: MON 87769
16: PV-GMPQ1972
17: No template
18: Conventional soybean DNA
19: MON 87769
20: Invitrogen 500 bp DNA ladder

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

[CBI CROSS REFERENCE 1]

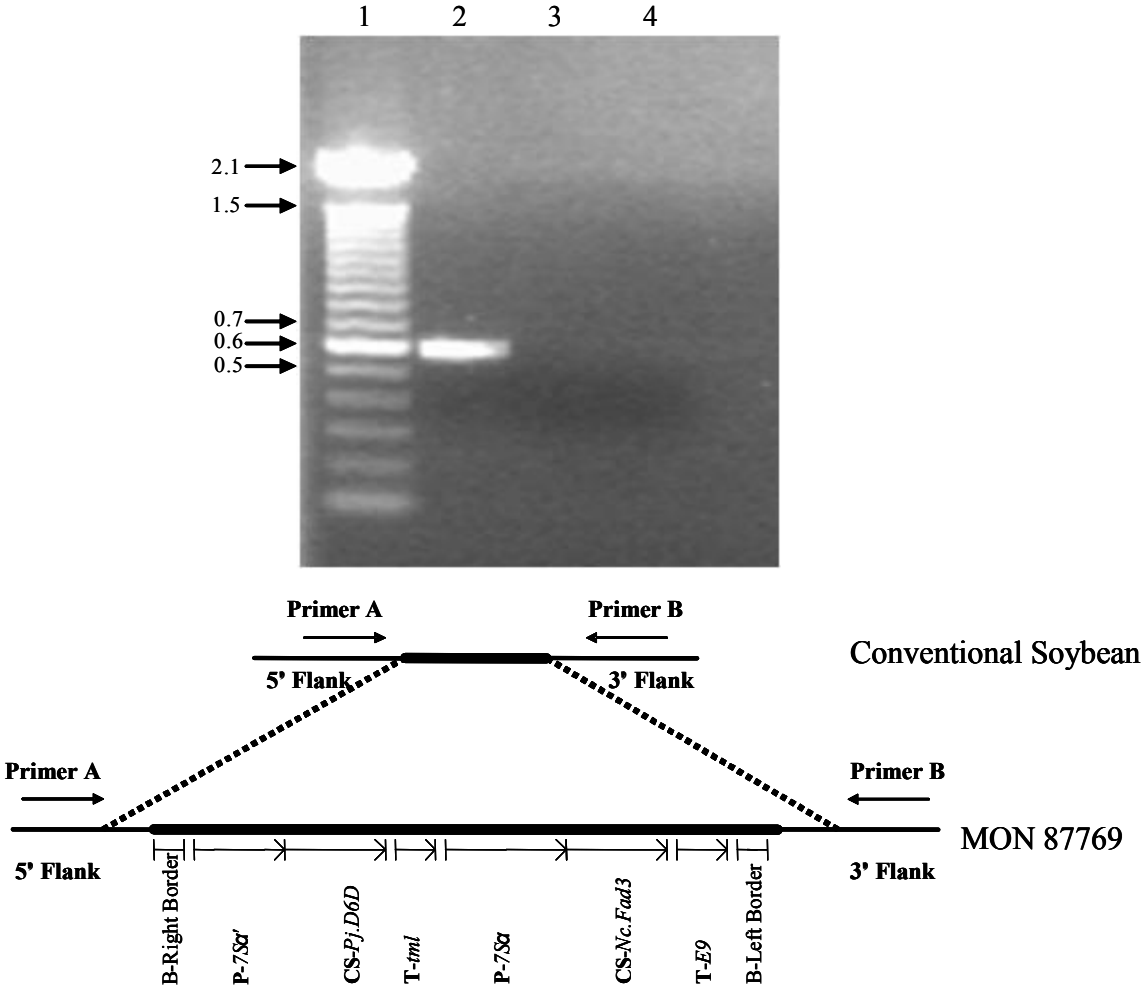


Figure 19. PCR Amplification of the Insertion Site in MON 87769

Depiction of the MON 87769 insertion locus in Conventional soybean (middle panel) and the MON 87769 insert (lower panel). PCR analysis demonstrating the insertion locus within the Conventional soybean genome is visualized by agarose gel. PCR amplification was performed using Primer A located in the 5' sequence flanking the insert and Primer B located in the 3' sequence flanking the insert to verify that the flanking sequences are native to the soybean genome. The PCR products were loaded and visualized on agarose gel. Lane designations are as follows:

Lane 1: 100 bp DNA ladder
2: Conventional soybean DNA
3: MON 87769
4: No template

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

[CBI CROSS REFERENCE 2]

APPENDIX 1

Standard Operating Procedures

BR-EQ-0065-02	Hoefer Scientific DyNA Quant 200 Fluorometer
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-PO-0573-02	Test, Control, and Reference Material Confirmation of Identity
BR-ME-1153-01	Quick CTAB DNA Extraction from Leaf Tissue

APPENDIX 2

Notes for Reviewer

MSL0021074 was amended to accurately reflect that there are 43 bp of Right Border sequence present in the insert, two molecular weight markers used in the study were added as reference materials, and typographical errors were corrected. These revisions are outlined in Appendix 2 as "Amendment 1". These changes do not impact the conclusions of this study.

Page Number in MSL0021926	Change
1	Added "Amended Report for MSL0021074:" to the title, "Amendment 1" after Study Completed, and revised report completion date.
4	Added "Amended Report Audit" to list of phases, changed study title, and deleted "Michelle Higgins" as the QAU personnel.
6	Changed the study title, changed the MSL number, added "Original" to Study Completion Date, added "Amendment 1 Report Completion Date".
8	Change page number for 4.3.3 to page 29.
9	Added "Appendix 2: Notes to Reviewer" to the Table of Contents.
13	Changed the sentence "The *Nc.Fad3* expression cassette consists of the *Nc.Fad3nno* coding sequence under the regulation of the *7Sα* promoter, and the *E9* 3' non-translated region." to "The *Nc.Fad3* expression cassette consists of the *Nc.Fad3* coding sequence under the regulation of the *7Sα* promoter, and the *E9* 3' non-translated region."
14	Added "100 bp" and "500 bp" molecular weight markers to the Reference Material section.
18	Added "5. The protocol was amended to reopen the study to correct a typographical error, and MSL0021074 was amended as outlined in Appendix 2."
18-19	Changed the sentence "5. Deviations to the protocol include: …conclusions of the study." to "6. Deviations to the protocol included: …conclusions to the study."
22	Changed the sentence "There are only 46 base pairs of the Right Border in the MON 87769 insert which were not detected in this analysis." to "This band was not detected because the probe only overlaps with 43 base pairs of the Right Border sequence within this fragment."
23	Changed the sentence "A band at 2.0 kb is also expected representing the Right Border sequence in the insert, however there are only 46 base pairs of the Right Border sequence in the MON 87769 insert which were not

	detected in this analysis." to "A band at 2.0 kb is also expected representing the Right Border sequence in the insert, however this band was not detected because the probe only overlaps with 43 base pairs of the Right Border sequence within this fragment."
29	Changed 46 bp to 43 bp.
61	Changed the sentence "Bases 343-489 (double underlined) represent genomic soybean DNA sequence that is identical to the 3' genomic DNA sequence flanking the MON 87769 insert (8309-9131 bp, Figure 18)." to "Bases 343-489 (double underlined) represent genomic soybean DNA sequence that is identical to the 3' genomic DNA sequence flanking the MON 87769 insert (8309-8455 bp, Figure 18)."
63-64	Added "Appendix 2: Notes to Reviewer" detailing the contents of Amendment 1.
Confidential Attachment Title Page	Changed Title from "Molecular Analysis of Soybean MON 87769" to "Amended Report for MSL0021074: Molecular Analysis of Stearidonic Acid Producing Soybean MON 87769", added "Amendment 1" after Study Completed, and revised report completion date.

CONFIDENTIAL ATTACHMENT