

**Study Title**

**Molecular Characterization of MON 87427**

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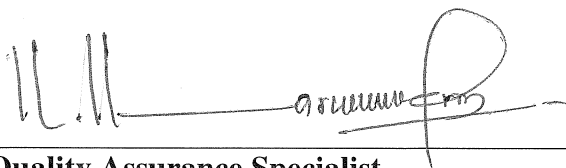
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**Quality Assurance Statement****Study Title: Molecular Characterization of MON 87427****Study Number: REG-08-575**

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.

<b>Dates Of Inspection / Audit</b>	<b>Phase</b>	<b>Date Reported To Study Director</b>	<b>Date Reported To Management</b>
02/23/2009	PCR/Sequence	02/26/2009	02/26/2009
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**Quality Assurance Specialist**  
**Monsanto Regulatory, Monsanto Company**

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**Study Information Page**

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**Title:** Molecular Characterization of MON 87427

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**Records Retention:** All study-specific raw data (including rejected data)  
protocol, final report and facility records will be  
retained at Monsanto Company, St. Louis, Missouri.

**Sample Storage:** Any study samples that are to be retained will be stored  
at Monsanto Company, St. Louis, Missouri

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**Study Certification Page**

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

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*5/24/2010*

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### Abbreviations<sup>1</sup> and Definitions

~	Approximately
<i>aadA</i>	Bacterial promoter, coding sequence, and 3' untranslated region for an aminoglycoside-modifying enzyme, 3''(9)- <i>O</i> -nucleotidyl-transferase from the transposon Tn7 that confers spectinomycin and streptomycin resistance
B-Left Border	DNA region from <i>Agrobacterium tumefaciens</i> containing the left border sequence used for transfer of the T-DNA
B-Right Border	DNA region from <i>Agrobacterium tumefaciens</i> containing the right border sequence used for transfer of the T-DNA
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase protein from <i>Agrobacterium</i> sp. strain CP4
CS- <i>cp4 epsps</i>	Codon-optimized coding sequence of the <i>aroA</i> gene from the <i>Agrobacterium</i> sp. strain CP4 encoding the CP4 EPSPS protein
CS- <i>rop</i>	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in <i>E. coli</i>
CTAB	Cetyltrimethylammonium bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dNTP	Deoxynucleoside triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
I- <i>Hsp70</i>	First intron from the maize heat shock protein 70 gene
OR- <i>ori-pBR322</i>	Origin of replication from pBR322 for maintenance of plasmid in <i>E. coli</i>
OR- <i>ori V</i>	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in <i>Agrobacterium</i>
P- <i>e35S</i>	Promoter and leader for the cauliflower mosaic virus (CaMV) 35S RNA containing the duplicated enhancer region
PCR	Polymerase chain reaction
sp.	Species
T-DNA	Transfer DNA
T- <i>nos</i>	3' Nontranslated region of the <i>nopaline synthase (nos)</i> gene from <i>Agrobacterium tumefaciens</i>
T <sub>m</sub>	Melting temperature
TS- <i>CTP2</i>	Targeting sequence from the <i>ShkG</i> gene encoding the chloroplast transit peptide region of <i>Arabidopsis thaliana</i> EPSPS that directs transport of the CP4 EPSPS protein to the chloroplast

<sup>1</sup>Standard abbreviations, e.g., units of measure, were used in this report according to format described in "Instructions to Authors" in the Journal of Biological Chemistry.

## 1.0 Summary

Monsanto Company has developed MON 87427, an inducible male sterile and glyphosate tolerant corn, to facilitate the production of viable hybrid corn seed. MON 87427 produces CP4 EPSPS protein via the incorporation of a *cp4 epsps* coding sequence. However, MON 87427 utilizes a specific promoter and intron combination (*e35S-Hsp70*) to drive CP4 EPSPS protein expression in vegetative and female reproductive tissues. Little to no CP4 EPSPS protein is expected to be produced in MON 87427 pollen, thus pollen from MON 87427 is not tolerant to glyphosate. Appropriately timed glyphosate applications produce a non-viable pollen phenotype and allow for specific cross pollinations to be made in corn without using traditional methods to control self pollination.

MON 87427 was produced by *Agrobacterium*-mediated transformation of corn tissue using the plasmid vector PV-ZMAP1043. This plasmid vector contains one T-DNA (transfer DNA), which is delineated by Right and Left Border sequences. The T-DNA contains an expression cassette consisting of the *cp4 epsps* coding sequence regulated by the *e35S* promoter, the *Hsp70* intron, and the *nos* 3' nontranslated region.

In this study, Southern blot analyses were used to confirm the copy number of the integrated T-DNA sequences in the genome and the presence or absence of backbone from the plasmid vector PV-ZMAP1043 in the genome of MON 87427. The data show that MON 87427 contains one copy of the insert at a single integration locus and that all expression elements are present in the insert. The data also show that MON 87427 does not contain detectable backbone from plasmid vector PV-ZMAP1043. The complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 87427 confirmed the integrity of the inserted *cp4* expression cassette and identified the 5' and 3' insert-to-flank junctions. Additional characterization of the insertion site in the conventional control LH198 × HiII confirmed that the flanking genomic DNA in MON 87427 is native to the corn genome and showed that 140 bases of genomic DNA were deleted at the insertion site in MON 87427. Additionally, there is a 41 base pair insertion at the 5' insert-to-flank junction, and a 24 base pair insertion at the 3' insert-to-flank junction that occurred during the *Agrobacterium*-mediated transformation in MON 87427. Furthermore, Southern blot analysis demonstrated that the DNA fingerprint of the T-DNA insert in MON 87427 has been maintained through five generations of breeding, thereby confirming the stability of the insert in multiple generations.

## 2.0 Introduction

### 2.1 Background

Monsanto Company has developed MON 87427, an inducible male sterile and glyphosate tolerant corn, to facilitate the production of viable hybrid corn seed. MON 87427 produces CP4 EPSPS protein via the incorporation of a *cp4 epsps* coding sequence. However, MON 87427 utilizes a specific promoter and intron combination (*e35S-Hsp70*) to drive CP4 EPSPS protein expression in vegetative and female reproductive tissues. Little to no CP4 EPSPS protein is expected to be produced in MON 87427 pollen, thus pollen from MON 87427 is not tolerant to glyphosate.

Appropriately timed glyphosate applications produce a non-viable pollen phenotype and allow for specific cross pollinations to be made in corn without using traditional methods to control self pollination.

## 2.2 Purpose

The purpose of this study was to characterize the integrated T-DNA in MON 87427. Genomic DNA was analyzed using a combination of Southern blot analyses and DNA sequencing methodologies. These methods were used to determine the number of insertion sites and copies of the integrated T-DNA as well as the presence or absence of plasmid vector backbone sequences. Insert stability analysis was performed to confirm the stability of the integrated T-DNA across five generations of MON 87427. The complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 87427 was also determined. In addition, PCR and DNA sequence analyses were performed with the conventional control LH198 × HiII to confirm that the genomic DNA sequences flanking the 5' and 3' ends of the insert in MON 87427 are native to the corn genome, and to characterize the wild type insertion site.

## 3.0 Materials and Methods

### 3.1 Test Substance

The test substance for the molecular characterization was the LH198 BC3F4 generation of MON 87427 in the LH198 × HiII genetic background. Genomic DNA was extracted from the seed samples listed in the table below for use in this study.

Test Substance	Generation	ORION <sup>2</sup> ID	Tissue Type	Seed Virgo Number
MON 87427	LH198 BC3F4	11214238-002	Seed	60070411411
		11214238-003		
		11223518-003		

Five generations of MON 87427 were used to assess the stability of the T-DNA insert and were derived from the conventional breeding of MON 87427. Genomic DNA was extracted from the leaf or seed samples listed in the table below for use in this study. Seed for the MON 87427 LH198 BC3F3, MON 87427 LH198 BC3F6, and MON 87427 LH198 BC3F7 generations were grown in the greenhouse production listed below to produce leaf tissue.

<sup>2</sup>ORION is a proprietary database used at Monsanto Company to track Regulatory plant samples.

Test Substance	Generation	ORION ID	Tissue Type	Seed Virgo Number	Production Plan
MON 87427	LH198 BC3F3	11227138-001	Leaf	60014785781	PPN-09-218
	LH198 BC3F4	11214238-003	Seed	60070411411	N/A
	LH198 BC3F6	11227139-001	Leaf	60083347483	PPN-09-218
	LH198 BC3F7	11227140-001	Leaf	60082580121	PPN-09-218
	[LH198 BC3F7 × LH287]F1	10001857-090	Seed	MIDAS number I000000140745 35617500001	N/A

### 3.2 Control Substances

The conventional control substances listed in the table below were used in this study. The control substance LH198 × HiII is the same genetic background as the test substance used in the molecular characterization. The control substance LH198 × LH287 is the same genetic background as the MON 87427 [LH198 BC3F7 × LH287]F1 generation used in the insert stability analysis. Genomic DNA was extracted from the seed samples listed below for use in this study.

Control Substances	ORION ID	Tissue Type	Seed Virgo Number
LH198 × HiII	11214241-002	Seed	60070416877
	11214241-003		
	11223519-003		
LH198 × LH287	10001859-092	Seed	MIDAS number I00000014074476635100001

### 3.3 Reference Substance

The reference substance, plasmid vector PV-ZMAP1043, was used to generate MON 87427. Digested whole plasmid vector and probe templates generated from this plasmid vector served as positive hybridization controls on Southern blot analyses. The identity of the plasmid vector was confirmed by restriction enzyme digestion prior to the study. Additionally, appropriate molecular weight markers from commercial sources were used for size estimations on Southern blots and agarose gels.

### 3.4 Characterization of Test, Control, and Reference Substances

The starting seed for the test and control substances used in this study was obtained from Monsanto Trait Development. The synthesis records for these materials are located in the Virgo, MIDAS, and ORION systems. No certificates of analysis (COA) or verification of identity (VOI) certificates were generated for these materials. The Study Director reviewed the chain of custody documentation to confirm the identity of the test, control, and reference substances prior to use of the materials in the study.

A molecular fingerprint of the test substance was generated in this study, and the identity of the test substance, as well as the absence of the test substance in the conventional control substances, was further confirmed by the methods defined in this study (i.e., Southern blot analyses). The test, control, and reference substances were

considered stable during storage because they yielded interpretable signals on the Southern blots and/or agarose gels, and did not appear visibly degraded on the ethidium bromide stained gels.

### 3.5 Genomic DNA Isolation

Genomic DNA samples were isolated from corn seed and leaf tissue from MON 87427 and the conventional controls LH198 × HiII and LH198 × LH287. Seed tissue was processed to a fine powder using a Harbil paint shaker according to SOP BR-ME-0878-02. Prior to extraction, leaf tissue was processed to a fine powder using a mortar and pestle in liquid nitrogen. Genomic DNA was extracted from seed from the LH198 × HiII and LH198 × LH287 conventional controls and from the MON 87427 LH198 BC3F4 and MON 87427 [LH198 BC3F7 × LH287]F1 generations according to SOP BR-ME-0094-02. Genomic DNA was extracted from leaf tissue of the MON 87427 LH198 BC3F3, MON 87427 LH198 BC3F6, and MON 87427 LH198 BC3F7 generations prior to use in the study according to SOP AG-ME-1153-02. All extracted DNA was stored in a 4°C refrigerator or a -20°C freezer.

### 3.6 DNA Quantification

Genomic DNA was quantified using a DyNA Quant 200 Fluorometer (Hoefer, Inc., Holliston, MA) according to SOP BR-EQ-0065-02.

### 3.7 Restriction Enzyme Digestion

Approximately ten micrograms of genomic DNA extracted from MON 87427 and the conventional controls LH198 × HiII and LH198 × LH287 were digested with the restriction enzymes *Nco* I or *Nsi* I (New England Biolabs, Ipswich, MA) according to SOP BR-ME-0316-01. All digests were conducted in 1X NEBuffer 3 (New England Biolabs) at 37°C in a total volume of ~500 µl using ~20 units or ~50 units of the appropriate enzyme. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from conventional control LH198 × HiII were digested and used as a matrix for the appropriate positive hybridization control(s).

### 3.8 Agarose Gel Electrophoresis

Digested DNA was resolved on 0.8% (w/v) agarose gels according to SOP BR-ME-0315-02. For all Southern blot analyses except for insert stability, individual digests containing ~10 µg each of MON 87427 and conventional control LH198 × HiII genomic DNA were loaded on the same gel in a long run/short run format. 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. The positive hybridization controls were only run in the short run format. For the insert stability analysis, individual digests of ~10 µg each of genomic DNA extracted from seed or leaf tissue across five generations of MON 87427 and conventional controls LH198 × HiII and LH198 × LH287 were loaded on the agarose gel in a single run format.

### 3.9 Probe Preparation

Probe templates were prepared by PCR amplification using plasmid vector PV-ZMAP1043 as the template according to SOP BR-ME-0486-01 and gel purified according to SOP BR-ME-0889-01. The probe templates were designed based on the nucleotide composition (% GC) of the sequence in order to optimize the detection of DNA sequences during hybridization. When possible, probes possessing a similar  $T_m$  were combined in the same Southern blot hybridization. Approximately 25 ng of each probe template were radiolabeled with either [ $\alpha$ - $^{32}$ P]-deoxycytidine triphosphate (dCTP) or [ $\alpha$ - $^{32}$ P]-deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using the random priming method (Invitrogen, Carlsbad, CA) according to SOP BR-ME-0611-01.

### 3.10 Southern Blot Analyses

Genomic DNA isolated from MON 87427 and conventional controls LH198  $\times$  HiII and LH198  $\times$  LH287 was digested and evaluated using Southern blot analyses according to SOP BR-ME-0317-02. In each Southern blot, the ~0.1 and ~1.0 genome equivalents of the appropriate probe templates were added to digested conventional control LH198  $\times$  HiII genomic DNA to serve as positive hybridization controls. Additionally, plasmid vector PV-ZMAP1043 DNA previously digested with *Sph* I (New England Biolabs) was added to digested conventional control LH198  $\times$  HiII genomic DNA to serve as a ~1.0 genome equivalent positive hybridization control. The DNA was separated by agarose gel electrophoresis and transferred onto a nylon membrane. Southern blots were hybridized and washed at 55°C, 60°C, or 65°C, depending on the calculated  $T_m$  of the probes that were used. The table below lists the hybridization and radiolabeling conditions of the probes used in this study. Multiple exposures of each blot were generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer.

Probe	DNA Probe	Labeling Method	Probe labeled with dNTP ( $^{32}$ P)	Hybridization/Wash Temperature (°C)
1	T-DNA Probe 1	RadPrime	dATP	60
2	T-DNA Probe 2	RadPrime	dATP	55
3	T-DNA Probe 3	RadPrime	dCTP	65
4	T-DNA Probe 4	RadPrime	dATP	60
5	Backbone Probe 5	RadPrime	dCTP	60
6	Backbone Probe 6	RadPrime	dCTP	60
7	Backbone Probe 7	RadPrime	dCTP	60

### 3.11 PCR and Sequence Analyses to Examine the Insert and Flanking Sequences in MON 87427

Overlapping PCR products, denoted as Product A, Product B, and Product C, were generated that span the insert and adjacent 5' and 3' flanking genomic DNA sequences in MON 87427. These products were sequenced to determine the nucleotide sequence of the insert in MON 87427 as well as that of the genomic 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 for Product A and Product B were conducted using 50 ng of genomic DNA template in a 50 µl reaction volume containing a final concentration of 2 mM MgSO<sub>4</sub>, 0.2 µM of each primer, 0.2 mM of each dNTP, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity (Invitrogen).

Product C was generated through a nested PCR strategy in order to acquire an adequate amount of template for sequencing. A primary PCR reaction was used in a secondary (nested) reaction to generate Product C. The primary PCR reaction (Product C') was conducted using 50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 2 mM MgSO<sub>4</sub>, 0.2 µM of each primer, 0.2 mM of each dNTP, 10% DMSO, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity (Invitrogen). The secondary (nested) reaction was conducted using 1 µl of a 1:10 or 1:100 dilution of Product C' as genomic DNA template in a 50 µl reaction volume containing a final concentration of 2 mM MgSO<sub>4</sub>, 0.2 µM of each primer, 0.2 mM of each dNTP, 10% DMSO, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity (Invitrogen).

The amplification of Product A and Product B was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 3.25 minutes; and one cycle at 68°C for 5 minutes. The amplification of Product C' was performed under the following touchdown cycling conditions: one cycle at 94°C for 2 minutes; 16 cycles at 94°C for 20 seconds, 62°C decreasing 1°C per cycle for 30 seconds, 68°C for 2 minutes; 20 cycles at 94°C for 20 seconds, 45°C for 30 seconds, 68°C for 2 minutes; and one cycle at 68°C for 7 minutes. The amplification of Product C was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1.5 minutes; and one cycle at 68°C for 5 minutes.

Aliquots of each PCR product were separated on 1.0% (w/v) agarose gels and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. To concentrate DNA prior to sequencing, some of the PCR reactions for Product B and Product C were combined separately, purified with the QIAquick PCR Purification Kit following the manufacturer's instructions (Qiagen, Valencia, CA), and quantified according to BR-EQ-0065-02. The PCR products were sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry (Applied Biosystems, Foster City, CA).

### **3.12 PCR and DNA Sequence Analyses to Examine the Integrity of the DNA Insertion Site in MON 87427**

To demonstrate that the DNA sequences flanking the insert in MON 87427 are native to the corn genome and to characterize the MON 87427 insertion site in the conventional control LH198 × HiII, PCR analyses were performed on genomic DNA from both MON 87427 and the conventional control LH198 × HiII. The product resulting from the PCR analysis on the conventional control LH198 × HiII was sequenced. The primers used in this analysis were designed from the genomic DNA sequences flanking the insert in MON 87427. One primer specific to the DNA region flanking the 5' end of the insertion site was paired with a second primer specific to the DNA region flanking the 3' end of the insertion site in the genomic DNA sequence.

The PCR analyses were performed according to SOP BR-ME-0486-01. The PCR analyses were conducted using 50 ng of MON 87427 and conventional control LH198 × HiII genomic DNA template in separate 50 µl reactions containing a final concentration of 2 mM MgSO<sub>4</sub>, 0.2 µM of each primer, 0.2 mM of each dNTP, 10% DMSO, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity (Invitrogen).

The amplification of the product was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 30 cycles at 94°C for 15 seconds, 64°C for 30 seconds, 68°C for 1.5 minutes; one cycle at 68°C for 5 minutes.

Aliquots of each PCR product were separated on 1.0% (w/v) agarose gels and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. To concentrate DNA prior to sequencing, some of the PCR reactions were purified with the QIAquick PCR Purification Kit following the manufacturer's instructions (Qiagen), and eluates were dried down in a vacufuge. 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 (Applied Biosystems).

### **3.13 Data Rejected**

Some Southern blot analyses conducted as part of this study were rejected. Criteria for rejection included the following: duplicate blots were generated; high levels of background signal on blots hindered the ability to draw accurate conclusions; or restriction enzyme digestion issues, such as partial digestion or star activity, affected the analyses. Some PCR analyses were rejected based on the following criteria: expected amplification products were not produced, the amounts of amplification products were too low for use as sequencing templates, or non-specific amplification was observed. Sequencing electropherograms were rejected if they were of unacceptable quality, particularly with respect to peak shape and intensity. None of the rejected data was inconsistent with the conclusions presented in this report.



### 3.14 Changes to the Study Protocol

During the course of the study, several changes were made to the original protocol. These changes were documented as either protocol amendments or protocol deviations and are summarized below. None of these changes had a negative impact on the conclusions of this study.

1. The protocol was amended to add an additional ORION number for the LH198 BC3F4 generation of the test substance and an additional ORION number for the LH198 × HiII control substance, because additional test and control seed substances were needed to complete the study analyses. All of the synthesis records are the same, so there was no impact on the study.
2. The protocol was amended to clarify a change in the procedure for the extraction of leaf genomic DNA from the LH198 BC3F3, LH198 BC3F6 and LH198 BC3F7 generations for the insert stability analysis. Genomic DNA extracted from the leaf tissue using the procedure outlined in SOP AG-ME-1153-02 was of higher quality than the DNA extracted using the study-specific procedure; therefore, there was a positive impact on the study.
3. In both the test and control substances sections (3.1 and 3.2 of this report, respectively), the name of the genetic background was misstated as LH198 × Hill instead of LH198 × HiII, so the correction provides a positive impact.
4. The statement in the test substance section (3.1 of this report) was changed to clarify that leaf tissue was required for the LH198 BC3F6 and LH198 BC3F7 generations due to depleted stocks of seed, and the production plan number was provided in the table. This same production plan provided additional leaf tissue for the LH198 BC3F3 generation, since the leaf tissue from the original production plan had been depleted. Since the original protocol was written, a business decision was made to drop the LH198 BC3F5 generation from the analysis, and include the [LH198 BC3F7 × LH287]F1 generation instead. There was a positive impact on the study, because it clarifies the generations used, the tissue source for the genomic DNA from each generation, and the location of their synthesis records.
5. For all generations the nomenclature was changed to reflect the actual backcross and filial generation name, rather than the R (reproductive stage) number. This nomenclature appears in the breeding tree and in pertinent text. There is a positive impact on the study because it provides the technical description of the test substances.
6. The [LH198 BC3F7 × LH287]F1 generation and its corresponding conventional control substance, LH198 × LH287, were added to the study. As a result, there was a positive impact on the study, because all necessary generations and appropriate controls were present to complete the insert stability analysis.

7. The method of DNA extraction for all test and control substances was clarified, and the change in the SOP number for the leaf tissue extraction method was recorded, providing a positive impact on the study.
8. A typographical error was corrected to clarify that digested plasmid vector would be spiked as ~1.0 genome equivalent only, not as ~0.1 and ~1.0 genome equivalents. There was no impact on the study, because all of the blots contained the appropriate controls. This was acknowledged as a protocol deviation.
9. A sentence was added to state that the probe template positive controls also indicate the sensitivity of the Southern blot analysis. This statement added clarity of the purpose for including these controls in the analysis, so there was a positive impact on the study.
10. The restriction enzyme for the insert stability analysis was changed from *Nco* I to *Nsi* I due to DNA quality issues affecting the *Nco* I restriction enzyme. The *Nsi* I restriction enzyme was not affected by DNA quality issues and consistently generated high quality DNA digests for Southern blot analyses, providing a positive impact on the study.
11. The protocol originally stated that the integrity and genomic organization of the insertion site in MON 87427 would be determined by performing PCR on the test and control substances using primers specific to the 5' and 3' genomic DNA sequences flanking the DNA insert in MON 87427. However, on multiple occasions, the PCR was set up without the MON 87427 template reaction. This was acknowledged as a protocol deviation. There was no impact, because the MON 87427 test substance is included in this analysis as a control reaction and was not sequenced.

#### 4.0 Results and Discussion

Genomic DNA from MON 87427 was analyzed by Southern blot analyses with probes that collectively spanned the complete plasmid vector PV-ZMAP1043 sequence. These analyses were designed to: 1) determine that the insert/copy number of the inserted T-DNA was stably integrated; 2) demonstrate that the Southern blot fingerprint of MON 87427 was stably maintained across five generations; 3) determine the presence or absence of plasmid vector PV-ZMAP1043 backbone sequences in MON 87427.

Sequencing of the insert and the flanking genomic DNA provided the following information: 1) the organization and intactness of the genetic elements within the insert; 2) the 5' and 3' insert-to-flank junctions and the complete DNA sequence of the insert and adjacent corn genomic DNA; 3) the genomic DNA sequences flanking the DNA insert in MON 87427 are native to the corn genome.

##### 4.1 Southern Blot Analyses of MON 87427

Southern blot analyses were used to characterize the T-DNA insert present in MON 87427. Genomic DNA from MON 87427 was digested using two restriction enzymes and subjected to Southern blot analyses utilizing probes that collectively

cover the entire sequence of the plasmid vector PV-ZMAP1043. The selection and design of the probes used in this study allowed for the determination of the copy number and presence or absence of all sequences from plasmid vector PV-ZMAP1043. Descriptions of the genetic elements and their locations within plasmid vector PV-ZMAP1043 are shown in Table 1. The genetic elements integrated in MON 87427 are summarized in Table 2. A map of plasmid vector PV-ZMAP1043 annotated with the probes used in the Southern analyses is presented in Figure 1. A linear map depicting the relative positions of the restriction sites within the inserted T-DNA sequence and the genomic DNA immediately flanking the insert in MON 87427 is shown in Figure 2 and is based on the sequence of the insert and adjacent genomic DNA discussed in Section 4.2. Based on the linear map of the insert and on the plasmid vector map, a table summarizing the expected DNA fragments for Southern analyses is presented in Table 3. The LH198 BC3F4 generation of MON 87427 was used for the molecular characterization analyses in this study and used to initiate commercial breeding. The generations used in this study are depicted in the breeding history diagram shown in Figure 3. The molecular weight markers on the left side of the Southern blot figures (Figure 4 through Figure 7) were used to estimate the sizes of bands present in the long run lanes. The molecular weight markers on the right side of the Southern blot figures (Figure 4 through Figure 7) were used to estimate the band sizes present in the short run lanes. The Southern blots (Figure 4 through Figure 7, and Figure 14) presented in this report are representative of the data generated in the study.

#### **4.1.1 Southern Blot Analyses to Determine Copy Number of the Inserted T-DNA**

The copy number and insertion sites of T-DNA sequences in the corn genome were evaluated by digesting the LH198 BC3F4 generation of MON 87427 and conventional control LH198 × HiII genomic DNA samples with the restriction enzymes *Nco* I and *Nsi* I in separate reactions. These enzymes cleave both within the T-DNA insert and within the known flanking sequences on either side of the insert (Figure 2) in MON 87427. *Nco* I and *Nsi* I each produce different restriction fragments containing portions of the insert and adjacent genomic DNA so that each enzyme generates a specific banding pattern on the Southern blots. If T-DNA sequences are present as a single copy at a single integration site in MON 87427, then hybridizing with probes against the T-DNA sequence should yield bands corresponding to the restriction fragments described in Table 3 and Figure 2. Any additional copies and/or integration sites would be detected as extra bands on the blots.

The Southern blots were hybridized with T-DNA probes that collectively span the entire inserted DNA sequence (Figure 1, Probe 1 through Probe 4). Each Southern blot contains a negative control and several positive controls. Conventional control LH198 × HiII genomic DNA digested with the appropriate restriction enzymes and spiked with either plasmid vector DNA or probe template(s) served as positive hybridization controls. In the Southern blots shown in Figure 4 through Figure 7, conventional control LH198 × HiII genomic DNA

digested with the appropriate restriction enzymes was used as a negative control.

#### 4.1.1.1 Probe 1 and Probe 4

Conventional control LH198 × HiII and MON 87427 genomic DNA digested with either *Nco* I or *Nsi* I (Figure 4) produced endogenous hybridization signals that were present in all lanes (Figure 4, lane 1 through lane 11). Each hybridization signal was produced in a test and conventional control lane; therefore, these signals are considered to be endogenous background hybridization and are not specific to the inserted DNA in MON 87427. These signals most likely resulted from hybridization of Probe 1 with the endogenous *Hsp70* sequence, because this probe contains a small portion of the *Hsp70* intron (Figure 1). Since the region of Probe 1 corresponding to the *Hsp70* intron sequence was small, the hybridization signals were relatively weak. These hybridization signals can be seen both in the form of the endogenous bands described below, and in the form of the background hybridization seen in each lane of the blot, and are not specific to the inserted DNA in MON 87427.

Conventional control LH198 × HiII genomic DNA digested with *Nco* I (Figure 4, lane 1 and lane 8) produced endogenous hybridization bands of ~6.1 kb and ~4.1 kb. Additional endogenous hybridization bands were visible with longer exposures of this blot (data not shown).

Conventional control LH198 × HiII genomic DNA digested with *Nsi* I (Figure 4, lane 3 and lane 10) produced endogenous hybridization bands of ~9.8 kb and ~4.3 kb. The presence of the observed endogenous bands is likely due to cross-hybridization of the probe with the genomic DNA samples. Additional endogenous hybridization bands were visible with longer exposures of this blot (data not shown).

Conventional control LH198 × HiII genomic DNA digested with *Nco* I and spiked with probe templates (Figure 1, Probe 1 and Probe 4) generated from plasmid vector PV-ZMAP1043 produced the expected bands at ~1.2 kb and ~0.7 kb (Figure 4, lane 5 and lane 6) in addition to the endogenous hybridization bands listed above.

Conventional control LH198 × HiII genomic DNA digested with *Nco* I and spiked with plasmid vector PV-ZMAP1043 DNA previously digested with *Sph* I produced the two expected bands at ~7.1 kb and ~1.8 kb (Figure 4, lane 7) in addition to the endogenous hybridization bands listed above. Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 87427 DNA digested with *Nco* I (Figure 4, lane 2 and lane 9) produced two bands (Table 3) of ~5.5 kb and ~2.2 kb in addition to the endogenous hybridization bands listed above. The ~5.5 kb band represents the 5' end of the inserted T-DNA and the adjacent flanking genomic DNA; this correlates with the expected border fragment size of ≥ 2.8 kb. The ~2.2 kb band represents the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert; this correlates with the expected border fragment size of ~2.2 kb.

MON 87427 DNA digested with *Nsi* I (Figure 4, lane 4 and lane 11) produced three bands (Table 3) of ~6.4 kb, ~2.0 kb, and ~1.7 kb in addition to the endogenous hybridization bands listed above. The ~1.7 kb band represents the 5' end of the inserted T-DNA and a small amount of adjacent flanking genomic DNA; this correlates with the expected border fragment size of ~1.7 kb. The ~2.0 kb band contains an internal portion of the inserted DNA; this correlates with the expected fragment size of ~2.0 kb. The ~6.4 kb band represents the 3' end of the inserted T-DNA and the adjacent genomic DNA flanking the 3' end of the insert; this correlates with the expected border fragment size of  $\geq 1.3$  kb.

The results presented in Figure 4 indicate that the sequences covered by Probe 1 and Probe 4 reside at a single detectable locus of integration in MON 87427.

#### 4.1.1.2 Probe 2

Conventional control LH198  $\times$  HiII and MON 87427 genomic DNA digested with *Nco* I and *Nsi* I (Figure 5) produced endogenous hybridization signals that were present in all lanes (Figure 5, lane 1 through lane 11). Each hybridization signal was produced in a test and conventional control lane; therefore, these signals are considered to be endogenous background hybridization and are not specific to the inserted DNA in MON 87427. These signals most likely resulted from hybridization of Probe 2 with the endogenous *Hsp70* sequence, because this probe encompasses the majority of the *Hsp70* intron in PV-ZMAP1043 (Figure 1). Since the region of Probe 2 corresponding to the *Hsp70* intron sequence was large, the hybridization signals were relatively strong. These hybridization signals are considered to be endogenous background hybridization and are not specific to the inserted DNA in MON 87427.

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nco* I (Figure 5, lane 1 and lane 8) produced an endogenous hybridization band of ~4.1 kb.

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nsi* I (Figure 5, lane 3 and lane 10) produced endogenous hybridization bands of ~5.2 kb and ~4.2 kb.

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nco* I and spiked with probe template (Figure 1, Probe 2) generated from plasmid vector PV-ZMAP1043 produced the expected band at ~1.0 kb (Figure 5, lane 5 and lane 6) in addition to the endogenous hybridization bands listed above.

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nco* I and spiked with plasmid vector PV-ZMAP1043 DNA previously digested with *Sph* I produced the expected band at ~7.1 kb (Figure 5, lane 7) in addition to the endogenous hybridization bands listed above. Detection of the positive controls indicates that the probe hybridized to its target sequences.

MON 87427 genomic DNA digested with *Nco* I (Figure 5, lane 2 and lane 9) produced two bands (Table 3) of ~5.5 kb and ~2.2 kb in addition to the endogenous hybridization bands listed above. The ~5.5 kb band represents the 5' end of the inserted T-DNA and the adjacent flanking genomic DNA; this

correlates with the expected border fragment size of  $\geq 2.8$  kb. The  $\sim 2.2$  kb band represents the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert; this correlates with the expected border fragment size of  $\sim 2.2$  kb.

MON 87427 DNA digested with *Nsi* I (Figure 5, lane 4 and lane 11) produced two bands (Table 3) of  $\sim 2.0$  kb and  $\sim 1.7$  kb in addition to the endogenous hybridization bands listed above. The  $\sim 1.7$  kb band represents the 5' end of the inserted T-DNA and a small amount of adjacent flanking genomic DNA, which correlates with the expected border fragment size of  $\sim 1.7$  kb. The  $\sim 2.0$  kb band represents an internal portion of the inserted T-DNA, which correlates with the expected fragment size of  $\sim 2.0$  kb.

The results presented in Figure 5 indicate that the sequence covered by Probe 2 resides at a single detectable locus of integration in MON 87427.

#### 4.1.1.3 Probe 3

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nco* I (Figure 6, lane 1 and lane 8) or digested with *Nsi* I (Figure 6, lane 3 and lane 10) showed no detectable hybridization bands, as expected for the negative control.

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nco* I and spiked with probe template (Figure 1, Probe 3) generated from plasmid vector PV-ZMAP1043 produced one expected band at  $\sim 1.5$  kb (Figure 6, lane 5 and lane 6).

Conventional control LH198  $\times$  HiII genomic DNA digested with *Nco* I and spiked with plasmid vector PV-ZMAP1043 DNA previously digested with *Sph* I produced one expected band at  $\sim 1.8$  kb (Figure 6, lane 7). Although the other *Sph* I fragment from the plasmid ( $\sim 7.1$  kb) contains a small portion of the Probe 3 sequence, it was not detected under these assay conditions. Detection of the positive controls indicates that the probe hybridized to its target sequences.

MON 87427 genomic DNA digested with *Nco* I (Figure 6, lane 2 and lane 9) produced one band (Table 3) of  $\sim 2.2$  kb. The  $\sim 2.2$  kb band represents the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert.

MON 87427 DNA digested with *Nsi* I (Figure 6, lane 4 and lane 11) produced one band (Table 3) of  $\sim 2.0$  kb. The  $\sim 2.0$  kb band represents an internal portion of the inserted T-DNA.

The results presented in Figure 6 indicate that the sequences covered by Probe 3 resides at a single detectable locus of integration in MON 87427.

#### 4.1.2 Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-ZMAP1043 Backbone Sequences

The presence or absence of plasmid vector backbone sequences in the corn genome was evaluated by digesting MON 87427 and conventional control LH198  $\times$  HiII genomic DNA samples with *Nco* I or *Nsi* I and hybridized simultaneously with

overlapping probes spanning the entire backbone sequence of PV-ZMAP1043 (Figure 1, Probe 5, Probe 6, and Probe 7). If backbone DNA sequences were present in MON 87427, then hybridizing with overlapping probes corresponding to the backbone sequence should result in the detection of hybridization bands on the Southern blot. The results of this analysis are shown in Figure 7.

#### 4.1.2.1 Probe 5, Probe 6 and Probe 7

Conventional control LH198 × HiII genomic DNA digested with *Nco* I (Figure 7, lane 1 and lane 10) or *Nsi* I (Figure 7, lane 3 and lane 12) showed no detectable hybridization bands, as expected for the negative control.

Conventional control LH198 × HiII genomic DNA digested with *Nco* I and spiked with probe templates (Figure 1, Probe 5 and Probe 6) generated from plasmid vector PV-ZMAP1043 produced two expected bands at ~1.8 kb and ~1.5 kb, respectively (Figure 7, lane 5 and lane 6). Conventional control LH198 × HiII genomic DNA digested with *Nco* I and spiked with probe template (Figure 1, Probe 7) generated from plasmid vector PV-ZMAP1043 produced one expected band at ~1.7 kb (Figure 7, lane 7 and lane 8).

Conventional control LH198 × HiII genomic DNA digested with *Nco* I and spiked with plasmid vector PV-ZMAP1043 DNA previously digested with *Sph* I produced one expected band of ~7.1 kb (Figure 7, lane 9). Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 87427 genomic DNA digested with *Nco* I (Figure 7, lane 2 and lane 11) or *Nsi* I (Figure 7, lane 4 and lane 13) produced no detectable bands.

The results presented in Figure 7 indicate that MON 87427 contains no detectable backbone elements from plasmid vector PV-ZMAP1043.

## 4.2 Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87427

The organization of the elements within the MON 87427 insert was confirmed using PCR analyses by amplifying and sequencing three overlapping regions of DNA that span the entire length of the insert and the associated flanking corn 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 8.

No PCR products were generated in negative control reactions containing no template DNA (Figure 8, lane 4, lane 7, and lane 10) or containing conventional control LH198 × HiII genomic DNA (Figure 8, lane 3, lane 6, and lane 9) with any of the primer sets. PCR products of the expected sizes were produced in reactions containing MON 87427 genomic DNA: ~2.9 kb for Product A (Figure 8, lane 2); ~3.0 kb for Product B (Figure 8, lane 5); and ~1.3 kb for Product C (Figure 8, lane 8). The generation of these overlapping PCR products confirms that the organization of the insert is as expected.

To determine the sequence of the insert in MON 87427 and genomic DNA flanking the insert, Product A, Product B, and Product C (Figure 8) were subjected to DNA sequencing. The consensus sequence representing the insert in MON 87427, including the genomic DNA flanking the insert, is shown in Figure 9 and is described in Table 2. This consensus sequence was generated by compiling numerous sequencing reactions using Product A, Product B, and Product C. The consensus sequence was then aligned to the sequence of PV-ZMAP1043 to determine the sequence of the T-DNA present in MON 87427. The amplification and sequencing of the insert and flanking DNA from MON 87427 establishes that the arrangement of genetic elements and nucleotide sequence within the T-DNA in the insert is as depicted in Figure 2 and Figure 9. The consensus DNA sequence of the MON 87427 insert is 3681 base pairs long, beginning at base 191 in the Left Border region of plasmid vector PV-ZMAP1043 and ending at base 3871 in the Right Border region of plasmid vector PV-ZMAP1043. PCR and sequence analyses of the DNA sequence of the MON 87427 insert and adjacent genomic DNA sequence demonstrate that the organization and sequence of the genetic elements in the insert are consistent with those in plasmid vector PV-ZMAP1043 (Figure 1).

The sequence of 1003 base pairs of DNA flanking the 5' end of the insert and 1092 base pairs of DNA flanking the 3' end of the insert were also determined (Figure 9). MON 87427 genomic DNA contains a poly(C) tract downstream of the insertion site (Figure 9, base 4951 through base 4960). The consensus sequence of this poly(C) tract indicates that it contains approximately 10 cytosines. However, the exact length of this region could not be determined using standard sequencing methods, because the PCR template generated for the region contained a mixed population of templates containing differing numbers of cytosines (Figure 10) (Fazekas et al., 2010).

#### **4.3 PCR and DNA Sequence Analysis to Examine the Integrity of the DNA Insertion Site in MON 87427**

PCR and sequence analyses were performed to demonstrate that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87427 are native to the corn genome and to examine the insertion site in MON 87427. PCR was performed using MON 87427 and conventional control LH198 × HiII genomic DNA as template. One primer specific to the genomic DNA sequence flanking the 5' end of the insert (Figure 11, Primer A) was paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure 11, Primer B). The results of the PCR analysis are shown in Figure 11.

No PCR product was generated in the control reaction containing no template DNA (Figure 11, lane 4), as expected. The reaction containing conventional control LH198 × HiII genomic DNA template (Figure 11, lane 2) generated a ~1.5 kb PCR product. As expected, a ~5.0 kb PCR product between Primer A and Primer B in MON 87427 (Figure 11, lane 3) was not amplified in this analysis, since the PCR conditions necessary to generate a product of this size were not used.



The ~1.5 kb PCR product generated from the conventional control LH198 × HiII genomic DNA was sequenced, and the resulting data are shown in Figure 12. Comparison of this sequence to the 5' and 3' genomic sequences flanking the insert (Figure 9) indicate that insertions at the 5' insert-to-flank junction and 3' insert-to-flank junction and a deletion of the genomic DNA occurred at the MON 87427 insertion site. A 41 base pair insertion (Figure 9, base 963 through base 1003) is located at the 5' end of the T-DNA. A 24 base pair insertion (Figure 9, base 4685 through base 4708) is located at the 3' end of the T-DNA. Base 1 through base 726 in Figure 12 match base 237 through base 962 of the 5' genomic DNA sequence flanking the MON 87427 insert as shown in Figure 9. Base 727 through base 866 of the conventional control LH198 × HiII DNA sequence (Figure 12) are not found in the 5' or 3' flanking sequences shown in Figure 9, indicating that 140 bases were most likely deleted upon T-DNA insertion. Base 867 through base 1340 in Figure 12 match base 4709 through base 5182 of the 3' genomic DNA sequence flanking the MON 87427 insert as shown in Figure 9.

This analysis confirms that the genomic sequences flanking the insert in MON 87427 are native to the corn genome. The analysis also indicated that a 140 base pair deletion, a 41 base pair insertion at the 5' insert-to-flank junction, and a 24 base pair insertion at the 3' insert-to-flank junction occurred during integration of the T-DNA sequence. Such deletions and insertions, presumably resulting from double-stranded break repair mechanisms in the plant, are common during the *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998).

MON 87427 and the conventional control LH198 × HiII genomic DNA each contain a poly(C) tract downstream of the insertion site (Figure 9, base 4951 through base 4960; Figure 12, base 1109 through base 1118). The consensus sequence of each poly(C) tract indicated that it contains approximately 10 cytosines. However, the exact length of this region could not be determined using standard sequencing methods, because the PCR template generated for the region contained a mixed population of templates containing differing numbers of cytosines (Figure 10 and Figure 13) (Fazekas et al., 2010). While the exact length of this poly(C) tract could not be determined, the sequence on either side of the tract is identical between MON 87427 and the conventional control LH198 × HiII. The fact that both MON 87427 and the conventional control LH198 × HiII have a poly(C) region confirmed that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87727 are native to the corn genome.

#### **4.4 Southern Blot Analysis to Examine Insert Stability in Five Generations of MON 87427**

In order to demonstrate the stability of the insert in MON 87427, Southern blot analysis was performed using genomic DNA obtained from five generations of MON 87427. For reference, the breeding history of MON 87427 and the generations that were tested are indicated in Figure 3. The MON 87427 LH198 BC3F4 generation was used for the molecular characterization analyses shown in Figure 4 through Figure 7. To analyze insert stability, four additional generations, MON 87427 LH198 BC3F3, LH198 BC3F6, LH198 BC3F7, and

[LH198 BC3F7 × LH287]F1, were evaluated by Southern blot analysis and compared to the LH198 BC3F4 generation. Genomic DNA, isolated from each of the selected generations of MON 87427, was digested with the restriction enzyme *Nsi* I (Figure 2) and hybridized with Probe 1 and Probe 4 (Figure 1). Probe 1 and Probe 4 are designed to detect both fragments generated by the *Nsi* I digest. Any instability associated with the insert would be detected as novel bands within the fingerprint on the Southern blot. This Southern blot contains the same controls as described in Section 4.1.1, including an additional conventional control LH198 × LH287 (Figure 14, lane 9). The conventional control LH198 × LH287 was provided as a reference for MON 87427 [LH198 BC3F7 × LH287]F1 generation, which is in the LH198 × LH287 background. The molecular weight markers on both sides of the Southern blot figure (Figure 14) were used to estimate the band sizes present in the single run. The results are shown in Figure 14.

Conventional controls LH198 × HiII and LH198 × LH287, and MON 87427 genomic DNA digested with *Nsi* I (Figure 14) produced hybridization signals resulting from endogenous targets residing in the corn genome. Each hybridization signal was produced in a test and conventional control lane; therefore, these signals are considered to be endogenous background hybridization and are not specific to the inserted DNA in MON 87427.

The conventional control LH198 × HiII (Figure 14, lane 4) digested with *Nsi* I displayed an endogenous hybridization band of ~4.3 kb. The conventional control LH198 × LH287 (Figure 14, lane 9) digested with *Nsi* I displayed the endogenous hybridization band of ~4.3 kb. In the conventional control LH198 × LH287 and MON 87427 [LH198 BC3F7 × LH287]F1 genomic DNA samples, (Figure 14, lane 9 and lane 10), the endogenous hybridization band appeared too faint on the blot, but the bands were visible with longer exposures of this blot (data not shown).

The conventional control LH198 × HiII genomic DNA digested with *Nsi* I and spiked with probe template (Figure 1, Probe 1 and Probe 4) generated from plasmid vector PV-ZMAP1043 produced the expected bands at ~1.2 kb and ~0.7 kb (Figure 14, lane 1 and lane 2) in addition to the endogenous hybridization band of ~4.3 kb.

The conventional control LH198 × HiII genomic DNA digested with *Nsi* I and spiked with plasmid vector PV-ZMAP1043 DNA previously digested with *Sph* I produced the two expected bands at ~7.1 kb and ~1.8 kb (Figure 14, lane 3) in addition to the endogenous hybridization band of ~4.3 kb. Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 87427 DNA extracted from generations LH198 BC3F3, LH198 BC3F4, LH198 BC3F6, LH198 BC3F7, and [LH198 BC3F7 × LH287]F1, and digested with *Nsi* I (Figure 14, lane 5, lane 6, lane 7, lane 8, and lane 10), produced three bands (Table 3) of ~6.4 kb, ~2.0 kb, and ~1.7 kb in addition to the endogenous hybridization bands listed above. The ~1.7 kb band represents the 5' end of the inserted T-DNA and a small amount of adjacent flanking genomic DNA; this correlates with the expected border fragment size of ~1.7 kb. The ~2.0 kb band contains an internal portion of the inserted DNA. The ~6.4 kb band represents the 3' end of the inserted T-DNA and the adjacent genomic DNA flanking the 3' end of the insert; this correlates with the expected border fragment size of ≥1.3 kb. The generations LH198 BC3F3, LH198 BC3F6, LH198 BC3F7, and [LH198 BC3F7 × LH287]F1 of MON 87427 (Figure 14, lane 5, lane 6, lane 7, lane 8, and lane 10) produced the same fingerprint as the LH198 BC3F4 generation (Figure 4, lane 4 and lane 11), indicating that the single copy of the T-DNA in MON 87427 is stably maintained across the selected generations.

## 5.0 Conclusions

Molecular characterization of MON 87427 by Southern blot analyses confirmed that the T-DNA was inserted into the corn genome at a single locus containing one copy of the *cp4 epsps* expression cassette. No elements were detected other than those associated with the reported insert. Moreover, no backbone DNA sequences from plasmid vector PV-ZMAP1043 were detected in MON 87427.

PCR and DNA sequence analyses confirmed the predicted organization and intactness of the genetic elements within the insert; determined the 5' and 3' insert-to-flank junctions; characterized the DNA sequence of the MON 87427 insert and adjacent genomic DNA sequence, with the exception of the poly(C) tract described above; determined the insertion site sequences in the conventional control LH198 × HiII; and confirmed that the genomic DNA sequences flanking the 5' and 3' ends of the MON 87427 insert are native to the corn genome. The PCR and DNA sequence analysis identified a 140 base pair deletion, a 41 base pair insertion, and a 24 base pair insertion that occurred at the insertion site.

Southern blot analysis of five MON 87427 generations demonstrated that the inserted DNA has been maintained through five generations of breeding, thereby, confirming the stability of the insert.

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Table 1. Summary of Genetic Elements in Plasmid Vector PV-ZMAP1043

Genetic Element	Location in Plasmid Vector	Function (Reference)
<b>T-DNA</b>		
<b>B<sup>1</sup>-Left Border</b>	1-442	DNA region from <i>Agrobacterium tumefaciens</i> containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	443-483	Sequences used in DNA cloning
<b>P<sup>2</sup>-e35S</b>	484-1104	Promoter for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells
<b>Intervening Sequence</b>	1105-1125	Sequences used in DNA cloning
<b>I<sup>3</sup>-Hsp70</b>	1126-1929	First intron from the maize heat shock protein 70 gene (Brown and Santino, 1997)
Intervening Sequence	1930-1953	Sequences used in DNA cloning
<b>TS<sup>4</sup>-CTP2</b>	1954-2181	Targeting sequence from the <i>ShkG</i> gene encoding the chloroplast transit peptide region of <i>Arabidopsis thaliana</i> EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
<b>CS<sup>5</sup>-cp4 epsps</b>	2182-3549	Codon-optimized coding sequence of the <i>aroA</i> gene from the <i>Agrobacterium</i> sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgett et al., 1996)
Intervening Sequence	3550-3555	Sequences used in DNA cloning
<b>T<sup>6</sup>-nos</b>	3556-3808	3' nontranslated region of the <i>nopaline synthase (nos)</i> gene from <i>Agrobacterium tumefaciens</i> that terminates transcription and directs polyadenylation (Bevan et al., 1983)
Intervening Sequence	3809-3835	Sequences used in DNA cloning
<b>B-Right Border</b>	3836-4192	DNA region from <i>Agrobacterium tumefaciens</i> containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)

**Table 1. Summary of Genetic Elements in Plasmid Vector PV-ZMAP1043 (Cont.)**

<b>Vector Backbone</b>		
Intervening Sequence	4193-4328	Sequences used in DNA cloning
<i>aadA</i>	4329-5217	Bacterial promoter, coding sequence, and 3' untranslated region for an aminoglycoside-modifying enzyme, 3''(9)- <i>O</i> -nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	5218-5747	Sequences used in DNA cloning
<b>OR<sup>7</sup>-ori-pBR322</b>	5748-6336	Origin of replication from pBR322 for maintenance of plasmid in <i>E. coli</i> (Sutcliffe, 1979)
Intervening Sequence	6337-6763	Sequences used in DNA cloning
<b>CS-rop</b>	6764-6955	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in <i>E. coli</i> (Giza and Huang, 1989)
Intervening Sequence	6956-8463	Sequences used in DNA cloning
<b>OR-ori V</b>	8464-8860	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in <i>Agrobacterium</i> (Stalker et al., 1981)
Intervening Sequence	8861-8946	Sequences used in DNA cloning

<sup>1</sup> B, Border<sup>2</sup> P, Promoter<sup>3</sup> I, Intron<sup>4</sup> TS, Targeting Sequence<sup>5</sup> CS, Coding Sequence<sup>6</sup> T, Transcription Termination Sequence<sup>7</sup> OR, Origin of Replication

Table 2. Summary of Genetic Elements in MON 87427

Genetic Element <sup>1</sup>	Location in Sequence <sup>2</sup>	Function (Reference)
Sequence flanking 5' end of the insert	1-1003	Corn genomic DNA and 41 base pair rearranged DNA at the site of insertion
B <sup>3</sup> -Left Border <sup>r1</sup>	1004-1255	DNA region from <i>Agrobacterium tumefaciens</i> containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	1256-1296	Sequences used in DNA cloning
P <sup>4</sup> - <i>e35S</i>	1297-1917	Promoter for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells
Intervening Sequence	1918-1938	Sequences used in DNA cloning
I <sup>5</sup> - <i>Hsp70</i>	1939-2742	First intron from the maize heat shock protein 70 gene (Brown and Santino, 1997)
Intervening Sequence	2743-2766	Sequences used in DNA cloning
TS <sup>6</sup> - <i>CTP2</i>	2767-2994	Targeting sequence from the <i>ShkG</i> gene encoding the chloroplast transit peptide region of <i>Arabidopsis thaliana</i> EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS <sup>7</sup> - <i>cp4 epsps</i>	2995-4362	Codon-optimized coding sequence of the <i>aroA</i> gene from the <i>Agrobacterium</i> sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgett et al., 1996)
Intervening Sequence	4363-4368	Sequences used in DNA cloning
T <sup>8</sup> - <i>nos</i>	4369-4621	3' nontranslated region of the nopaline synthase ( <i>nos</i> ) gene from <i>Agrobacterium tumefaciens</i> which terminates transcription and directs polyadenylation (Bevan et al., 1983)



**Table 2. Summary of Genetic Elements in MON 87427 (Cont.)**

Intervening Sequence	4622-4648	Sequences used in DNA cloning
<b>B-Right Border</b> <sup>r1</sup>	4649-4684	DNA region from <i>Agrobacterium tumefaciens</i> containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
<b>Sequence flanking 3' end of the insert</b>	4685-5776	Corn genomic DNA and 24 base pair rearranged DNA at the site of insertion

<sup>1</sup> Although flanking sequences and intervening sequences are not functional genetic elements, they comprise a portion of the sequence reported in Figure 9.

<sup>2</sup> Numbering refers to the sequence from Figure 9 that includes the insert in MON 87427 and adjacent genomic DNA.

<sup>3</sup> B, Border

<sup>4</sup> P, Promoter

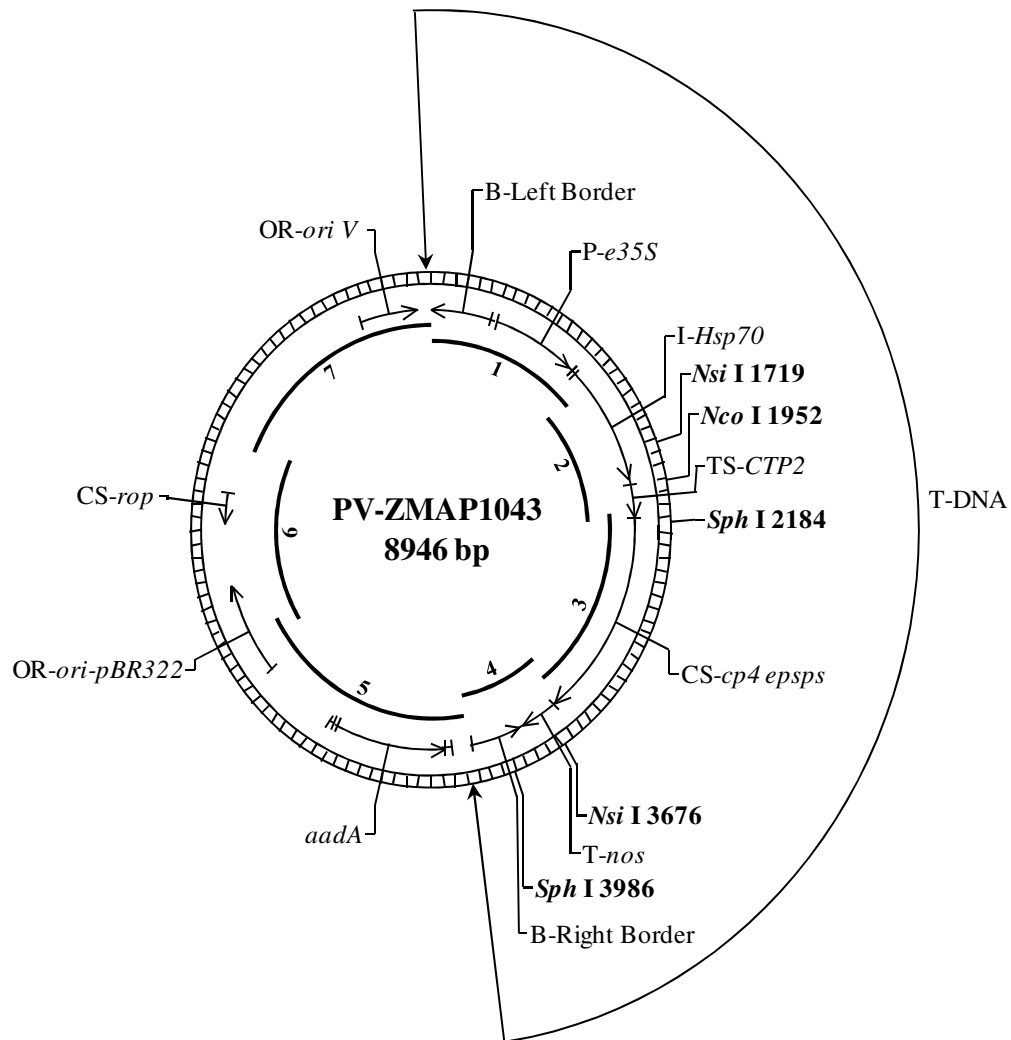
<sup>5</sup> I, Intron

<sup>6</sup> TS, Targeting Sequence

<sup>7</sup> CS, Coding Sequence

<sup>8</sup> T, Transcription Termination Sequence

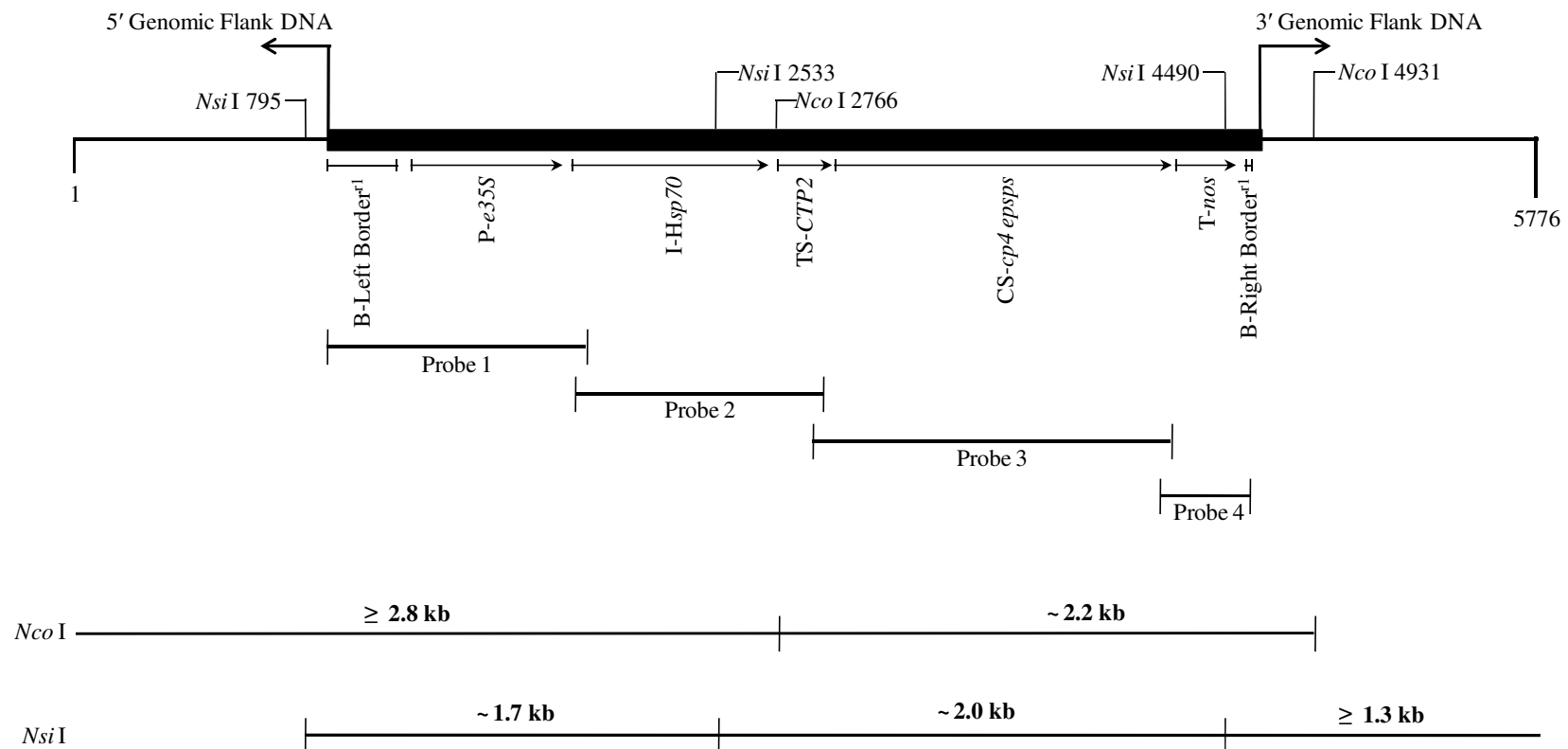
<sup>r1</sup> Superscripts in Left and Right Borders indicate that the sequences in MON 87427 were truncated compared to the sequences in the plasmid vector PV-ZMAP1043.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	T-DNA Probe 1	1	1200	1.2
2	T-DNA Probe 2	1150	2150	1.0
3	T-DNA Probe 3	2100	3550	1.5
4	T-DNA Probe 4	3500	4192	0.7
5	Backbone Probe 5	4193	5942	1.8
6	Backbone Probe 6	5864	7368	1.5
7	Backbone Probe 7	7290	8946	1.7

**Figure 1. Map of Plasmid Vector PV-ZMAP1043 Showing Probe 1 through Probe 7**

A circular map of the plasmid vector PV-ZMAP1043 used to develop MON 87427 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map. PV-ZMAP1043 contains a single T-DNA.



**Figure 2. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87427**

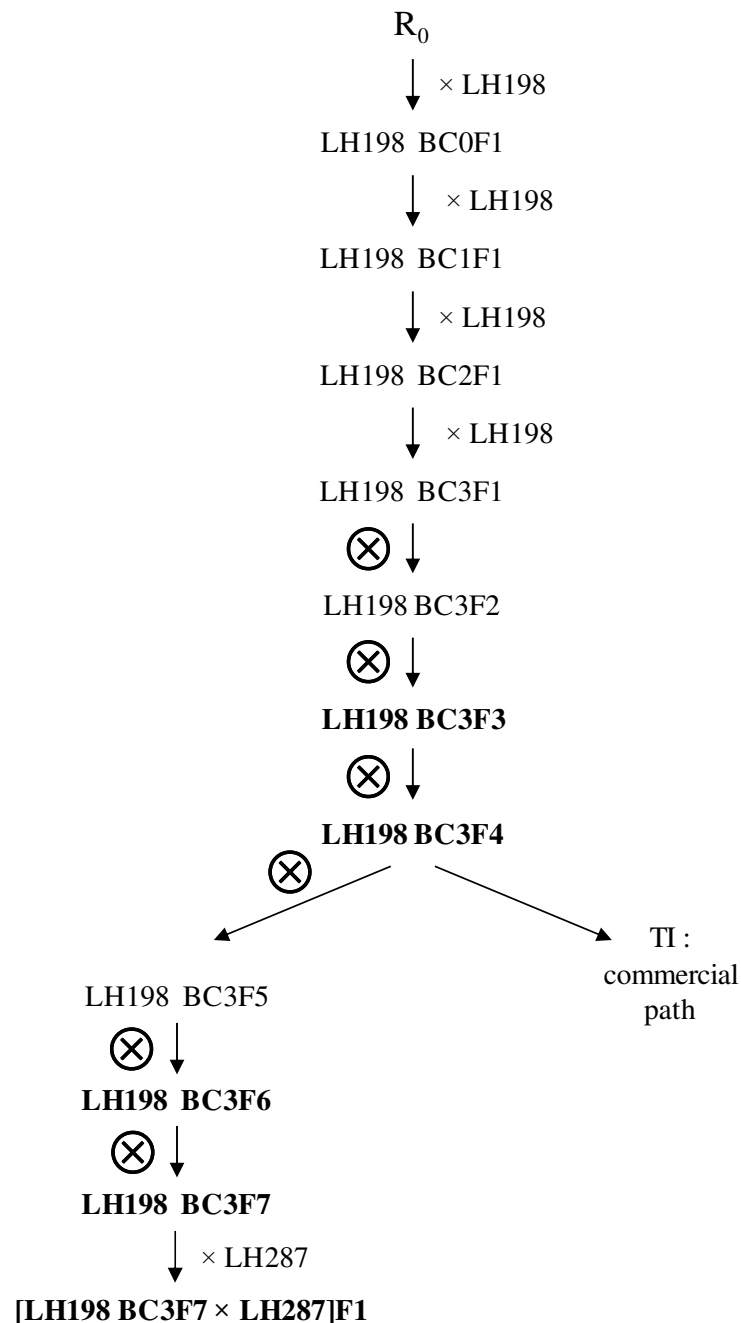
DNA derived from the T-DNA of plasmid vector PV-ZMAP1043 integrated into MON 87427. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking sequence. Identified on the linear map are genetic elements within the insert, as well as restriction enzyme sites with positions relative to the size of the DNA sequence (genomic flanks and insert) used in the Southern analyses. The relative sizes and locations of the T-DNA probes and the expected sizes of restriction fragments are indicated. This schematic diagram is not drawn to scale. Locations of genetic elements, restriction sites, and T-DNA probes are approximate. Probes are described in Figure 1.

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

Southern Blot Analysis		T-DNA			Backbone	Insert Stability
Figure Number		4	5	6	7	14
Probes Used		1, 4	2	3	5, 6, 7	1, 4
Probing Target	Digestion Enzyme	Expected Band Sizes on each Southern Blot				
Plasmid Vector PV-ZMAP1043	<i>Sph</i> I	~7.1 kb ~1.8 kb	~7.1 kb	~7.1 kb ~1.8 kb	~7.1 kb	~7.1 kb ~1.8 kb
Probe Templates <sup>1</sup>		~1.2 kb ~0.7 kb	~1.0 kb	~1.5 kb	~1.8 kb ~1.5 kb ~1.7 kb	~1.2 kb ~0.7 kb
MON 87427	<i>Nco</i> I	≥ 2.8 kb ~2.2 kb	≥ 2.8 kb ~2.2 kb	~2.2 kb	No band	-- <sup>2</sup>
	<i>Nsi</i> I	~1.7 kb ~2.0 kb ≥ 1.3 kb	~2.0 kb ~1.7 kb	~2.0 kb	No band	~1.7 kb ~2.0 kb ≥ 1.3 kb

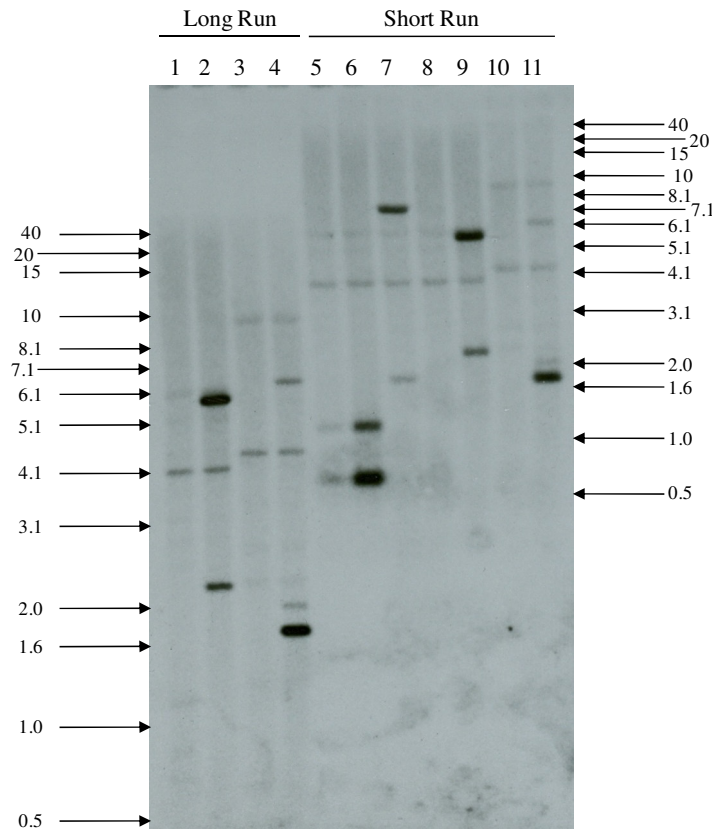
<sup>1</sup> probe template spikes were used as positive hybridization controls in Southern blot analyses

<sup>2</sup> '--' indicates that the particular restriction enzyme was not used in the analysis



**Figure 3. MON 87427 Breeding History Diagram**

The LH198 BC3F4 generation was used for the molecular characterization of MON 87427. Generations used for insert stability Southern blot analysis are indicated in bold text.  $R_0$  corresponds to the transformed plant. F# is the filial generation. ⊗ designates self-pollination. BC# is the backcross generation, and TI corresponds to trait integration for commercial seed development.



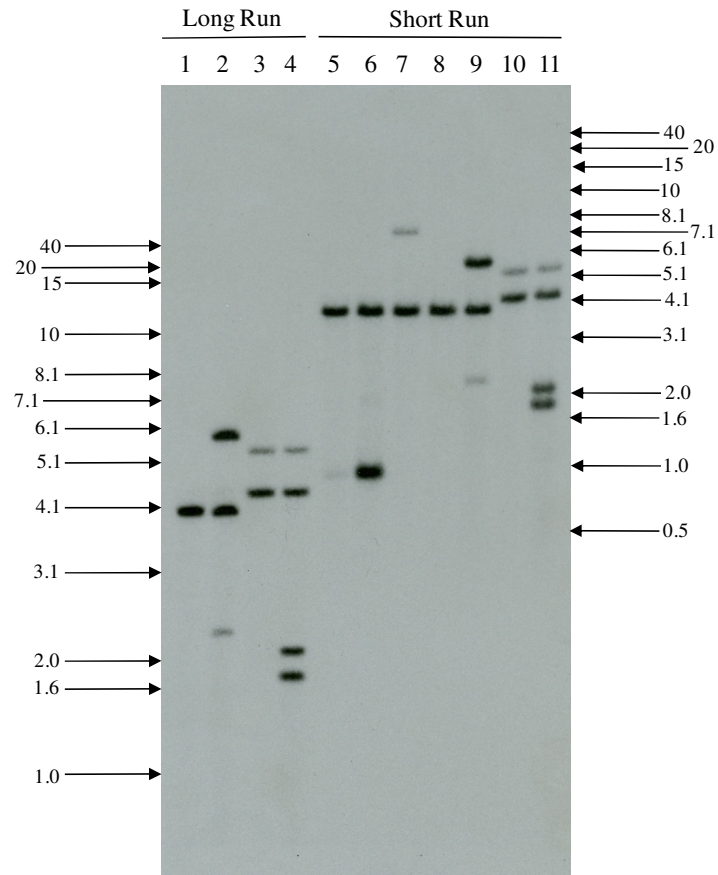
**Figure 4. Southern Blot Analysis of MON 87427: T-DNA Analysis (Probe 1 and Probe 4)**

The blot was hybridized with two  $^{32}\text{P}$ -labeled probes that spanned portions of the T-DNA sequence (Figure 1, Probe 1 and Probe 4). Each lane contains ~10  $\mu\text{g}$  of digested genomic DNA isolated from corn seed. Lane designations are as follows:

**Lane**

- 1 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 2 MON 87427 (*Nco* I)
- 3 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 4 MON 87427 (*Nsi* I)
- 5 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 1 and Probe 4 [~0.1 genome equivalent]
- 6 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 1 and Probe 4 [~1.0 genome equivalent]
- 7 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
- 8 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 9 MON 87427 (*Nco* I)
- 10 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 11 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.



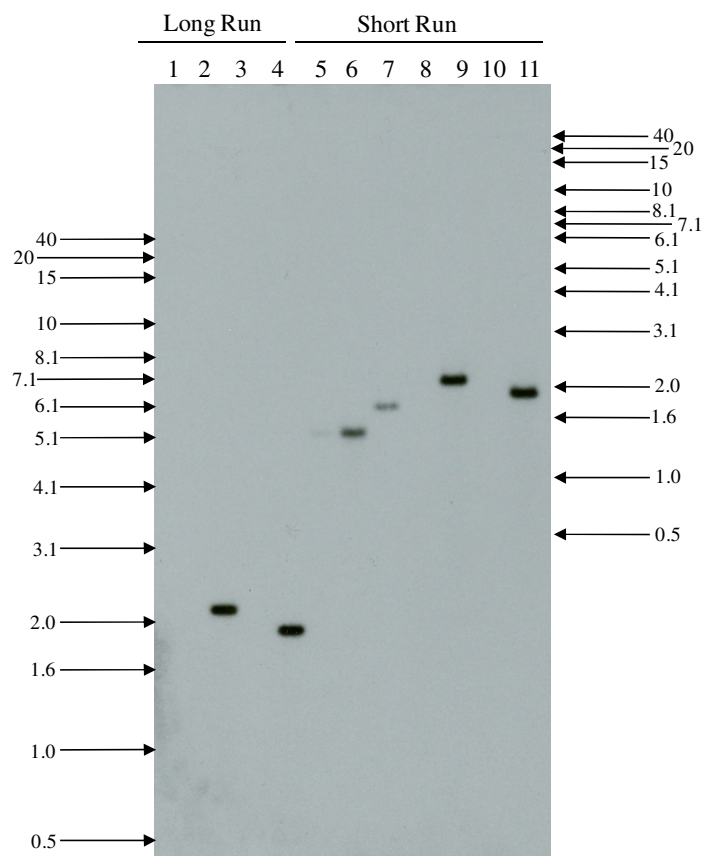
**Figure 5. Southern Blot Analysis of MON 87427: T-DNA Analysis (Probe 2)**

The blot was hybridized with one  $^{32}\text{P}$ -labeled probe that spanned a portion of the T-DNA sequence (Figure 1, Probe 2). Each lane contains ~10  $\mu\text{g}$  of digested genomic DNA isolated from corn seed. Lane designations are as follows:

**Lane**

- 1 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 2 MON 87427 (*Nco* I)
- 3 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 4 MON 87427 (*Nsi* I)
- 5 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 2 [~0.1 genome equivalent]
- 6 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 2 [~1.0 genome equivalent]
- 7 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
- 8 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 9 MON 87427 (*Nco* I)
- 10 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 11 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.



**Figure 6. Southern Blot Analysis of MON 87427: T-DNA Analysis (Probe 3)**

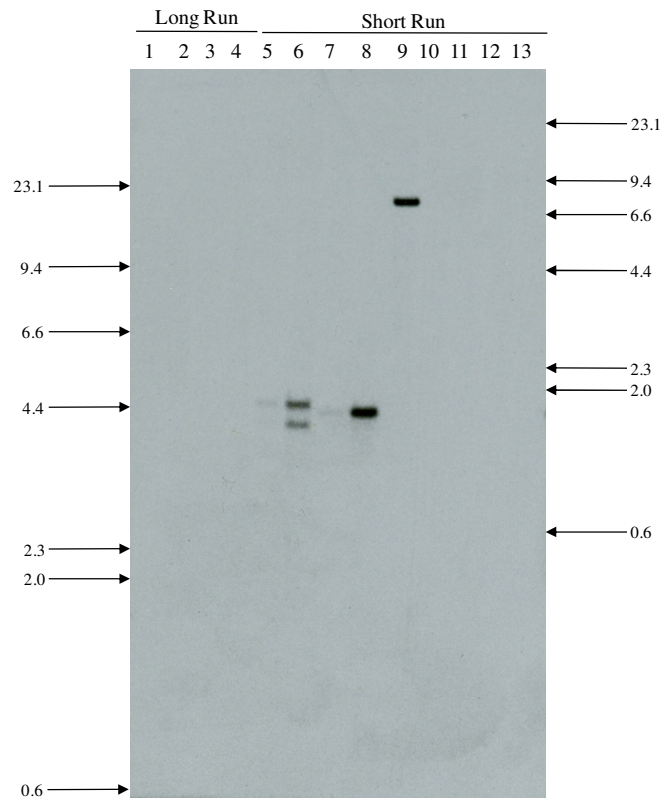
The blot was hybridized with one  $^{32}\text{P}$ -labeled probe that spanned a portion of the T-DNA sequence (Figure 1, Probe 3). Each lane contains ~10  $\mu\text{g}$  of digested genomic DNA isolated from corn seed. Lane designations are as follows:

**Lane**

- 1 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 2 MON 87427 (*Nco* I)
- 3 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 4 MON 87427 (*Nsi* I)
- 5 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 3 [~0.1 genome equivalent]
- 6 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 3 [~1.0 genome equivalent]
- 7 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
- 8 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 9 MON 87427 (*Nco* I)
- 10 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 11 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.





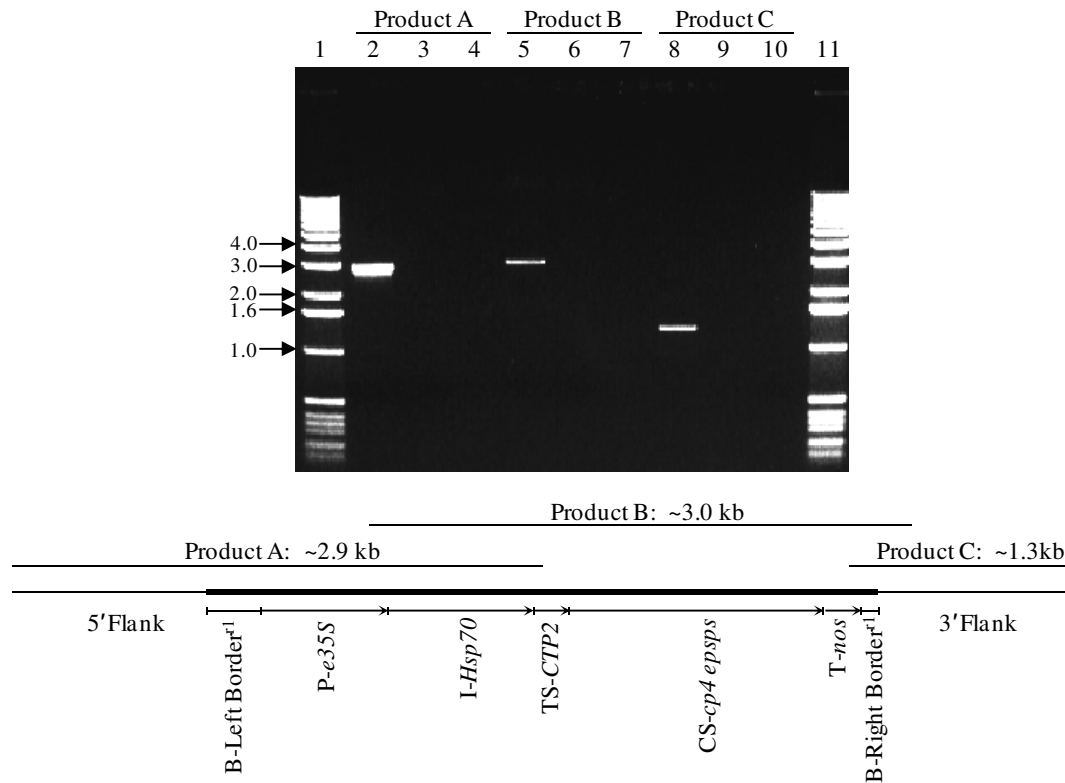
**Figure 7. Southern Blot Analysis of MON 87427: PV-ZMAP1043 Backbone Sequence Analysis (Probe 5, Probe 6 and Probe 7)**

The blot was hybridized with three overlapping  $^{32}\text{P}$ -labeled probes that spanned the entire PV-ZMAP1043 backbone sequence (Figure 1, Probe 5, Probe 6, and Probe 7). Each lane contains ~10  $\mu\text{g}$  of digested genomic DNA isolated from corn seed. Lane designations are as follows:

**Lane**

- 1 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 2 MON 87427 (*Nco* I)
- 3 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 4 MON 87427 (*Nsi* I)
- 5 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 5 and Probe 6 [~0.1 genome equivalent]
- 6 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 5 and Probe 6 [~1.0 genome equivalent]
- 7 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 7 [~0.1 genome equivalent]
- 8 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with Probe 7 [~1.0 genome equivalent]
- 9 Conventional Control LH198  $\times$  HiII (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
- 10 Conventional Control LH198  $\times$  HiII (*Nco* I)
- 11 MON 87427 (*Nco* I)
- 12 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 13 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the  $\lambda$  DNA /*Hind* III Fragments (Invitrogen) on the ethidium bromide stained gel.



**Figure 8. Overlapping PCR Analysis Across the Insert in MON 87427**

PCR was performed on conventional control LH198 × HiII genomic DNA isolated from corn seed and on MON 87427 genomic DNA isolated from corn seed. Only lanes containing PCR reactions are shown in the figure. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87427 that appears at the bottom of the figure. Four to nine microliters of each of the PCR reactions were loaded on the gel. PCR products reported in this figure are representative of the study data.

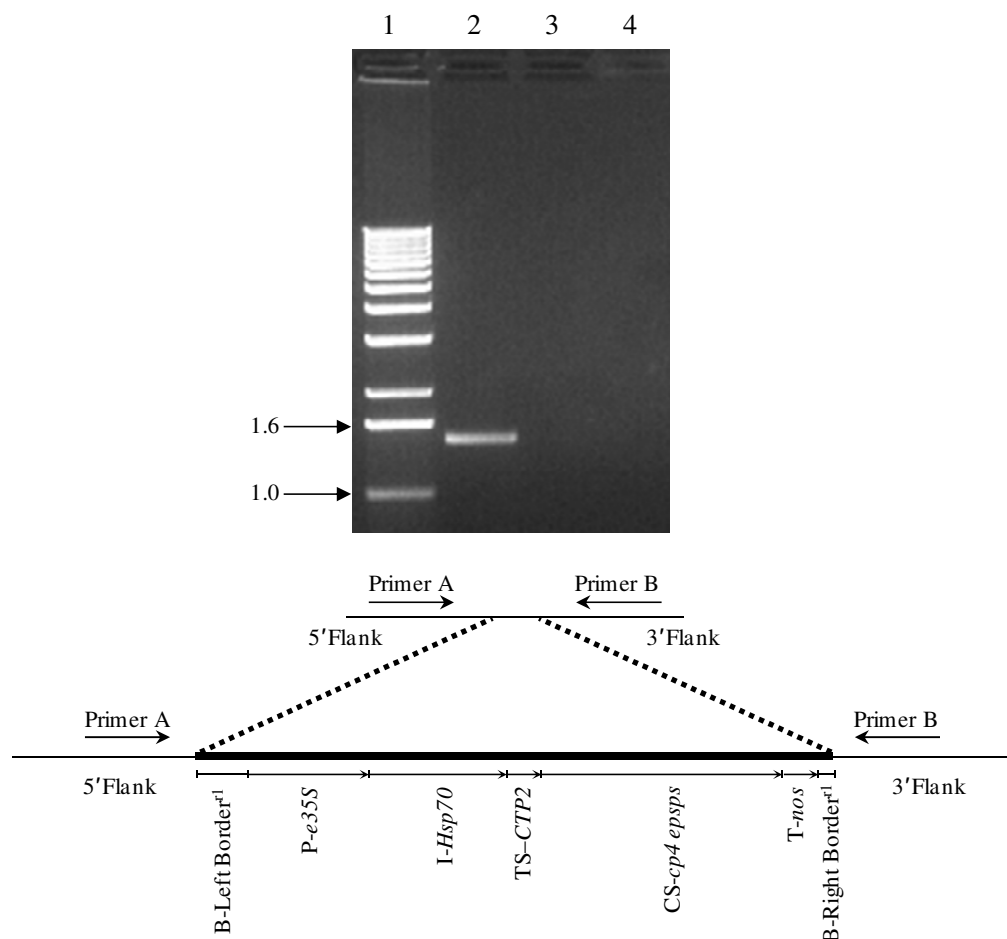
**Lane**

- 1 1 Kb DNA Ladder
- 2 MON 87427
- 3 Conventional Control LH198 × HiII
- 4 No template DNA control
- 5 MON 87427
- 6 Conventional Control LH198 × HiII
- 7 No template DNA control
- 8 MON 87427
- 9 Conventional Control LH198 × HiII
- 10 No template DNA control
- 11 1 Kb DNA Ladder

The arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Ladder (Invitrogen) on the ethidium bromide stained gel.

**[CBI CROSS REFERENCE 1]**

**[CBI CROSS REFERENCE 2]**



**Figure 11. PCR Amplification of the MON 87427 Insertion Site in the Conventional Control LH198 × HiII**

PCR was performed on conventional control LH198 × HiII genomic DNA isolated from corn seed and on MON 87427 genomic DNA isolated from corn seed. Only lanes containing PCR reactions are shown in the figure. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. Depiction of the MON 87427 insertion site in conventional control LH198 × HiII (upper panel) and the MON 87427 insert (lower panel). PCR amplification was performed using Primer A in the 5' flanking sequence and Primer B in the 3' flanking sequence of the insert in MON 87427 to verify that the flanking sequences are native to the corn genome. Five microliters of each of the PCR reactions were loaded on the gel. Lane designations are as follows:

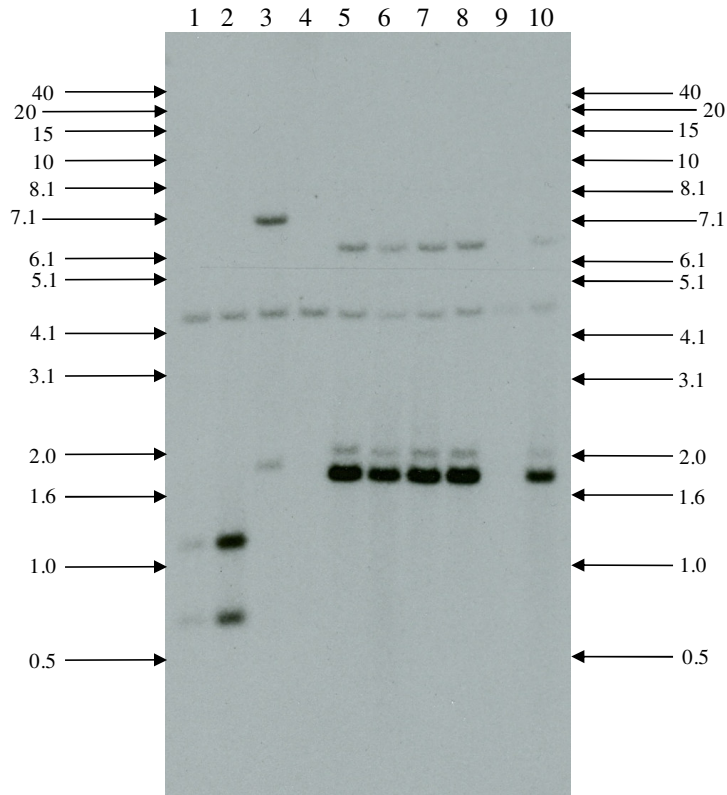
**Lane**

- 1 1 Kb DNA Ladder
- 2 Conventional Control LH198 × HiII
- 3 MON 87427
- 4 No template DNA control

The arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Ladder (Invitrogen) on the ethidium bromide stained gel.

**[CBI CROSS REFERENCE 3]**

**[CBI CROSS REFERENCE 4]**



**Figure 14. Insert Stability of MON 87427: T-DNA (Probe 1 and Probe 4)**

The blot was hybridized with two  $^{32}\text{P}$ -labeled probes that spanned portions of the T-DNA sequence (Figure 1, Probe 1 and Probe 4). Each lane contains ~10  $\mu\text{g}$  of digested genomic DNA isolated from corn seed with the exception of MON 87427 LH198 BC3F3, MON 87427 LH198 BC3F6, and MON 87427 LH198 BC3F7, which were isolated from leaf tissue. Lane designations are as follows:

**Lane**

- 1 Conventional Control LH198  $\times$  HiII (*Nsi* I) spiked with Probe 1 and Probe 4 [~0.1 genome equivalent]
- 2 Conventional Control LH198  $\times$  HiII (*Nsi* I) spiked with Probe 1 and Probe 4 [~1.0 genome equivalent]
- 3 Conventional Control LH198  $\times$  HiII (*Nsi* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
- 4 Conventional Control LH198  $\times$  HiII (*Nsi* I)
- 5 MON 87427 (LH198 BC3F3) (*Nsi* I)
- 6 MON 87427 (LH198 BC3F4) (*Nsi* I)
- 7 MON 87427 (LH198 BC3F6) (*Nsi* I)
- 8 MON 87427 (LH198 BC3F7) (*Nsi* I)
- 9 Conventional Control LH198  $\times$  LH287 (*Nsi* I)
- 10 MON 87427 ([LH198 BC3F7  $\times$  LH287]F1) (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

### **Appendix 1. Standard Operating Procedures**

BR-EQ-0065-02	DyNA Quant 200 Fluorometer
BR-ME-0094-02	DNA Extraction from Plant Tissues (Based on Procedure of Rogers and Bendich)
BR-ME-0315-02	Procedure for Agarose Gel Electrophoresis
BR-ME-0316-01	Procedure for Restriction Enzyme Digestion of DNA
BR-ME-0317-02	Procedure for Southern Blot Analysis
BR-ME-0486-01	Polymerase Chain Reaction
BR-ME-0611-01	Radiolabeling of Nucleic Acids
BR-ME-0878-02	Plant Tissue Processing and Analyte Extraction in Various Matrices Using the Harbil <sup>®</sup> 5G High-Speed Paint Shaker
BR-ME-0889-01	Purification of DNA from an Agarose Gel (Adaptation of Qiagen QiaQuick Gel Extraction Kit)
AG-ME-1153-02	Quick CTAB DNA Extraction from Leaf Tissue