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 within a Single Segregating Generation

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

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STUDY COMPLETED ON

24-Sept-2009

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
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LABORATORY STUDY ID

091071

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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 acidovoran* and a *pat* gene from *Streptomyces viridochromogenes*. The *aad*-12 encodes aryloxyalkanoate dioxygenase-12 (AAD-12) which provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D), fluroxypyr, and triclopyr-based herbicides. The *pat* gene encodes phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate-based herbicides and was used as a selectable marker during 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.

A F2 segregating population of soybean event DAS-68416-4 was used in this study. AAD-12 protein expression testing of the individual plants in the F2 segregating population displayed a 3:1 phenotypic segregation of AAD-12 protein expression. Southern blot data generated with various restriction enzyme digests and probes also produced a ratio of 3:1 between the numbers of hybridization positive and negative plants (segregated nulls), matching the phenotypic observation from the protein expression testing. In addition, the Southern hybridization pattern across all the *aad-12* positive plants is identical. These results demonstrated the typical Mendelian inheritance pattern of a single transgene insert expressing the AAD-12 protein in the soybean event DAS-68416-4.

STUDY TITLE

Molecular Characterization of AAD-12 Soybean Event DAS-68416-4 within a Single Segregating Generation

DATA REQUIREMENTS

Not Applicable

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STUDY COMPLETED ON

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-12

Title: Molecular Characterization of AAD-12 Soybean Event DAS-68416-4 within a Single Segregating Generation

*STATEMENT OF DATA CONFIDENTIALITY CLAIMS:

Information claimed as confidential has been removed to a confidential attachment.

No claims or markings on the document or any attachments, other than these statements and attachments submitted in accordance with 40 CFR 158.33(c)(3) will be recognized as asserting a claim of confidentiality.

Company: Dow AgroSciences LLC

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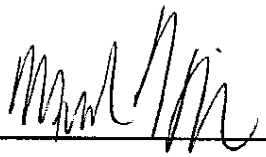
Signature: 

Date: 18 August 2009

*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 within a Single Segregating Generation

Study Initiation Date: 04/March/09

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

Organisation 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 standards (Digoxigenin (DIG)-labeled DNA Molecular Size Marker II and 1kb plus DNA ladder) was unknown.

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Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 091071

Title: Molecular Characterization of AAD-12 Soybean Event DAS-68416-4 within a Single Segregating Generation

Study Initiation Date: 4 March 2009 **Study Completion Date:** 24 September 2009

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
4 March 2009	4 March 2009	Protocol Review
10 March 2009	10 March 2009	Planting
2 & 3 June 2009	3 June 2009	Agarose gel loading and transfer
15, 16, 17, 21 September 2009	21 September 2009	Report & Raw Data Review, Test Substance Container 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.

C-L Dudley — 24 September 2009

Date

Dow AgroSciences, Quality Assurance

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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 aryloxyalkanoate dioxygenase-12 (AAD-12) which provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D), fluroxypyr, and triclopyr-based herbicides. The *pat* gene encodes phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate-based herbicides and was used as a selectable marker during 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.

A F2 segregating population of soybean event DAS-68416-4 was used in this study. AAD-12 protein expression testing of the individual plants in the F2 segregating population displayed a 3:1 phenotypic segregation of AAD-12 protein expression. Southern blot data generated with various restriction enzyme digests and probes also produced a ratio of 3:1 between the numbers of *aad-12* hybridization positive and negative plants (segregated nulls), matching the phenotypic observations from the protein expression testing. In addition, the Southern hybridization pattern across all the *aad-12* positive plants is identical. These results demonstrated the typical Mendelian inheritance pattern of a single transgene insert expressing the AAD-12 protein in the soybean 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 aryloxyalkanoate dioxygenase-12 (AAD-12) which provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D), fluroxypyr, and triclopyr-based herbicides. The *pat* gene encodes phosphinothricin acetyl transferase (PAT) which provides tolerance to glufosinate-based herbicides and was used as a selectable marker during 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 was to demonstrate the Mendelian inheritance pattern of the transgene insert as a single locus in event DAS-68416-4 through protein expressing testing and Southern blot analysis of individual plants in a F2 segregating population. Lateral Flow Strip (LFS) assay specific to the AAD-12 protein, probes of *aad*-12 and *pat* combined with different restriction enzyme digestions, were used to generate data. The Mendelian inheritance pattern was statistically evaluated based on the ratio between individual plants carrying the transgene and the segregated nulls.

MATERIALS AND METHODS

Test Substance/Test System

The test substance in this study is the genomic DNA extracted from DAS-68416-4 responsible for producing the AAD-12 protein. The soybean genomic DNA was extracted from leaf tissue harvested from individual plants (Test System) in a segregating F2 population of soybean event DAS-68416-4. Soybean seeds from a F2 generation of event DAS-68416-4, along with the source identification, were provided by the Department of TG&T, Dow AgroSciences (Table 1). Test substance was labeled with unique IDs associated with event number and plant number.

Control Substance

Negative control

The control substances used in this study are gDNA extracted from leaf tissue harvested from individual plants of the non-transgenic soybean variety "Maverick" (source ID: SGN080003-061-0001. The unmodified plants were used to produce the transgenic event and therefore have a genetic background representative of the test substance line, but do not contain *aad*-12 and *pat* genes. The control seeds were provided by the Department of TG&T, 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 and *pat* in the Southern blot hybridization. The reference plasmid was 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 #: 11218590910) 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 200-300 base pairs 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 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 the F2 generation, 150 pots, one seed per pot, were planted. Ten pots, one seed per pot, were planted for the non-transgenic control Maverick. Emerged plants were labeled accordingly and were grown at least 2 weeks prior to AAD-12 expression verification analysis.

AAD-12 Protein Expression Testing

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 manufacturer's instructions. Each leaf punch sample was given a score of + or – for the presence or absence of AAD-12, respectively. The protein expression data was analyzed by Chi-square (χ^2) fitness testing for the 3:1 segregation ratio of AAD-12 protein positive versus negative plants and compared with the results of Southern blot analysis to confirm consistency.

Leaf Sample Collection

Leaf samples from all the individual plants in the F2 generation and non transgenic controls "Maverick" were collected for DNA extraction or stored at approximately -80°C for future use.

Genomic DNA Extraction from Leaf Tissue

DNA extraction was performed based on the modified CTAB method. Briefly, leaf samples were individually ground in liquid nitrogen followed by the addition of extraction buffer (~5:1 ratio milliliter CTAB extraction buffer: gram leaf tissue) and RNase-A (>10 µL) (Qiagen, Valencia, Catalog #: 1007885). After approximately 2 hours of incubation at ~65 °C with gentle shaking, samples were spun down and the supernatants were extracted with equal volume of chloroform : octonal = 24:1 (chloroform, Fisher Scientific, Catalog #: 4440-8; octanol, Sigma, Catalog #: O4504-100mL). DNA was precipitated by mixing the supernatants with equal volume of precipitation buffer (1% CTAB, Sigma, Catalog #: H6269-250G; 50 mM Tris-HCl, Invitrogen, Catalog #: 15568-025; 10 mM EDTA, AcruGene, Catalog #: 51234). The precipitated DNA was dissolved in high salt TE buffer (1X TE pH8.0, Thermo Scientific, Catalog #: 17290; 1.0M NaCl, AccuGene, Catalog #: 51202) followed by precipitation with isopropyl alcohol (EMD, Catalog #: PX-1834-1). The precipitated DNA was rinsed with 70% ethanol, air-dried, then dissolved in appropriate volume of 1× TE buffer (pH8.0). 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, diluted from 10X TBE, Fisher Scientific, Catalog #: BP1333-4). 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 all the individual plants from the F2 generation, and 9 plants from negative control, Maverick, were used for Southern analysis. Approximately nine micrograms (µg) of genomic DNA from each transgenic sample of the F2 population 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 was 400 µL. Each sample was incubated at approximately 37°C overnight. The restriction enzymes, *Nco* I, *Pst* I, and *Xho* I, were used for the digests (New England Biolabs, Cat #: R0193L, R0140L, R0146L, 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 #: BP1333-4) at approximately 50-60 volts for approximately 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide (~1 µg/mL) (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* (1) 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 about 30 minutes. Southern transfer was performed overnight onto Gene Screen Plus nylon membranes (Roche Applied Sciences, Cat #: 11417240001) 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* and *pat* genes 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 2 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 #: 1603558001). 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 #: 11585762001) 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. 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.

Southern Data Analysis

The Southern blot data were compared with the AAD-12 protein expression testing data and analyzed by Chi-square (χ^2) fitness testing for the 3:1 segregation ratio between the number of individual plants carrying the transgenic insert and the number of segregated nulls.

RESULTS AND DISCUSSION

AAD-12 Protein Expression

Out of 150 pots planted with seeds from the F2 generation of soybean AAD-12 event DAS-68416-4, seeds in 3 pots did not germinate, resulting in a total of 147 seedlings for testing of AAD-12 protein expression. Among the 147 tested plants, 102 plants were positive for AAD-12 protein expression, and 45 plants were negative (segregated null) (Table 3). All of the plants from non-transgenic control soybean variety Maverick were negative for AAD-12 protein expression. Statistical analysis using χ^2 fitness testing indicated the phenotypic segregation

ratio of the plants with positive AAD-12 protein expression versus negative fit the 3:1 segregation ratio characteristic of the Mendelian inheritance pattern of a single dominate trait ($\chi^2 = 2.469$, $P > 0.05$).

Southern Blot Analysis

Among 147 emerged plants, 4 of them (2 positive and 2 negative for AAD-12 protein expression) died prior to proceeding with DNA extraction. To further confirm if the phenotypic segregation matches the genotypic makeup of the tested F2 population, genomic DNA samples from each of these 143 plants, along with DNA sample from non-transgenic control soybean variety Maverick, were analyzed by Southern blot through various combinations of restriction enzyme digestions and probes. All the DNA samples from AAD-12 expression positive plants displayed a ~5500 bp expected single band including the 3' border of the transgene insert when digested by *Nco* I and hybridized with either the *aad*-12 or *pat* probes (Table 3, Figure1, 2-11). Moreover, a single expected band of 2868 bp for the AAD-12 PTU, released by *Pst* I digestion, and 1928 bp for PAT-PTU, released by *Pst* I/*Xho* I digestion, were detected in all the samples from the AAD-12 protein expression positive plants (Table 3, Figure 1, 12-21). None of the DNA samples from AAD-12 protein expression negative plants and non-transgenic control Maverick showed any hybridization bands. The Southern blot analysis data matches what was observed in the AAD-12 protein expression testing, *i.e*., individual plant tested positive for AAD-12 expression displayed expected hybridization bands, while negative plants (segregated nulls) for AAD-12 protein expression didn't have any hybridization signals. In addition, the hybridization pattern in all plants tested positive for AAD-12 protein expression was consistent. As observed in the protein expression testing, the ratio of Southern *aad-12* positive versus negative plants in the F2 population also fits the expected 3:1 segregation ratio characteristic of the Mendelian inheritance pattern of a single gene (insert) ($\chi^2 = 1.960$, $P > 0.05$).

Previous molecular characterization study across multiple generations has demonstrated that AAD-12 soybean event DAS-68416-4 contained a single insert with intact PTUs for both *aad-12*

and *pat* (2). Hybridization patterns generated in this study are identical to what were observed in the previous study. In conclusion, AAD-12 soybean event DAS-68416-4 carries a single insert including one intact PTU for each AAD-1 and PAT. The insert acts as a single locus with a typical Mendelian inheritance pattern.

CONCLUSION

Southern blot analysis and *aad*-12 protein expression testing of individual plants from a F2 segregating population of soybean event AAD-12 DAS-68416-4 were conducted. The result demonstrated that the segregation ratio between the number of plants carrying the *aad*-12 transgenic insert and the segregated nulls was 3:1 characteristic of the Mendelian inheritance pattern of a single gene, indicating that the insert is inherited as a single locus.

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

Chi-square (χ^2) Analysis of AAD-12 Protein Expression Data

A Chi-square (χ^2) fitness test was conducted to determine if the observed segregation ratio fits to the expected ratio of 3:1 as a single insert/locus. The analysis was carried out using the FRET procedure in SAS Version 9.2.

Status	Observed Frequency	Observed Percent	Test Percent	Cumulative Frequency	Cumulative Percent
AAd-12 negative	45	30.61	25.00	45	30.61
AAD-12 positive	102	69.39	75.00	147	100.00

Chi-Square Test
for Specified Proportions

Chi-Square	2.4694
DF	1
Pr > ChiSq	0.1161

Sample Size = 147

Chi-Square (χ^2) Analysis of Southern Blot Data

Status	Observed Frequency	Observed Percent	Test Percent	Cumulative Frequency	Cumulative Percent
Southern negative	43	30.07	25.00	43	30.07
Southern positive	100	69.93	75.00	143	100.00

Chi-Square Test
for Specified Proportions

Chi-Square	1.9604
DF	1
Pr > ChiSq	0.1615

Sample Size = 143

REFERENCES

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

2. Song P., Cruse J., Thomas A. 2009. Molecular characterization of AAD-12 soybean Event DAS-68416-4. Dow AgroSciences Internal Report, 2009.

Table 1. List of Seed Sources

Name	Generation	Source ID	Purpose
397.TCL/pDAB4468(4)-0416.001-523-3-2979	F2	GX08KX037025.007	Test 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

Table 3. AAD-12 Protein Expression and Southern Hybridization in a F2 population of Event DAS-68416-4

Plant / Sample	**AAD-12 Expression Test**	***Nco* I**				***Pst* I**		***Pst* I/*Xho* I**	
		***aad*-12 probe (>4043 bp)**	**Southern Blot Figure**	***pat* probe (> 4043 bp)**	**Southern Blot Figure**	***aad*-12 probe (2868 bp)**	**Southern Blot Figure**	***pat* probe (1928 bp)**	**Southern Blot Figure**
Plasmid pDAB4468	N/A	7429	2-6	7429	7-11	2868	12-16	1928	17-21
416-1	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-2	–	–	2A	–	7A	–	12A	–	17A
416-3	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-4	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-5	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-6	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-7	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-8	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-9	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-10	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-11	–	–	2A	–	7A	–	12A	–	17A
416-12	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-13	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-14	–	–	2A	–	7A	–	12A	–	17A
416-15	–	–	2A	–	7A	–	12A	–	17A
416-16	+	~5500	2A	~5500	7A	2868	12A	1928	17A
416-17	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-18	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-19	–	–	2B	–	7B	–	12B	–	17B
416-20	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-21	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-22	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-23	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-24	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-25	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-26	N/G	N/A		N/A		N/A		N/A	
416-27	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-28	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-29	+	~5500	2B	~5500	7B	2868	12B	1928	17B

Plant / Sample	AAD-12 Expression Test	*Nco* I				*Pst* I		*Pst* I/*Xho* I	
		aad-12 probe (>4043 bp)	Southern Blot Figure	*pat* probe (> 4043 bp)	Southern Blot Figure	*aad*-12 probe (2868 bp)	Southern Blot Figure	*pat* probe (1928 bp)	Southern Blot Figure
416-30	–	–	2B	–	7B	–	12B	–	17B
416-31	+	~5500	2B	~5500	7B	2868	12B	1928	17B
416-32	–	–	2B	–	7B	–	12B	–	17B
416-33	–	–	2B	–	7B	–	12B	–	17B
416-34	+	~5500	3A	~5500	8A	2868	13A	1928	18A
416-35	+	~5500	3A	~5500	8A	2868	13A	1928	18A
416-36	–	–	3A	–	8A	–	13A	–	18A
416-37	+	~5500	3A	~5500	8A	2868	13A	1928	18A
416-38	+	~5500	3A	~5500	8A	2868	13A	1928	18A
416-39	+	~5500	3A	~5500	8A	2868	13A	1928	18A
416-40	–	–	3A	–	8A	–	13A	–	18A
416-41	–	–	3A	–	8A	–	13A	–	18A
416-42	–	–	3A	–	8A	–	13A	–	18A
416-43	–	–	3A	–	8A	–	13A	–	18A
416-44	+	~5500	3A	~5500	8A	2868	13A	1928	18A
416-45	–	–	3A	–	8A	–	13A	–	18A
416-46	–	–	3B	–	8B	–	13B	–	18B
416-47	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-48	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-49	–	–	3B	–	8B	–	13B	–	18B
416-50	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-51	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-52	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-53	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-54	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-55	–	–	3B	–	8B	–	13B	–	18B
416-56	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-57	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-58	–	–	3B	–	8B	–	13B	–	18B
416-59	–	–	3B	–	8B	–	13B	–	18B
416-60	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-61	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-62	+	~5500	3B	~5500	8B	2868	13B	1928	18B

Plant / Sample	AAD-12 Expression Test	*Nco* I				*Pst* I		*Pst* I/*Xho* I	
		***aad*-12 probe (>4043 bp)**	**Southern Blot Figure**	***pat* probe (> 4043 bp)**	**Southern Blot Figure**	***aad*-12 probe (2868 bp)**	**Southern Blot Figure**	***pat* probe (1928 bp)**	**Southern Blot Figure**
416-63	–	–	3B	–	8B	–	13B	–	18B
416-64	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-65	+	~5500	3B	~5500	8B	2868	13B	1928	18B
416-66	–	–	4A	–	9A	–	14A	–	19A
416-67	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-68	–	–	4A	–	9A	–	14A	–	19A
416-69	–	–	4A	–	9A	–	14A	–	19A
416-70	–	–	4A	–	9A	–	14A	–	19A
416-71	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-72	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-73	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-74	–	–	4A	–	9A	–	14A	–	19A
416-75	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-76	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-77	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-78	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-79	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-80	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-81	N/S	N/A		N/A		N/A		N/A	
416-82	+	~5500	4A	~5500	9A	2868	14A	1928	19A
416-83	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-84*	–	N/A		N/A		N/A		N/A	
416-85	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-86	–	–	4B	–	9B	–	14B	–	19B
416-87	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-88	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-89	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-90	–	–	4B	–	9B	–	14B	–	19B
416-91	–	–	4B	–	9B	–	14B	–	19B
416-92	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-93	–	–	4B	–	9B	–	14B	–	19B
416-94	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-95	–	–	4B	–	9B	–	14B	–	19B

Plant / Sample	AAD-12 Expression Test	*Nco* I				*Pst* I		*Pst* I/*Xho* I	
		***aad*-12 probe (>4043 bp)**	**Southern Blot Figure**	***pat* probe (> 4043 bp)**	**Southern Blot Figure**	***aad*-12 probe (2868 bp)**	**Southern Blot Figure**	***pat* probe (1928 bp)**	**Southern Blot Figure**
416-96	–	–	4B	–	9B	–	14B	–	19B
416-97*	+	N/A		N/A		N/A		N/A	
416-98	–	–	4B	–	9B	–	14B	–	19B
416-99	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-100	+	~5500	4B	~5500	9B	2868	14B	1928	19B
416-101	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-102	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-103	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-104	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-105	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-106	–	–	5A	–	10A	–	15A	–	20A
416-107	–	–	5A	–	10A	–	15A	–	20A
416-108	–	–	5A	–	10A	–	15A	–	20A
416-109	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-110	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-111	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-112	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-113	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-114	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-115	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-116	+	~5500	5A	~5500	10A	2868	15A	1928	20A
416-117	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-118	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-119	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-120*	+	N/A		N/A		N/A		N/A	20B
416-121	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-122	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-123*	–	N/A		N/A		N/A		N/A	20B
416-124	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-125	–	–	5B	–	10B	–	15B	–	20B
416-126	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-127	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-128	+	~5500	5B	~5500	10B	2868	15B	1928	20B

Plant / Sample	AAD-12 Expression Test	*Nco* I				*Pst* I		*Pst* I/*Xho* I	
		aad-12 probe (>4043 bp)	Southern Blot Figure	*pat* probe (> 4043 bp)	Southern Blot Figure	*aad*-12 probe (2868 bp)	Southern Blot Figure	*pat* probe (1928 bp)	Southern Blot Figure
416-129	N/G	N/A		N/A		N/A		N/A	
416-130	–	–	5B	–	10B	–	15B	–	20B
416-131	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-132	NG	N/A		N/A		N/A		N/A	
416-133	–	–	5B	–	10B	–	15B	–	20B
416-134	–	–	5B	–	10B	–	15B	–	20B
416-135	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-136	+	~5500	5B	~5500	10B	2868	15B	1928	20B
416-137	–	–	6	–	11	–	16	–	21
416-138	–	–	6	–	11	–	16	–	21
416-139	–	–	6	–	11	–	16	–	21
416-140	+	~5500	6	~5500	11	2868	16	1928	21
416-141	+	~5500	6	~5500	11	2868	16	1928	21
416-142	+	~5500	6	~5500	11	2868	16	1928	21
416-143	+	~5500	6	~5500	11	2868	16	1928	21
416-144	+	~5500	6	~5500	11	2868	16	1928	21
416-145	+	~5500	6	~5500	11	2868	16	1928	21
416-146	+	~5500	6	~5500	11	2868	16	1928	21
416-147	+	~5500	6	~5500	11	2868	16	1928	21
416-148	+	~5500	6	~5500	11	2868	16	1928	21
416-149	+	~5500	6	~5500	11	2868	16	1928	21
416-150	–	–	6	–	11	–	16	–	21
Total	**AAD-12 Expression Positive**	**102**	**AAD-12 Expression Negative**	**45**		**Southern Positive**	**100**	**Southern Negative**	**43**

N/A = not applicable; N/G = Seed not germinated; N/S = no seed in the pot"*" = Plants dead before DNA extraction; "+" = Test positive for AAD-12 protein expression; "–" = Test negative for AAD-12 protein expression or no hybridization signal in Southern blot; (Note: Seeds in 3 pots didn't germinate;
4 seedlings (2 positive and 2 negative for AAD-12 protein expression) died before sample collection and DNA extraction.)

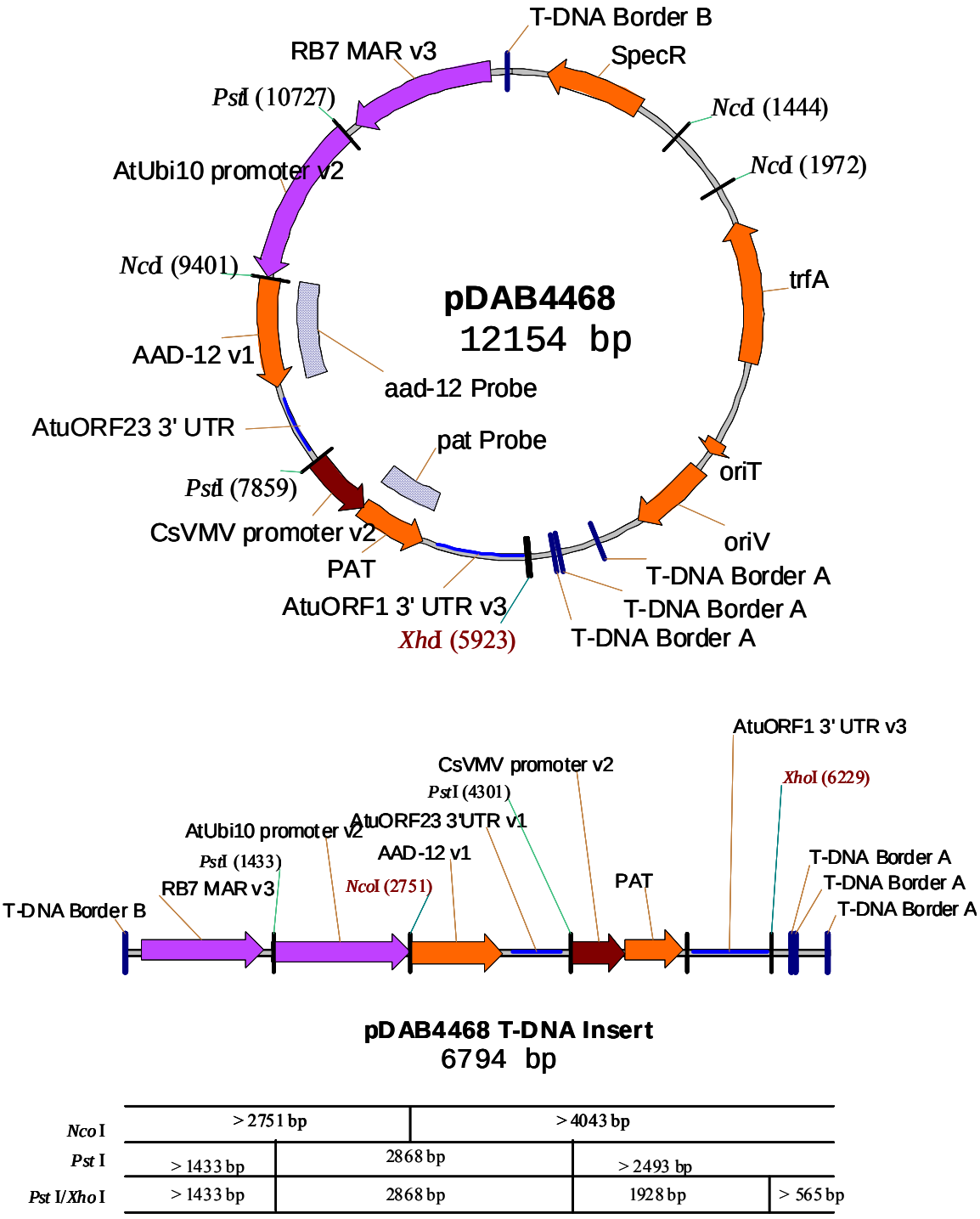
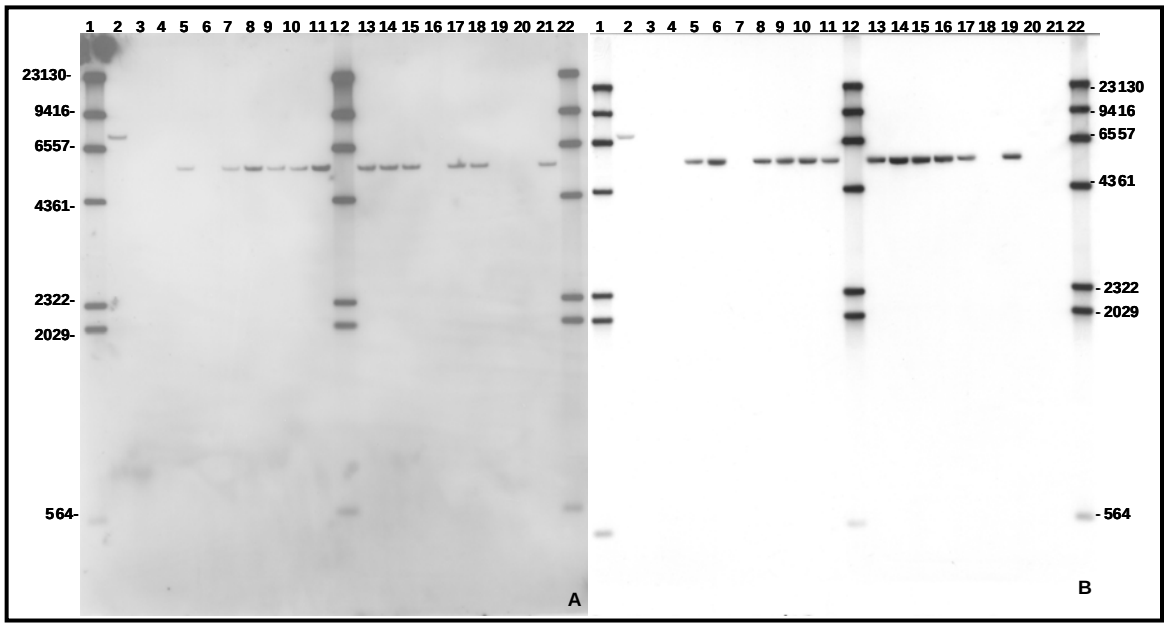


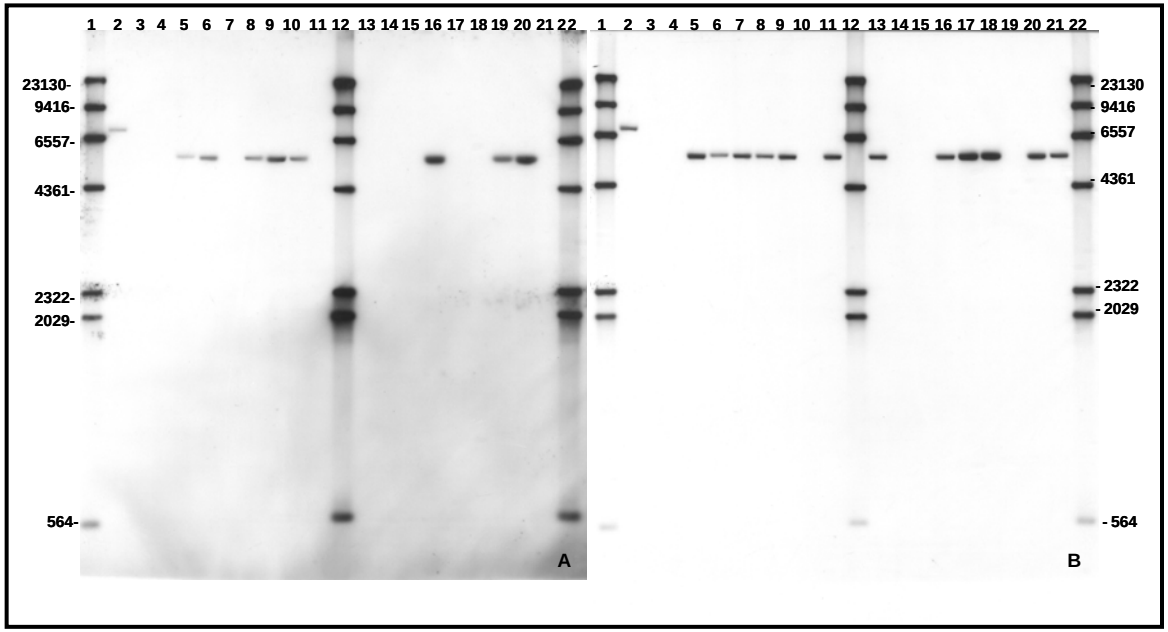
Figure 1. Plasmid Map of pDAB4468 and Its T-DNA Insert



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-7	1, 12, 22	Molecular Marker	11	416-23
2	pDAB4468+ Maverick C6	13	416-8	2	pDAB4468+ Maverick C2	13	416-24
3	Maverick C6	14	416-9	3	Maverick C2	14	416-25
4	Maverick C10	15	416-10	4	Maverick C3	15	416-27
5	416-1	16	416-11	5	416-17	16	416-28
6	416-2	17	416-12	6	416-18	17	416-29
7	416-3	18	416-13	7	416-19	18	416-30
8	416-4	19	416-14	8	416-20	19	416-31
9	416-5	20	416-15	9	416-21	20	416-32
10	416-6	21	416-16	10	416-22	21	416-33

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

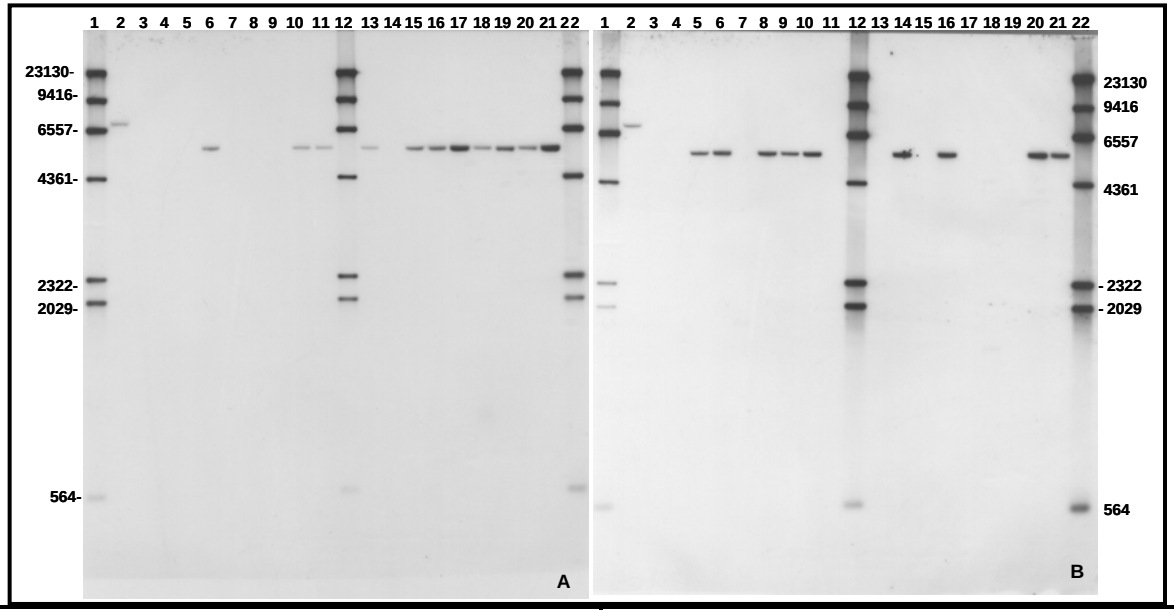
Figure 2. Southern blot analysis of *Nco* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-40	1, 12, 22	Molecular Marker	11	416-56
2	pDAB4468+ Maverick C2	13	416-41	2	pDAB4468+ Maverick C2	13	416-57
3	Maverick C2	14	416-42	3	Maverick C2	14	416-58
4	Maverick C3	15	416-43	4	Maverick C3	15	416-59
5	416-34	16	416-44	5	416-50	16	416-60
6	416-35	17	416-45	6	416-51	17	416-61
7	416-36	18	416-46	7	416-52	18	416-62
8	416-37	19	416-47	8	416-53	19	416-63
9	416-38	20	416-48	9	416-54	20	416-64
10	416-39	21	416-49	10	416-55	21	416-65

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

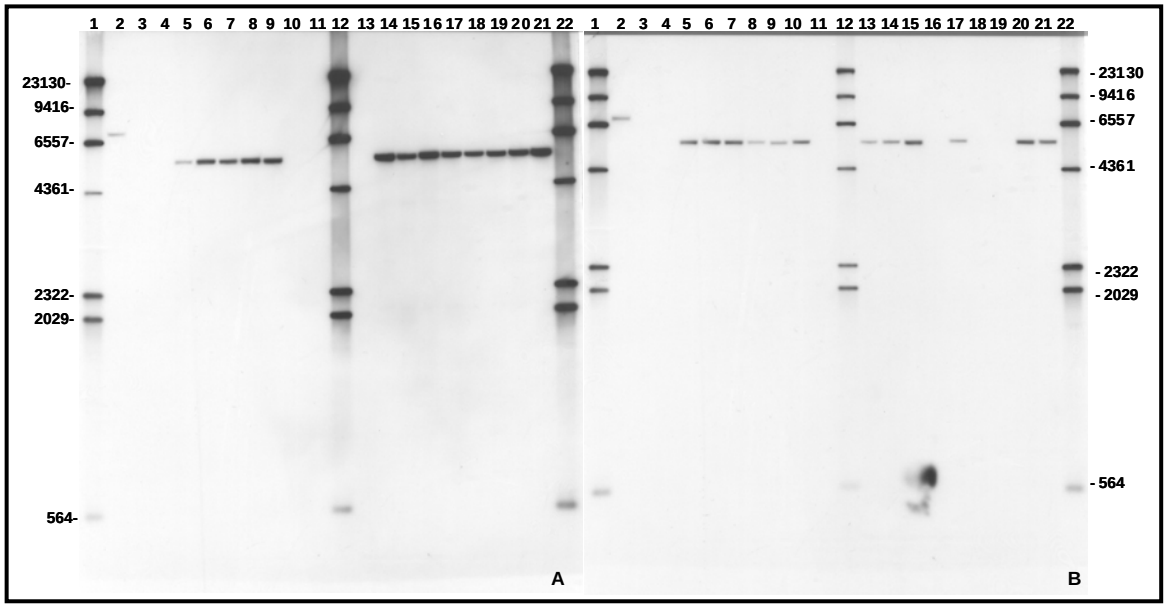
Figure 3. Southern blot analysis of *Nco* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-72	1, 12, 22	Molecular Marker	11	416-90
2	pDAB4468+ Maverick C2	13	416-73	2	pDAB4468+ Maverick C2	13	416-91
3	Maverick C2	14	416-74	3	Maverick C2	14	416-92
4	Maverick C3	15	416-75	4	Maverick C3	15	416-93
5	416-66	16	416-76	5	416-83	16	416-94
6	416-67	17	416-77	6	416-85	17	416-95
7	416-68	18	416-78	7	416-86	18	416-96
8	416-69	19	416-79	8	416-87	19	416-98
9	416-70	20	416-80	9	416-88	20	416-99
10	416-71	21	416-82	10	416-89	21	416-100

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

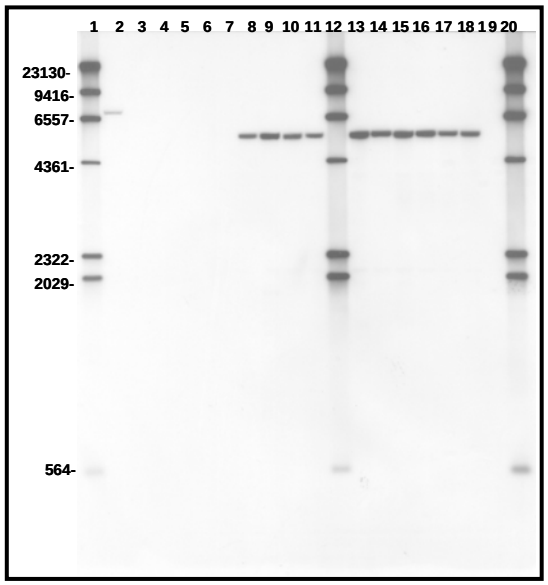
Figure 4. Southern blot analysis of *Nco* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-107	1, 12, 22	Molecular Marker	11	416-125
2	pDAB4468+ Maverick C2	13	416-108	2	pDAB4468+ Maverick C2	13	416-126
3	Maverick C2	14	416-109	3	Maverick C2	14	416-127
4	Maverick C3	15	416-110	4	Maverick C3	15	416-128
5	416-101	16	416-111	5	416-117	16	416-130
6	416-102	17	416-112	6	416-118	17	416-131
7	416-103	18	416-113	7	416-119	18	416-133
8	416-104	19	416-114	8	416-121	19	416-134
9	416-105	20	416-115	9	416-122	20	416-135
10	416-106	21	416-116	10	416-124	21	416-136

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: the spot in Panel B is an unspecific signal caused by unknown reason)

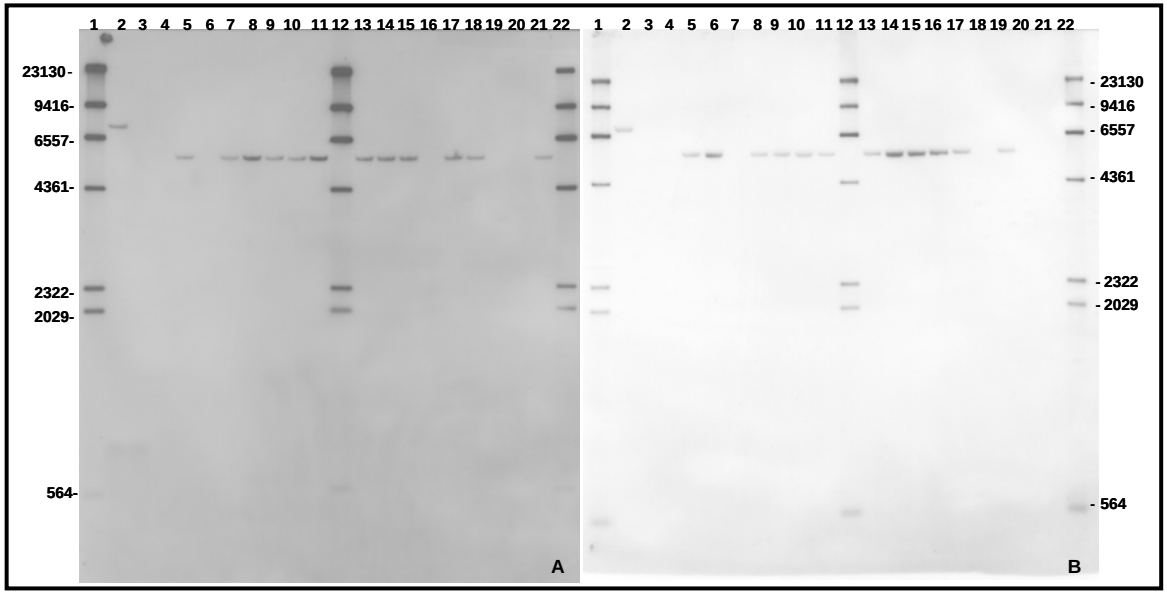
Figure 5. Southern blot analysis of *Nco* I digest with *aad*-12 probe



Panel A			
Lane	Sample	Lane	Sample
1, 12, 20	Molecular Marker	10	416-142
2	pDAB4468+ Maverick C2	11	416-143
3	Maverick C2	13	416-144
4	Maverick C3	14	416-145
5	416-137	15	416-146
6	416-138	16	416-147
7	416-139	17	416-148
8	416-140	18	416-149
9	416-141	19	416-150

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

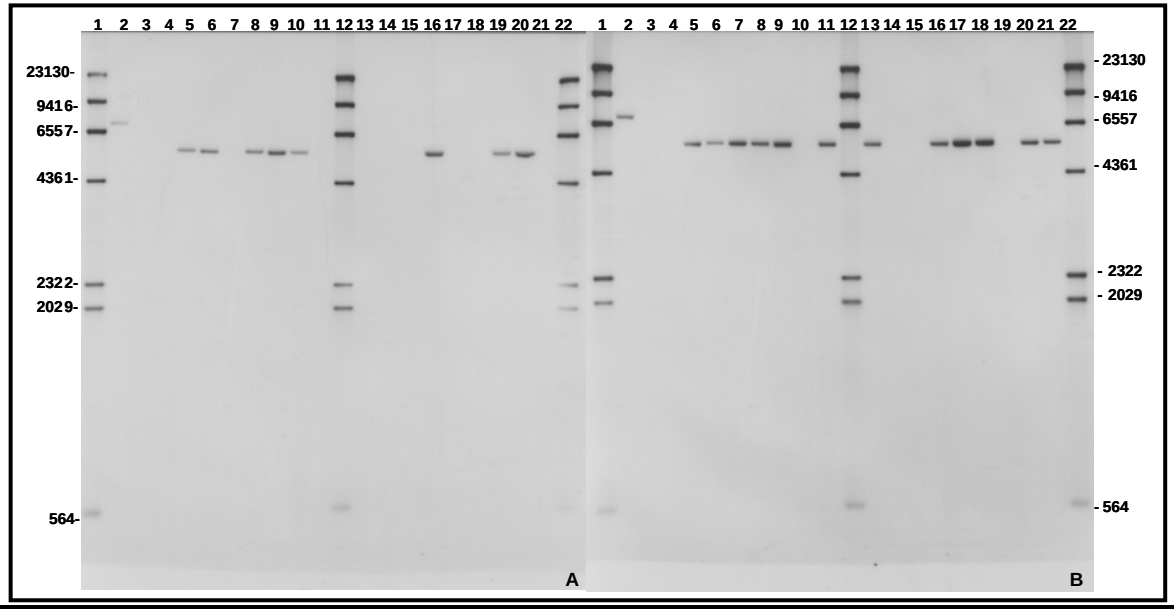
Figure 6. Southern blot analysis of *Nco* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-7	1, 12, 22	Molecular Marker	11	416-23
2	pDAB4468+ Maverick C6	13	416-8	2	pDAB4468+ Maverick C2	13	416-24
3	Maverick C6	14	416-9	3	Maverick C2	14	416-25
4	Maverick C10	15	416-10	4	Maverick C3	15	416-27
5	416-1	16	416-11	5	416-17	16	416-28
6	416-2	17	416-12	6	416-18	17	416-29
7	416-3	18	416-13	7	416-19	18	416-30
8	416-4	19	416-14	8	416-20	19	416-31
9	416-5	20	416-15	9	416-21	20	416-32
10	416-6	21	416-16	10	416-22	21	416-33

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

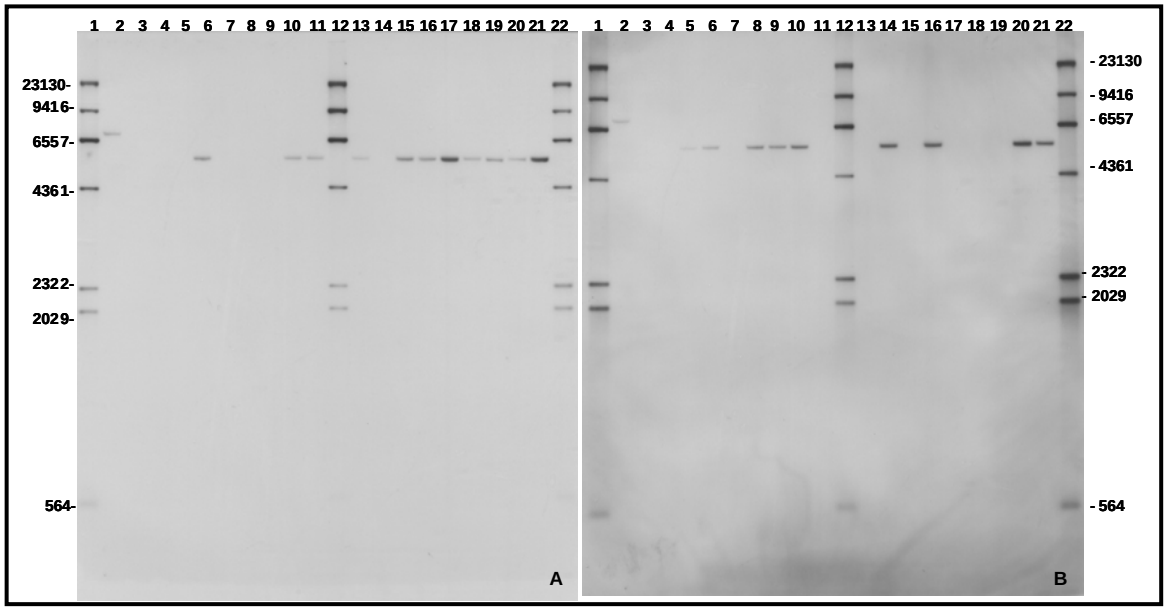
Figure 7. Southern blot analysis of *Nco* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-40	1, 12, 22	Molecular Marker	11	416-56
2	pDAB4468+ Maverick C2	13	416-41	2	pDAB4468+ Maverick C2	13	416-57
3	Maverick C2	14	416-42	3	Maverick C2	14	416-58
4	Maverick C3	15	416-43	4	Maverick C3	15	416-59
5	416-34	16	416-44	5	416-50	16	416-60
6	416-35	17	416-45	6	416-51	17	416-61
7	416-36	18	416-46	7	416-52	18	416-62
8	416-37	19	416-47	8	416-53	19	416-63
9	416-38	20	416-48	9	416-54	20	416-64
10	416-39	21	416-49	10	416-55	21	416-65

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

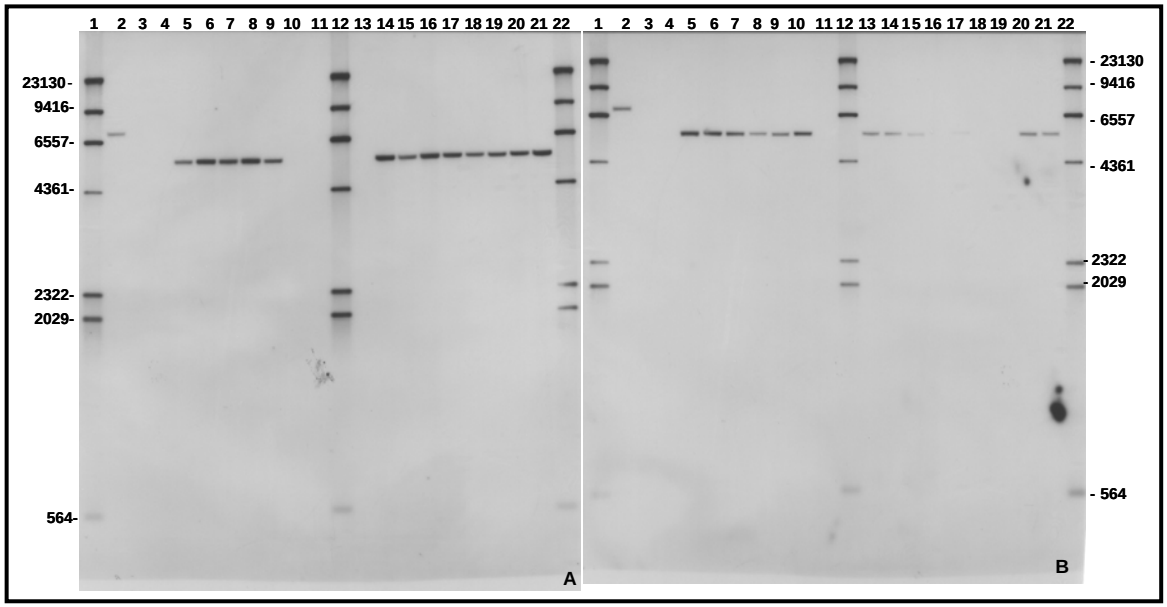
Figure 8. Southern blot analysis of *Nco* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-72	1, 12, 22	Molecular Marker	11	416-90
2	pDAB4468+ Maverick C2	13	416-73	2	pDAB4468+ Maverick C2	13	416-91
3	Maverick C2	14	416-74	3	Maverick C2	14	416-92
4	Maverick C3	15	416-75	4	Maverick C3	15	416-93
5	416-66	16	416-76	5	416-83	16	416-94
6	416-67	17	416-77	6	416-85	17	416-95
7	416-68	18	416-78	7	416-86	18	416-96
8	416-69	19	416-79	8	416-87	19	416-98
9	416-70	20	416-80	9	416-88	20	416-99
10	416-71	21	416-82	10	416-89	21	416-100

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

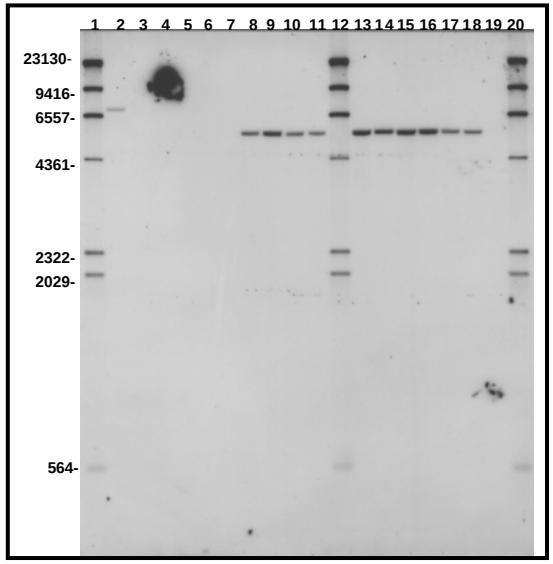
Figure 9. Southern blot analysis of *Nco* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-107	1, 12, 22	Molecular Marker	11	416-125
2	pDAB4468+ Maverick C2	13	416-108	2	pDAB4468+ Maverick C2	13	416-126
3	Maverick C2	14	416-109	3	Maverick C2	14	416-127
4	Maverick C3	15	416-110	4	Maverick C3	15	416-128
5	416-101	16	416-111	5	416-117	16	416-130
6	416-102	17	416-112	6	416-118	17	416-131
7	416-103	18	416-113	7	416-119	18	416-133
8	416-104	19	416-114	8	416-121	19	416-134
9	416-105	20	416-115	9	416-122	20	416-135
10	416-106	21	416-116	10	416-124	21	416-136

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: no specific signals in Lane 20, 21 are dirty spots occurred in the membrane process for unknown reason.)

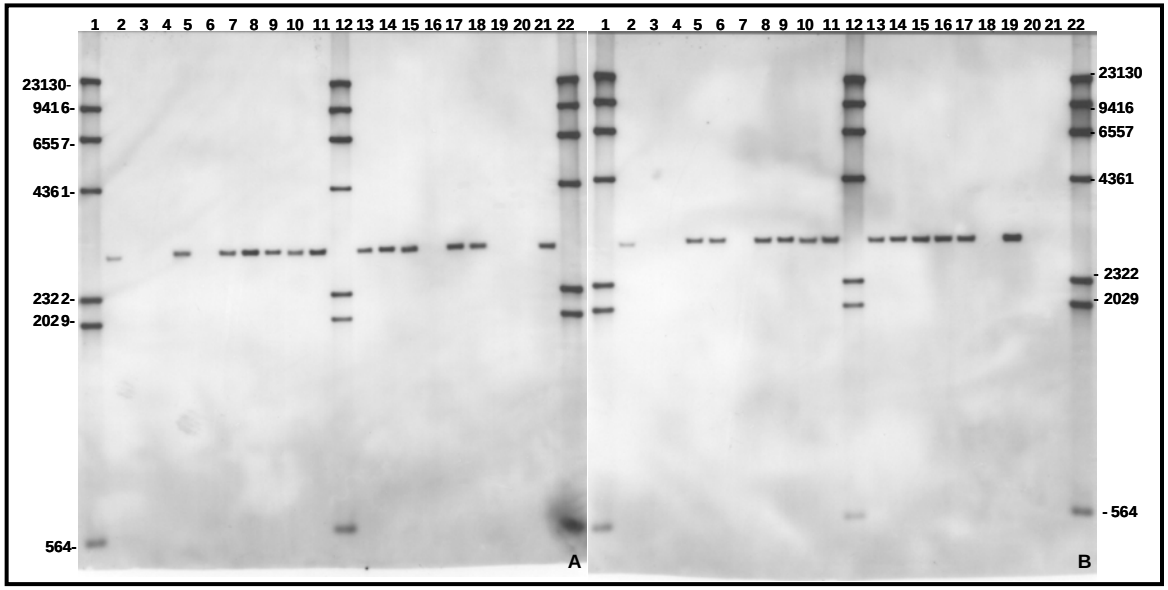
Figure 10. Southern blot analysis of *Nco* I digest with *pat* probe



Panel A			
Lane	Sample	Lane	Sample
1, 12, 20	Molecular Marker	10	416-142
2	pDAB4468+ Maverick C2	11	416-143
3	Maverick C2	13	416-144
4	Maverick C3	14	416-145
5	416-137	15	416-146
6	416-138	16	416-147
7	416-139	17	416-148
8	416-140	18	416-149
9	416-141	19	416-150

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: no specific signals on Lane 3-5, 19 are blobs occurred in the membrane process for unknown reasons.)

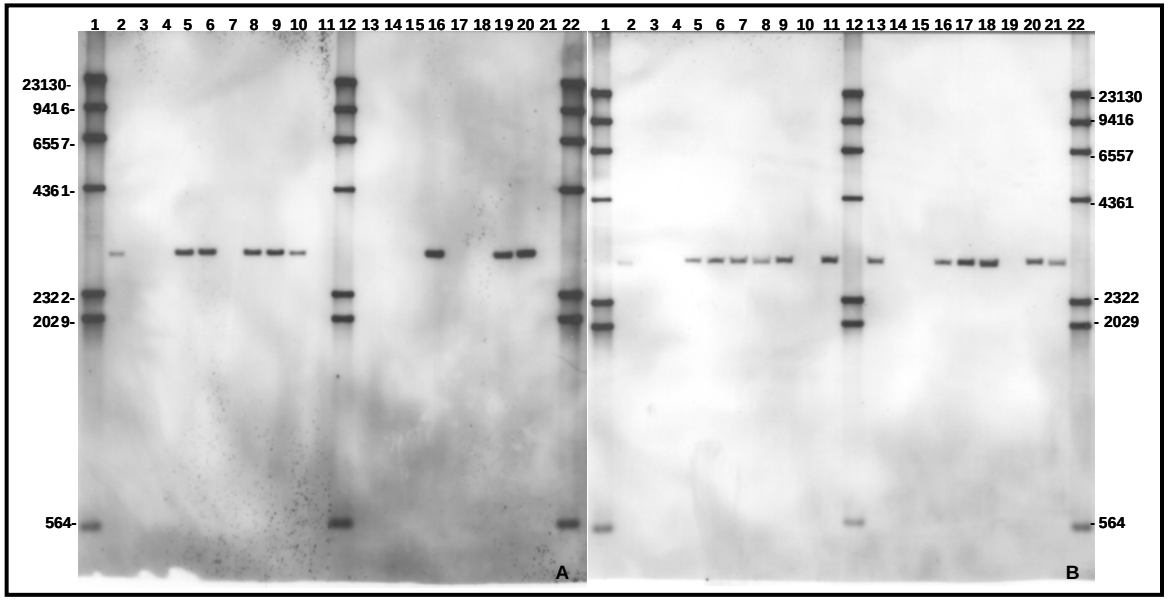
Figure 11. Southern blot analysis of *Nco* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-7	1, 12, 22	Molecular Marker	11	416-23
2	pDAB4468+ Maverick C4	13	416-8	2	pDAB4468+ Maverick C4	13	416-24
3	Maverick C4	14	416-9	3	Maverick C4	14	416-25
4	Maverick C5	15	416-10	4	Maverick C5	15	416-27
5	416-1	16	416-11	5	416-17	16	416-28
6	416-2	17	416-12	6	416-18	17	416-29
7	416-3	18	416-13	7	416-19	18	416-30
8	416-4	19	416-14	8	416-20	19	416-31
9	416-5	20	416-15	9	416-21	20	416-32
10	416-6	21	416-16	10	416-22	21	416-33

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

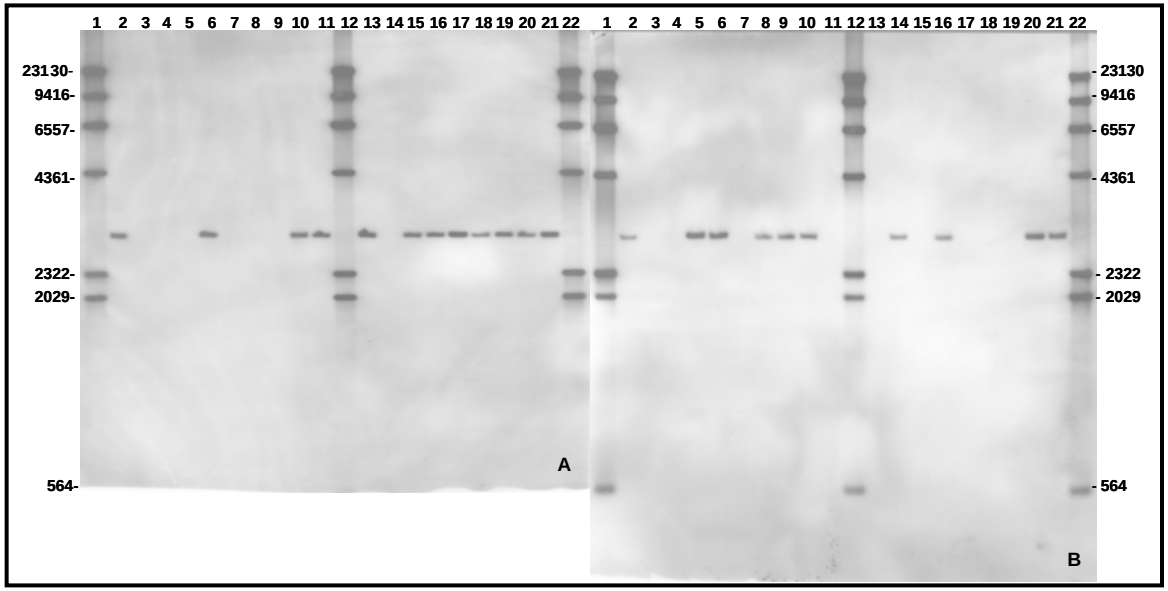
Figure 12. Southern blot analysis of *Pst* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-40	1, 12, 22	Molecular Marker	11	416-56
2	pDAB4468+ Maverick C4	13	416-41	2	pDAB4468+ Maverick C4	13	416-57
3	Maverick C4	14	416-42	3	Maverick C4	14	416-58
4	Maverick C5	15	416-43	4	Maverick C5	15	416-59
5	416-34	16	416-44	5	416-50	16	416-60
6	416-35	17	416-45	6	416-51	17	416-61
7	416-36	18	416-46	7	416-52	18	416-62
8	416-37	19	416-47	8	416-53	19	416-63
9	416-38	20	416-48	9	416-54	20	416-64
10	416-39	21	416-49	10	416-55	21	416-65

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

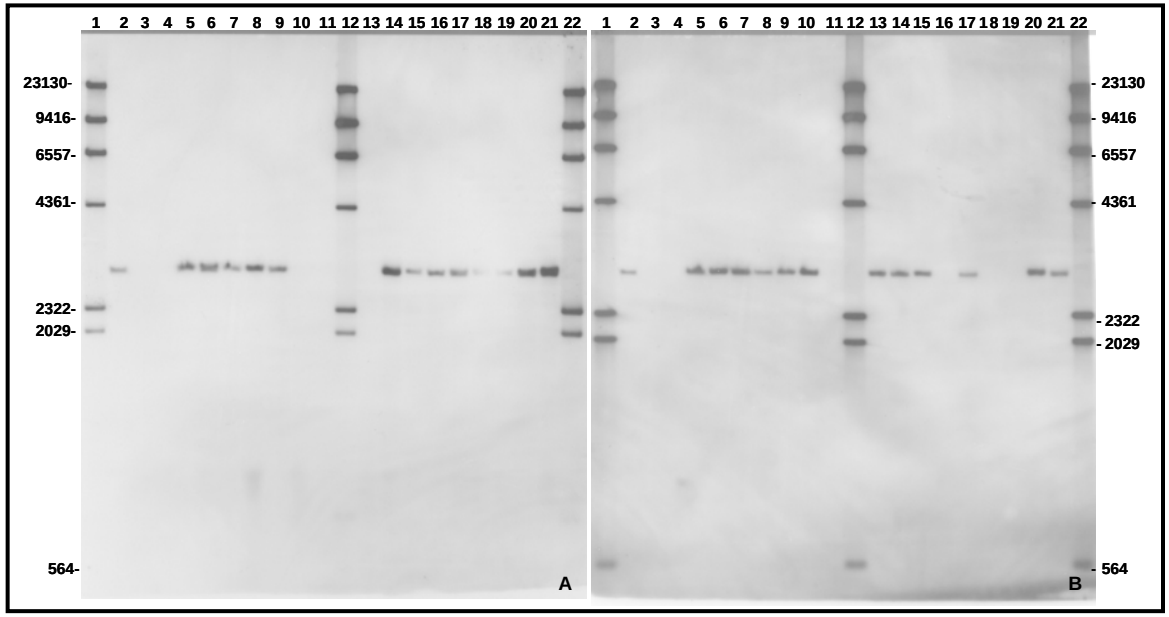
Figure 13. Southern blot analysis of *Pst* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-72	1, 12, 22	Molecular Marker	11	416-90
2	pDAB4468+ Maverick C10	13	416-73	2	pDAB4468+ Maverick C4	13	416-91
3	Maverick C10	14	416-74	3	Maverick C4	14	416-92
4	Maverick C5	15	416-75	4	Maverick C5	15	416-93
5	416-66	16	416-76	5	416-83	16	416-94
6	416-67	17	416-77	6	416-85	17	416-95
7	416-68	18	416-78	7	416-86	18	416-96
8	416-69	19	416-79	8	416-87	19	416-98
9	416-70	20	416-80	9	416-88	20	416-99
10	416-71	21	416-82	10	416-89	21	416-100

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: The blot for Panel A was broken below the 564 bp marker, but it won't affect the Southern result since hybridization with other probes did not indicate any additional fragments of *aad*-12 transgene.)

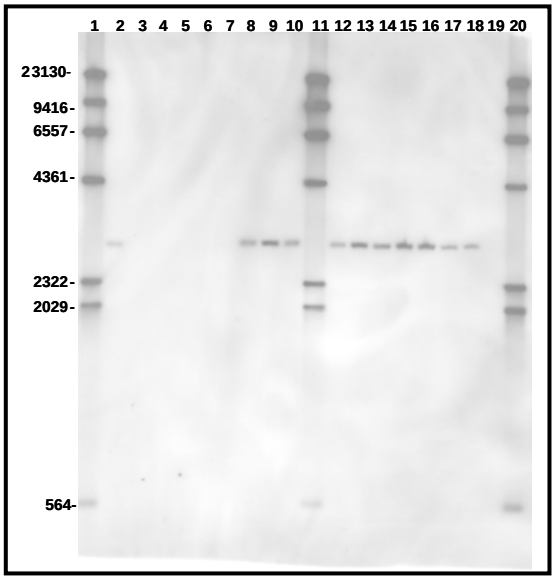
Figure 14. Southern blot analysis of *Pst* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-107	1, 12, 22	Molecular Marker	11	416-125
2	pDAB4468+ Maverick C6	13	416-108	2	pDAB4468+ Maverick C6	13	416-126
3	Maverick C6	14	416-109	3	Maverick C6	14	416-127
4	Maverick C10	15	416-110	4	Maverick C5	15	416-128
5	416-101	16	416-111	5	416-117	16	416-130
6	416-102	17	416-112	6	416-118	17	416-131
7	416-103	18	416-113	7	416-119	18	416-133
8	416-104	19	416-114	8	416-121	19	416-134
9	416-105	20	416-115	9	416-122	20	416-135
10	416-106	21	416-116	10	416-124	21	416-136

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

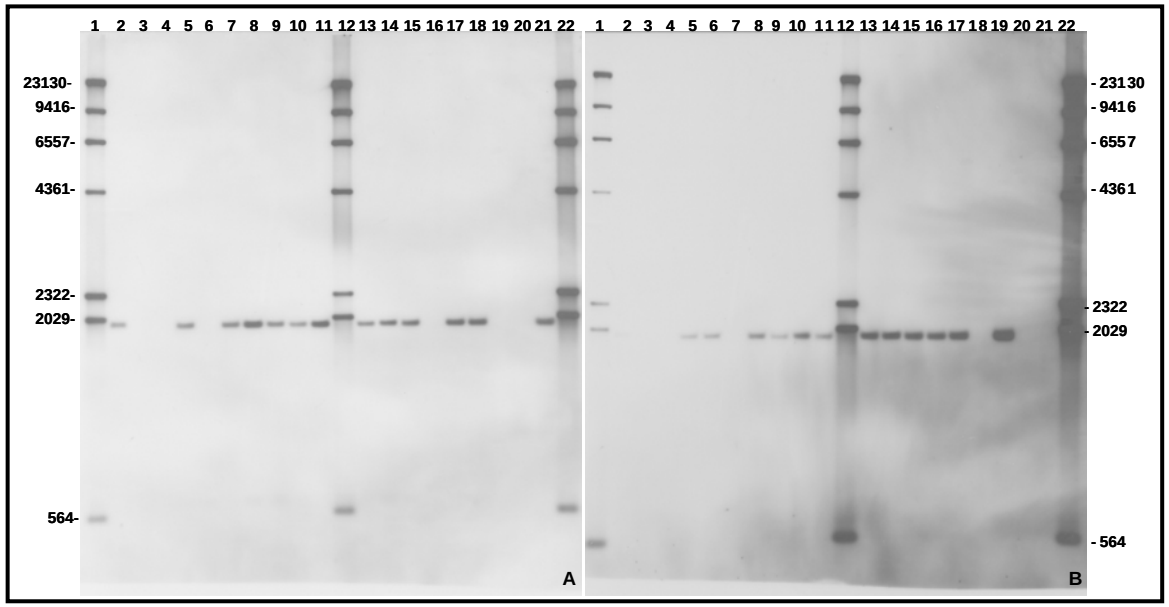
Figure 15. Southern blot analysis of *Pst* I digest with *aad*-12 probe



Panel A			
Lane	Sample	Lane	Sample
1, 11, 20	Molecular Marker	10	416-142
2	pDAB4468+ Maverick C7	12	416-143
3	Maverick C7	13	416-144
4	Maverick C9	14	416-145
5	416-137	15	416-146
6	416-138	16	416-147
7	416-139	17	416-148
8	416-140	18	416-149
9	416-141	19	416-150

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

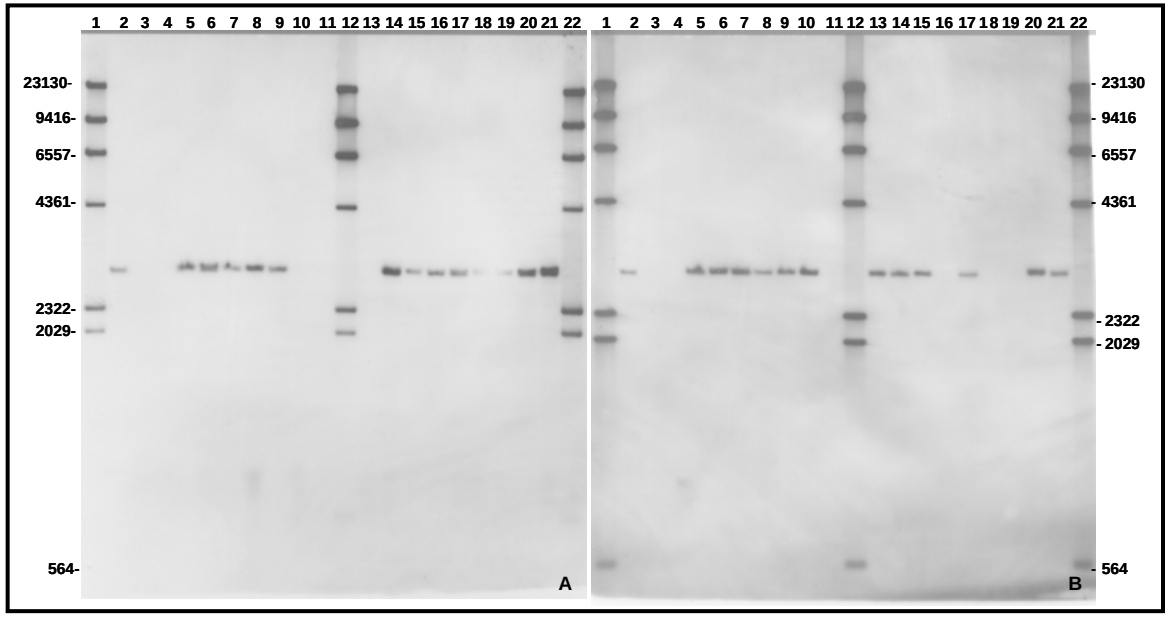
Figure 16. Southern blot analysis of *Pst* I digest with *aad*-12 probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-7	1, 12, 22	Molecular Marker	11	416-23
2	pDAB4468+ Maverick C8	13	416-8	2	pDAB4468+ Maverick C8	13	416-24
3	Maverick C8	14	416-9	3	Maverick C8	14	416-25
4	Maverick C9	15	416-10	4	Maverick C9	15	416-27
5	416-1	16	416-11	5	416-17	16	416-28
6	416-2	17	416-12	6	416-18	17	416-29
7	416-3	18	416-13	7	416-19	18	416-30
8	416-4	19	416-14	8	416-20	19	416-31
9	416-5	20	416-15	9	416-21	20	416-32
10	416-6	21	416-16	10	416-22	21	416-33

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

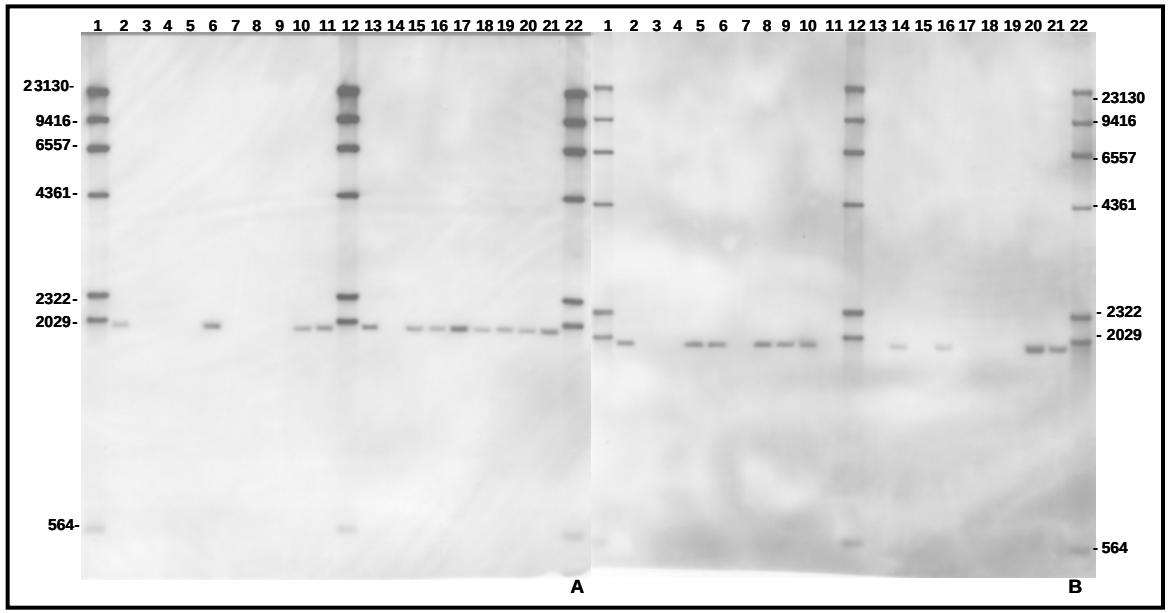
Figure 17. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-40	1, 12, 22	Molecular Marker	11	416-56
2	pDAB4468+ Maverick C6	13	416-41	2	pDAB4468+ Maverick C10	13	416-57
3	Maverick C6	14	416-42	3	Maverick C10	14	416-58
4	Maverick C10	15	416-43	4	Maverick C9	15	416-59
5	416-34	16	416-44	5	416-50	16	416-60
6	416-35	17	416-45	6	416-51	17	416-61
7	416-36	18	416-46	7	416-52	18	416-62
8	416-37	19	416-47	8	416-53	19	416-63
9	416-38	20	416-48	9	416-54	20	416-64
10	416-39	21	416-49	10	416-55	21	416-65

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: The blots below the 564 bp marker did not show up due to over run of electrophoresis, but it won't affect the Southern result since hybridization with other probes didn't indicate the presence of additional *pat* transgene.)

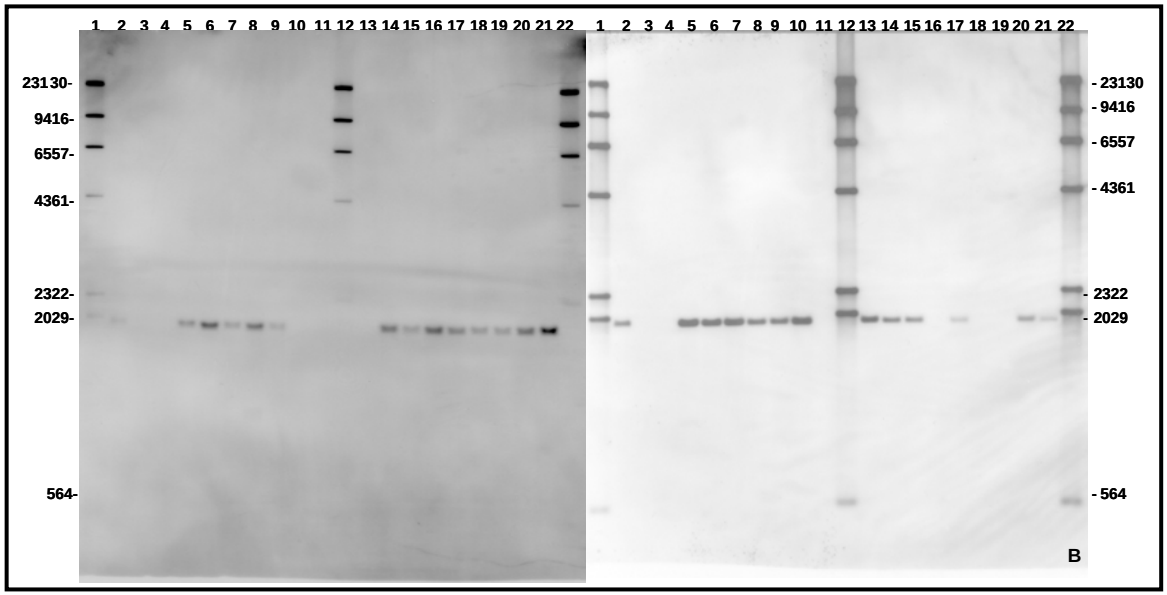
Figure 18. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-72	1, 12, 22	Molecular Marker	11	416-90
2	pDAB4468+ Maverick C10	13	416-73	2	pDAB4468+ Maverick C8	13	416-91
3	Maverick C10	14	416-74	3	Maverick C8	14	416-92
4	Maverick C9	15	416-75	4	Maverick C9	15	416-93
5	416-66	16	416-76	5	416-83	16	416-94
6	416-67	17	416-77	6	416-85	17	416-95
7	416-68	18	416-78	7	416-86	18	416-96
8	416-69	19	416-79	8	416-87	19	416-98
9	416-70	20	416-80	9	416-88	20	416-99
10	416-71	21	416-82	10	416-89	21	416-100

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome.

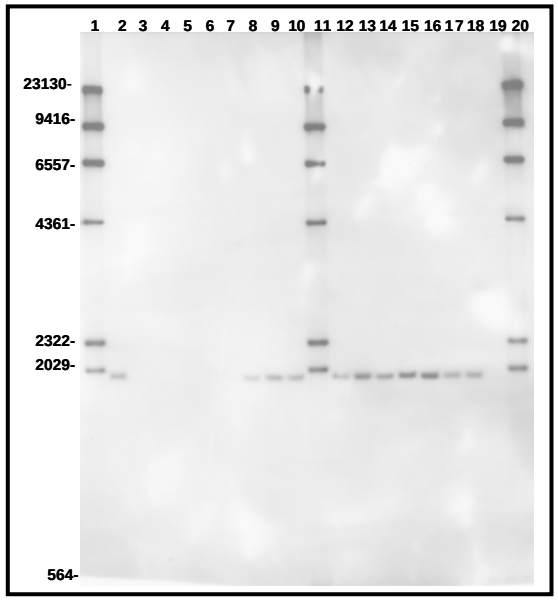
Figure 19. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe



Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-107	1, 12, 22	Molecular Marker	11	416-125
2	pDAB4468+ Maverick C3	13	416-108	2	pDAB4468+ Maverick C8	13	416-126
3	Maverick C3	14	416-109	3	Maverick C8	14	416-127
4	Maverick C7	15	416-110	4	Maverick C9	15	416-128
5	416-101	16	416-111	5	416-117	16	416-130
6	416-102	17	416-112	6	416-118	17	416-131
7	416-103	18	416-113	7	416-119	18	416-133
8	416-104	19	416-114	8	416-121	19	416-134
9	416-105	20	416-115	9	416-122	20	416-135
10	416-106	21	416-116	10	416-124	21	416-136

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: the faint bands on the top of Lane 15-21 are non-specific blobs.)

Figure 20. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe



Panel A			
Lane	Sample	Lane	Sample
1, 11, 20	Molecular Marker	10	416-142
2	pDAB4468+ Maverick C7	11	416-143
3	Maverick C7	13	416-144
4	Maverick C6	14	416-145
5	416-137	15	416-146
6	416-138	16	416-147
7	416-139	17	416-148
8	416-140	18	416-149
9	416-141	19	416-150

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was 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 transgenic copy per soybean genome. (Note: The blot was broken below the 564 bp marker, but it won't affect the Southern result since hybridization with other probes didn't indicate the presence of additional *pat* transgene.)

Figure 21. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe