Title

Amended Report for MSL0022670: Molecular Analysis of Dicamba-Tolerant Soybean MON 87708

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Sponsor/ Testing Facility

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Study REG-08-182
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STATEMENT OF COMPLIANCE

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

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

Submitter	Date
Melinda C. McCann	4 Mar 2011
Sponsor Representative	Date
Zihong Song	03/04/2011
Study Director	Date

QUALITY ASSURANCE STATEMENT

Study Title: Amended Report for MSL0022670: Molecular Analysis of Dicamba-Tolerant Soybean MON 87708

Study Number: REG-08-182

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

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

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
05/22/2008	PCR/Sequence	05/22/2008	05/22/2008
06/26/2008	Southern Blot	06/28/2008	06/28/2008
04/30/2009	Draft Report and Data Audit	04/30/2009	04/30/2009
06/18/2009	Draft Report Amendment Review	06/19/2009	06/19/2009
06/29/2010	Draft Report Amendment Review	06/30/2010	06/30/2010
02/28/2011	Draft Report Amendment Review	03/04/2011	03/04/2011

Kanthasamy Karunanandaa, Ph.D.
Quality Assurance Specialist
Monsanto Regulatory, Monsanto Company

MARCH 4th, 2011
Date

STUDY CERTIFICATION PAGE

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

Zihong Song 03/04/2011

Zihong Song, M.S. — Date
Study Director
Regulatory Product Characterization Center

Qing Tian 3/4/2011

Qing Tian, M.D., Ph.D. — Date
Molecular Characterization Platform Lead
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STUDY INFORMATION PAGE

Study Number:	REG-08-182
MSL Number:	0023278
Title:	Amended Report for MSL0022670: Molecular Analysis of Dicamba-Tolerant Soybean MON 87708
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:	Melinda C. McCann, M.S.
Study Director:	Zihong Song, M.S.
Supervisory Personnel:	Qing Tian, M.D., Ph.D.
Contributors:	Zihong Song and Kim D. Lawry
Study Initiation Date:	April 18, 2008
Original Study Completion Date:	April 30, 2009
Amendment 3 Completion Date:	March 4, 2011
Records Retention:	The protocol, all raw data, documentation, records, and the final report for this study are retained at Monsanto Company
Sample Storage:	Any study samples that are to be retained will be stored at Monsanto Company, St. Louis, Missouri.

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ABBREVIATIONS[1] AND DEFINITIONS

aadA	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3" (9)-O-nucleotidyltransferase from the transposon Tn7 that confers spectinomycin and streptomycin resistance
B-Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
B-Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
bp	Base pair
BSA	Bovine serum albumin
CS-*cp4 epsps*	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein
CS-*dmo*	Coding sequence for the dicamba mono-oxygenase from *Stenotrophomonas maltophilia*
CS-*rop*	Coding sequence for repressor of primer protein used for maintenance of plasmid copy number in *E. coli*
CTAB	Hexadecyltrimethylammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dNTP	Deoxynucleoside triphosphate
E. coli	*Escherichia coli*
GLP	Good Laboratory Practice
kb	Kilobase
L-*DnaK*	5' non-translated leader sequence from the *Petunia hybrida Hsp70* gene that is involved in regulating gene expression
L-*TEV*	5' non-translated region from the Tobacco Etch RNA virus genome that is involved in regulating gene expression
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-*FMV*	Promoter for the figwort mosaic virus (FMV) 35S RNA
P-*PC1SV*	Promoter for the full length transcript of peanut chlorotic streak virus that directs transcription in plant cells
PCR	Polymerase chain reaction

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

ABBREVIATIONS AND DEFINITIONS (CONTINUED)

PV-GMHT4355	Plasmid vector used to develop MON 87708
SOP	Standard operating procedure
T-DNA	Transfer(ed) DNA
T-*E9*	3′ non-translated region of the pea rbcS2 gene, which functions to direct polyadenylation of the mRNA
TS-*CTP2*	Sequences encoding the transit peptide region of *Arabidopsis thaliana* EPSPS used to direct proteins into chloroplasts
TS-*RbcS*	Sequences encoding the transit peptide region of the *RbcS* gene of *Pisum sativum* that directs transport the DMO protein to the chloroplast
w/v	weight per volume

1.0 SUMMARY

Monsanto Company has developed herbicide-tolerant soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses the dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide.

MON 87708 was developed through *Agrobacterium*-mediated transformation of a conventional soybean variety A3525 with the 2T-DNA transformation plasmid PV-GMHT4355 (Figure 1). The plasmid contains two separate T-DNAs that are delineated by right and left border sequences. The first T-DNA, designated as T-DNA I, contains the dicamba mono-oxygenase (*dmo*) coding region under regulation of the *PC1SV* promoter and the *E9* 3′ non-translated region. The second T-DNA, designated as T-DNA II, contains the *cp4 epsps* coding sequence under the regulation of the *FMV* promoter and the *E9* 3′ non-translated region. During transformation, both T-DNAs were inserted into the soybean genome. Subsequently, conventional breeding and segregation were used to isolate those plants that contain the *dmo* expression cassette (T-DNA I) but do not contain the *cp4 epsps* expression cassette (T-DNA II), resulting in the production of marker-free, Dicamba-tolerant soybean MON 87708.

In this study, Southern blot analyses determined the copy number of T-DNA I sequences and the absence of the *cp4 epsps* expression cassette (T-DNA II) and transformation vector backbone sequences in the genome of MON 87708. The data indicate that MON 87708 contains one copy of the insert at a single integration locus and all expression elements are present in the T-DNA I insert. These data also demonstrate that MON 87708 does not contain detectable T-DNA II sequences other than the *E9* 3′ non-translated region identical with the same region in T-DNA I, or backbone sequences from plasmid PV-GMHT4355. The complete DNA sequence of the insert and adjacent DNA sequence in MON 87708 confirmed the integrity of the inserted *dmo* expression cassette within the inserted sequences and identified the 5' and 3' insert-to-flank DNA junctions. Additional analysis of the insertion site in conventional soybean and MON 87708 determined that an 899 bp deletion and two insertions of 128 bp and 35 bp occurred at the site of insertion during the formation of MON 87708. Furthermore, Southern blot fingerprint analysis demonstrated that the insert in MON 87708 has been maintained through five generations of breeding, thereby confirming the stability of the insert over multiple generations.

2.0 INTRODUCTION

2.1 Background

Monsanto Company has developed dicamba-tolerant soybean MON 87708 that produces the dicamba mono-oxygenase (DMO) protein from the *dmo* gene derived from *Stentrophomonas maltophilia*. The DMO protein deactivates dicamba to form

3,6-dichloro-2-hydroxybenzoic acid (DCSA), a non-herbicidal salicylic acid derivative, thereby allowing in-crop applications of dicamba herbicide to MON 87708 for the control of broad-leaf weeds, including glyphosate-resistant broad-leaf weeds. MON 87708 was developed through *Agrobacterium*-mediated transformation of a conventional soybean variety with the transformation plasmid PV-GMHT4355 which contains two separate T-DNAs. The T-DNAs can segregate through conventional breeding and allow the isolation of those plants that contain the *dmo* expression cassette but do not contain the *cp4 epsps* expression cassette thus producing the marker-free, dicamba-tolerant soybean MON 87708.

2.2 Purpose

The purpose of this study was to characterize the integrated DNA in MON 87708. Genomic DNA from MON 87708 was analyzed using Southern blot and PCR analyses. The insert and copy number of the inserted *dmo* expression cassette (T-DNA I) and the presence or absence of the *cp4 epsps* expression cassette (T-DNA II) and plasmid backbone sequences were evaluated by Southern analyses. Southern analyses were also performed on five generations of MON 87708 to confirm the stability of the inserted DNA in MON 87708. The DNA sequence and integrity of the insert and adjacent DNA sequences were determined by PCR and DNA sequence analyses. In addition, PCR and DNA sequence analyses were performed on the conventional soybean A3525 and MON 87708 to examine the DNA insertion site in MON 87708.

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance is MON 87708. Genomic DNA was extracted from leaf tissue harvested from production plan 07-01-B4-01, which was generated from seed lot GLP-0707-18882-S. The DNA was used for Southern blot and PCR analyses in this study.

Generation[2]	Production Plan	Seed Lot Number	Virgo Number	Sample ID
R_3	07-01-B4-01	GLP-0707-18882-S	60083541687	0701B401-00015

Additional samples from the conventional breeding of MON 87708 were used to assess the stability of test substance across generations. Genomic DNA extracted from the

[2] See Figure 14.

leaf tissues listed below was used for Southern blot analyses in this study.

Generation	Production Plan	Orion[3] ID	Source ID	Container ID
R_2	PPN-08-179	10002634	10002226	10002634-003
R_4	PPN-08-179	10002635	10002227	10002635-001
R_5	PPN-08-179	10002636	10002228	10002636-001
R_6	PPN-08-179	10002637	10002229	10002637-001

3.2 Control Substance

The control substance is conventional soybean variety A3525, which has the same genetic background as the test substance. Genomic DNA was extracted from leaf tissue harvested from production plan 07-01-B4-01, which was generated from seed lot GLP-0707-18884-S. The DNA was used for Southern blot and PCR analyses in this study.

Production Plan	Seed Lot Number	Virgo Number	Sample ID
07-01-B4-01	GLP-0707-18884-S	60083545902	0701B401-00002

3.3 Reference Substances

The reference substance was plasmid PV-GMHT4355 (Figure 1), which was used as the transformation vector to develop MON 87708. Plasmid PV-GMHT4355 DNA was isolated prior to the study and its identity was confirmed by restriction enzyme digestion. Digested plasmid and/or probe templates generated from this plasmid were used as positive hybridization controls in Southern analyses. As additional references, the 1 kb DNA extension ladder and λ DNA/*Hin*d III fragments from Invitrogen (Carlsbad, CA) were used for size estimations on agarose gels for Southern analyses. The GeneRuler 1 kb Plus DNA ladder from Fermentas (Hanover, MD) was used for size estimations on agarose gels for PCR analyses.

3.4 Characterization of Test, Control and Reference Substances

The identity of the test substance, including the leaf materials from additional generations of the test substance, and the control substances was determined by event-specific PCR prior to use in the study. The Study Director reviewed the chain-of-custody documentation for the test and control substances prior to use of the materials in the study and the raw data was archived with the study. Genomic DNA extracted from those materials was stored in a 4°C refrigerator or a -20°C freezer. The test, control, and reference substances were considered stable if they did not seem degraded on ethidium bromide stained agarose gels and/or if they yielded

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

interpretable signals on Southern blots and/or produced specific PCR products.

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, except that the amount of processed leaf tissue was scaled up and the volumes of reagents used were scaled up accordingly. This was acknowledged in the raw data as a SOP deviation. All extracted DNA was stored in a 4°C refrigerator or a -20°C freezer.

3.6 Quantification of DNA

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

3.7 Restriction Enzyme Digestion of Genomic DNA

Approximately 10 µg of genomic DNA extracted from the test and control substances were digested with appropriate combinations of restriction enzymes *Bsp1286* I /*Pvu* II or *Hpa* I /*Kpn* I (New England Biolabs, Inc.) according to SOP BR-ME-0316-01. All digests were conducted in 1X NEBuffer 4 (New England Biolabs, Inc.) plus 1X BSA (New England Biolabs, Inc.) at 37°C in a total volume of ~500 µl with ~50 units of each restriction enzyme. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from the control substance was digested with the combination of *Hpa* I/*Kpn* I or *Bsp1286* I /*Pvu* II restriction enzymes and the appropriate positive hybridization control(s) were added to these digests.

3.8 Agarose Gel Electrophoresis

Digested DNA was resolved on ~0.8% (w/v) agarose gels according to SOP BR-ME-0315-02. Individual digests of the test and control DNA were loaded on the same gel in a long-run/short-run format. For the long-run format, a set of digested genomic DNA, including the conventional and MON 87708 genomic DNA digested with *Bsp1286* I /*Pvu* II or *Hpa* I /*Kpn* I, was loaded onto an agarose gel along with an appropriate DNA ladder and subjected to electrophoresis at an appropriate voltage. The electrophoresis was stopped when the blue dye from the 10X BlueJuice Gel Loading Buffer (Invitrogen, Carlsbad, CA) had migrated approximately halfway through the gel and then the second set of the digested genomic DNA along with an appropriate DNA ladder was loaded. This was referred to as the short-run format. The electrophoresis for both runs was carried out until the blue dye from the short-run lanes reached about the middle of the gel. The long-run allows for greater resolution of large molecular weight DNA, whereas the short-run allows for the detection of small molecular weight DNA. For the insert stability analysis, individual digests of genomic DNA extracted from leaf tissue across multiple generations were loaded on the agarose

gel in a single short-run format. The positive hybridization controls were only run in the short-run format.

3.9 DNA Probe Preparation for Southern Blot Analyses

Probe templates were prepared by PCR amplification from plasmid PV-GMHT4355 according to SOP BR-ME-0486-01. Approximately 25 ng of each probe template were radiolabeled with ^{32}P-deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) or ^{32}P-deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA) according to SOP BR-ME-0611-01. Probe locations relative to the genetic elements in plasmid PV-GMHT4355 are depicted in Figure 1.

3.10 Southern Blot Analyses of Genomic DNA

Digested genomic DNA isolated from the test and control substances was evaluated using Southern blot analyses according to SOP BR-ME-0317-02. The PV-GMHT4355 plasmid DNA digested with *Aat* II/*Nde* I was added to conventional soybean genomic DNA pre-digested with *Hpa* I/*Kpn* I or *Bsp1286* I /*Pvu* II to serve as positive hybridization controls. When multiple probes were hybridized simultaneously to one Southern blot, the appropriate probe templates were mixed with pre-digested conventional soybean genomic DNA to serve as additional positive hybridization controls. The digested DNA was then separated by agarose gel electrophoresis and transferred onto a membrane. Southern blots were hybridized and washed at 55°C, 60°C, or 65°C, depending on the melting temperature of the probes. The table below lists the radiolabeling conditions and hybridization temperatures of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film in conjunction with one or two Kodak Biomax MS intensifying screen(s) in a -80°C freezer. It was acknowledged in the raw data as a SOP deviation when using two Kodak Biomax MS intensifying screens.

Probe Number	Probe Type	Probe Name	Labeling Method	Probe labeled with dNTP (^{32}P)	Hybridization Temperature (°C)
1	Backbone Probe	B1	RadPrime	dCTP	60
2	Backbone Probe	B2	RadPrime	dCTP	60
3	Backbone Probe	B3	RadPrime	dCTP	60
4	T-DNA II Probe	TII-1	RadPrime	dATP	55
5	T-DNA II Probe	TII-2	RadPrime	dCTP	60
6	T-DNA II Probe	TII-3	RadPrime	dATP	55
7	Backbone Probe	B4	RadPrime	dCTP	60
8	T-DNA I Probe	TI-1	RadPrime	dATP	60
9	T-DNA I Probe	TI-2	RadPrime	dCTP	65
10	T-DNA I Probe	TI-3	RadPrime	dATP	55

3.11 PCR and Sequence Analyses to Examine the Insert and Flanking Sequences in MON 87708

Overlapping PCR products that span the insert and adjacent 5' and 3' flanking DNA sequences in MON 87708 (Figure 10) were generated. These products were sequenced to determine the nucleotide sequence of the insert in MON 87708 as well as the nucleotide sequence of the DNA flanking the 5' and 3' ends of the insert.

The PCR analyses were performed according to SOP BR-ME-0486-01. The PCR analyses were conducted using 50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 1 M Betaine, 1 mM $MgSO_4$, 0.8 µM of each primer, 0.2 mM of each dNTP, and 0.5 units of KOD Hot Start DNA polymerase (Novagen, Madison, WI). The amplification of Product A (Figure 10) was performed under the following cycling conditions: 1 cycle at 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60.2°C for 45 seconds, 72°C for 5 minutes; 1 cycle at 72°C for 10 minutes. The amplification of Product B (Figure 10) was performed under the following cycling conditions: 1 cycle at 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60.8°C for 45 seconds, 72°C for 5 minutes; 1 cycle at 72°C for 10 minutes.

Following PCR amplification, exonuclease I (Exo)/ shrimp alkaline phosphatase (SAP) purification of the PCR products used for sequencing was performed in a 21 µl reaction volume containing 15 µl of the PCR product and a final concentration of 0.1 units/µl of Exo (US-Biochemicals, Cleveland, OH) and 0.1 units/µl of SAP (US-Biochemicals, Cleveland, OH). The reaction was incubated at 37ºC for 15 minutes, followed by 80ºC for an additional 15 minutes.

Prior to sequencing, aliquots of untreated and Exo/SAP-treated PCR product were separated on 0.8 % (w/v) agarose E-gels (Invitrogen, Carlsbad, CA) and visualized by ethidium bromide staining to verify that the products were of the expected size. The PCR products were sequenced using multiple primers including primers used for PCR amplification and primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry (ABI, Foster City, CA).

3.12 PCR and DNA Sequence Analyses to Examine the DNA Insertion Site in MON 87708

To examine the DNA insertion site in MON 87708, PCR analyses were performed on genomic DNA from both MON 87708 and the conventional soybean control. The primers used in this analysis were designed from the genomic DNA sequences flanking the insert in MON 87708 (Figure 12). 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 analysis was conducted using approximately 50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 1 M Betaine, 1 mM $MgSO_4$, 0.8 µM of each primer, 0.2 mM of each dNTP, and 0.5 units of KOD Hot Start DNA polymerase. The amplification of the product was performed under the following cycling conditions: 1 cycle at 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60.2°C for 45 seconds, 72°C for 5 minutes; 1 cycle at 72°C for 10 minutes.

Following PCR amplification, Exo/SAP purification of the PCR products used for sequencing was performed in a 21 µl reaction volume containing 15 µl of the PCR product and a final concentration of 0.1 units/µl of Exo and 0.1 units/µl of SAP. The reaction was incubated at 37ºC for 15 minutes, followed by 80ºC for an additional 15 minutes.

Prior to sequencing, aliquots of untreated and Exo/SAP-treated PCR product were separated on 0.8 % (w/v) agarose E-gels and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing (Figure 12). The PCR products were sequenced using multiple primers, including primers used for PCR amplification and primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry.

3.13 Data Rejected or Not Reported

Some Southern blot analyses conducted as part of this study were not reported because the results obtained were not of report quality. No Southern analysis data was rejected during the Southern blot analysis of MON 87708. PCR analyses were rejected if the amplification was not sufficient for sequencing or if the expected amplification product was not produced. Sequencing electropherograms were rejected if they were of unacceptable quality, particularly with respect to peak shape and intensity. Nothing in the "Data Rejected" or "Data Not Reported" was inconsistent with the conclusions presented in this report.

3.14 Changes to the Study Protocol

During the course of the study, three changes to the original protocol were required. These changes were documented as protocol amendments and are summarized below. None of these changes negatively impacted the study.

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

2. Leaf materials from different generations of the test substance MO N87708 for the generational stability analysis were finalized after the protocol was signed. The protocol was amended to include those leaf materials and clarify the study procedures used for the Southern blot analysis.

3. The protocol was amended to change the Study Director.

4.0 RESULTS AND DISCUSSION

Genomic DNA from MON 87708 was analyzed using Southern blot analyses and sequencing to characterize the insert and adjacent DNA in MON 87708. Southern analyses examined the MON 87708 genome for the number of insertions and copies of T-DNA I and the presence or absence of all the other sequences from PV-GMHT4355, including T-DNA II and backbone sequences. Southern analysis also examined the stability of the integrated DNA in MON 87708 in multiple generations produced by conventional breeding. PCR and DNA sequence analyses confirmed the intactness of all genetic elements within the *dmo* expression cassette and examined the organization of the DNA insertion site in MON 87708.

4.1 Southern Blot Analyses to Determine the Insert and Copy Number of T-DNA I and the Presence or Absence of T-DNA II and Plasmid PV-GMHT4355 Backbone Sequences in MON 87708

Southern blot analysis was used to characterize the insert present in MON 87708. Genomic DNA from MON 87708 was digested with appropriate restriction enzymes and subjected to Southern blot analyses utilizing probes that cover the entire sequence of the transformation vector. The probes used allow for a determination of the copy number of all sequences from PV-GMHT4355. Therefore, it can be determined if any elements within the insert are present at multiple loci or if any T-DNA II or backbone sequences are present in MON 87708. A map of the plasmid vector PV-GMHT4355, which is annotated with the probes used in the Southern analyses, is presented in Figure 1. A description of the genetic elements within the plasmid PV-GMHT4355 and MON 87708 is shown in Table 1 and Table 2, respectively. A linear map depicting the restriction sites within the insert DNA sequence, as well as within the DNA sequences immediately flanking the insert in MON 87708 is shown in Figure 2. A summarization of the expected band sizes for Southern analyses is presented in Table 3. The molecular weight markers on the left side of the figures were used for estimating the sizes of bands present in the long-run lanes of Southern blots. The molecular weight markers on the right side of the figures were used for estimating the band sizes present in the short-run lanes. In most of the Southern blots, the migration of the genomic DNA is slightly different when compared to the migration of the molecular weight markers and, in some instances, there are slight migration differences between different DNA preparations. These altered migrations

are likely the result of a difference in salt concentrations between the genomic DNA samples and the molecular weight marker (Sambrook and Russell, 2001). The Southern blots (Figures 3–9 and 15) presented in this report are representative of the data generated in the study.

4.1.1 Southern Blot Analyses to Confirm the Insert and Copy Number of T-DNA I in MON 87708

The number of copies and insertion sites of T-DNA I sequences in the soybean genome were evaluated by digesting the test and control genomic DNA samples with two sets of restriction enzymes, *Bsp1286* I /*Pvu* II and *Hpa* I /*Kpn* I. The combination of *Bsp1286* I/*Pvu* II restriction enzymes cleaves once within the inserted DNA and within the known DNA flanking the 5' and 3' ends of the insert (Figure 2). The combination of *Hpa* I/*Kpn* I restriction enzymes cleaves once within the inserted DNA and within the known DNA flanking the 3' end of the insert (Figure 2). The enzymes used generate restriction fragments containing T-DNA I and adjacent flanking DNA so that each insert would generate a unique banding pattern. If T-DNA I sequences are present at a single integration site in MON 87708, then probing with the sequence from T-DNA I should result in the hybridization bands as described in Figure 2 and Table 3. Any additional integration sites would be detected as additional bands. Each blot was hybridized with one of the overlapping T-DNA I probes taken together spanning the entire inserted DNA sequence (Figure 1, Probes 8, 9, and10). Each Southern blot contained several controls. Genomic DNA isolated from the conventional soybean, A3525, was used as a negative control to determine if the probes hybridized to any endogenous sequences. Conventional soybean genomic DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid DNA pre-digested with *Aat* II/*Nde* I was used as a positive hybridization control and to demonstrate sensitivity of the Southern blots. The results of these analyses are shown in Figures 3, 4, and 5.

4.1.1.1 Probe 8

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 3, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 3, lanes 3 and 7) and hybridized with Probe 8 (Figure 1) produced no detectable hybridization bands as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced a band at ~7.4 kb (Figure 3, lanes 10 and 11) with the ~1 and ~1/10 genomic equivalent spikes, respectively. The band is consistent with the expected band (Figure 1). Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II and hybridized with Probe 8 (Figure 1) produced the expected band at ~2.6 kb (Figure 2; Figure 3, lanes 2 and 6). MON 87708 DNA digested with *Hpa* I/*Kpn* I and hybridized with Probe 8

(Figure 1) produced a unique band at ~5.6 kb (Figure 3, lanes 4 and 8), which is consistent with the expected >2.7 kb band (Figure 2). The results presented in Figure 3 indicate that the sequences covered by Probe 8 reside at a single detectable locus of integration as a single copy in MON 87708.

4.1.1.2 Probe 9

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 4, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 4, lanes 3 and 7) and hybridized with Probe 9 showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced a band at ~7.4 kb (Figure 4, lanes 10 and 11) with the ~1 and ~1/10 genomic equivalent spikes, respectively. The band is consistent with the expected band (Figure 1). Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II and hybridized with Probe 9 (Figure 1) produced the expected bands at ~1.5 kb and ~2.6 kb (Figure 2; Figure 4, lanes 2 and 6). MON 87708 DNA digested with *Hpa* I/*Kpn* I and hybridized with Probe 9 (Figure 1) produced two bands at ~1.7 kb and ~5.6 kb, which are consistent with the expected ~1.7 kb and >2.7 kb bands (Figure 2). The results presented in Figure 4 indicate that the sequences covered by Probe 9 reside at a single detectable locus of integration as a single copy in MON 87708.

4.1.1.3 Probe 10

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 5, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 5, lanes 3 and 7) and hybridized with Probe 10 showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced two bands at ~4.0 kb and ~7.4 kb (Figure 5, lanes 10 and 11) with the ~1 and ~1/10 genomic equivalent spikes, respectively. These bands are consistent with expected bands (Figure 1). Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II and hybridized with Probe 10 (Figure 1) produced the expected band at ~1.5 kb (Figure 2; Figure 5, lanes 2 and 6). MON 87708 DNA digested with *Hpa* I/*Kpn* I and hybridized with Probe 10 (Figure 1) produced the expected band at ~1.7 kb (Figure 2; Figure 5, lanes 4 and 8). The results presented in Figure 5 indicate that the sequences covered by Probe 10 reside at a single detectable locus of integration as a single copy in MON 87708.

The results presented in Figures 3, 4, and 5 indicate that the T-DNA I sequence, which is covered by the three overlapping probes (Figure 1, Probes 8, 9 and 10), resides at a single detectable locus of integration as a single copy in MON 87708.

4.1.2 Southern Blot Analyses to Determine the Presence or Absence of T-DNA II Sequences in MON 87708

The presence or absence of T-DNA II sequences in MON 87708 genome was evaluated by digesting the test and control genomic DNA samples with two sets of restriction enzymes, *Bsp1286* I/*Pvu* II and *Hpa* I/*Kpn* I. Each blot was hybridized with one of the three overlapping T-DNA II probes spanning the T-DNA II sequence other than the two border regions (Figure 1, Probes 4, 5 and 6). The border sequences of T-DNA II share 100% identity to those of T-DNA I and were previously covered by probes 8 and 10, thus, not included in the T-DNA II probes. If T-DNA II sequences are present in MON 87708, then probing with the T-DNA II sequences should result in hybridizing bands. The results of this analysis are shown in Figures 6, 7, and 8. Each Southern blot contains the same controls as described in Section 4.1.1.

4.1.2.1 Probe 4

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 6, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 6, lanes 3 and 7) and hybridized with Probe 4 showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced two bands at ~4.0 kb and ~7.4 kb (Figure 6, lanes 10 and 11) with the ~1 and ~1/10 genomic equivalent spikes, respectively. These bands are consistent with the two expected bands (Figure 1). Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II and hybridized with Probe 4 (Figure 1) produced one unique band at ~1.5 kb (Figure 6, lanes 2 and 6). MON 87708 DNA digested with *Hpa* I/*Kpn* I and hybridized with Probe 4 (Figure 1) produced one unique band at ~1.7 kb (Figure 6, lanes 4 and 8). Probe 4 contains the *E9* 3′ non-translated region sequence that is also contained in T-DNA I and covered by Probe 10 (Figure 1). Therefore, Probe 4 was expected to hybridize to the ~1.5 kb and ~1.7 kb fragments (Figure 2) derived from the T-DNA I insert by digesting with *Bsp1286* I/*Pvu* II and *Hpa* I/*Kpn* I, respectively. Those bands were also detected by Probe 10 (Figure 5, lanes 2 and 6, and lanes 4 and 8). Any T-DNA II sequences other than those associated with T-DNA I would be detected as novel bands. No unexpected bands were detected indicating that MON 87708 contains no detectable T-DNA II elements covered by Probe 4.

4.1.2.2 Probe 5

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 7, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 7, lanes 3 and 7) and hybridized with Probe 5 (Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced two bands at ~4.0 kb and ~7.4 kb (Figure 7, lanes 10 and 11) with the ~1 and ~1/10 genomic equivalent spikes, respectively. These bands are consistent with the two expected bands (Figure 1). Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II (Figure 7, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure 7, lanes 4 and 8) and hybridized with Probe 5 produced no detectable hybridization bands. These results indicate that MON 87708 contains no detectable T-DNA II elements covered by Probe 5.

4.1.2.3 Probe 6

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 8, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure 8, lanes 3 and 7) and hybridized with Probe 6 (Figure 1) showed no detectable hybridization bands, as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced a unique band at ~7.4 kb (Figure 8, lanes 10 and 11) with the ~1 and ~1/10 genomic equivalent spikes, respectively. The band is consistent with the expected band (Figure 1). Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II (Figure 8, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure 8, lanes 4 and 8) and hybridized with Probe 6 produced no detectable hybridization bands. These results indicate that MON 87708 contains no detectable T-DNA II elements covered by Probe 6.

The results presented in Figures 6, 7, and 8 indicate that there is no T-DNA II element other than those associated with T-DNA I present in the genome of MON 87708.

4.1.3 Southern Blot Analysis to Determine the Presence or Absence of Plasmid PV-GMHT4355 Backbone Sequences in MON 87708

The presence or absence of the plasmid PV-GMHT4355 backbone sequences in MON 87708 soybean genome was evaluated by digesting the test and control genomic DNA with two sets of restriction enzymes, *Bsp1286* I/*Pvu* II and *Hpa* I/*Kpn* I, and hybridizing the blot simultaneously with three overlapping

backbone probes spanning the main backbone sequence (Figure 1, Probes 1, 2, and 3) and one individual probe covering the backbone sequence between T-DNA I and T-DNA II (Figure 1, Probe 7). If backbone sequences are present in MON 87708, then probing with backbone probes should result in hybridizing bands. The results of this analysis are shown in Figure 9.

4.1.3.1 Probes 1, 2, 3, and 7

Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II (Figure 9, lanes 1 and 5) or *Hpa* I /*Kpn* I (Figure 9, lanes 3 and 7) and hybridized simultaneously with the four probes (Figure 1, Probes 1, 2, 3, and 7) spanning the entire backbone sequence of PV-GMHT4355 showed no detectable hybridization bands, as expected for the negative control. Conventional soybean DNA digested with *Hpa* I/*Kpn* I and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced two bands at ~ 4.0 kb and ~7.4 kb (Figure 9, lane 10) with the ~1 genomic equivalent spike. These bands are consistent with the two expected bands (Figure 1). In addition, there are two faint hybridization bands at ~ 4.5 kb and ~11 kb (Figure 9, lane 10). The ~4.5 kb bands was likely due to an artifact that occurred during the electrophoresis, and the ~11 kb band was likely due to undigested plasmid DNA or an artifact that occurred during the electrophoresis. Since these faint bands appeared only in the plasmid spike and the expected bands were observed, they have no negative impact on the conclusions made from this blot. Pre-digested conventional soybean DNA spiked with probe templates of Probes 1, 2, 3, and 7 (Figure 1) produced the expected bands at ~0.2 kb, ~0.9 kb, ~1.5 kb, and ~1.8 kb (Figure 9, lane 11) when ~1 genomic equivalent of the probe templates were spiked. When ~1/10 genomic equivalent of those probe templates were spiked, the expected bands at ~0.9 kb, ~1.5 kb, and ~1.8 kb were observed (Figure 9, lane 12), but not the one at ~0.2 kb on the reported blot (Figure 9, lane 12) due to less radioactive materials associated with it than with the bigger bands. The 0.2 kb band was observed on the same blot with a longer exposure (data not shown). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87708 DNA digested with *Bsp1286* I/*Pvu* II (Figure 9, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure 9, lanes 4 and 8) and hybridized with Probes 1, 2, 3, and 7 produced no detectable bands. The data indicates that MON 87708 contains no detectable backbone elements from the transformation vector PV-GMHT4355.

4.2 Organization and Sequence of the Insert and Adjacent DNA in MON 87708

The organization of the elements within the MON 87708 insert was confirmed by amplifying and sequencing two overlapping regions of DNA that span the entire length of the insert and the associated flanking DNA. The positions of the PCR products relative to the insert, as well as the results of the PCR analyses, are shown in Figure 10.

As expected, no product was obtained with either the control reactions containing no template DNA (Figure 10, lanes 4 and 7) or the conventional soybean DNA template (Figure 10, lanes 2 and 5) with any of the primer sets. The reactions containing genomic DNA from MON 87708 produced the expected bands at ~ 3.2 kb (Figure 10, lane 3) and ~ 3.0 kb (Figure 10, lane 6).

To determine the sequence of the insert in MON 87708 and adjacent DNA flanking the insert, the PCR product A (Figure 10, lane 3) and product B (Figure 10, lane 6) were subjected to DNA sequencing. The consensus sequence representing the insert in MON 87708, including the DNA flanking the ends of the insert, is shown in Figure 11 and is described in Table 2. This consensus sequence was generated by compiling multiple sequencing reactions performed on the overlapping PCR products which spanned the length of the insert and the 5' and 3' flanking DNA. The amplification and sequencing of the insert and flanking DNA from MON 87708 establishes that the arrangement and linkage of elements in the insert are consistent with those in plasmid PV-GMHT4355 (Figure 1) and are as depicted in Figure 2.

This analysis determined that the DNA sequence of the MON 87708 insert is 3003 base pairs long (Figure 11, bases 1049-4051, sequence not underlined), beginning at base 8604 in the Right Border region and ending at base 254 in the Left Border region of PV-GMHT4355 (Figure 1). A1048 base pair DNA sequence flanking the 5' end of the insert (Figure 11, bases 1-1048, sequence single-underlined) and a 1271 base pair DNA sequence flanking the 3' end of the insert (Figure 11, bases 4052-5322, sequence double-underlined) were also determined. The sequence confirmed the presence and organization of the integrated genetic elements as described in Table 2 and depicted in Figure 2.

4.3 Organization of the DNA Insertion Site in MON 87708

PCR and sequence analysis were performed on genomic DNA extracted from MON 87708 and conventional soybean control, A3525, to examine the DNA insertion site in MON 87708. The PCR was performed with one primer specific to the DNA sequence flanking the 5' end of the insert (Figure 12, Primer A) paired with a second primer specific to the DNA sequence flanking the 3' end of the insert (Figure 12, Primer B). The results of the PCR analysis are shown in Figure 12. The control

PCR containing no template DNA did not generate a PCR product (Figure 12, lane 4), as expected. The reaction using the conventional genomic DNA as template generated a PCR product at ~3.3 kb (Figure 12, lane 2). The reaction using the MON 87708 genomic DNA as template generated a PCR product at ~5.6 kb (Figure 12, lane 3), as expected.

The ~3.3 kb PCR product generated from the conventional genomic DNA was sequenced and the resulting data are shown in Figure 13. A comparison between the sequence generated from MON 87708 (Figure 11) and the sequence generated from the conventional soybean (Figure 13) was conducted. Bases 1-920 of the MON 87708 genomic DNA sequence at the 5' end of the insert (Figure 11) are identical to bases 9-928 of the conventional soybean genomic DNA (Figure 13, sequence single-underlined). Bases 4087-5322 of the MON 87708 genomic DNA sequence at the 3' end of the insert (Figure 11) are identical to bases 1828-3063 of the conventional soybean genomic DNA (Figure 13, sequence double-underlined). There was an 899 bp deletion (Figure 13, bases 929-1827), a 128 bp insertion (Figure 11, bases 921-1048) just 5' to the MON 87708 insert, and a 35 bp insertion (Figure 11, bases 4052-4086) just 3' to the MON 87708 insert. These most likely occurred in MON 87708 upon insertion of the T-DNA (Salomon and Puchta, 1998).

This analysis determines that an 899 bp (Figure 13) deletion and two insertions of 128 bp and 35 bp (Figure 11) occurred at the site of insertion during the formation of MON 87708.

4.4 Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87708

In order to demonstrate the stability of MON 87708 through multiple generations, Southern blot analysis was performed using DNA obtained from five generations of MON 87708. For reference, the breeding history of MON 87708 is presented in Figure 14. The specific generations tested are indicated in the legends of Figure 15. The R_3 generation was used for the molecular characterization analyses shown in Figures 3 through 9. To analyze stability, four additional generations were evaluated by Southern analysis and compared to the R_3 generation. Genomic DNA, isolated from each of the selected generations of MON 87708, was digested with the restriction enzymes *Bsp1286* I/*Pvu* II (Figure 2) and hybridized with Probe 9 (Figure 1), which was designed to detect both fragments generated by the *Bsp1286* I/*Pvu* II digestion. Any instability associated with the insert would be detected as extra bands within the fingerprint on the Southern blot. The results are shown in Figure 15. The Southern blot has the same controls as described in Section 4.1.1.

4.4.1 Probe 9

Conventional soybean DNA digested with restriction enzymes *Bsp1286* I/*Pvu* II produced no hybridization signals (Figure 15, Lane 1) as expected for the negative control. Conventional soybean DNA digested with *Bsp1286* I/*Pvu* II and spiked with plasmid PV-GMHT4355 DNA, previously digested with *Aat* II/*Nde* I (Figure 1), produced a band at ~7.4 kb (Figure 15, lanes 8 and 9) with the ~1 and ~1/10 genomic equivalent spikes, respectively. The band is consistent with the expected band (Figure 1). Additionally, there were two very faint hybridization bands in the ~1 genomic equivalent plasmid spike at ~4.3 kb and ~6.5 kb observed in a longer exposure of the Southern blot illustrated in Figure 15 (data not shown). These bands were likely due to an artifact that occurred during the electrophoresis or star activity of the enzymes used to digest the plasmid or conventional genomic DNA. Since these faint bands appeared only in the plasmid spike and the expected ~7.4 kb band was observed, they do not have any negative impact on the conclusions from this Southern blot analysis. Detection of the spiked controls indicates that the probe is recognizing its target sequences.

MON 87708 genomic DNA digested with *Bsp1286* I/*Pvu* II and hybridized with Probe 9 (Figure 1) is expected to produce two bands at ~1.5 kb and ~2.6 kb (Figure 2 and Table 3). The fingerprint of the Southern signals from multiple generations, R_2, R_4, R_5, and R_6 (Figure 15, lanes 2, 4, 5, and 6), of MON 87708 is consistent with that from the fully characterized generation R_3 (Figure 15, lane 3), indicating that MON 87708 contains one copy of T-DNA I residing at a single locus of integration that is stable across the selected generations.

5.0 CONCLUSIONS

Molecular characterization of MON 87708 by Southern blot analyses demonstrated that the introduced DNA was inserted into the soybean genome at a single locus and contains one intact copy of T-DNA I. All expression elements are present in the inserted DNA and no additional elements were detected other than those associated with the respective insert. No T-DNA II sequences were detected other than those associated with T-DNA I. No plasmid backbone sequences were detected. Southern analysis of multiple MON 87708 generations demonstrated that the introduced DNA was stably maintained across the generations tested.

PCR and DNA sequence analyses were performed on MON 87708, 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 DNA sequences in MON 87708, and determined that an 899 bp deletion and two insertions of 128 bp and 35 bp occurred at the site of insertion during the formation of MON 87708.

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Table 1. Summary of Genetic Elements in Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
T-DNA I		
B[1]-Right Border	8290-8646	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening sequence	8647-8691	Sequence used in DNA cloning
P[2]-*PC1SV*	8692-9124	Promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) (Maiti and Shepherd, 1998) that directs transcription in plant cells
Intervening sequence	9125-9144	Sequence used in DNA cloning
L[3]-*TEV*	9145-9276	5' non-translated region from the Tobacco Etch virus (*TEV*) genome (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening sequence	9277	Sequence used in DNA cloning
TS[4]-*RbcS*	9278-9520	Sequences encoding the transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport to the DMO protein to the chloroplast
Intervening Sequence	9521-9529	Sequence used in DNA cloning
CS[5]-*dmo*	9530-10552	Coding sequence for the dicamba mono-oxygenase from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	10553-10620	Sequence used in DNA cloning
T[6]-*E9*	10621-11263	3′ non-translated region from the *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	11264-11352	Sequence used in DNA cloning
B-Left Border	1-442	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Vector Backbone (Not present in MON 87708)		
Intervening Sequence	443-528	Sequence used in DNA cloning
OR[7]-*ori V*	529-925	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	926-1662	Sequence used in DNA cloning

B[1]-Border; P[2]-Promoter; L[3]-Leader; TS[4]-Targeting Sequence; CS[5]-Coding Sequence; T[6]-3' non-translated transcriptional termination sequence and polyadenylation signal sequences; OR[7]-Origin of Replication

Table 1. Continued

CS-*rop*	1663-1854	Coding sequence for repressor of primer protein derived from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	1855-2281	Sequence used in DNA cloning
OR-*ori-pBR322*	2282-2870	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	2871-3400	Sequence used in DNA cloning
aadA	3401-4289	Bacterial promoter, coding and 3′ UTR sequences for an aminoglycoside-modifying enzyme, 3″ (9)-O-nucleotidyltransferase from transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	4290-4384	Sequence used in DNA cloning
T-DNA II (Not present in MON 87708)		
B-Left Border	4385-4795	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	4796-4809	Sequence used in DNA cloning
T-*E9*	4810-5452	3′ non-translated sequence from *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	5453-5458	Sequence used in DNA cloning
CS-*cp4 epsps*	5459-6826	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Padgette et al., 1996; Barry et al., 1997)
TS-*CTP2*	6827-7054	Sequences encoding the chloroplast transit peptide region from the *shkG* gene of *Arabidopsis thaliana* encoding EPSPS (Klee et al., 1987; Herrmann,1995) that directs transport of the CP4 EPSPS protein to the chloroplast
Intervening Sequence	7055-7063	Sequence used in DNA cloning
L-*DnaK*	7064-7159	5' non-translated leader sequence from the *Petunia hybrida Hsp70* gene (Rensing and Maier, 1994) that is involved in regulating gene expression
Intervening Sequence	7160-7162	Sequence used in DNA cloning
P-*FMV*	7163-7714	Promoter for the 35S RNA from figwort mosaic virus (*FMV*) (Rogers, 2000) that directs transcription in plant cells
Intervening Sequence	7715-7761	Sequence used in DNA cloning
B-Right Border	7762-8118	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector Backbone (Not present in MON 87708)		
Intervening Sequence	8119-8289	Sequence used in DNA cloning

Table 2. Summary of Genetic Elements in MON 87708

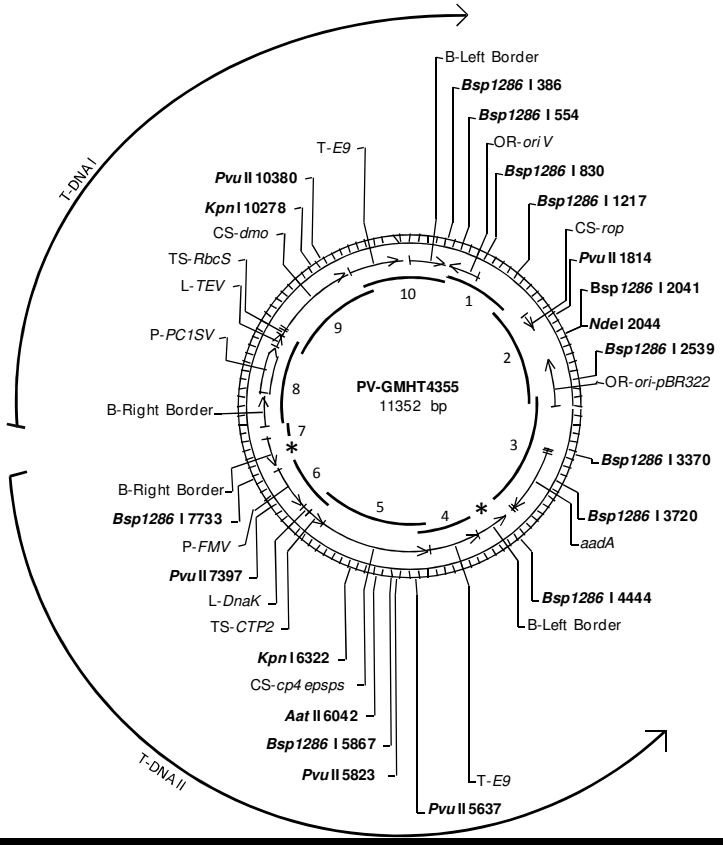
Genetic Element	Location *	Function (Reference)
5' flanking Sequences	1-1048	DNA sequence adjacent to the 5' end of the insertion site
B[1]-Right Border **	1049-1091	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening sequence	1092-1136	Sequence used in DNA cloning
P[2]-*PC1SV*	1137-1569	Promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) (Maiti and Shepherd, 1998) that directs transcription in plant cells
Intervening sequence	1570-1589	Sequence used in DNA cloning
L[3]-*TEV*	1590-1721	5' non-translated region from the Tobacco Etch virus (*TEV*) genome (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening sequence	1722-1722	Sequence used in DNA cloning
TS[4]-*RbcS*	1723-1965	Sequences encoding the transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport to the DMO protein to the chloroplast
Intervening Sequence	1966-1974	Sequence used in DNA cloning
CS[5]-*dmo*	1975-2997	Coding sequence for the dicamba mono-oxygenase from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	2998-3065	Sequence used in DNA cloning
T[6]-*E9*	3066-3708	3′ non-translated region from the *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	3709-3797	Sequence used in DNA cloning
B-Left Border**	3798-4051	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
3' Flanking Sequences	4052-5322	DNA sequence adjacent to the 3' end of the insertion site

* Numbering in the second column refers to the sequence reported in Figure 11.
**These borders are truncated.
B[1]-Border; P[2]-Promoter; L[3]– Leader; TS[4]-Targeting Sequence; CS[5]-Coding Sequence; T[6]-3' non-translated transcriptional termination sequence and polyadenylation signal sequences

Table 3. Summary of the Expected Band Sizes (kb) on Southern Blots Based on Restriction Enzymes and Probes Used in MON 87708 Analysis

Southern Blot Figure		3	4	5	6	7	8	9	15
Probe Used		8	9	10	4	5	6	1, 2, 3, and 7	9
Probing Target	**Digestion Enzyme**	**Expected Band Sizes (kb) on Each Southern Blot**							
Plasmid PV-GMHT4355	***Aat* II /*Nde* I**	~7.4	~7.4	~4.0 ~7.4	~4.0 ~7.4	~4.0 ~7.4	~7.4	~4.0 ~7.4	~7.4
Probe Templates [1]	**N/A**	~~[2]	~~[2]	~~[2]	~~[2]	~~[2]	~~[2]	~0.2 ~0.9 ~1.5 ~1.8	~~[2]
A3525	***Bsp1286* I/*Pvu* II**	None	None	None	None	None	None	None	None
	***Hpa* I/*Kpn* I**	None	None	None	None	None	None	None	None
MON 87708	***Bsp1286* I/*Pvu* II**	~2.6	~2.6 ~1.5	~1.5	~1.5	None	None	None	~2.6 ~1.5
	***Hpa* I/*Kpn* I**	>2.7	>2.7 ~1.7	~1.7	~1.7	None	None	None	--[3]

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



Probe Number	Probe Type	Probe Name	Start Position (bp)	End Position (bp)	Total Length (bp)
1	Backbone Probe	B1	443	1328	886
2	Backbone Probe	B2	1250	2754	1505
3	Backbone Probe	B3	2625	4384	1760
4	T-DNA II Probe	TII-1	4796	5637	842
5	T-DNA II Probe	TII-2	5575	7021	1447
6	T-DNA II Probe	TII-3	6937	7761	825
7	Backbone Probe	B4	8119	8289	171
8	T-DNA I Probe	TI-1	8290	9523	1234
9	T-DNA I Probe	TI-2	9448	10668	1221
10	T-DNA I Probe	TI-3	10610	442	1185

Figure 1. Physical Map of Vector PV– GMHT4355 Showing the Genetic Elements and All Probes Used for Southern Analyses

A physical map of the plasmid vector PV–GMHT4355 used to develop soybean MON 87708 is shown. PV– GMHT4355 contains two T-DNA regions designated as T-DNA I and T-DNA II above. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map.

* The left and right border sequences of T-DNA II share 100% identity to those of T-DNA I, which were covered by probes 8 and 10 and thus not included in the T-DNA II probes.

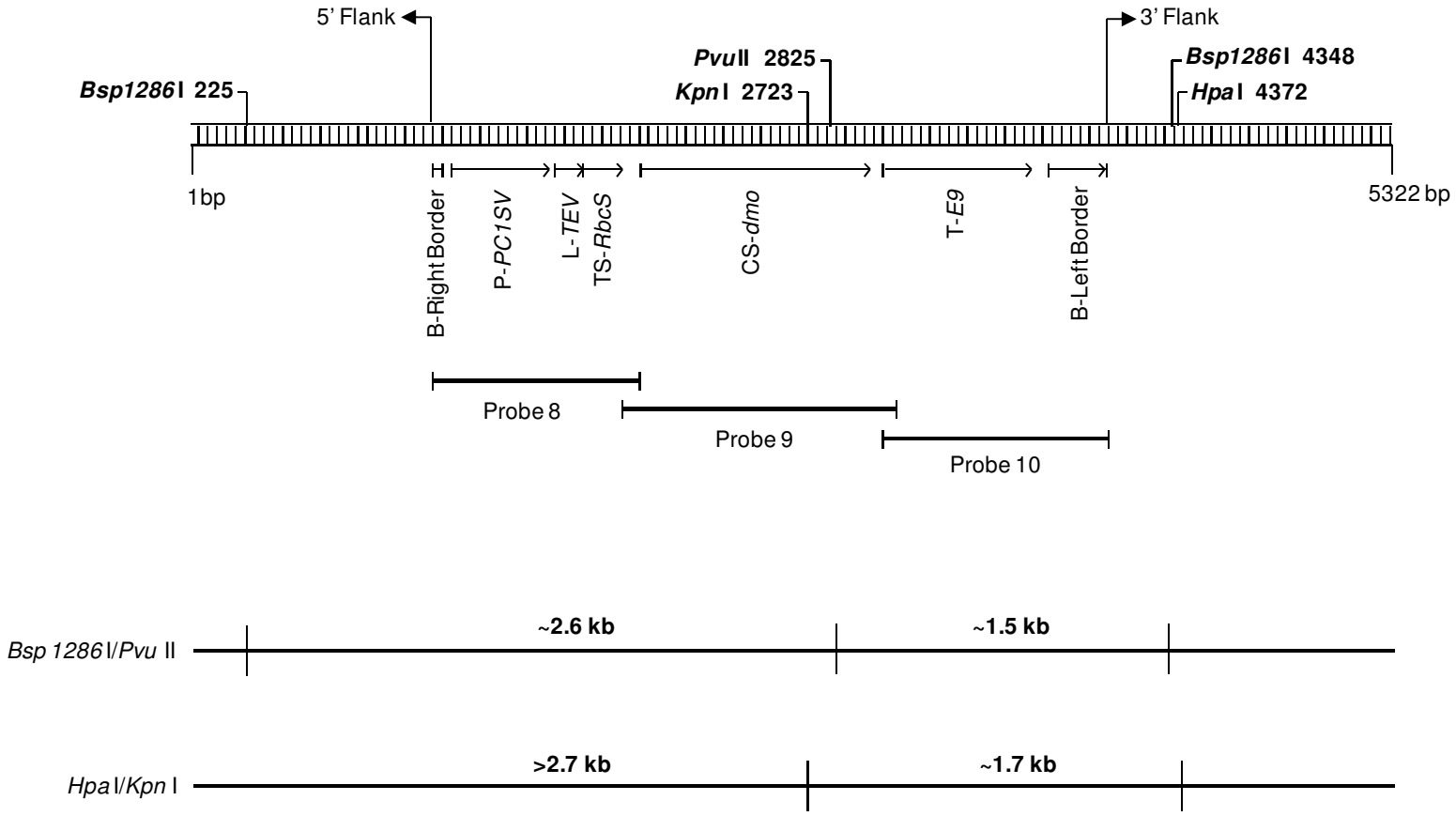


Figure 2. Schematic Representation of the Insert and DNA Flanking the Insert in MON 87708
A linear map of the insert and DNA flanking the insert in MON 87708 is shown. Identified on the linear map are genetic elements within the insert, the restriction enzymes used in the Southern analyses, and their positions relative to the first base of the sequence reported in Figure 11. The relative sizes and locations of the T-DNA I probes, which are described in Figure 1, are shown on the middle portion. Shown on the lower portion of the map are the expected sizes of the DNA fragments generated by the digestions with respective combination of restriction enzymes. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking DNA. Arrows indicate the sequence direction of the elements in MON 87708. This schematic diagram is not drawn to scale.

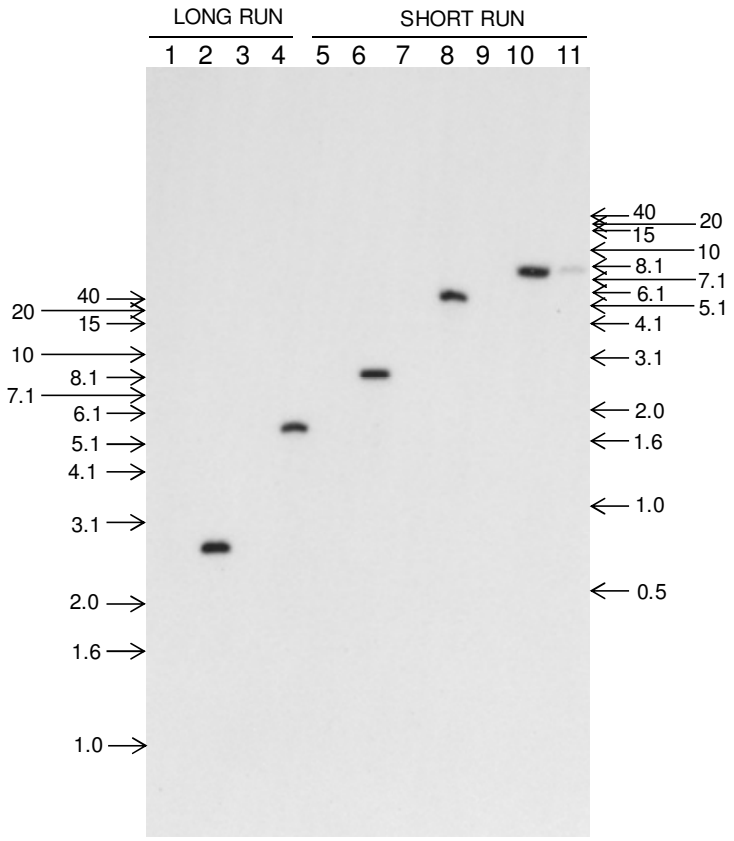


Figure 3. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 8

The blot was hybridized with a ^{32}P-labeled T-DNA I probe that spans a portion of theT-DNA I sequence (Figure 1, Probe 8). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

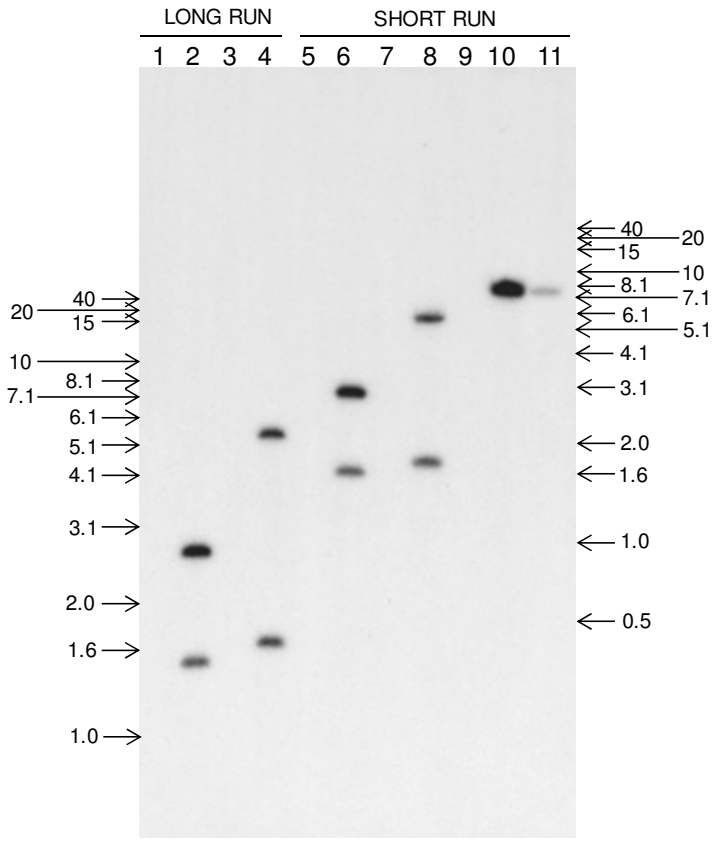


Figure 4. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 9

The blot was hybridized with a ^{32}P-labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Figure 1, Probe 9). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

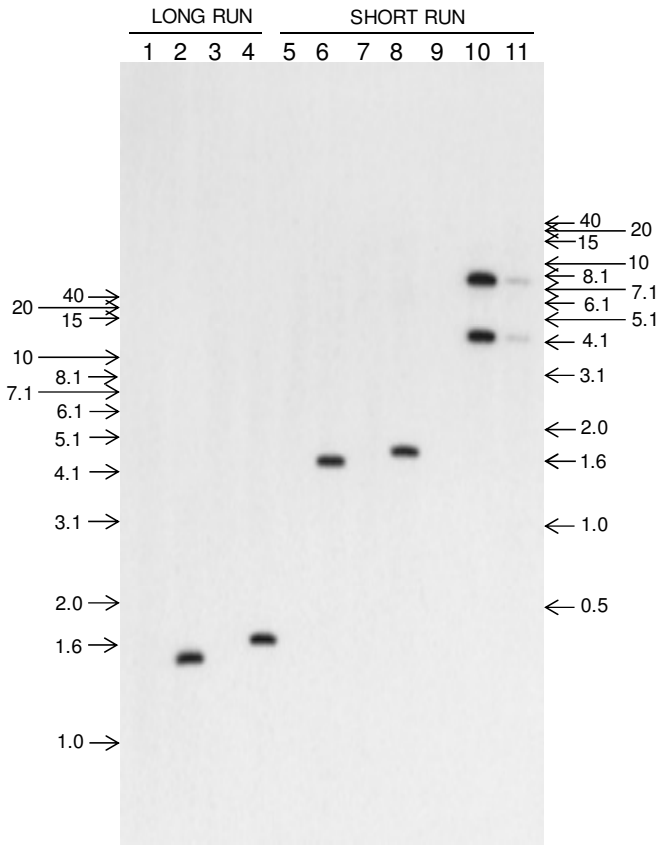


Figure 5. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 10

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

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

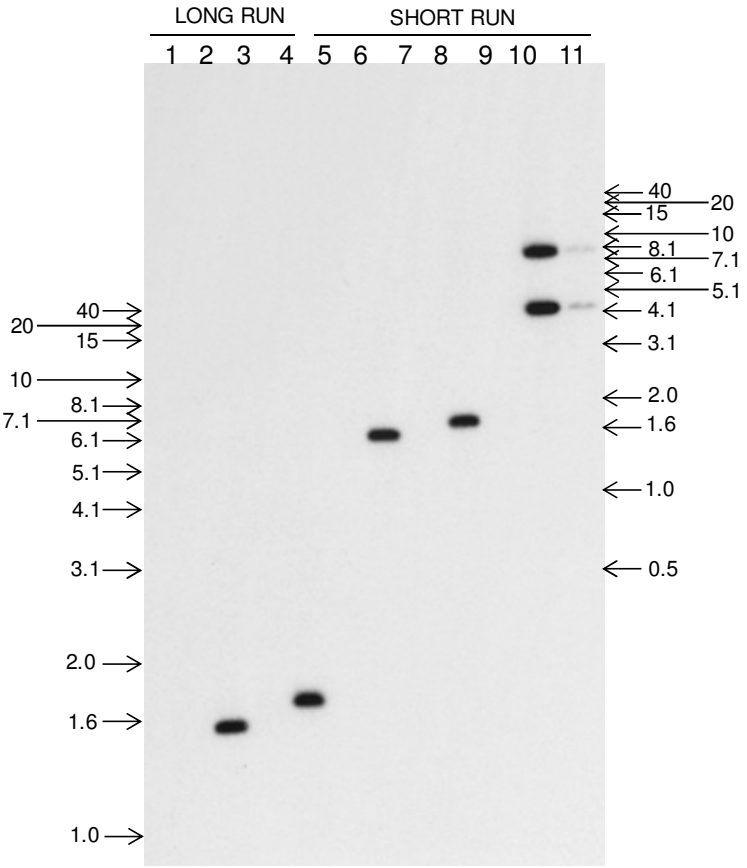


Figure 6. Southern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 4

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

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

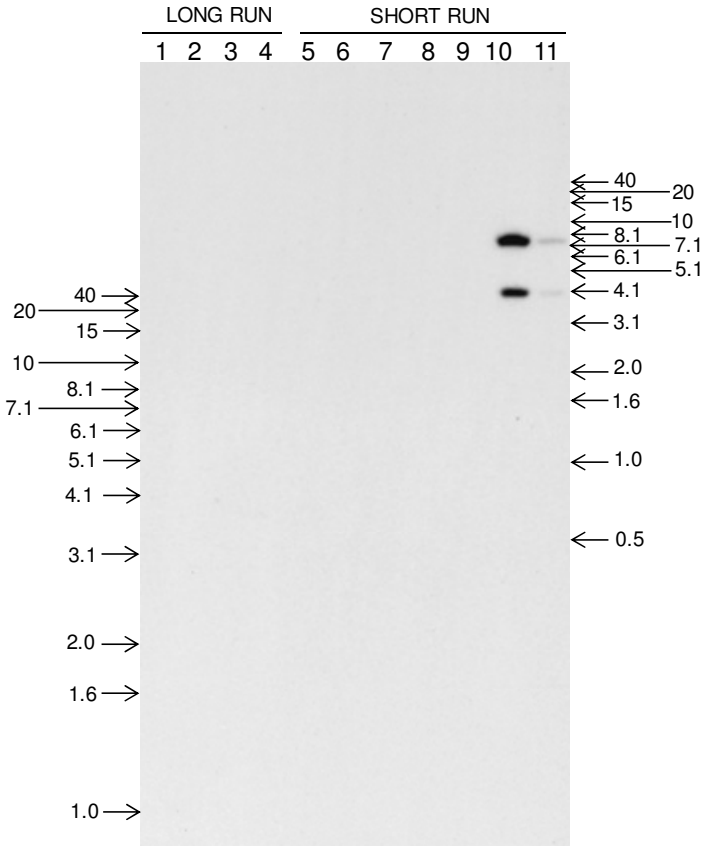


Figure 7. Southern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 5

The blot was hybridized with a ^{32}P-labeled T-DNA II probe that spans the coding region of the T-DNA II sequence (Figure 1, Probe 5). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

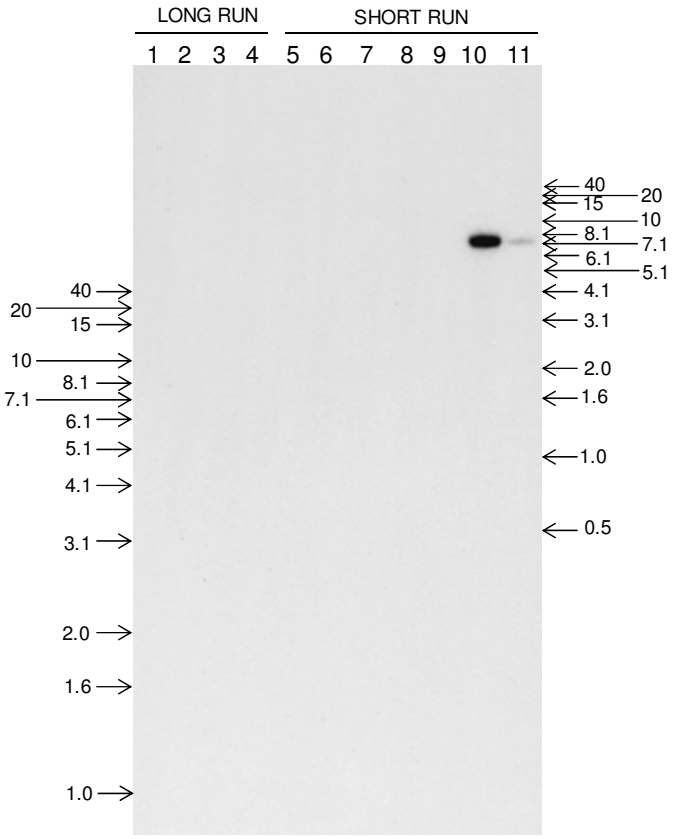


Figure 8. Southern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 6

The blots were hybridized with a ^{32}P-labeled T-DNA II probe that spans a portion of the T-DNA II sequence (Figure 1, Probe 6). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

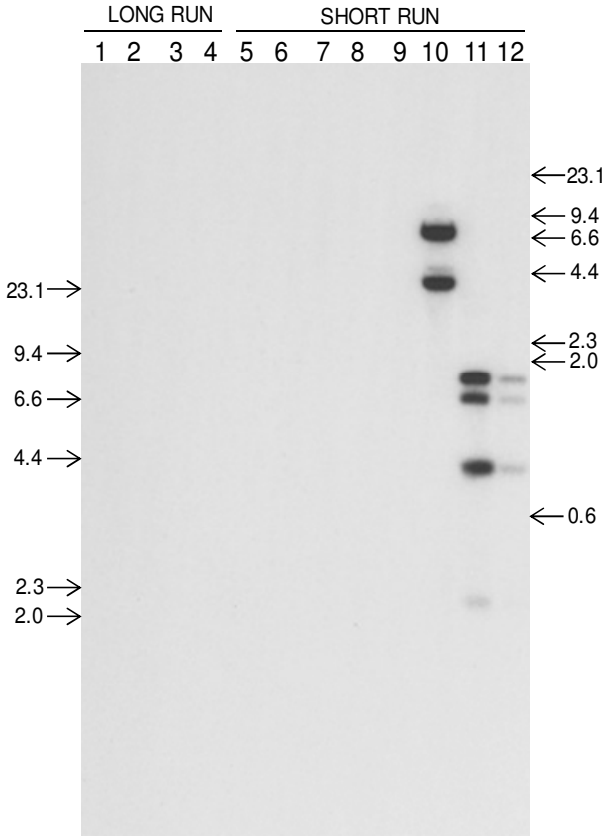


Figure 9. Southern Blot Analysis to Detect the Presence or Absence of the Plasmid PV-GMHT4355 Backbone Sequences in MON 87708: Probes 1, 2, 3, and 7

The blot was hybridized simultaneously with four backbone probes (Figure 1, Probes 1, 2, 3, and 7). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. MON 87708 (*Bsp1286* I/*Pvu* II)
3. Conventional soybean (*Hpa* I/*Kpn* I)
4. MON 87708 (*Hpa* I/*Kpn* I)
5. Conventional soybean (*Bsp1286* I/ *Pvu* II)
6. MON 87708 (*Bsp1286* I/*Pvu* II)
7. Conventional soybean (*Hpa* I/*Kpn* I)
8. MON 87708 (*Hpa* I/*Kpn* I)
9. Blank
10. Conventional soybean (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/ *Nde* I) [~1 genomic equivalent]
11. Conventional soybean (*Hpa* I/*Kpn* I) spiked with probe templates [~1 genomic equivalent]
12. Conventional soybean (*Hpa* I/*Kpn* I) spiked with probe templates [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

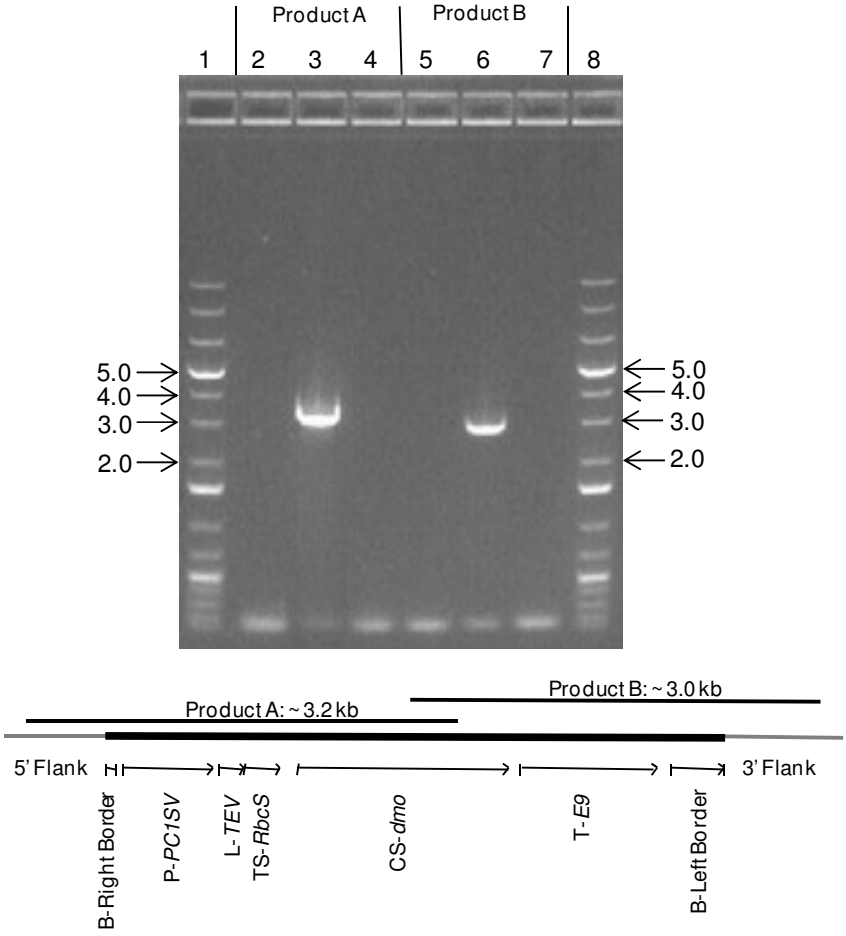


Figure 10. Overlapping PCR Analysis Across the Insert in MON 87708
PCR analyses were performed on MON 87708 genomic DNA extracted from leaf (Lanes 3 and 6). Lanes 2 and 5 contain reactions with conventional soybean control DNA while lanes 4 and 7 are reactions containing no template DNA. Lanes 1 and 8 contain Fermentas GeneRuler™ 1 kb Plus DNA Ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87708 that appears at the bottom of the figure. Five microliters of each of the PCR products was loaded on the gel. This figure is representative of the data generated in the study; however the specific bands from this gel were not excised and sequenced.

Lane
1. GeneRuler™ 1 kb Plus DNA Ladder
2. Conventional soybean control DNA
3. MON 87708 genomic DNA
4. No template DNA control
5. Conventional soybean control DNA
6. MON 87708 genomic DNA
7. No template DNA control
8. GeneRuler™ 1 kb Plus DNA Ladder

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

[CBI CROSS REFERENCE 1]

Deleted Figure 11.
Deleted pages 44 – 45 are found in the Confidential Attachment, pages 3 – 4.

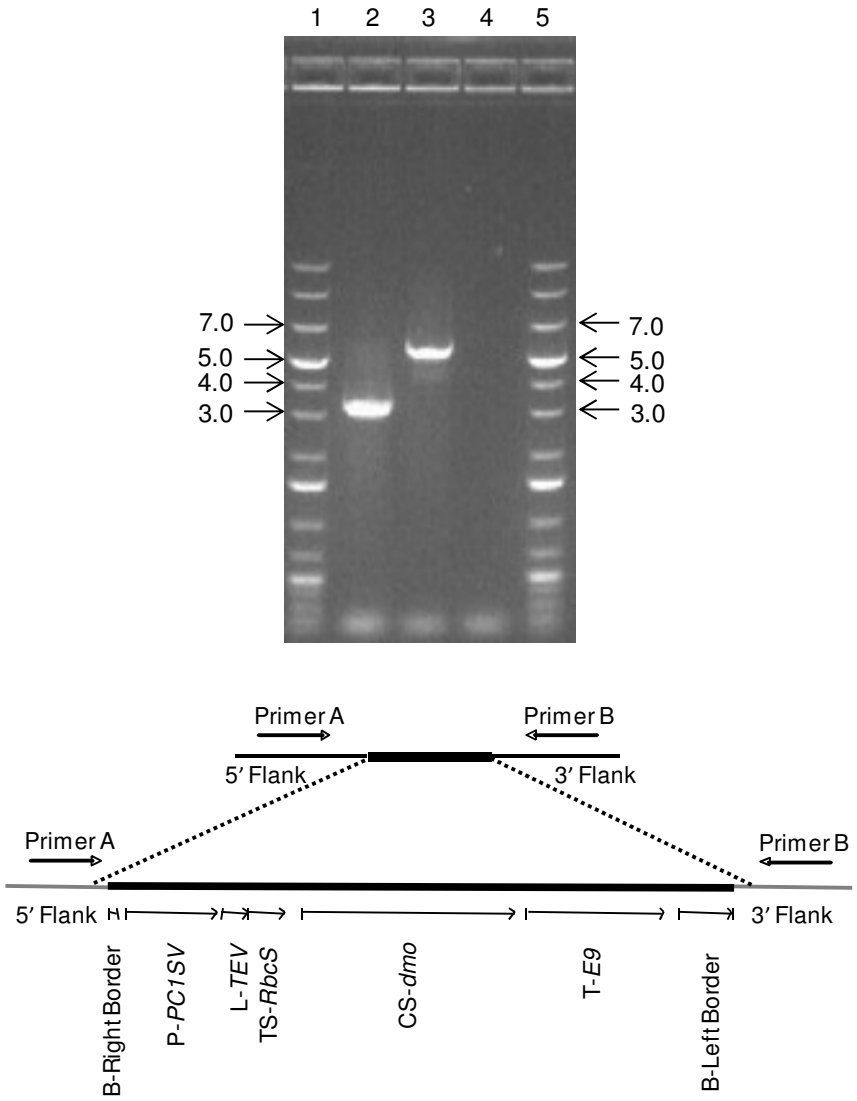


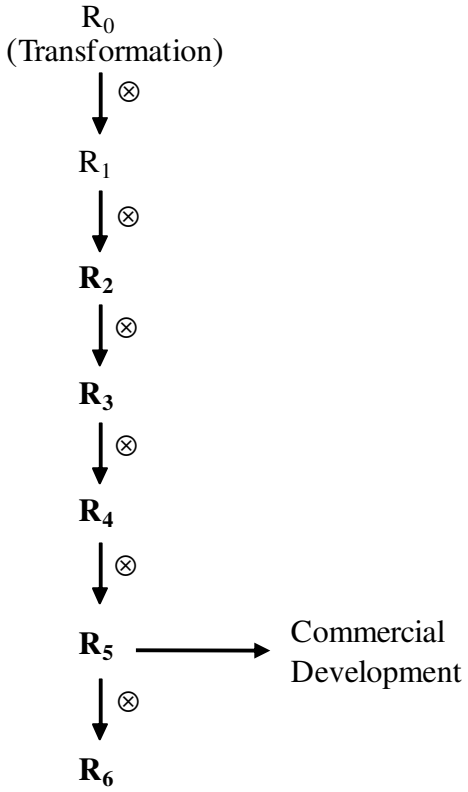
Figure 12. PCR Amplification of the MON 87708 Insertion Site in Conventional Soybean. Depiction of the MON 87708 insertion locus in conventional (upper panel) and MON 87708 soybean (lower diagram). PCR amplification was performed using Primer A in the 5' flanking sequence and Primer B in the 3' flanking sequence of the insert in MON 87708. Lane designations are as follows:

Lane
1. GeneRuler™ 1 kb Plus DNA Ladder
2. Conventional soybean
3. MON 87708
4. No template DNA control
5. GeneRuler™ 1 kb Plus DNA Ladder

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel

[CBI CROSS REFERENCE 2]

Deleted Figure 13.
Deleted pages 47 – 48 are found in the Confidential Attachment, pages 5 – 6.



R_0 –originally transformed plant; ⊗ –self pollinated

Figure 14. Breeding History of MON 87708
The R_3 generation was used for the molecular analyses reported in Figures 3-9 and is referred to as MON 87708 in all Southern blot figures. The R_5 generation was used for development of all commercial products. MON 87708 from generations R_2, R_3, R_4, R_5, and R_6 (bolded in the breeding tree) were used for analyzing the stability of the insert across generations.

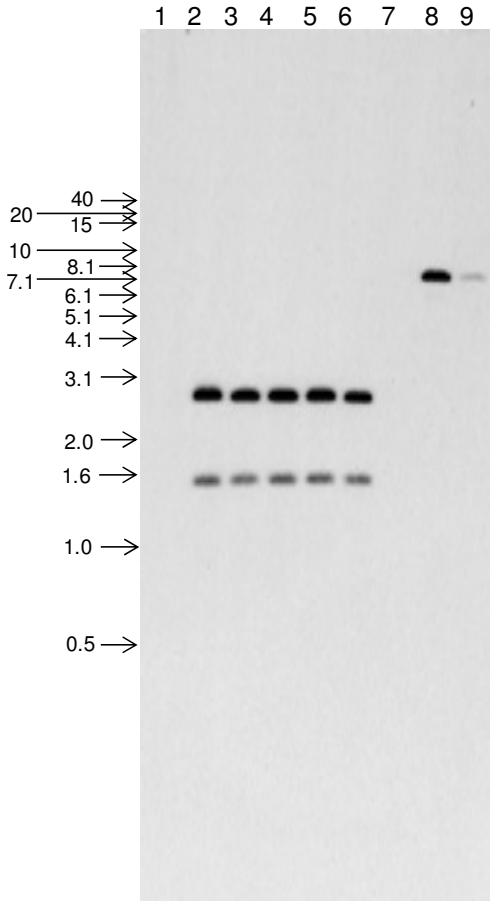


Figure 15. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87708: Probe 9

The blot was hybridized with a ^{32}P-labeled T-DNA I probe that spans the coding region of the T-DNA I (Figure 1, Probe 9). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane
1. Conventional soybean (*Bsp1286* I/*Pvu* II)
2. R_2 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
3. R_3 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
4. R_4 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
5. R_5 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
6. R_6 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
7. Blank
8. Conventional soybean (*Bsp1286* I and *Pvu* II) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genomic equivalent]
9. Conventional soybean (*Bsp1286* I and *Pvu* II) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genomic equivalent]

→ Symbol denotes sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

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-ME-1153-01	Quick CTAB DNA Extraction from Leaf Tissue

APPENDIX 2

Notes for Reviewer

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

Page Number in MSL0023278	Change
1	Added "Amended Report for MSL0021418:" to the title, "Amendment 1" after Study Completed, and revised report completion date. Updated report title, MSL number, and report completetion date, changed "Amendment 1" to "Amendment 2".
2	Removed the statement "The inclusion of this page in all studies is for quality assurance purposes and does not necessarily indicate that this study has been submitted to the U.S. EPA." from the "Statement of Data Confidentiality Claim" and added it to the section prior to the claim. Removed the statement "A claim of data confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d) (1) (A), (B), or (C)." from the "Statement of Data Confidentiality Claim". Changed the statement of "Information claimed confidential on the basis of … in the body of the report" to "Information claimed confidential on the basis of … in the body of the study"
4	Added "Amended Report Audit" to list of phases, and changed the study title. Updated the study title and the list of reviews for Amendment 2.
5	Changed the Study Director from "James F. Rice, Ph. D." to "Zihong Song" and the "Molecular Team Lead" to "Molecular Characterization and TCR Platform Lead" for Qing Tian.
6	Changed the study title and the MSL number, added "Original" to "Study Completion Date", added "Amendment 1 Report Completion Date". Changed the study title, the MSL number, the name for the Study Director, updated the amendment number and the date of completion, revised the statements for "Record Retention"and copy rights for Amendment 2.
7, 8, and 9	Revised "Table of Contents" to reflect changes in pagination and in titles for sections 3.12, 4.1, 4.1.1, 4.2 and 4.3, as well as Figure 2.
9	Added "Appendix 2: Notes for Reviewer" to the "Table of Contents".
10, 30, 32, 34, 35, 43, and 46	Changed "CS-*DMO*" to "CS-*dmo*".
10	Corrected a typographical error by changing "3'(9)-O-nucleotidyltransferase" to "3" (9)-O-nucleotidyltransferase" for *aadA*. Changed "Coding region for…" to "Coding sequence for…" for CS-*dmo*
10 and 31	Changed "Codon modified coding sequence of …" to "Codon optimized coding sequence of…" for CS-*cp4 epsps*

12	Changed the sentence "Monsanto company has...dicamba (3,6-dichloro-2-methosybenzoic methoxybenzoic acid) herbicide." to "Monsanto company has...dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide." in the first paragraph of Section 1.0. Changed the last sentence "Subsequently, traditional breeding…" to "Subsequently, conventional breeding…" in the second paragraph of Section 1.0. Changed "adjacent genomic DNA" to "adjacent DNA" and "insert-to-genomic" to "insert-to-flank" in the third paragraph of Section 1.0. Changed the sentence in the third paragraph of Section 1.0 "Additional analysis of the insertion site in conventional soybean confirmed that the genomic DNA flanking the T-DNA insert is native to the soybean genome." to "Additional analysis of the insertion site in conventional soybean and MON 87708 determined that an 899 bp deletion and two insertions of 128 bp and 35 bp occurred at the site of insertion during the formation of MON 87708." Changed the first sentence in Section 2.1 from "…from the *dmo* gene derived from *Psuedomonas maltophilia* (reclassified as *Stentrophomonas maltophilia).*" to "…from the *dmo* gene derived from *Stentrophomonas maltophilia*.".
13	Changed the last sentence in Section 2.1 "The T-DNAs can segregate through traditional breeding…" to "The T-DNAs can segregate through conventional breeding…". Changed "adjacent genomic DNA" to "adjacent DNA" in Section 2.2, changed the last sentence in Section 2.2 "In addition, PCR and DNA sequence analyses were performed on the conventional soybean A3525 to confirm that the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87708 are native to the soybean genome." to "In addition, PCR and DNA sequence analyses were performed on the conventional soybean A3525 and MON 87708 to examine the DNA insertion site in MON 87708."
15	Changed the last sentence in Section 3.7 "For the purpose of …digested with the combination of *Hpa* I/*Kpn* I restriction enzymes…" to "For the purpose of …digested with the combination of *Hpa* I/*Kpn* I or *Bsp1286* I /*Pvu* II restriction enzymes…".
17	Revised the first paragraph of Section 3.11. Revised the title for Section 3.12 and changed the first sentence in the first paragraph "To demonstrate that the DNA sequences flanking the insert in MON 87708 are native to the soybean genome, …" to "To examine the DNA insertion site in MON 87708, …".
18 and 19	Changed the sentence "During the course of the study, two changes to the original protocol were required." to "During the course of the study, three changes to the original protocol were required.", added a protocol amendment summary to Section 3.14 "Changes to the Study Protocol".
19	Revised the summary paragraph under Section 4.0 by changing "adjacent genomic DNA" to "adjacent DNA" in the first sentence and changing the last sentence "PCR and DNA sequence analyses confirmed…, confirmed the genomic DNA…are native to the soybean genome, and determined the integration site in conventional soybean." to "PCR and DNA sequence analyses confirmed…and examined the organization of the DNA insertion site in MON 87708." Changed "soybean genomic DNA" to "DNA sequences" in the summary paragraph of Section 4.1.

20	Changed "known genomic DNA" to "known DNA" and "adjacent plant genomic DNA" to "adjacent flank DNA" in the summary paragraph of Section 4.1.1.
21 and 22	Revised the last sentence in the second paragraph under each of the sections 4.1.1.1, 4.1.1.2, and 4.1.1.3, revised the last paragraph under Section 4.1.1.3
25	Revised the title of Section 4.2, changed "flanking genomic DNA" in the first paragraph to "flanking DNA", changed "genomic DNA" and "soybean genomic DNA" in the third paragraph to "DNA", and revised the last paragraph in Section 4.2.
25 and 26	Revised the title of Section 4.3, the first and second sentences of the first paragraph, and the last paragraph under this section.
27	Changed the last sentence under Section 4.4.1 "The fingerprint of …, indicating that MON 87708 contains one copy of T-DNA I that is stable across the selected generations." to "The fingerprint of …, indicating that MON 87708 contains one copy of T-DNA I residing at a single locus of integration that is stable across the selected generations." Revised the second paragraph under Section 5.0.
28	Corrected a typographical error by changing "3'(9)-O-nucleotidyltransferase" to "3" (9)-O-nucleotidyltransferase" for Fling reference. Added "Herrmann, K. M. 1995. The Shikimate Pathway: Early Steps in the Biosynthesis of Aromatic Compounds. *The Plant Cell* **7**(7): 907-919." to the Reference section.
29	Corrected a typographical error by changing "Maiti, I.B. and R.J. Shepperd. 1998" to "Maiti, I.B. and R.J. Shepherd. 1998" Corrected the year from 1978 to 1979 and the page numbers from 77-103 to 77-90 for the Sutcliffe reference. Added "Zambryski, P., A. Depicker, K. Kruger, and H. M. Goodman. 1982. Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. J. Molec. Appl. Genet. 1(4): 361-370."
30, 31, and 32	Moved a few rows of Table 1 from the first portion to the "Continued" portion to accommodate additional text, added "(Not present in MON 87708)" to the Vector Backbone and T-DNA II headers, Added "(pea)" after *Pisum sativum* in the TS-*RbcS* and T-*E9* element discriptions for Table 1 and Table 2. Changed the sentence "DNA region from *Agrobacterium*…" to "DNA region from *Agrobacterium tumefaciens*…" in B-Right Border and B-Left Border descriptions, added "Zambryski et al., 1982" for genetic element B-Right border.
30 and 32	Corrected a typographical error by changing "Maiti, et al., 1998" to "Maiti and Shepherd, 1998" for genetic element P-*PC1SV*. Changed the sentence "Coding region for the dicamba mono-oxygenase" to "Modified coding region for the dicamba mono-oxygenase". Changed the sentence "Modified coding region for the dicamba…" to "Coding sequence for the dicamba…" for the genetic element CS-*dmo*.
31	Corrected a typographical error by changing "OR-*ori-PBR322*" to "OR-*ori-pBR322*". Corrected the year from 1978 to 1979 for the Sutcliffe reference for the genetic element OR-*ori-pBR322*. Deleted a blank row after the row of OR-*ori-pBR322* in Table 1. Changed the Sentence "Sequences encoding the transit peptide region of *Arabidopsis*

	thaliana (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast" to "Sequences encoding the chloroplast transit peptide region from the *shkG* gene of *Arabidopsis thaliana* encoding EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast" in Amendment 1. Added "Herrmann, 1995" after "Klee et al., 1987" for the genetic element "TS-*CTP2"* in Amendment 2. Defined the "Intervening Sequence" immediately after the "B-Right Border" of T-DNA II as "Vector Backbone".
32	Corrected two typographical errors by changing 5**'** and 3**'** to 5' and 3'. Revised the description for the 5' and 3' Flanking Sequences under the header of "Function (Reference)" of Table 2.
34	Corrected two typographical errors by changing *Bsp 1286*I to *Bsp 1286* I at position 2041bp of the plasmid map and changing "OR-*ori-PBR322*" to "OR-*ori-pBR322*". Extended the arch/arrow to cover the left border of T-DNA I.
35	Added the relative sizes and locations of the T-DNA I probes to the diagram, moved the names of the restriction enzymes from the inside of the lower portion of the map to the left side, removed letters A and B from the diagram, and updated the figure legend accordingly.
49	Corrected an error by changing the generation used for "Commercial Development" from R_3 to R_5 in the diagram of the breeding history and updated the figure legend accordingly.
52, 53, 54, and 55	Added "Appendix 2: Notes to Reviewer" detailing the contents of Amendment 1. Revised appendix 2 to reflect all the changes for Amendment 2.
Confidential Attachment Page 1 of 6	Changed Title from "Molecular Analysis of Dicamba-Tolerant Soybean MON 87708" to "Amended Report for MSL0021418: Molecular Analysis of Dicamba-Tolerant Soybean MON 87708", added "Amendment 1" after Study Completed, and revised report completion date. Updated report title, MSL number, and report completetion date, changed "Amendment 1" to "Amendment 2".
Confidential Attachment Page 2 of 6	Changed the page number "45-46" to "44-45" for Figure 11 and "48-49" to "47-48" for Figure 13, revised the title for Fgure 11.
Confidential Attachment Page 3 of 6	Revised the figure title, changed the sentence "…soybean genomic DNA flanking…" to "…DNA sequence flanking…" in the figure legend.
Confidential Attachment Page 5 of 6	Corrected two typographical errors in the figure legend by changing 5**'** and 3**'** to 5' and 3', changed "soybean genomic DNA" in the last sentence of the figure legend to "DNA sequences".