SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

AMENDED REPORT

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

P. Song, J. Cruse, A. Thomas

STUDY COMPLETED ON

19-June-2009

Amended Report Date: November 8, 2010

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

081087

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Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

SUMMARY

Soybean (*Glycine max*) event DAS-68416-4 was generated by Agrobacterium-mediated transformation of a variety "Maverick" with plasmid pDAB4468, followed by conventional breeding. Plasmid pDAB4468 contains a T-DNA insert including two expression cassettes, an *aad*-12 gene from common soil bacterium *Delftia acidovorans* and a *pat* gene from *Streptomyces viridochromogenes*. The *aad*-12 encodes an aryloxyalkanoate dioxygenase-12 (AAD-12) which provides tolerance to 2,4-dicholorophenoxyacetic acid (2,4-D), fluroxypyr, and triclopyr-based herbicides. The *pat* gene encodes a phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate based herbicides and was used as a selectable marker during the transformation of soybean variety "Maverick". In addition, RB7, a matrix attachment region from tobacco (*Nicotiana tabacuum*), is located at the 5'end of the T-DNA insert to facilitate gene expression. The initial transgenic event DAS-68416-4, carrying the insert from pDAB4468, has been self-pollinated several generations to stabilize the agronomic performance.

Southern blot analysis of individual plants from T3 to T5 generations was conducted using multiple enzyme digestions according to the restriction map of pDAB4468 and probes derived from the genetic elements in the construct. The results demonstrated that soybean event DAS-68416-4 contains a single T-DNA insert of pDAB4468. The insert includes the intact genetic elements consisting of *aad*-12 and *pat* PTU (plant transcription unit) along with a RB7 matrix attachment region at the 5' end of the insert. The hybridization pattern is identical across all four generations, indicating that the insert is stable in the soybean genome. Hybridization with probes flanking the T-DNA insert of pDAB4468 confirms that no vector backbone sequences have been incorporated into the event DAS-68416-4.

STUDY TITLE

Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

AMENDED REPORT

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

P. Song 317-337-3434
[psong@dow.com]
J. Cruse, A. Thomas

STUDY COMPLETED ON

19-June-2009

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Total number of pages is 52 including 4.1R2 of 52.

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-12

Title: Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).*

Company: Dow AgroSciences LLC

Company Agent: M. Krieger

Title: Regulatory Manager

Signature: 

Date: 26 October 2010

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

Study Initiation Date: 08-Sept-2008

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organization for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160, with the following exceptions: The preparation of plasmid DNA used in the positive control samples and the generation of template DNA for probes were conducted in a non-GLP laboratory. The GLP status of the commercial reference standard (Digoxigenin (DIG)-labeled DNA Molecular Size Marker II and 1kb Plus DNA Ladder) was unknown.

M. Krieger
Sponsor
Dow AgroSciences LLC

26 October 2010
Date

M. Krieger
Submitter
Dow AgroSciences LLC

26 October 2010
Date

P. Song
Study Director/Author
Dow AgroSciences LLC

8-Nov-2010
Study Completion Date

*** This report has been amended. The original report was completed and signed on 19-June-2009. The study director's signature and date reflect the date of the report amendment.**

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 081087

Title: Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

Study Initiation Date: 08-Sept-2008 **Study Completion Date:** 19-June-2009
***Amended Report Date:** 08-Nov-2010

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
03-Sept-2008	05-Sept-2009	Protocol Review
16-Sept-2008	16-Sept-2008	Initiation: Planting, Labeling, and Documentation
17-Oct-2008	20-Oct-2008	Sample Collection and DNA Extraction
19-Nov-2008	19-Nov-2008	Final Harvest of Plants
07, 08, 11, 12, 18, 19-May-2009	21-May-2009	Raw Data and Report Review; Test Substance Container and Sample Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

Gaya Wickremsinhe 08 Nov 2010

Gaya Wickremsinhe, RQAP-GLP
Dow AgroSciences, Quality Assurance

Date

***The original report was completed and signed on 19-Jun-2009. The new signature and date reflect the date of the report amendment.**

Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

Summary of amendment changes:
Summary Page: Page 1**R2** of 2, the phrase AMENDED REPORT and Amended Report date were added. "T6" was changed to "T5".
Title page: Page 1**R2** of 52, the phrase AMENDED REPORT and Amended Report date, Total number of pages, were added.
Page 3 **R2** of 52: the report amended date and a statement describing the original signature dates were added.
Page 4 **R2** of 52: A new QA page was added.
Page 4.1 **R2** of 52: This page is added to describe the changes.

Throughout the whole report, wherever "T5" appears, it was replaced with "T5-A". Wherever "T6" appears, it was replaced with either "T5-B" or "T5" depending on the context in the sentence. Those changed pages were Page 8 - 10, 13-15, 18-20, 23, and 28-52.

Page 23 of 52: In Table 2, "AtuORF3 3' UTR" was changed to "AtuORF23 3' UTR" due to a misspelled word. The probe position in pDAB4468 was changed from "9974 – 10744" to "9974 – 10733" due to a mismarked position in pDAB4468, which resulted in the size of AtuUbi10 promoter probe being changed from "771" to "760".
Page 25: In Table 3, "4197, 7957" was changed to "7429, 4197, 528" due to a missed *Nco* I site was added in the vector backbone.
Page 26: In Figure 1, a missed *Nco* I site "*Nco* I (1972)" was added.

Reason for Amendment:
It was discovered that the generation number of seeds was not correct due to an error in Variety database used to generate the generation number of DHT soybean event DAS-68416-4 from pedigree information.

Impact on Study:
There is no impact on the study because the nature of the seeds and the probes has not changed.

Signatures:

P. Song, Study Director — Date: 08-NOV-2010

K. A. Clayton, Global Leader, Biotechnology Regulatory Sciences — Date: 08 NOV 2010

G. N. Wickremsinhe
Quality Assurance — Date: 08 Nov 2010

SIGNATURE PAGE

P. Song
Author
Dow AgroSciences LLC

22-May-2009
Date

J. Cruse
Co-author
Dow AgroSciences LLC

21-April-09
Date

A. Thomas
Co-author
Dow AgroSciences LLC

21 April 09
Date

M. Zhuang
Peer Reviewer
Dow AgroSciences LLC

April. 21. 2009
Date

K.A. Clayton
Group Leader
Dow AgroSciences LLC

4/21/09
Date

G. Shang @ SP 21-Apr-09
Science Leader
Dow AgroSciences LLC

21 April 2009
Date

STUDY PERSONNEL

Title: Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

Study director: P. Song

Analysts: J. Cruse, A. Thomas

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Molecular Characterization of AAD-12 Soybean Event DAS-68416-4

ABSTRACT

Soybean (*Glycine max*) event DAS-68416-4 was generated by Agrobacterium-mediated transformation of a variety "Maverick" with plasmid pDAB4468, followed by conventional breeding. Plasmid pDAB4468 contains a T-DNA insert including two expression cassettes, an *aad*-12 gene from common soil bacterium *Delftia acidovoran* and a *pat* gene from *Streptomyces viridochromogenes*. The *aad*-12 encodes an aryloxyalkanoate dioxygenase-12 (AAD-12) which provides tolerance to 2,4-dicholrophenoxyacetic acid (2,4-D), fluroxypyr, and triclopyr-based herbicides. The *pat* gene encodes a phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate-based herbicides and was used as a selectable marker during the transformation of soybean variety "Maverick". In addition, there is a RB7, a matrix attachment region from tobacco (*Nicotiana tabacuum*), located at the 5'end of the T-DNA insert to facilitate gene expression. The initial transgenic event DAS-68416-4, carrying the insert from pDAB4468, has been self-pollinated several generations to stabilize the insert and agronomic performance.

Southern blot analysis of individual plants from T3 to T5 generations was conducted using multiple enzyme digestions according to the restriction map of pDAB4468 and probes derived from the genetic elements in the construct. The results demonstrated that soybean event DAS-68416-4 contains a single T-DNA insert of pDAB4468. The insert includes the intact genetic elements consisting of *aad*-12 and *pat* PTU (plant transcript unit) along with a RB7 matrix attachment region at the 5' end of the insert. The hybridization pattern is identical across all four generations, indicating that the insert is stable in the soybean genome. Hybridization with probes flanking the T-DNA insert of pDAB4468 confirms that no vector backbone sequences have been incorporated into the event DAS-68416-4.

INTRODUCTION

Soybean (*Glycine max*) event DAS-68416-4 was generated by Agrobacterium-mediated transformation of a variety "Maverick" with plasmid pDAB4468, followed by conventional breeding. Plasmid pDAB4468 contains a T-DNA insert including two expression cassettes, an *aad*-12 gene from common soil bacterium *Delftia acidovoran* and a *pat* gene from *Streptomyces viridochromogenes*. The *aad*-12 encodes an aryloxyalkanoate dioxygenase-12 (AAD-12) which provides tolerance to 2,4-dicholrophenoxyacetic acid (2,4-D), fluroxypyr, and triclopyr-based herbicides. The *pat* gene encodes a phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate-based herbicides and was used as a selectable marker during the transformation of soybean variety "Maverick". In addition, RB7, a matrix attachment region from tobacco (*Nicotiana tabacuum*), is located at the 5'end of the T-DNA insert to facilitate gene expression. The initial transgenic event DAS-68416-4, carrying the insert from pDAB4468, has been self-pollinated several generations to stabilize the agronomic performance.

The purpose of this study is to characterize the insert in the event DAS-68416-4 using Southern blot analysis. The characterization study will use the genetic elements from pDAB4468 as probes to determine the number of insert and its structure, and compare the hybridization patterns of these probes among four generations, thus confirming the stable inheritance of the insert across different generations. In addition, the absence of transformation vector backbone sequences will be demonstrated by Southern blot analysis using probes covering the whole backbone regions flanking the T-DNA insert.

MATERIALS AND METHODS

Test Substance/Test System

The test substance in this study is the inserted portion of DNA in the soybean genome responsible for production of the AAD-12 protein. The soybean genomic DNA was extracted from leaf tissue harvested from individual plants (Test System) carrying AAD-12 event DAS-68416-4. Transgenic soybean seeds from four generations of event DAS-68416-4, along with the source identification, were provided by the Department of TG&T in Dow AgroSciences (Table 1). Test substance was labeled with unique IDs associated with event number, plant generation, and plant number.

Control Substance

Negative control

The control substances used in this study are DNA extracted from leaf tissue harvested from individual plants of the non-transgenic soybean variety "Maverick" (source ID: YX07KX002114 and SGN080003-061-0001) used to produce the transgenic event. The unmodified plants have the genetic background representative of the test substance line, but do not contain *aad*-12 and *pat* gene. The control seeds were provided by the Department of TG&T in Dow AgroSciences (Table 1). Control substance was labeled with unique IDs associated with trait name and plant number.

Reference Substance

Hybridization Reference/Positive Control

Plasmid pDAB4468 was used as the transformation vector to generate AAD-12 event DAS-68416-4. The plasmid therefore serves as a reference for the *aad*-12, *pat*, and other genetic elements in the Southern blot hybridization. The reference plasmids were mixed with DNA samples from non-transgenic soybean plants "Maverick" (negative controls) to serve as positive controls during hybridization.

DNA Size Reference

Digoxigenin (DIG)-labeled DNA Molecular Size Marker II (Roche Diagnostics, Indianapolis, IN, catalog # 1-218-590) served as a size reference for Southern blot hybridization. The markers are a mixture of fragments prepared by cleavage of λ-DNA with *Hind* III. For these DIG labeled DNA markers, a photodigoxigenin has been introduced at approximately every 200^{th} to 300^{th} base in the DNA fragment by the manufacturer. In addition, a 1 kb plus DNA ladder (Invitrogen, Carlsbad, CA, catalog #: 10787-018) containing a mixture of DNA fragments with different sizes was served as a size reference for agarose gel electrophoresis.

Planting of Test and Control Seeds

Seeds were planted in a Dow AgroSciences Indianapolis greenhouse with the pots uniquely identified by labeled stakes following the DAS procedure SOP-ECL-32a. The plants were grown under typical greenhouse conditions for soybean. For generation T3 –T5-A, twelve pots, one seed per pot, were planted for each transgenic generation. For generation T5-B, ten pots, one seed per pot, were planted. Six pots, one seed per pot, were planted for the non-transgenic control Maverick. Due to poor germination rate of the first batch of Maverick seeds, additional 6

pots, one seed per pot, were planted. Emerged plants were labeled accordingly and were grown at least 2 weeks prior to AAD-12 expression verification analysis.

Verification of AAD-12 Protein Expression

Prior to leaf sample harvest, leaf punches were taken from individual plants and analyzed by AAD-12 specific Lateral Flow Strip (LFS) testing (American Bionostica, Inc., Item no. 702K100) assay to check the expression of the AAD-12 protein. This assay was conducted in accordance with the manufacturers' instructions. Each leaf punch sample was given a score of + or – for the presence or absence of AAD-12, respectively. Only positive plants from four generations of event DAS-68416-4 were subject to leaf sample collection.

Leaf Sample Collection

Leaf samples from AAD-12 protein expression positive plants in T3, T4, T5-A, and T5-B generations, and non transgenic controls "Maverick", were collected for DNA extraction or stored in approximately -80°C freezer for future use.

Genomic DNA Extraction from Leaf Tissue

DNA extraction was performed based on the method of Guillement (1). Briefly, leaf samples were ground individually in liquid nitrogen, and then extraction buffer was added to samples at a ratio of about 3:1 plus 10 µL of RNase-A (Qiagen, Valencia, catalog # 1007885). After precipitation using isopropyl alcohol, crude DNA samples were purified using PCI (phenol:chloroform:isoamyl alcohol = 25:24:1, Sigma, St. Louis, MO, catalog #: P2069) and CI (chloroform:isoamyl alcohol = 24:1, Sigma, St. Louis, MO, catalog # C0549) extraction. DNA was precipitated again by addition of 1/10 volume of 3 M NaOAc and equal volume of isopropyl alcohol. The precipitated DNA was rinsed with 70% ethanol, then dissolved in appropriate volume of 0.1× TE buffer. To check the quality of the resultant genomic DNA, an aliquot of the

DNA samples was electrophoretically separated on a 1% agarose gel containing ethidium bromide (~1 µg/mL) with 1× TBE buffer (89 mM Tris-Borate, 20 mM EDTA, pH 8.3). The gel was visualized under ultraviolet (UV) light to confirm that the DNA was not degraded and that the RNA had been removed by the RNase-A. The concentration of DNA in solution was determined by a picogreen kit (Invitrogen, Carlsbad, CA, catalog # P7589) in a fluorometer (Bio-TEK, FLX800).

DNA Digestion and Separation

Genomic DNA of 4 plants from each T4, T5-A, and T5-B generations, and 3 plants from T3 generations were used for molecular characterization. Approximately nine micrograms (µg) of genomic DNA from each transgenic sample and the non-transgenic control were digested by mixing with approximately 100 units of selected restriction enzyme and the corresponding reaction buffer. The total volume of the digest reaction is 400 µL. Each sample was incubated at approximately 37°C overnight. The restriction enzymes *Nco* I, *Pst* I, *Xho* I, *Sph* I, *Bam*H I, and *Nhe* I were used for the digests (New England Biolabs, Cat #: R0193L, R0140L, R0146L, R0182L, R0136L, and R0131L, respectively). The positive control sample for hybridization was prepared by combining pDAB4468 plasmid DNA with non transgenic genomic DNA (Maverick) at a ratio of approximately equivalent to 1 copy of transgene per soybean genome. This positive control mixture was digested using the same procedures and restriction enzyme as the test samples. DNA from the conventional soybean control (Maverick) was digested using the same procedures and restriction enzyme as the test samples to serve as a negative control.

The digested DNA samples were precipitated with Quick-Precip (Edge BioSystems, Cat #:72641) and resuspended in 1× Blue Juice gel buffer (Invitrogen, Cat#: 10816-015) to achieve the desired volume for gel loading. The DNA samples and molecular weight markers were then electrophoresed through 1×TBE 0.8% agarose (Invitrogen, Cat# 15510-019) gels submerged in 1×TBE buffer (Fisher Scientific, Cat #: BP13334-4) at approximately 50-55 volts for approximately 18-22 hours to achieve fragment separation. The gels were stained with ethidium

bromide (Invitrogen, Cat #: 15585-011) and the DNA was visualized under ultraviolet (UV) light. A photographic record was made for each stained gel.

Southern Transfer and Membrane Treatment

Southern blot analysis was performed essentially as described by Memelink, *et al* (2) and Dow AgroSciences SOPs ECL-30b. Briefly, following electrophoresis separation and visualization of the DNA fragments, the gels were depurinated by 0.25N HCl (Fisher Scientific, Cat #: 5A48-1) for about 15 minutes, and then exposed to a denaturing solution (Sigma, Cat #: N1531-4L) for approximately 30 minutes followed by neutralizing solution (AccuGENE, Cat #: 51230) for at around 30 minutes. Southern transfer was performed overnight onto Gene Screen Plus nylon membranes (Roche Diagnostics, Cat #: 1417240) using a wicking system with 10×SSC buffer (Sigma, Cat #: S6639). After transfer, the membranes were washed in a 2×SSC solution (Sigma, Cat #: S6639), followed by UV crosslinking treatment to immobilize DNA. This process resulted in Southern blot membranes ready for hybridization.

DNA Probes

DNA fragments specific to the *aad-12*, *pat,* genetic elements, and vector backbone sequences flanking the T-DNA insert were produced from plasmid pDAB4468 by polymerase chain reaction (PCR) amplification and used as templates for probe generation. Probe sizes and sequence locations are described in Table 2.

DNA Probe Labeling and Hybridization

Labeled probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, from fragments generated by primers specific to genetic elements and vector backbone in plasmid pDAB4468. Probe size and position in pDAB4468 are

described in Table 1 and Figure 1. Generation of DNA probes by PCR synthesis was carried out using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Cat #: 11636090910) following the manufacturer recommended procedures.

Labeled probes were analyzed by agarose gel electrophoresis to determine their quality and quantity. A desired amount of labeled probe was then used for hybridization to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG Easy Hyb Solution (Roche Diagnostics, Cat #: 1603558). Briefly, nylon membrane blots were briefly washed in 2×SSC and pre-hybridized in 20-25 mL of pre-warmed DIG Easy Hyb solution at 50°C for a minimum of 30 minutes in a hybridization oven. The pre-hybridization solution was then decanted and replaced with 20 mL of pre-warmed DIG Easy Hyb solution containing a desired amount of specific probes pre-denatured by boiling in water for ~5 minutes. The hybridization was then conducted at ~50°C overnight in the hybridization oven.

Detection

At the end of the probe hybridization, DIG Easy Hyb solutions containing the probes were transferred into sterile tubes and stored at -20°C. These probes could be reused 2-3 times according to the manufacturer's procedure. The membrane blots were rinsed briefly and washed twice in clean plastic containers with low stringency wash buffer (2×SSC, 0.1%SDS) for about 5 minutes at room temperature, followed by washing twice with high stringency wash buffer (0.1×SSC, 0.1% SDS) for about15 minutes at 65°C. The membrane blots were then transferred to other clean plastic containers and briefly washed with 1×washing buffer from the DIG Wash and Block Buffer Set (Roche Diagnostics, Cat #: 1585762) for approximately 2 minutes, proceeded to blocking in 1× blocking buffer for a minimum of 30 minutes, followed by incubation with anti-DIG-AP antibody (Roche Diagnostics, Cat #: 11093274910, 1:5,000 dilution) in 1× blocking buffer for a minimum of 30 minutes. After 2-3 washes with 1× washing buffer, specific DNA probes remain bound to the membrane blots and DIG-labeled DNA standards were visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System

(Roche Diagnostics, Cat #: 11759051001) following the manufacturer's recommendation. Blots were exposed to chemiluminescent film (Roche Diagnostics, Cat #: 1666657) for one or more time points to detect hybridizing fragments and to visualize molecular size marker standards. Films were then developed with an All-Pro 100 Plus film developer and images were scanned for documentation. The number and sizes of detected bands were documented for each probe. DIG-labeled DNA Molecular Weight Marker II (MWM DIG II), visible after DIG detection as described below, was used to determine hybridizing fragment size on the Southern blots.

Probe Stripping

DNA probes were stripped off the membrane blots after the Southern hybridization data were recorded, and the membrane blots were reused for hybridization with a different DNA probe. Briefly, after signal detection and film exposure, membrane blots were thoroughly rinsed with sterile water and followed by washing twice in stripping buffer (0.2N NaOH, 0.1% SDS) for approximately 15 minutes at room temperature. The membrane blots were then briefly washed in 2×SSC and exposed to chemiluminescent film to ensure the entire DNA probes were completely removed before proceeding to the next hybridization. Afterwards, the membrane blots were ready for pre-hybridization and hybridization with another DNA probe or stored in a refrigerator for the next hybridization.

RESULTS AND DISCUSSION

Number of Insertion Sites

Restriction enzymes that only have a single site in the T-DNA insert of pDAB4468 were selected to digest genomic DNA extracted from non-transgenic controls and individual transgenic plants (Figure 2). When digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with *aad*-12 probe, each individual plants from T3 to T5 generations showed one single band around 5500 bp,

8500 bp, and 7200 bp, respectively (Table 3, Figure 3-6). These bands are corresponding to the predicted band size, >4043 bp, >6229 bp, and > 6229 bp since the second restriction site in each of these digestions is located in the border regions outside of the insert. No hybridization bands were detected in the non-transgenic controls. In addition, the hybridization pattern across each generation is identical. When the *aad*-12 probe was removed and re-hybridized with *pat* probe, the hybridization pattern remains the same as observed when the *aad*-12 probe was used (Figure 7-10). The result indicates that soybean event DAS-68416-4 contains a single T-DNA insert derived from pDAB4468.

Structure of the Insert and Its Genetic Elements

According to the restriction map of the T-DNA insert in pDAB4468, *Pst* I, and *Pst* I/*Xho* I, and *Bam*H I/*Nco* I were selected to release the plant transcription unit (PTU) of *aad*-12, *pat,* and the RB7 matrix attachment region along with the AtUbi10 promoter (Figure 2). When digested with *Pst* I and hybridized with *aad*-12 probe, each individual plants from T3 to T5 generation along with the positive control displayed a single band around 2868 bp, the predicted size of *aad*-12 PTU (Table 3, Figure 11, 14A). After the aad-12 probe was removed from the blots, they were re-hybridized with re-hybridized with AtUbi10 or AtuORF23 UTR probes and the same 2868 bp band was detected (Figure 12, 13, 14B, 14C). Furthermore, the hybridization pattern is identical across different generations.

When digested with *Pst* I/*Xho* I and hybridized with *pat* probe, each individual plant from T3 to T5 generations along with the positive control displayed a single band around 1928 bp, the predicted size of *pat* PTU (Table 3, Figure 15, 18A). Similar to the *pat* probe was used, same hybridization band was detected across different generations when the blot was re-hybridized with CsVMV or AtuORF1 probes after complete removal of previously hybridized probes (Figure 16, 17, 18B, 18C).

When digested with *Bam*H I/*Nco* I and hybridized with RB7 probe, each individual plant from T3 to T5 generations along with the positive control displayed a single band around 2617 bp, the

predicted size of RB7 plus AtUbi10 promoter (Table 3, Figure 19, 21A). Similar to the RB7 probe was applied, the same 2617 bp hybridization band was detected across different generations when the blot was re-hybridized with AtUbi10 probe after complete removal of previously hybridized RB7 probe (Figure 20, 21B).

Taking together the Southern blot analysis results, it is clear that the insert in soybean event DAS-68416-4 contains intact PTUs for *aad*-12 and *pat* along with a matrix attachment region RB7 at its 5' end of the insert.

Absence of Backbone Sequences

In order to verify that no plasmid vector backbone sequences exist in event DAS-68416-4, 6 probes covering the whole backbone region of pDAB4468 were used to hybridize the blots from digestions with *Nco* I and *Sph* I/*Xho* I (Table 2, Figure 1). For T5-B generation, a blot from digestion with *Nhe* I/*Xho* I was also hybridized with backbone probes. The probes were grouped into 2 sets for hybridization purposes. Probe Set 1 included backbone1, flanking A, and *Spec*R, and Probe Set 2 included backbone 2, flanking B, and *Ori-Rep* (Figure 1, Table 2). The blots were hybridized with Probe Set 1, and then followed by Probe Set 2 after complete removal of previously deployed probes. The hybridization results demonstrated that no hybridization signals had been detected in any samples across T3 to T5 generation (Table 3, Figure 22, 23, 24, 25, 26, 27) except for the positive controls, indicating no backbone sequences from pDAB4468 has been incorporated into event DAS-68416-4.

CONCLUSIONS

Southern blot analysis of individual plants from T3 to T5 generations was conducted with multiple enzymes digestions and probes. The results demonstrated that soybean event DAS-68416-4 contains a single T-DNA insert of pDAB4468. The insert includes intact genetic elements consisting of *aad*-12 and *pat* PTUs along with a RB7 matrix attachment region at the 5'

end of the insert. The hybridization pattern is identical across 4 generations, indicating the insert is stable in the soybean genome. Hybridization with probes covering the entire vector backbone of pDAB4468 confirms that no vector backbone sequences have been incorporated into the event DAS-68416-4.

ARCHIVING

The raw data and the original version of the final report are all filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, IN 46268-1054.

STATISTICAL TREATMENT OF DATA

No statistical methods are used in this study.

REFERENCES

1. Guillemant, P. (1992) Isolation of Plant DNA: A Fast, Inexpensive, and Reliable Method. Plant Molecular Biology Reporter 10(1): 60-65.

2. Memelink, J.; Swords, K.; Harry J.; Hoge, C. (1994) Southern, Northern, and Western Blot Analysis. Plant Mol. Biol. Manual F1:1-23.

Table 1. List of Seed Sources

Name	Generation	Source ID	Purpose
pDAB4468(4)-0416.001-517-9	T3	YW07EW020708.009	Test Substance
pDAB4468(4)-0416.001-519-4-1	T4	YX07KX000160.001	Test Substance
pDAB4468(4)-0416.001-523-3-8-01	T5-A	YX08CX010827.001	Test Substance
pDAB4468-416	T5-B	SGN080003-061-0007	Test Substance
Maverick	N/A	YX07KX002114	Control Substance
Maverick	N/A	SGN080003-061-0001	Control Substance

Table 2. List of Probes and Their Positions in Plasmid pDAB4468

Probe Name	Size (bp)	Location in pDAB4468
aad-12	882	8522 – 9403
pat	552	6784 – 7335
RB7	1010	10840 – 11849
AtUbi10	760	9974 – 10733
CsVMV	478	7346 – 7823
AtuORF1 3’ UTR	684	5998 – 6681
AtuORF23 3’ UTR	413	7981 – 8393
T-DNA Flanking A	339	5024 – 5362
T-DNA Flanking B	302	12113-261
Spec R	789	275 -1063
Backbone 1	1310	992 – 2301
Backbone 2	1728	2271 – 3998
Ori-Rep	1068	3977 – 5044

Table 3. Event DAS-68416-4 Predicted and Observed Sizes of Hybridizing Fragments

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
aad-12	*Nco* I	Plasmid pDAB4468	3, 6	7957	7957
		DAS-68416-4	3, 6	> 4043*	~5500*
		Maverick	3, 6	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	4, 6	12146	12146
		DAS-68416-4	4, 6	> 6229*	~8500*
		Maverick	4, 6	none	none
	Nhe I/*Xho* I	Plasmid pDAB4468	5, 6	12148	12148
		DAS-68416-4	5, 6	> 6229*	~7200*
		Maverick	5, 6	none	none
pat	*Nco* I	Plasmid pDAB4468	7, 10	7957	7957
		DAS-68416-4	7, 10	> 4043*	~5500*
		Maverick	7, 10	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	8, 10	12146	12146
		DAS-68416-4	8, 10	> 6229*	~8500*
		Maverick	8, 10	none	none
	Nhe I/*Xho* I	Plasmid pDAB4468	9, 10	12148	12148
		DAS-68416-4	9, 10	> 6229*	~7200*
		Maverick	9, 10	none	none
aad-12	*Pst* I (Release PTU)	Plasmid pDAB4468	11, 14A	2868	2868
		DAS-68416-4	11, 14A	2868	2868
		Maverick	11, 14A	none	none
AtUbi10		Plasmid pDAB4468	12, 14B	2868	2868
		DAS-68416-4	12, 14B	2868	2868
		Maverick	12, 14B	none	none
AtuORF23		Plasmid pDAB4468	13, 14C	2868	2868
		DAS-68416-4	13, 14C	2868	2868
		Maverick	13, 14C	none	none
pat	*Pst* I/*Xho* I (Release PTU)	Plasmid pDAB4468	15, 18A	1928	1928
		DAS-68416-4	15, 18A	1928	1928
		Maverick	15, 18A	none	none
CsVMV		Plasmid pDAB4468	16, 18B	1928	1928
		DAS-68416-4	16, 18B	1928	1928
		Maverick	16, 18B	none	none
AtuORF1		Plasmid pDAB4468	17, 18C	1928	1928
		DAS-68416-4	17, 18C	1928	1928
		Maverick	17, 18C	none	none
RB7	*Bam*H I/*Nco* I	Plasmid pDAB4468	19, 21A	2617	2617
		DAS-68416-4	19, 21A	2617	2617
		Maverick	19, 21A	none	none
AtUbi10		Plasmid pDAB4468	20, 21B	2617	2617
		DAS-68416-4	20, 21B	2617	2617
		Maverick	20, 21B	none	none

Table 2 (continued)

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
T-DNA A Flanking	*Nco* I	Plasmid pDAB4468	22, 26	7957	7957
		DAS-68416-4	22, 26	none	none
		Maverick	22, 26	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	24, 27	12146	12146
		DAS-68416-4	24, 27	none	none
		Maverick	24, 27	none	none
Backbone1	*Nco*I	Plasmid pDAB4468	22, 26	7429, 4197, 528	7429,4197,528
		DAS-68416-4	22, 26	none	none
		Maverick	22, 26	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	24, 27	12146	12146
		DAS-68416-4	24, 27	none	none
		Maverick	24, 27	none	none
SpecR	*Nco* I	Plasmid pDAB4468	22, 26	4197	4197
		DAS-68416-4	22, 26	none	none
		Maverick	22, 26	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	24, 27	12146	12146
		DAS-68416-4	24, 27	none	none
		Maverick	24, 27	none	none
T-DNA B Flanking	*Nco* I	Plasmid pDAB4468	23, 26	4197	4197
		DAS-68416-4	23, 26	none	none
		Maverick	23, 26	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	25, 27	12146	12146
		DAS-68416-4	25, 27	none	none
		Maverick	25, 27	none	none
Backbone 2	*Nco* I	Plasmid pDAB4468	23, 26	7957	7957
		DAS-68416-4	23, 26	none	none
		Maverick	23, 26	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	25, 27	12146	12146
		DAS-68416-4	25, 27	none	none
		Maverick	25, 27	none	none
Ori-Rep	*Nco* I	Plasmid pDAB4468	23, 26	7957	7957
		DAS-68416-4	23, 26	none	none
		Maverick	23, 26	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	25, 27	12146	12146
		DAS-68416-4	25, 27	none	none
		Maverick	25, 27	none	none

Note: * These bands include border region of soybean genome;

1. Expected fragment sizes are based on the plasmid map of the pDAB4468 and its T-DNA insert as shown in Figure 1 and 2
2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker II fragments. Due to the incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

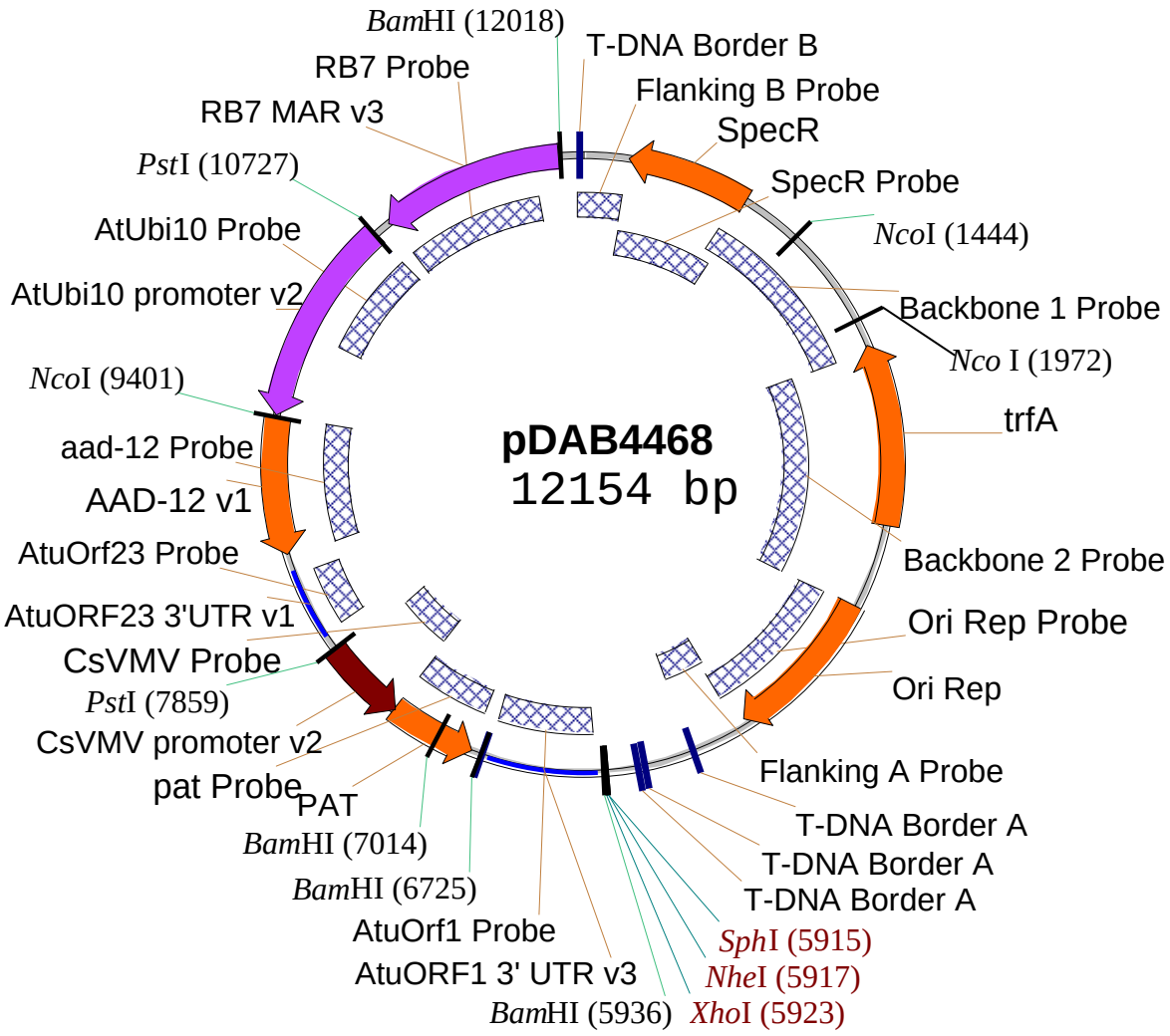
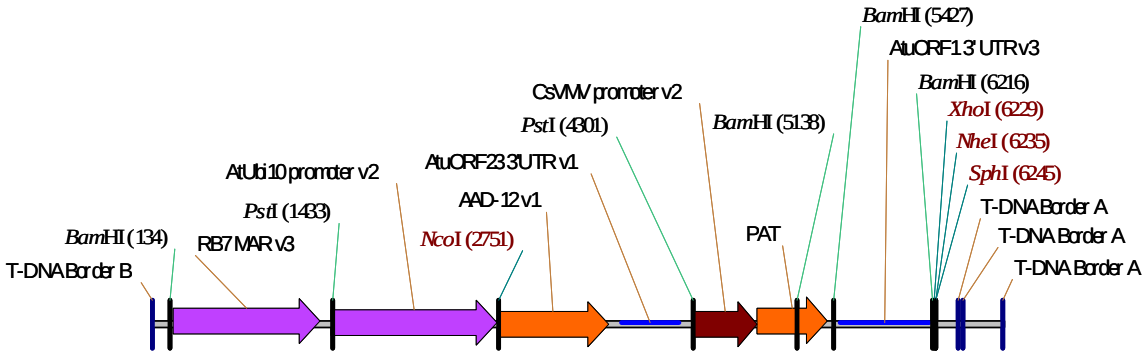


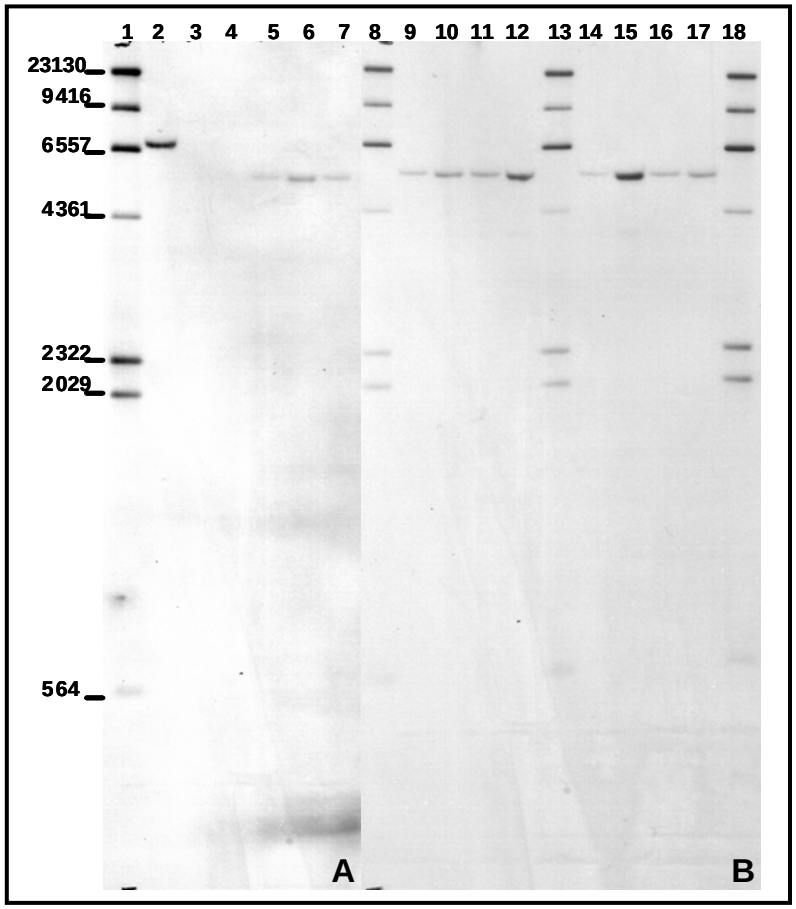
Figure 1. Plasmid Map of pDAB4468 and Probe Positions



pDAB4468 T-DNA Insert
6794 bp

Nco I	> 2751 bp	> 4043 bp		
Nhe I/*Xho* I	> 6229 bp			> 559 bp
Sph I/*Xho* I	> 6229 bp			> 549bp
Pst I	> 1433 bp	2868 bp	> 2493 bp	
Pst I/*Xho* I	> 1433 bp	2868 bp	1928 bp	> 565 bp
*Bam*H I/*Nco* I	2617 bp	2387 bp	1078bp	> 578 bp

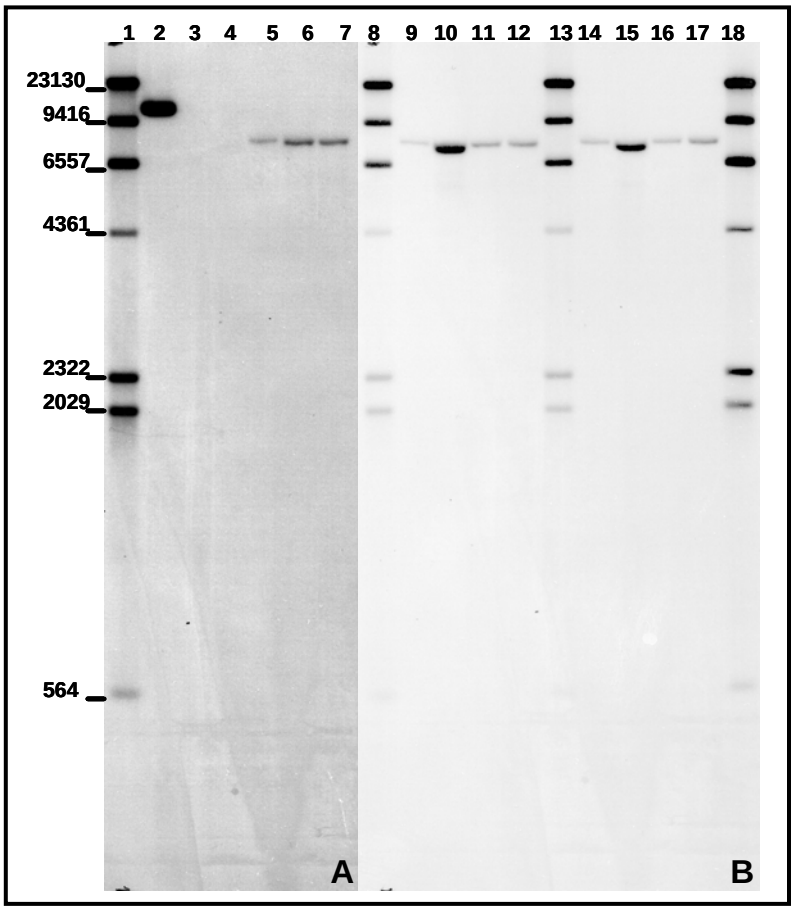
Figure 2. pDAB4468 T-DNA Insert, Restriction Enzymes Used in DNA Digestion and Expected Hybridization Bands



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signal in Lane15 was due to the more DNA amount probably occurred during DNA recovery after digestion. Panel A and B were from the same blot and hybridized in the same container.)

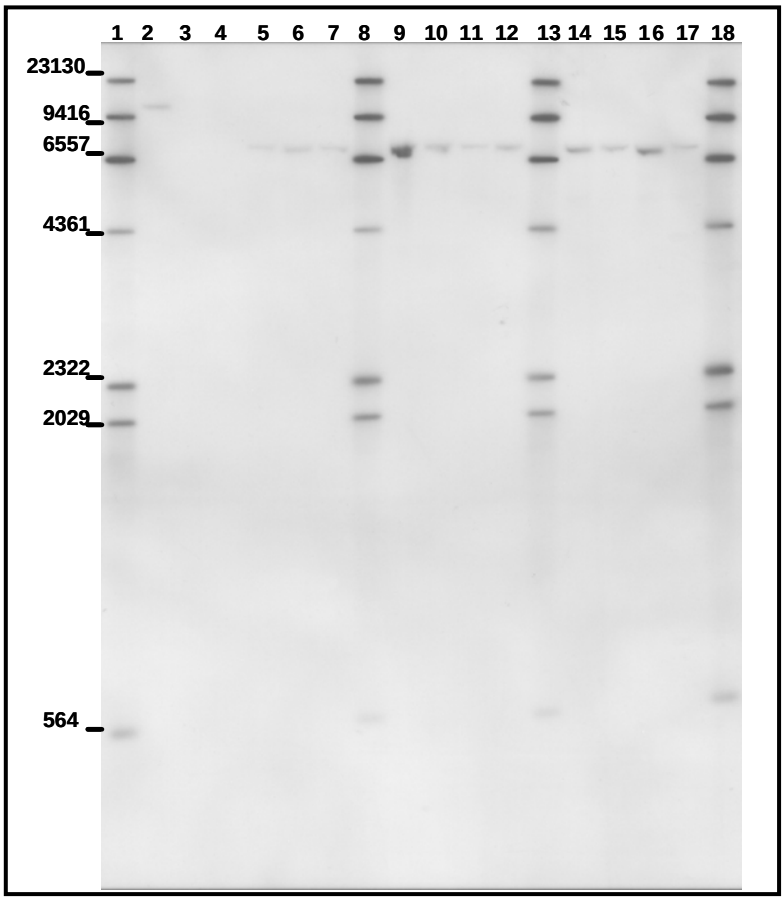
Figure 3. Southern Blot Analysis of *Nco* I Digest with *aad*-12 Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signals in Lane 10 and 15 were due to the more DNA amount probably occurred during DNA recovery after digestion, which is also the cause of the faint band in Lane 10 and 15; Panel A and B were from the same blot and hybridized in the same container.)

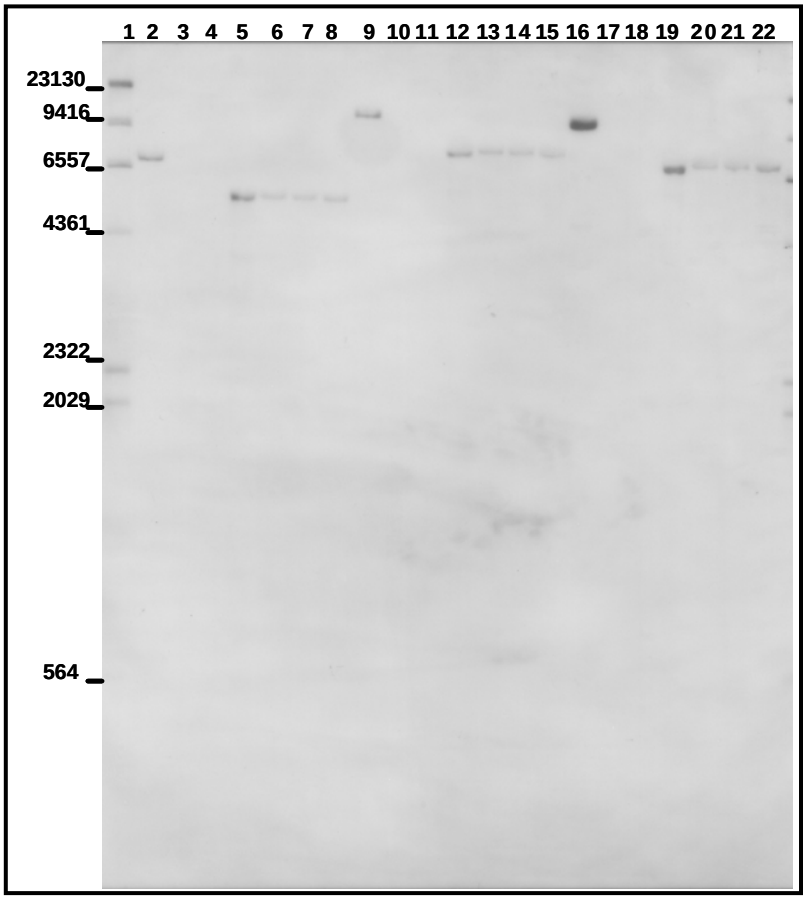
Figure 4. Southern Blot Analysis of *Sph* I/*Xho* I Digest with *aad*-12 Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Nhe* I/*Xho* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signal in Lane 9 was due to the more DNA amount probably occurred during DNA recovery after digestion.)

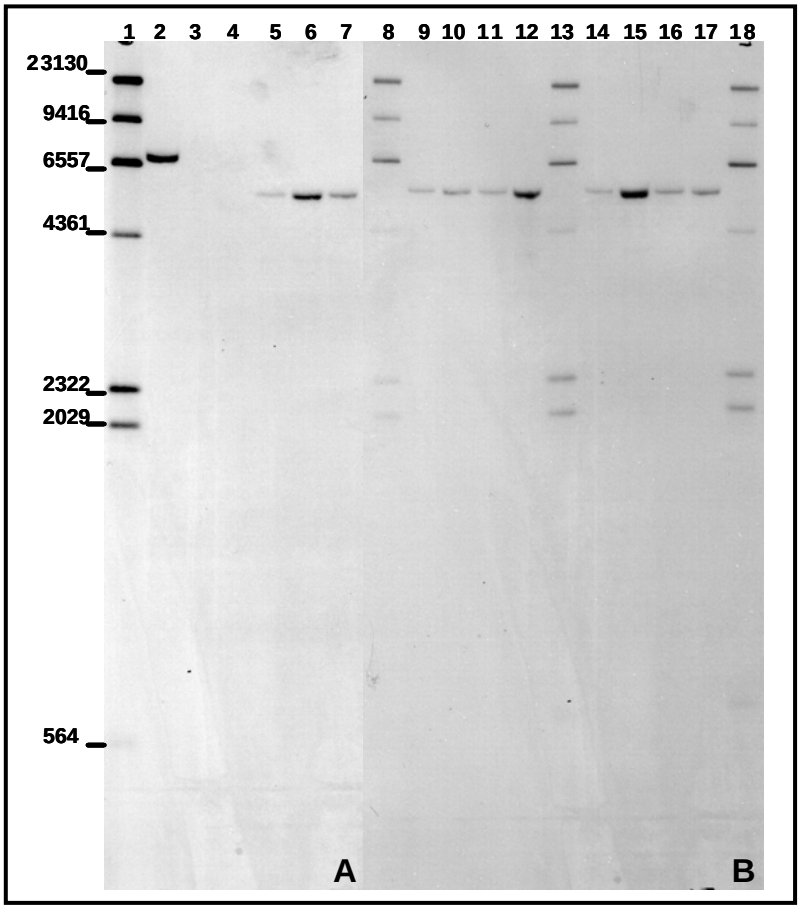
Figure 5. Southern Blot Analysis of *Nhe* I/*Xho* I Digest with *aad*-12 Probe



Lane	Description	Enzyme	Lane	Description	Enzyme
1	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/ *Xho* I
2	pDAB4468 + Non-transgenic control #2	*Nco* I	13	T5-B #4	
3	Non-transgenic control #2		14	T5-B #6	
4	Non-transgenic control #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + Non-transgenic control #2	*Nhe* I/ *Xho* I
6	T5-B #4		17	Non-transgenic control #2	
7	T5-B #6		18	Non-transgenic control #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control #4	*Sph* I/ *Xho* I	20	T5-B #4	
10	Non-transgenic control #4		21	T5-B #6	
11	Non-transgenic control #5		22	T5-B #8	

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signal in Lane 9 was due to the more DNA amount probably occurred during DNA recovery after digestion.)

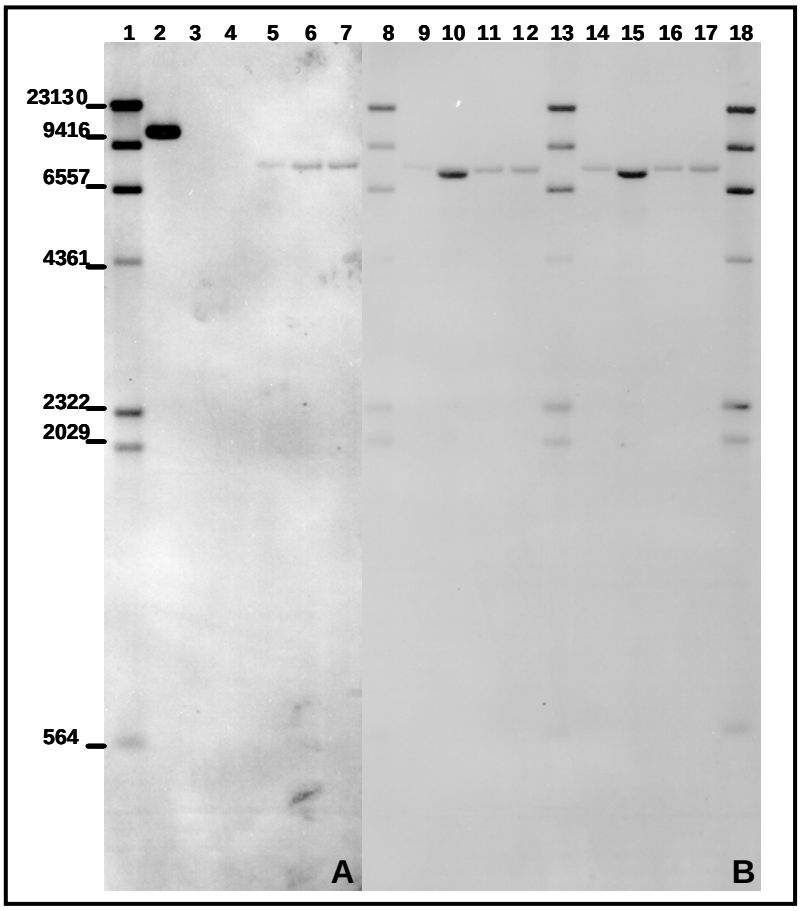
Figure 6. Southern Blot Analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5-B generation with *aad*-12 Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signals in Lane 12 and 15 were due to the more DNA amount probably occurred during DNA recovery after digestion. Panel A and B were from the same blot and hybridized in the same container)

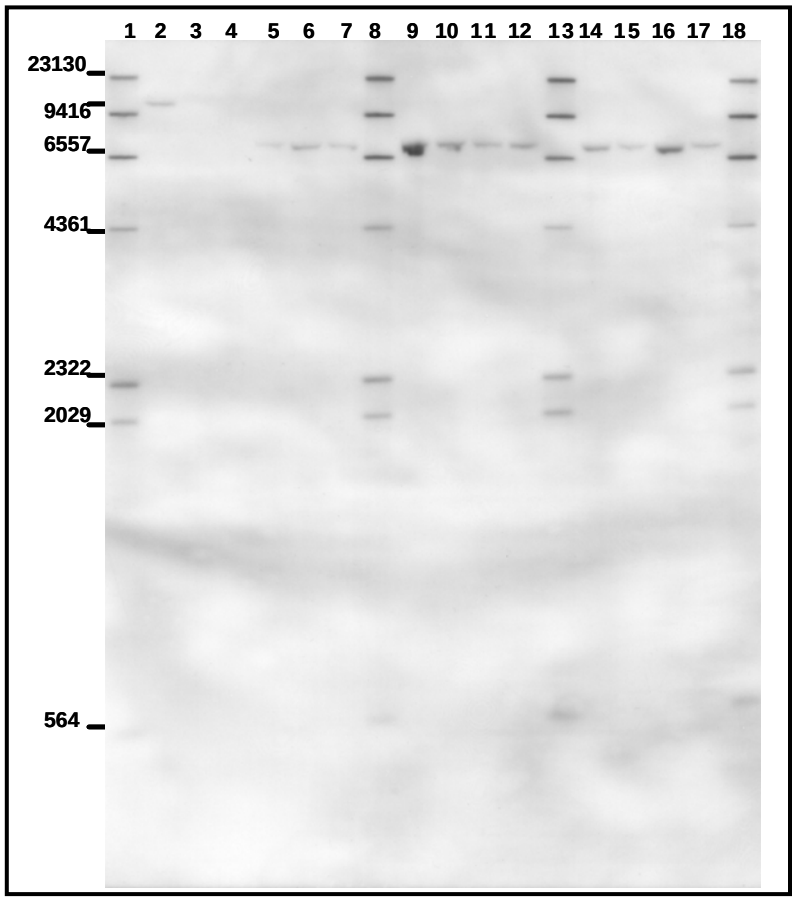
Figure 7. Southern Blot Analysis of *Nco* I Digest with *pat* Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signals in Lane 10 and 15 were due to the more DNA amount probably occurred during DNA recovery after digestion. Panel A and B were from the same blot and hybridized in the same container.)

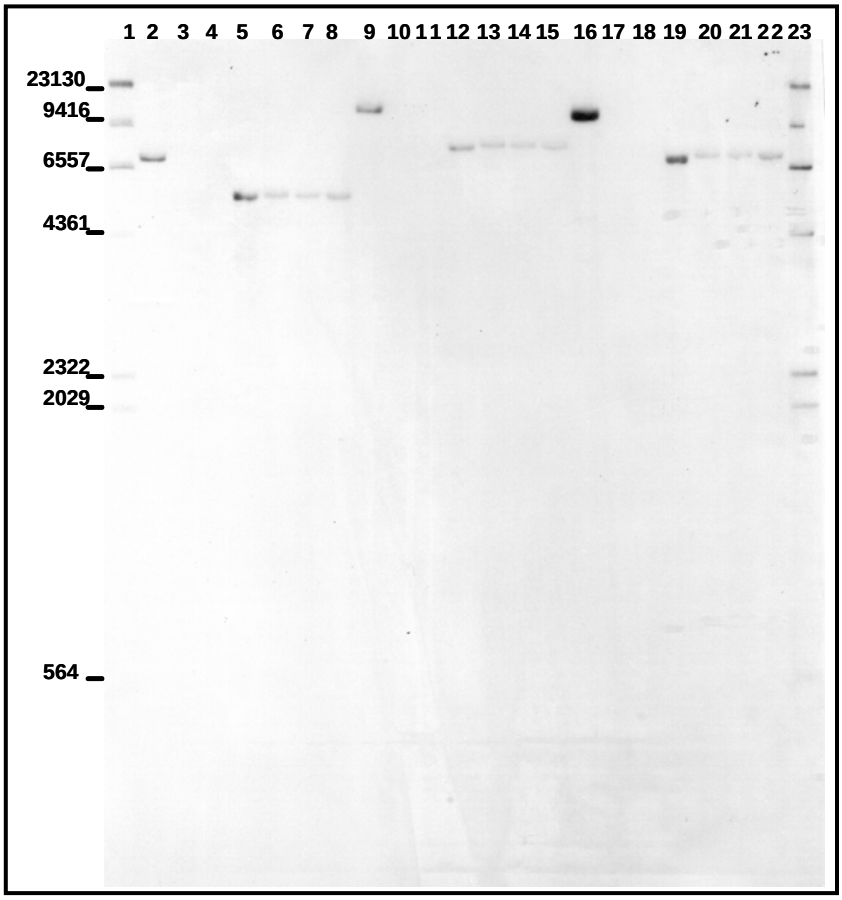
Figure 8. Southern Blot Analysis of *Sph* I/*Xho* I digest with *pat* Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Nhe* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signal in Lane 9 was due to the more DNA amount probably occurred during DNA recovery after digestion.)

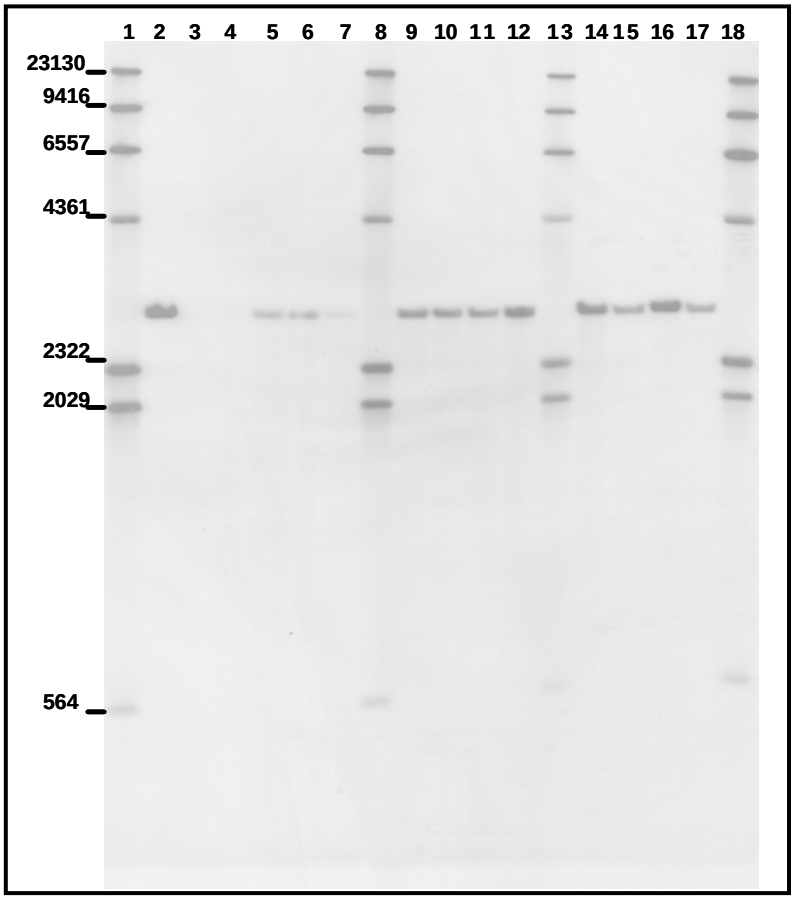
Figure 9. Southern Blot Analysis of *Nhe* I/*Xho* I digest with *pat* Probe



Lane	Description	Enzyme	Lane	Description	Enzyme
1, 23	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/ *Xho* I
2	pDAB4468 + non-transgenic control #2	*Nco* I	13	T5-B #4	
3	Non-transgenic control #2		14	T5-B #6	
4	Non-transgenic control #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + non-transgenic control #2	*Nhe* I/ *Xho* I
6	T5-B #4		17	Non-transgenic control #2	
7	T5-B #6		18	Non-transgenic control #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control #4	*Sph* I/ *Xho* I	20	T5-B #4	
10	Non-transgenic control #4		21	T5-B #6	
11	Non-transgenic control #5		22	T5-B #8	

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative strong signal in Lane 19 was due to the more DNA amount probably occurred during DNA recovery after digestion. The faint band in Lane 16 is probably degraded plasmid DNA.)

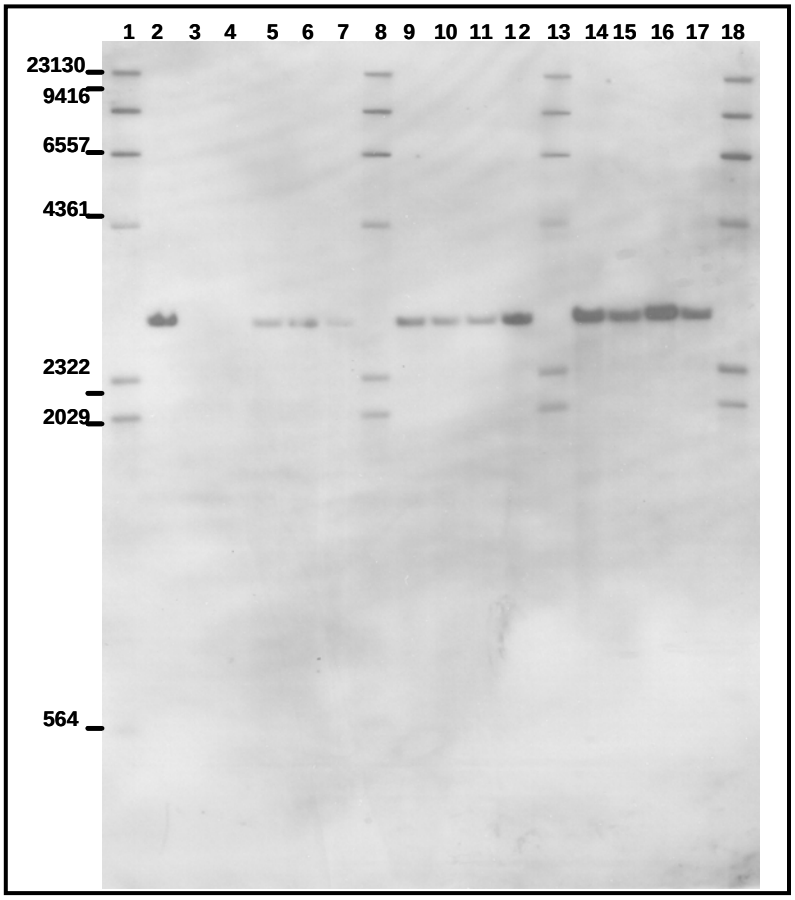
Figure 10. Southern Blot Analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5-B generation with *pat* Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I and hybridized with *aad*-12 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative weak signal in Lane 7 was due to the less DNA amount probably occurred during DNA recovery after digestion.)

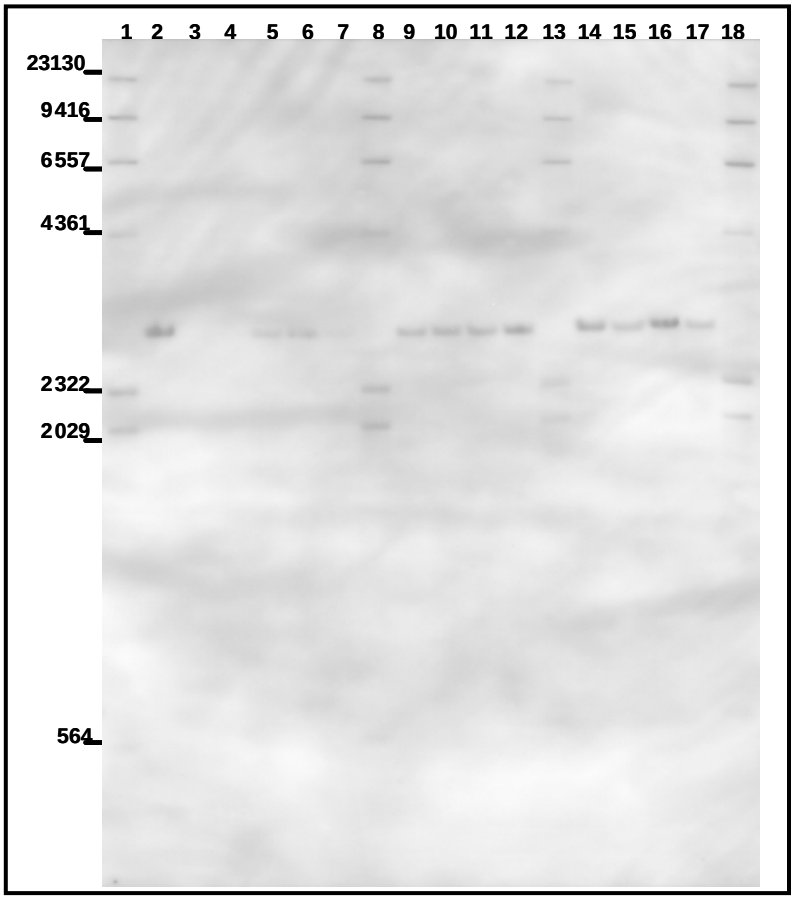
Figure 11. Southern Blot Analysis of *Pst* I digest with *aad*-12 Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative weak signal in Lane 7 was due to the less DNA amount probably occurred during DNA recovery after digestion.)

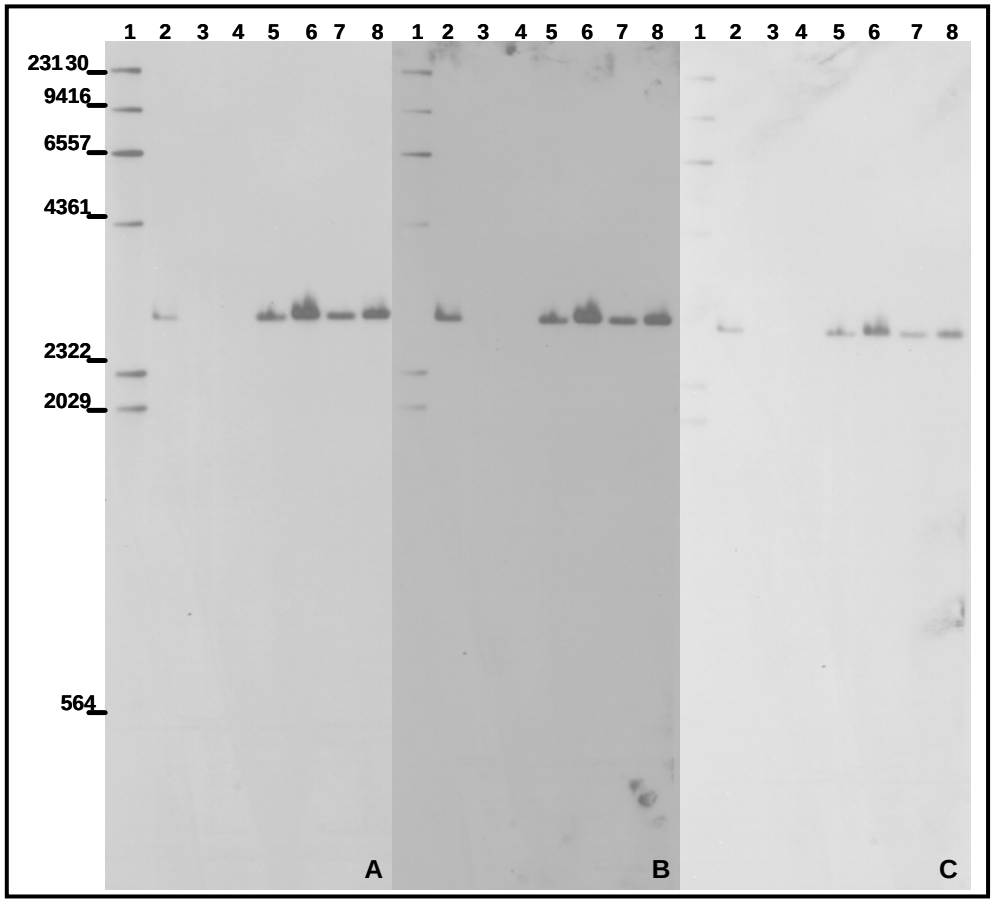
Figure 12. Southern Blot Analysis of *Pst* I digest with AtUbi10 Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I and hybridized with AtuORF23 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relative weak signal in Lane 7 was due to the less DNA amount probably occurred during DNA recovery after digestion.)

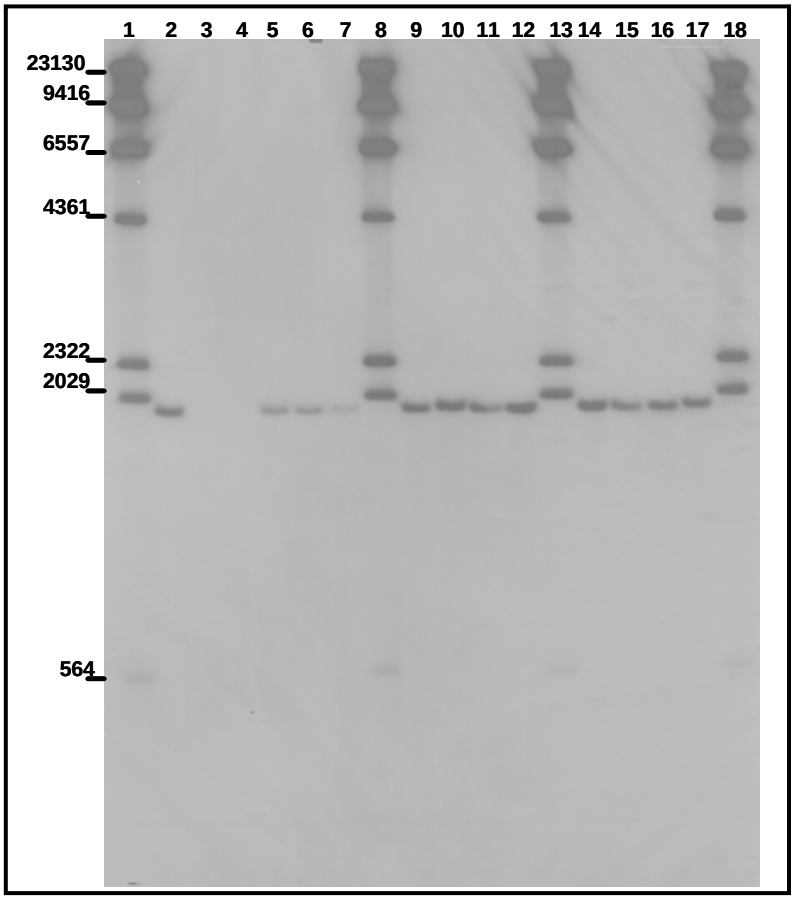
Figure 13. Southern Blot Analysis of *Pst* I digest with AtuORF23 Probe



Lane	Description
1	DNA molecular marker (bp)
2	pDAB4468 +Non-transgenic control #2
3	Non-transgenic control #2
4	Non-transgenic control #3
5	T5-B #1
6	T5-B #4
7	T5-B #6
8	T5-B #8

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B generation and the non-transgenic control were digested with *Pst* I and hybridized with *aad*-12 (Panel A), AtUbi10 (Panel B), and ORF23 probes (Panel C). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

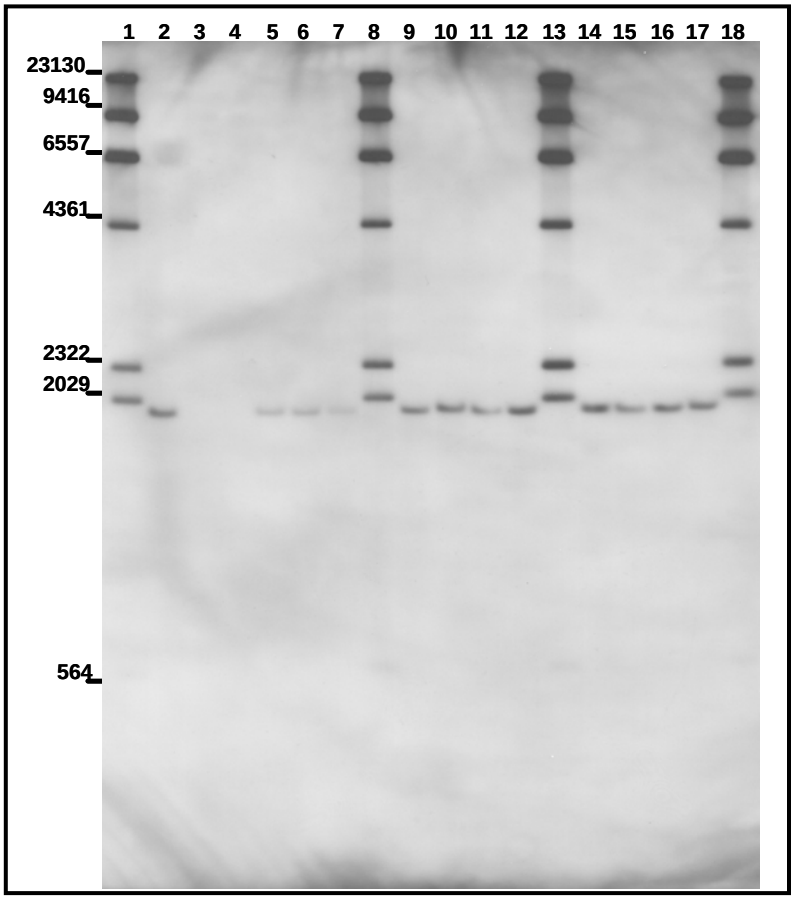
Figure 14. Southern Blot Analysis of *Pst* I digest of T5-B Generation with *aad*-12, Atubi10, and AtuORF23 Probes



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #3	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

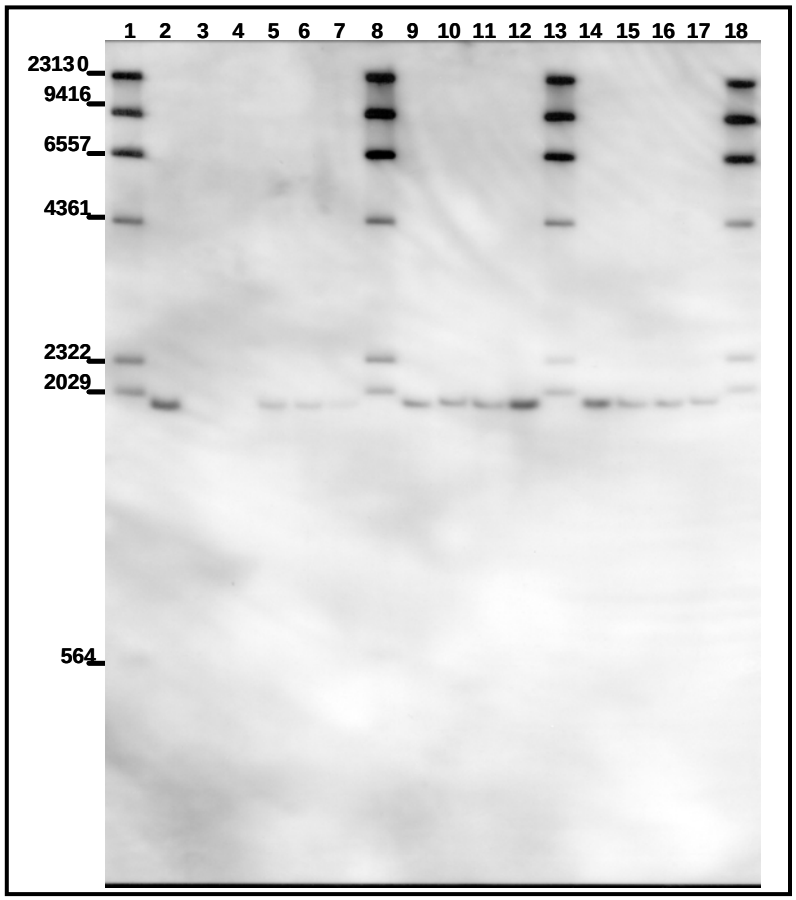
Figure 15. Southern Blot Analysis of *Pst* I/*Xho* I Digest with *pat* Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #3	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with CsVMV probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

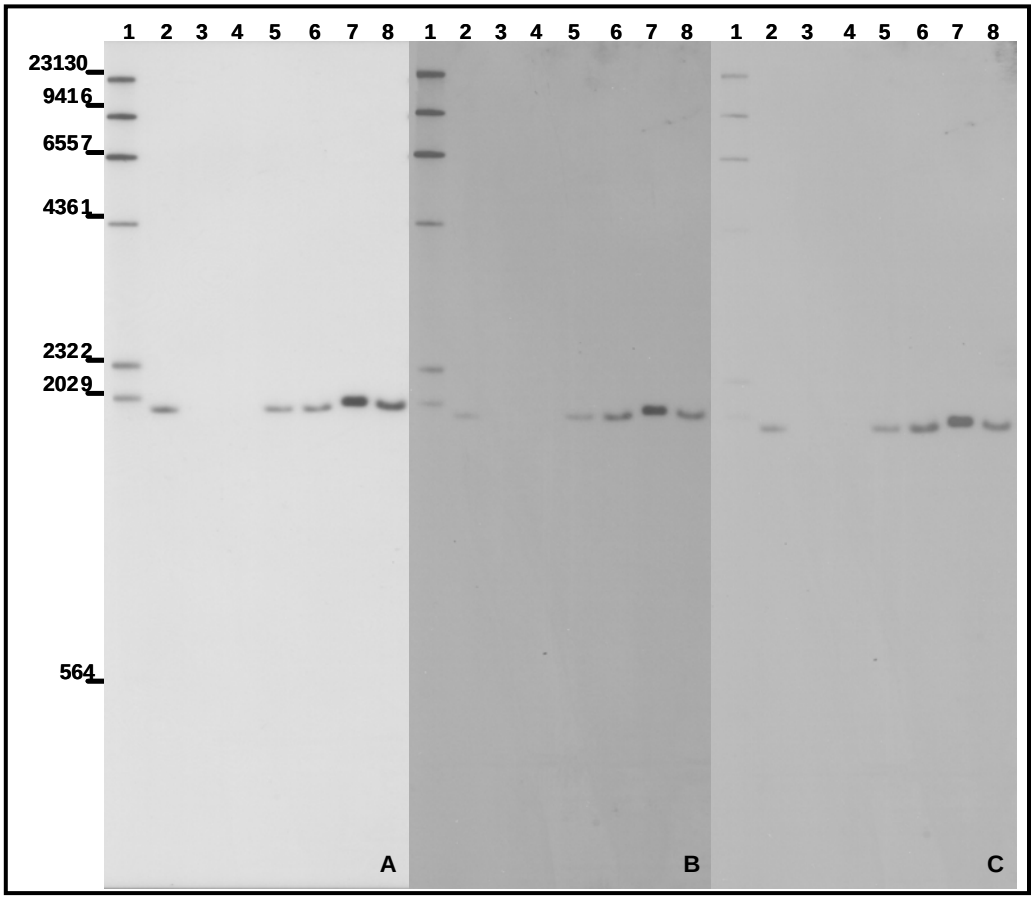
Figure 16. Southern Blot Analysis of *Pst* I/*Xho* I Digest with CsVMV Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #3	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with AtuORF1 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

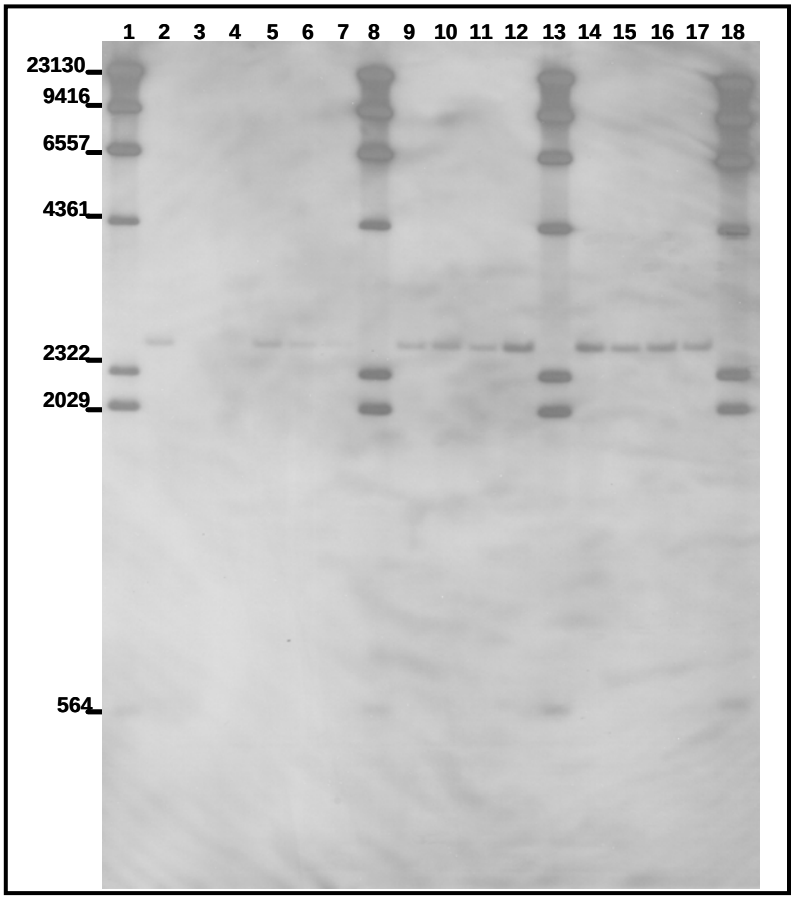
Figure 17. Southern Blot Analysis of *Pst* I/*Xho* I Digest with AtuORF1 Probe



Lane	Description
1	DNA molecular marker (bp)
2	pDAB4468 +Non-transgenic control #2
3	Non-transgenic control #3
4	Non-transgenic control #3
5	T5-B #1
6	T5-B #4
7	T5-B #6
8	T5-B #8

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B generation and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with *pat* (Panel A), CsVMV (Panel B), and AtuORF1 probes (Panel C). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

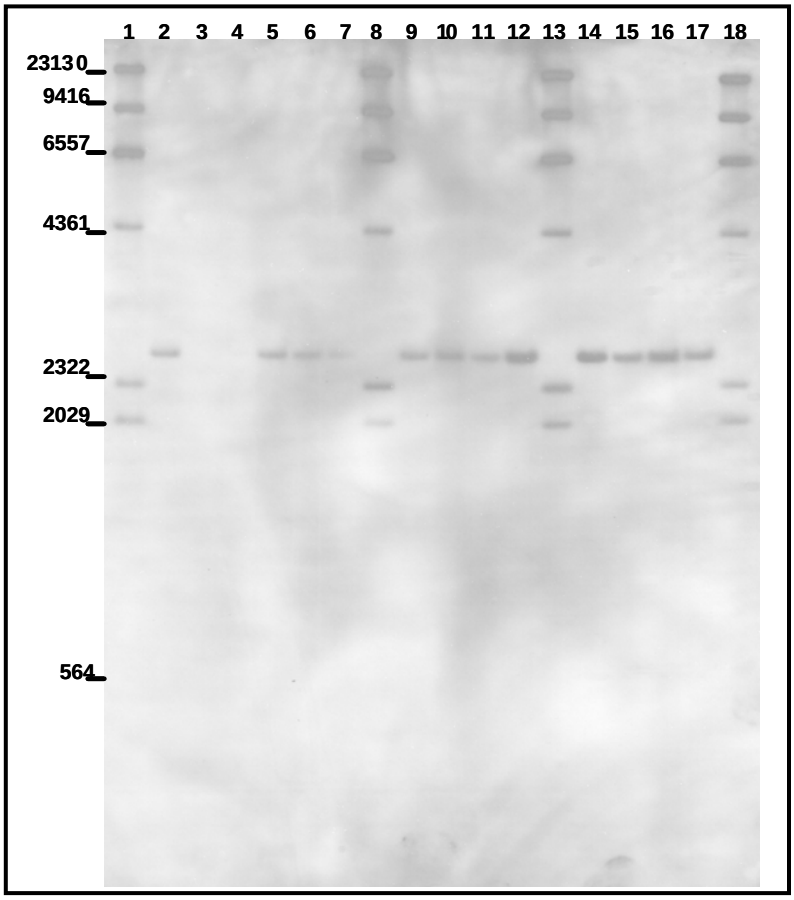
Figure 18. Southern Blot Analysis of *Pst* I/*Xho* I Digest of T5-B Generation with *pat*, CsVMV, and AtuORF1 Probes



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Bam*H I/*Nco* I and hybridized with RB7 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. ((Note: The relative weak signals in Lane 6 and 7 were due to the less DNA amount probably occurred during DNA recovery after digestion.)

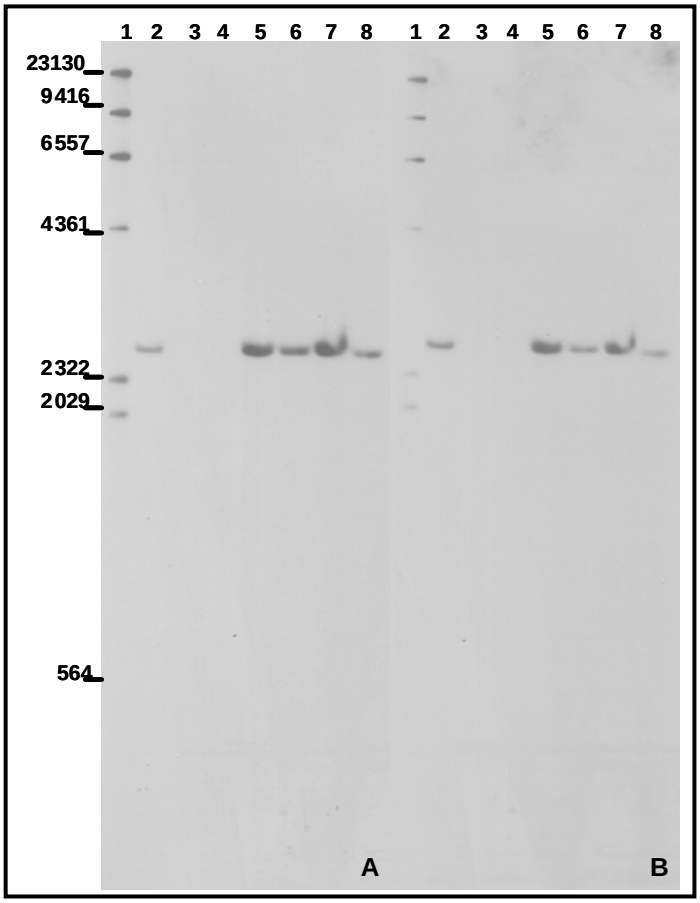
Figure 19. Southern Blot Analysis of *Bam*H I/*Nco* I Digest with RB7 Probe



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3, T4, T5-A and the non-transgenic control were digested with *Bam* HI/*Nco* I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

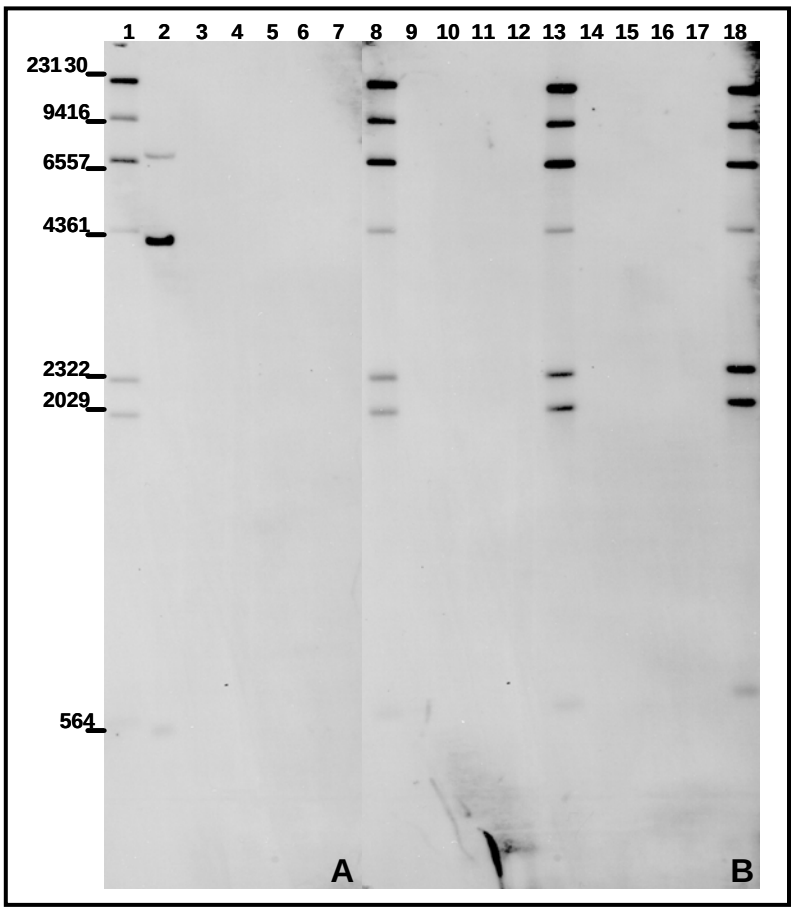
Figure 20. Southern Blot Analysis of *Bam*H I/*Nco* I Digest with AtUbi10 Probe



Lane	Description
1	DNA molecular marker (bp)
2	pDAB4468 +Non-transgenic control #2
3	Non-transgenic control #3
4	Non-transgenic control #3
5	T5-B #1
6	T5-B #4
7	T5-B #6
8	T5-B #8

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B generation and the non-transgenic control were digested with *Bam*H I/*Nco* I and hybridized with RB7 (Panel A), AtUbi10 (Panel B. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

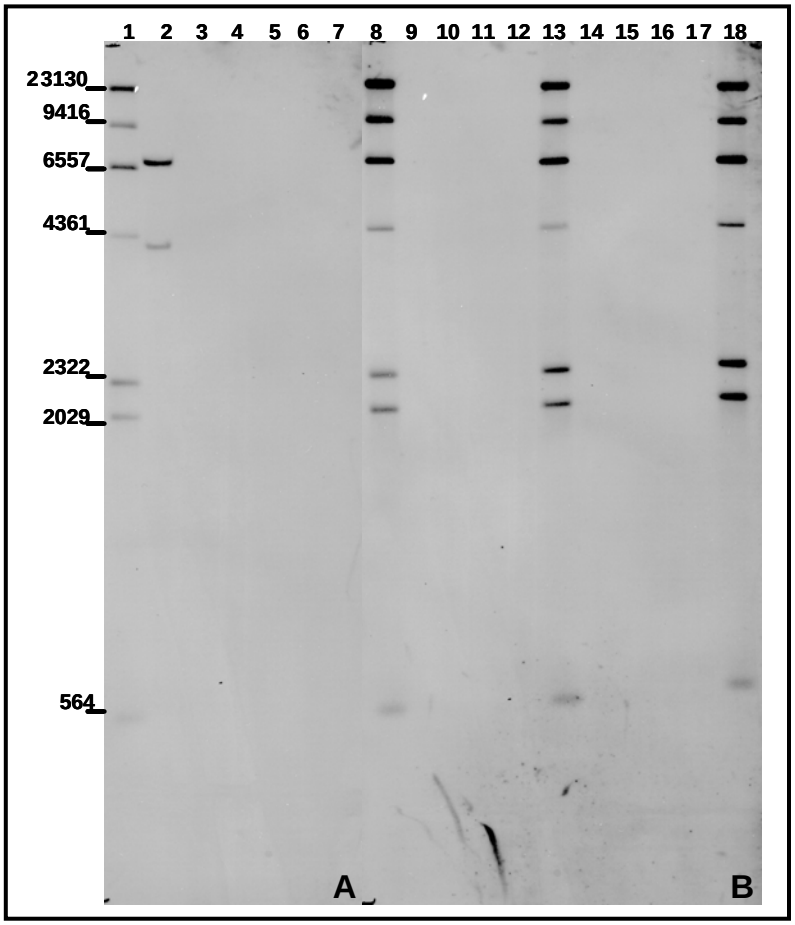
Figure 21. Southern Blot Analysis of *Bam*H I/*Nco* I Digest of T5-B Generation with RB7 and AtUbi10 Probes



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container.)

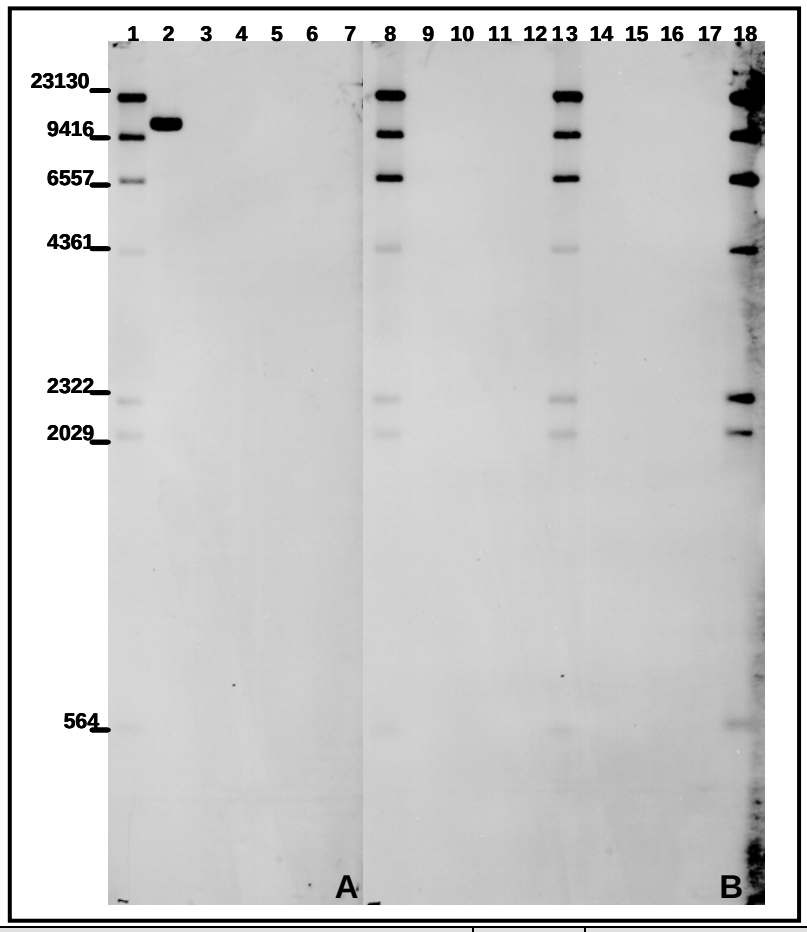
Figure 22. Southern Blot Analysis of *Nco* I Digest with Backbone Probe Set 1 from Plasmid pDAB4468 Vector Backbone



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*) probes. Nine (9) μg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 μg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container.)

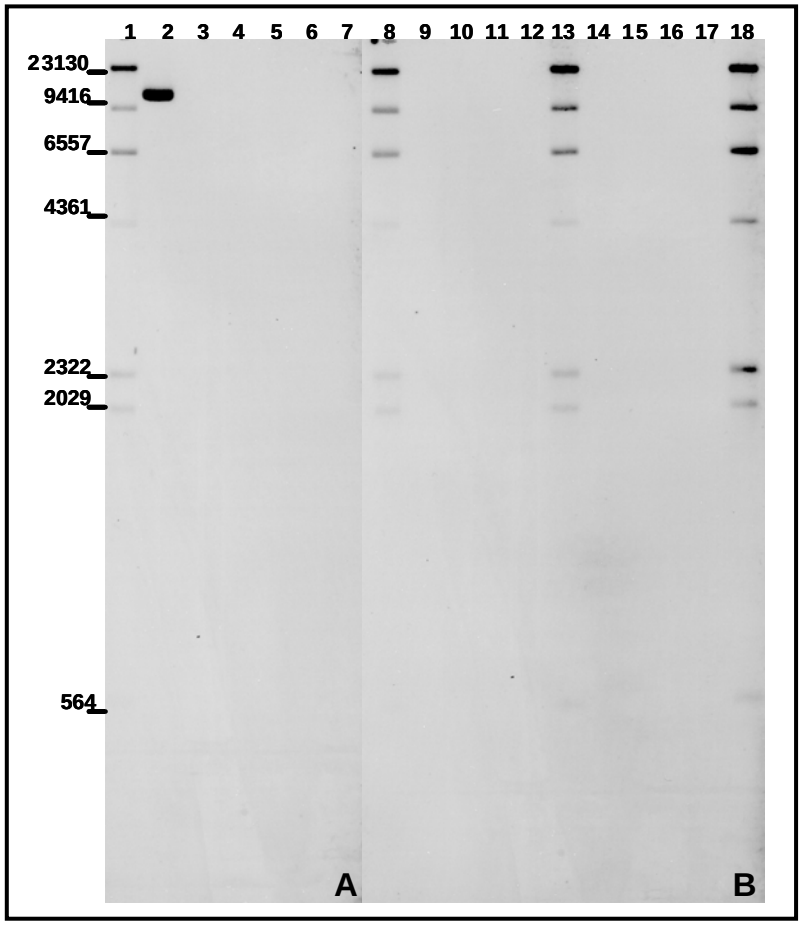
Figure 23. Southern Blot Analysis of *Nco* I Digest with Backbone Probe Set 2 from Plasmid pDAB4468 Vector Backbone



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container.)

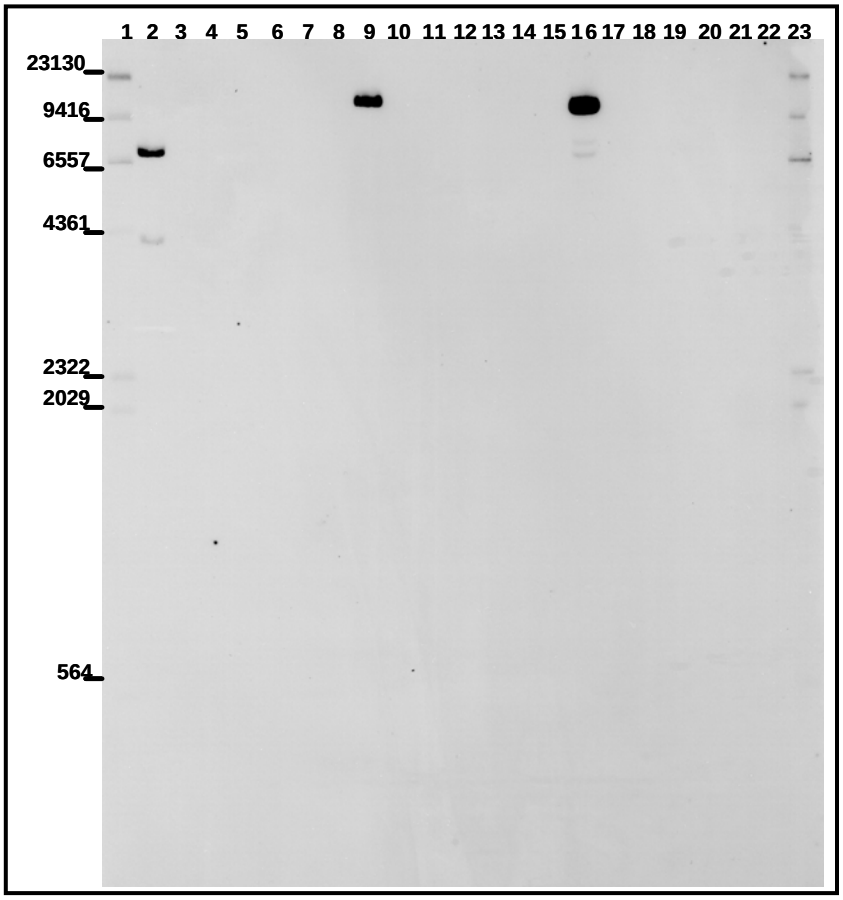
Figure 24. Southern Blot Analysis of *Sph* I/*Xho* I Digest with Backbone Probe Set 1 from Plasmid pDAB4468 Vector Backbone



Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 +Non-transgenic control #2	11	T4 #9
3	Non-transgenic control #3	12	T4 #10
4	Non-transgenic control #2	13	DNA molecular marker (bp)
5	T3 #2	14	T5-A #2
6	T3 #7	15	T5-A #4
7	T3 #8	16	T5-A #5
8	DNA molecular marker (bp)	17	T5-A #7
9	T4 #3	18	DNA molecular marker (bp)

DNA isolated from individual plants of soybean event DAS-68416-4 generation T3 (Panel A), T4, T5-A (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container.)

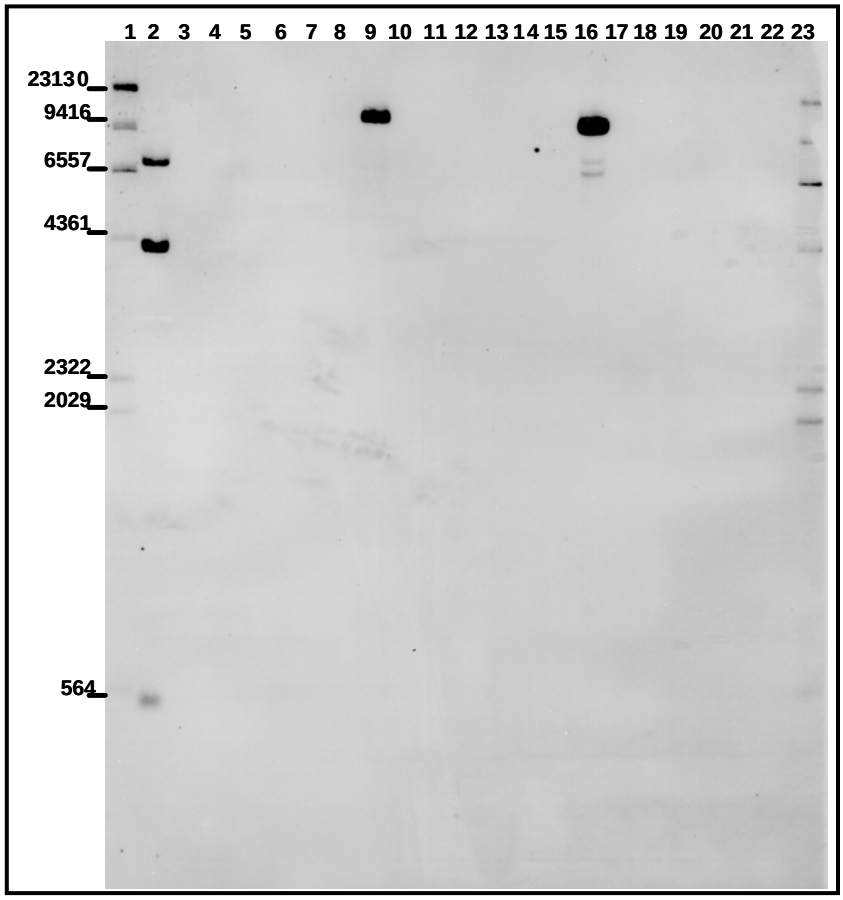
Figure 25. Southern Blot Analysis of *Sph* I/*Xho* I Digest with Backbone Probe Set 2 Plasmid pDAB4468 Vector Backbone



Lane	Description	Enzyme	Lane	Description	Enzyme
1, 23	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/*Xho* I
2	pDAB4468 + control #2	*Nco* I	13	T5-B #4	
3	Non-transgenic control #2		14	T5-B #6	
4	Non-transgenic control #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + control #2	*Nhe* I/*Xho* I
6	T5-B #4		17	Non-transgenic control #2	
7	T5-B #6		18	Non-transgenic control #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control #4	*Sph* I/*Xho* I	20	T5-B #4	
10	Non-transgenic control #4		21	T5-B #6	
11	Non-transgenic control #5		22	T5-B #8	

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: faint bands in lane 16 could be a result of hybridization to the degraded plasmid DNA. The expected 528bp band was not observed in plasmid control due to less amount of plasmid DNA.)

Figure 26. Southern Blot Analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I Digests of T5-B Generations with the Backbone Probe Set 1 from pDAB4468 Vector Backbone



Lane	Description	Enzyme	Lane	Description	Enzyme
1, 23	DNA molecular marker (bp)		12	T5-B #1	*Sph* I/*Xho* I
2	pDAB4468 + control #2	*Nco* I	13	T5-B #4	
3	Non-transgenic control #2		14	T5-B #6	
4	Non-transgenic control #3		15	T5-B #8	
5	T5-B #1		16	pDAB4468 + control #2	*Nhe* I/*Xho* I
6	T5-B #4		17	Non-transgenic control #2	
7	T5-B #6		18	Non-transgenic control #3	
8	T5-B #8		19	T5-B #1	
9	pDAB4468 + control #4	*Sph* I/*Xho* I	20	T5-B #4	
10	Non-transgenic control #4		21	T5-B #6	
11	Non-transgenic control #5		22	T5-B #8	

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5-B and the non-transgenic control was digested with *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Faint bands in lane 16 could be a result of hybridization to the degraded plasmid DNA.

Figure 27. Southern Blot Analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I Digests of T5-B Generation with the Backbone Probe Set 2 from pDAB4468 Vector Backbone