SUMMARY

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STUDY TITLE

Cloning and Characterization of the DNA Sequence for the Insert and Flanking Border Regions of AAD-12 Soybean Event DAS-68416-4

DATA REQUIREMENTS

Not Applicable

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STUDY COMPLETED ON

July 15, 2009

PERFORMING LABORATORY

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LABORATORY STUDY ID

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Cloning and Characterization of the DNA Sequence for the Insert and Flanking Border Regions of AAD-12 Soybean Event DAS-68416-4

SUMMARY

Transgenic AAD-12 soybean (*Glycine max* L.) event DAS-68416-4 (pDAB4468-416) was generated by *Agrobacterium*-mediated transformation using plasmid pDAB4468. The transgene (T-DNA) of plasmid pDAB4468 contains two expression cassettes, the *aad*-12 expression cassette and the *pat* expression cassette, arranged as tail-to-head, with the RB7 (a matrix attachment region (MAR) element from *Nicotiana tabacuum*) at the 5' end to facilitate gene expression. The *aad*-12 gene is a synthetic gene from *Delftia acidovorans* that encodes an aryloxyalkanoate dioxygenase enzyme capable of deactivating several herbicides with an aryloxyalkanoate moiety, including phenoxy auxin (e.g., 2,4-D, MCPA) and pyridyloxy auxins (e.g., fluroxypyr, triclopyr*).* The *pat* gene is from *Streptomyces viridochromogenes* and provides tolerance to the glufosinate-ammonium herbicide.

To characterize the inserted DNA and its flanking regions in the genomic insertion site, the sequence of the DNA insert and flanking genomic DNA border regions of AAD-12 soybean event DAS-68416-4 were determined. A total of 10212 bp of event DAS-68416-4 genomic sequence was confirmed, comprising 2730 bp of 5' flanking border sequence, 1082 bp of 3' flanking border sequence, and 6400 bp of T-DNA insert. Sequence analysis confirmed that AAD-12 soybean event DAS-68416-4 contains a single-copy of intact transgene including the RB7 element, the *aad*-12 expression cassette, and the *pat* expression cassette with no sequence variation compared to the expected T-DNA insert.

PCR amplification based on the AAD-12 soybean event DAS-68416-4 insert and border sequences confirmed that the border regions were of soybean origin and that the junction regions could be used for event-specific identification of event DAS-68416-4. Analysis of the sequence spanning the junction regions, as well as the flanking border sequences themselves, did not indicate any homologies in a BLAST search or any novel open reading frames resulting from the

T-DNA insertion. Additionally, the T-DNA insertion site was characterized by cloning a genomic fragment from the non-transgenic soybean genome corresponding to the region of the identified flanking border sequences. The resulting sequence revealed a 55 bp deletion from the original locus and a 9 bp insertion at 3' integration junction of the event. Overall, the characterization of the insert and border sequence of AAD-12 soybean event DAS-68416-4 indicated that a single, intact copy of the T-DNA was present in the soybean genome and the sequence information can be further utilized for event-specific tests.

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DATA REQUIREMENTS

Not Applicable

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Aryloxyalkanoate Dioxygenase-12 (AAD-12) and Phosphinothricin N-Acetyltransferase (PAT)

Title: Cloning and Characterization of the DNA Sequence for the Insert and Flanking Border Regions 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).*

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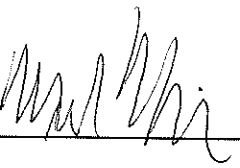
Signature: 

Date: 13 July 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: Cloning and Characterization of the DNA Sequence for the Insert and Flanking Border Regions of AAD-12 Soybean Event DAS-68416-4

Study Initiation Date: 10/08/2008

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

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Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

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No aspect of this study is subject to Good Laboratory Practice Standards.

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ABSTRACT

Transgenic AAD-12 soybean (*Glycine max* L.) event DAS-68416-4 (pDAB4468-416) was generated by *Agrobacterium*-mediated transformation using plasmid pDAB4468. The transgene (T-DNA) of plasmid pDAB4468 contains two main constructs: the *aad*-12 expression cassette and the *pat* expression cassette, arranged as tail-to-head, with a MAR (matrix attachment region) element at the 5' end. The *aad*-12 gene is a synthetic gene from *Delftia acidovorans* that encodes an aryloxyalkanoate dioxygenase enzyme capable of deactivating several herbicides with an aryloxyalkanoate moiety, including phenoxy auxin (e.g., 2,4-D, MCPA) and pyridyloxy auxins (e.g., fluroxypyr, triclopyr*).* The *pat* gene is from *Streptomyces viridochromogenes* and provides tolerance to the glufosinate-ammonium herbicide.

To characterize the inserted DNA and describe the genomic insertion site, the sequence of the DNA insert and flanking genomic DNA border regions of AAD-12 soybean event DAS-68416-4 were determined. In total, 10212 bp of event DAS-68416-4 genomic sequence were confirmed, comprising 2730 bp of 5' flanking border sequence, 1082 bp of 3' flanking border sequence, and 6400 bp of T-DNA insert. Sequence analysis confirmed that AAD-12 soybean event DAS-68416-4 contains a single-copy of intact transgene containing the MAR element, the *aad*-12 expression cassette, and the *pat* expression cassette with no sequence variation from the expected T-DNA insert.

PCR amplification based on the AAD-12 soybean event DAS-68416-4 insert and border sequences confirmed that the border regions were of soybean origin and that the junction regions could be used for event-specific identification of event DAS-68416-4. Analysis of the sequence spanning the junction regions, as well as the flanking border sequences themselves, did not indicate any homologies in a BLAST search or any novel open reading frames resulting from the T-DNA insertion. Additionally, the T-DNA insertion site was characterized by cloning a genomic fragment from the non-transgenic soybean genome corresponding to the region of the

identified flanking border sequences. The resulting sequence revealed a 55 bp deletion from the original locus and a 9 bp insertion at 3' integration junction of the event. Overall, the characterization of the insert and border sequence of AAD-12 soybean event DAS-68416-4 indicated that a single, intact copy of the T-DNA was present in the soybean genome and the sequence information can be further utilized for event-specific tests.

INTRODUCTION

Transgenic AAD-12 soybean (*Glycine max* L.) event DAS-68416-4 (pDAB4468-416) was generated by *Agrobacterium*-mediated transformation using plasmid pDAB4468. The transgene (T-DNA) of plasmid pDAB4468 contains two expression cassettes, the *aad*-12 expression cassette and the *pat* expression cassette, arranged as tail-to-head, with RB7 (a matrix attachment region (MAR) element from *Nicotiana tabacuum*) at the 5' end to facilitate gene expression (Figure 1). The *aad*-12 gene is a synthetic gene from *Delftia acidovorans* that encodes an aryloxyalkanoate dioxygenase enzyme capable of deactivating several herbicides with an aryloxyalkanoate moiety, including phenoxy auxin (e.g., 2,4-D, MCPA) and pyridyloxy auxins (e.g., fluroxypyr, triclopyr*).* The *pat* gene is from *Streptomyces viridochromogenes* and the encoded PAT protein provides tolerance to the glufosinate-ammonium based herbicide. The *pat* gene was also used as a selectable marker in the process of transgenic soybean regeneration.

Southern analysis indicated that AAD-12 soybean event DAS-68416-4 contained an intact copy of the transgene including both the *aad*-12 and *pat* expression cassettes (Song et al, 2009). Cloning and sequence analysis of the transgene insertion and its flanking border regions allows to verify the DNA sequence and identify the transgene location in the soybean genome. The junction regions between the insert and its flanking border sequences can be utilized to develop event-specific identification method to detect the presence of AAD-12 soybean event DAS-68416-4. This report describes the cloning and characterization of the DNA sequence in the transgene insertion and its flanking border regions of AAD-12 soybean event DAS-68416-4.

MATERIALS AND METHODS

Test Substance

The test substance in this study was genomic DNA extracted from leaves of individual AAD-12 soybean event DAS-68416-4 plants in T4 or T5 generation. The source of the seeds is listed in Table 1.

Control Substance

The negative control substances used in this study were the genomic DNA isolated from leaves of the non-transgenic soybean line, Maverick and four other independent transgenic AAD-12 soybean lines. Those transgenic soybean lines included AAD-12 soybean events pDAB4468-309, pDAB4464-1104, pDAB4464-209, and pDAB4472-1606 (Table 1).

Reference Substance

E-Gel® Low Range Quantitative DNA Ladder (Invitrogen, Carlsbad, CA, catalog # 12373-031) and 1 Kb Plus DNA Ladder (Invitrogen, Carlsbad, CA, catalog # 10787-018) were used as reference substances to estimate the size of PCR amplified products.

Genomic DNA Extraction and Quantification

Genomic DNA was extracted from lyophilized or freshly ground leaf tissues using a modified CTAB method. Following genomic DNA extraction, DNA samples were dissolved in 1× TE (10mM Tris pH8.0, 1mM EDTA, Fluka catalog # 93283) and quantified with the Pico Green method according to manufacturer's instructions (Molecular Probes, Eugene, OR, catalog # P7581). For PCR analysis, DNA samples were diluted with molecular biology grade water (5 PRIME, Gaithersburg, MD, catalog # 2500020) to result in a concentration of 10-100 ng/µL.

PCR Primers

Table 1 in the Confidential Appendix lists the primer sequences that were used to clone the insert and flanking border regions of AAD-12 soybean event DAS-68416-4 and are marked in Figures 2, 3 and 5. Table 2 in the Confidential Appendix lists the primer sequences that were used to confirm the cloned border sequences. Primer positions are marked in Figure 4. All primers were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Primers were dissolved in water (5 PRIME, Gaithersburg, MD, catalog #2500020 or Sigma, St. Louis, MO, catalog # W4502) to a concentration of 100 μM as the stock solution.

Genome Walking

The GenomeWalker™ Universal Kit (Clontech Laboratories, Inc., Mountain View, CA, catalog #638904) was used to clone the 5' and 3' flanking border regions of AAD-12 soybean event DAS-68416-4 following manufacturer's instructions. Specifically, approximately 2 μg of genomic DNA from AAD-12 soybean event DAS-68416-4 was digested overnight with *Eco*R V and *Pvu* II (provided in the kit) (Figure 2). DNA digests were purified using the DNA Clean & Concentrator™-25 (ZYMO Research, Orange, CA, catalog # D4006) followed by ligation to GenomeWalker™ adaptors to construct GenomeWalker™ libraries. Each GenomeWalker™ library was used as DNA template for primary PCR amplification with adaptor primer AP1 provided in the kit and a construct-specific primer ES_LEnd03 or ES_PATEnd03 (Table 2 and 3). One microliter of 1:25 dilution of primary PCR reaction was then used as template for the secondary PCR amplification with the nested adaptor primer AP2 provided in the kit and a nested construct-specific primer ES_LEnd04 or ES_PATEnd04 (Table 2, 3 and Figure 2).

Conventional PCR

Standard PCR was used to clone the insert of AAD-12 soybean event DAS-68416-4 and confirm the border sequences, as well as, the insertion site in the host genome. TaKaRa LA Taq™ (Takara Bio Inc, Shiga, Japan, catalog# RR002M), HotStarTaq DNA Polymerase (Qiagen, Valencia, CA, catalog# 203207), High Fidelity PCR Kit (Roche Diagnostics, Inc, catalog#

11732641001), or the Easy-A High Fidelity Polymerase Kit (Stratagene, catalog# 600400) was used for conventional PCR amplification according to the manufacturer's recommended procedures. Specific PCR conditions and amplicon descriptions are listed in Table 2, 4 and 5.

PCR Product Detection and Purification

PCR products were inspected by electrophoresis with a 1.2 % or 2 % E-gel (Invitrogen, Carlsbad, CA, catalog # G5018) according to product instructions. Fragment size was estimated by comparison with the DNA markers. If necessary, PCR fragments were purified by excising the fragments from a 1% agarose gel in 1× TBE (89mM Tris-Borate, 2mM EDTA, pH 8.3) stained with ethidium bromide using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, catalog # 28706).

Sub-cloning of PCR Products

PCR fragments were sub-cloned into the pCR®4-TOPO® vector using TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, catalog # K4575-01) according to the product instructions. Specifically, two to five microliters of the TOPO® [c] cloning reaction was transformed to One Shot chemically competent TOP10 cells following manufacturer's instruction. Cloned fragments were verified by minipreparation of the plasmid DNA (QIAprep Spin Miniprep Kit, Qiagen, CA, catalog # 27106) followed by restriction digestion with *Eco*R I or by direct colony PCR using T3 and T7 primers. Plasmid DNA or glycerol stock of selected colonies was then sent for sequencing.

DNA Sequencing

After sub-cloning, putative target PCR products were sequenced initially to confirm that the expected DNA fragments had been cloned. Once confirmed, the colonies were chosen to complete double-strand full length sequencing by primer walking. All sequencing was performed by Cogenics (Houston, TX) with a Phred score of 30.

DNA Sequencing Analysis

Final assembly of insert and border sequences was done using Sequencher® software (Gene Codes Corporation, Ann Arbor, MI). Annotation of the insert and its flanking border sequences of AAD-12 soybean event DAS-68416-4 was done with Vector NTI (Version 10 and 11, Invitrogen, Carlsbad, CA).

Homology searching was performed in-house using the BLAST program against the GenBank non-redundant nucleotide database. Open reading frame (ORF) analysis using Vector NTI (Version 11, Invitrogen) was performed to identify ORFs (>= 150 aa) in the junction regions, flanking border sequences, and the original locus where the transgene was inserted.

RESULTS AND DISCUSSION

5' End Border Sequence

A DNA fragment was amplified from each AAD-12 soybean event DAS-68416-4 GenomeWalker™ library using the specific nested primer set for the 5' end of transgene. The fragment is ~1.8 kb from the *Eco*R V GenomeWalker™ library and ~ 3 kb from the *Pvu* II GenomeWalker™ library, respectively (Figure 6). These 2 fragments were cloned into pCR®4-TOPO® vector. Five colonies for each library were randomly picked for end sequencing at both strands. The colonies containing the sequences of both PCR primers were selected to obtain the full sequences by primer walking. Sequence analysis revealed that the clone amplified from AAD-12 soybean event DAS-68416-4 *Eco*R V GenomeWalker™ library contained a 1744 bp DNA fragment and the clone amplified from AAD-12 soybean event DAS-68416-4 *Pvu* II GenomeWalker™ library contained a 3047 bp DNA fragment with a single *Eco*R V site. Sequence analysis revealed that the DNA fragment obtained from the *Eco*R V GenomeWalker™ library overlapped with the DNA fragment obtained from the *Pvu* II GenomeWalker™ library clone at regions between primer ES_LEnd04 and the *Eco*R V site. These DNA fragments all contained the 5' end junction of T-DNA border B in the transgene, indicating that they were amplified from the same region of the 5' end transgene insert and its flanking border in AAD-12 soybean event DAS-68416-4. The resultant 2730 bp soybean genomic sequence was found to have no significant homologies with the sequences in GenBank.

3' End Border Sequence

A DNA fragment with size of about 1.3 kb was amplified from AAD-12 soybean event DAS-68416-4 *Eco*R V GenomeWalker™ library using the specific nested primer set for 3' end of transgene (Figure 6). The DNA fragment was then cloned into pCR®4-TOPO® vector. Five colonies were randomly picked for end sequencing at both strands. All five clones contained the sequences of both Primer AP2 and Primer ES_PATEnd04. Complete sequencing of those clones resulted in a 1359 bp consensus DNA fragment. Sequence analysis disclosed that the 1359-bp

fragment comprised of a 277 bp fragment from the 3' end region of T-DNA Border A and a 1082 bp fragment from soybean genomic DNA. BLAST search did not identify any significant homologies between this 1082 bp soybean DNA sequence and the sequences in GenBank.

T-DNA Insert and Flanking Border Sequence

The entire DNA insert and flanking border regions were cloned from AAD-12 soybean event DAS-68416-4 using conventional PCR. The 5' and 3' flanking border sequences and the expected transgene sequence were used to design the PCR primers listed in Table 5 and Confidential Appendix Table 1. In total, four overlapping DNA fragments (Amplicon 1 of 978 bp, Amplicon 2 of 2414 bp, Amplicon 3 of 1834 bp, and Amplicon 4 of 1705 bp) were cloned and sequenced (Figure 3). The whole insert and flanking border sequences were assembled based on overlapping sequence among the four fragments. Analysis of the final assembled sequence confirmed the presence of a 6400 bp fragment derived from the transgene of pDAB4468, and no base changes of the inserted DNA sequence were encountered when compared to the expected sequences from plasmid pDAB4468.

Confirmation of Soybean Genomic Sequences

To confirm the insertion site of AAD-12 soybean event DAS-68416-4 transgene in the soybean genome, PCR was carried out with different pairs of primers (Figure 4). Genomic DNA from AAD-12 soybean event DAS-68416-4 and other transgenic or non-transgenic soybean lines was used as templates. The *aad*-12 gene from *Delftia acidovorans* and the *pat* gene from *Streptomyces viridochromogenes* were target genes during event selection in previous generations. Thus, to confirm if the obtained 5' end border sequences are correct, two *aad*-12 specific primers, namely AIILEnd05 and AIILEnd06, and two primers designed according to the 5' end border sequence, designated 16LEndG01 and 16LEndG02, were used for amplifying the DNA segment that spans the *aad*-12 gene to 5' end border sequence. Similarly, for confirmation of the cloned 3' end border sequence, a *pat* specific primer, namely PAT-End06, and two primers designed according to the 3' end border sequence, designated 16PATG01 and 16PATG02, were used for amplifying DNA segment that spans the *pat* gene to 3' end border

sequence. As shown in Figure 7, DNA fragments with predicted sizes were amplified only from the genomic DNA of AAD-12 soybean event DAS-68416-4 with each primer pair, one primer located on the flanking border of AAD-12 soybean event DAS-68416-4 and one transgene specific primer, but not from DNA samples from other transgenic soybean lines or non-transgenic control. The results indicate that the cloned 5' and 3' border sequences are indeed the flanking border sequences of the T-DNA insert in AAD-12 soybean event DAS-68416-4.

Confirmation of the insertion site in the soybean genome was obtained by PCR amplification spanning the two soybean sequences, two primers designed according to the 5' end border sequence, namely 16LEndG03 and 16LEndG04, and two primers for the 3' end border sequence, namely 16PATG03 and 16PATG04, were used to amplify DNA segments that contain the entire transgene, the 5' end border sequence, and the 3' border sequence (Figure 8). As predicted, PCR amplification with the primer pair of 16LEndG03 and 16PATG03 (Figure 8. Gel A) amplified an approximately 9 kb DNA fragment from the genomic DNA of AAD-12 soybean event DAS-68416-4 and a 2.7 kb DNA fragment from the non-transgenic soybean controls and other soybean transgenic lines. Similarly, PCR reaction with the primer pair of 16LEndG04 and 16PATG04 (Figure 8. Gel B) produced an approximately 9 kb DNA fragment from the sample of AAD-12 soybean event DAS-68416-4 and a 2.8 kb DNA fragment from all the other soybean control lines, correspondingly. It is noted that a faint band with size of about 6 kb was visible in all the soybean samples except AAD-12 soybean event DAS-68416-4 when both primer pairs were used for PCR, suggesting that this faint band resulted from nonspecific amplification in soybean genome with this pair of primers, particularly in the region where the transgene of AAD-12 soybean event DAS-68416-4 was integrated.

The 2.7 kb and 2.8 kb amplified DNA fragments, using the primer pair of 16LEndG03 and 16PATG03 or the primer pair of 16LEndG04 and 16PATG04, from non-transgenic soybean line Maverick were cloned and sequenced. Their sequences were matched with each other and aligned with the cloned 5' and 3' border sequences from AAD-12 soybean event DAS-68416-4. This demonstrated that the cloned DNA sequence contained the locus where the AAD-12 soybean event DAS-68416-4 was integrated. Alignment analysis also revealed a 55 bp deletion

from the original locus and a 9 bp insertion at 3' integration junction (Figure 5 and in the Confidential Appendix, Figure 2). No open reading frames (>/=450 bp, 150 aa) were identified in the soybean genomic region of the original locus that was cloned.

Based on the sequence in the 5'-integration junction of AAD-12 soybean event DAS-68416-4, PCR primers Soy416-R and Soy416-F were designed to specifically amplify this insert-to-genome segment. As expected, the desired DNA fragment was only generated in the genomic DNA from AAD-12 soybean event DAS-68416-4 but not from any other transgenic or non-transgenic soybean lines tested (Figure 9). Therefore, this primer pair can be used as an event-specific identifier for AAD-12 soybean event DAS-68416-4.

CONCLUSIONS

In conclusion, 10212 bp of insert and flanking border sequences in AAD-12 soybean event DAS-68416-4 were cloned and sequenced (Confidential Appendix Figure 1). The insert contains a single intact T-DNA transgene from pDAB4468. PCR analysis confirmed that the cloned 5' and 3' flanking border sequences were indeed from the soybean genome. The locus where AAD-12 soybean event DAS-68416-4 was integrated was also described. Based on sequences near the 5' insert junction regions, a PCR amplicon specific to AAD-12 soybean event DAS-68416-4 was generated, which confirms that the junction regions between the insert and its flanking borders can be used for development of event-specific PCR detection.

REFERENCES

1. Song, P.; Cruse, J.; Thomas, A. (2009). Molecular Characterization of AAD-12 Soybean Event DAS-68416-4. Dow AgroSciences unpublished report 081087.

Table 1. Test and Control Substances Used in the Study

Genomic DNA	Description	Source ID
AAD-12 Soybean Event DAS-68416-4 (pDAB4468-416)	Transgenic AAD-12 Soybean	Sequencing - YX08CX010827.001 Genome Walking - YX07KX000191.039
Maverick	Non Transgenic Soybean	SGN080003-61-0001
AAD-12 Soybean Event pDAB4468-309	Transgenic AAD-12 Soybean	YX07KX000100.001
AAD-12 Soybean Event pDAB4464-1104	Transgenic AAD-12 Soybean	YX07KX000065.021
AAD-12 Soybean Event pDAB4464-209	Transgenic AAD-12 Soybean	YX07KX000310.009
AAD-12 Soybean Event pDAB4472-1606	Transgenic AAD-12 Soybean	YX07KX000515.017

Table 2. PCR Mixture for Standard PCR Amplification of the Border Regions and Event-specific Sequences in AAD-12 Soybean Event DAS-68416-4

PCR Mixture A		**PCR Mixture B**		**PCR Mixture C**	
Reagent	1 x reaction (µL)	Reagent	1 x reaction (µL)	PCR Mix	1 x reaction (µL)
H20	29	H20	30.5	H2O	31
10X PCR buffer II (Mg-plus)	5	10X PCR buffer II (Mg-plus)	5	10xQIA buffer	5
$MgCl_2$[25mM]	1.5	$MgCl_2$[25mM]	0	$MgCl_2$	1.5
dNTP[2.5mM]	8	dNTP[2.5mM]	8	dNTP[2.5mM]	8
primer1 (10µM)	1	primer1 (10µM)	1	primer1 (10µM)	1
primer2 (10µM)	1	primer2 (10µM)	1	primer2 (10µM)	1
DNA[10ng/uL]	4	DNA[10ng/uL]	4	DNA[10ng/uL]	4
LA Taq (5U/ul)	0.5	LA Taq (5U/ul)	0.5	QIA Hstaq(5U/ul)	0.5
rxn vol:	50	rxn vol:	50	rxn vol:	50
PCR Mixture D		**PCR Mixture E**		**PCR Mixture F**	
Reagent	1 x reaction (µL)	PCR Mix	1 x reaction (µL)	Reagent	1 x reaction (µL)
H20	40.25	H2O	22	H20	32
10X PCR buffer II (Mg-plus)	5	Easy-A 2x Master Mix	25	10X PCR buffer II (Mg-plus)	5
$MgCl_2$	0	$MgCl_2$	0	$MgCl_2$[25mM]	1.5
dNTP[10mM]	1	dNTP[2.5mM]	0	dNTP[2.5mM]	8
primer1 (100µM)	1	primer1 (100µM)	1	primer1 (10µM)	1
primer2 (100µM)	1	primer2 (100µM)	1	primer2 (10µM)	1
DNA[100ng/uL]	1	DNA[10ng/uL]	1	DNA Template	1
Expand High Fidelity Taq (5U/ul)	0.75	rxn vol:	50	LA Taq (5U/ul)	0.5
rxn vol:	50			rxn vol:	50

Table 3. Conditions for Genome Walking of AAD-12 Soybean Event DAS-68416-4 to Amplify the Flanking Border Regions

Target Sequence	**Primer Set**	**PCR Mixture**	**Pre-denature (°C/min)**	**Denature (°C/sec.)**	**Anneal (°C/sec.)**	**Extension (°C /min:sec)**	**Denature (°C /sec.)**	**Anneal (°C /sec.)**	**Extension (°C /min:sec)**	**Final Extension (°C /min)**
5' border	ES_LEnd03 / AP1	F	95/3	95/30	68-0.5/cycle→64 /30	68/10:00	95/30	64/30	68/10:00	72/10
				8 cycles			22 cycles			
5' border (nested)	ES_LEnd04 / AP2	F	95/3	95/30	68-0.5/cycle→64 /30	68/10:00	95/30	64/30	68/10:00	72/10
				8 cycles			24 cycles			
3' border	ES_PATEnd03 / AP1	F	95/3	95/30	68-0.5/cycle→64 /30	68/10:00	95/30	64/30	68/10:00	72/10
				8 cycles			22 cycles			
3'border (nested)	ES_PATEnd04 / AP2	F	95/3	95/30	68-0.5/cycle→64 /30	68/10:00	95/30	64/30	68/10:00	72/10
				8 cycles			24 cycles			

Table 4. Conditions for Standard PCR Amplification of the Border Regions and Event-specific Sequences in AAD-12 Soybean Event DAS-68416-4

Target Sequence	Primer Set	PCR Mixture	Pre-denature (°C/min)	Denature (°C/sec.)	Anneal (°C/sec.)	Extension (°C/min:sec)	Final Extension (°C/min)
5' border	16LEndG01 / AllLEnd05	B	95/3	95/30	60/30	68/5:00	72/10
				35 cycles			
5' border	16LEndG02 / AllLEnd06	B	95/3	95/30	60/30	68/5:00	72/10
				35 cycles			
specific sequence in 5' end insert junction	Soy416-F / Soy416-R	C	95/15	94/30	60/30	72/1:00	72/10
				35 cycles			
3'border	16PATG01 / PATEnd06	B	95/3	95/30	60/30	68/5:00	72/10
				35 cycles			
3' border	16PATG02 / PATEnd06	B	95/3	95/30	60/30	68/5:00	72/10
				35 cycles			
Across the insert locus	16LEndG03 / 16PATG03	A	95/3	95/30	60/30	68/5:00	72/10
				35 cycles			
Across the insert locus	16LEndG04 / 16PATG04	A	95/3	95/30	60/30	68/5:00	72/10
				35 cycles			

Table 5. Primer Description for Amplicons 1-4 for T-DNA Insert

Target Sequence	Primer Set	PCR Mixture	Pre-denature (°C/min)	Denature (°C/sec.)	Anneal (°C/sec.)	Extension (°C/min:sec)	Final Extension (°C/min)
5' Genomic DNA/ DNA insert (978bp)	416-5-1 / 4468-1R	D	95/2	94/60	55/60	72/2:00	72/15
				35 cycles			
DNA insert (2414bp)	4468-1 / 4468-2R	E	95/2	94/60	55/60	72/2:00	72/15
				35 cycles			
DNA insert (1834bp)	4468-2 / 4468-3R	E	95/2	94/60	55/60	72/2:00	72/15
				35 cycles			
DNA insert/ 3' Genomic DNA (1705bp)	4468-3 / 416-3-1R	E	95/2	94/60	55/60	72/2:00	72/15
				35 cycles			
DNA Insertion Site (~470bp)	416-5-1 / 416-3-1R	E	95/2	94/60	55/60	72/1:30	72/15
				35 cycles			

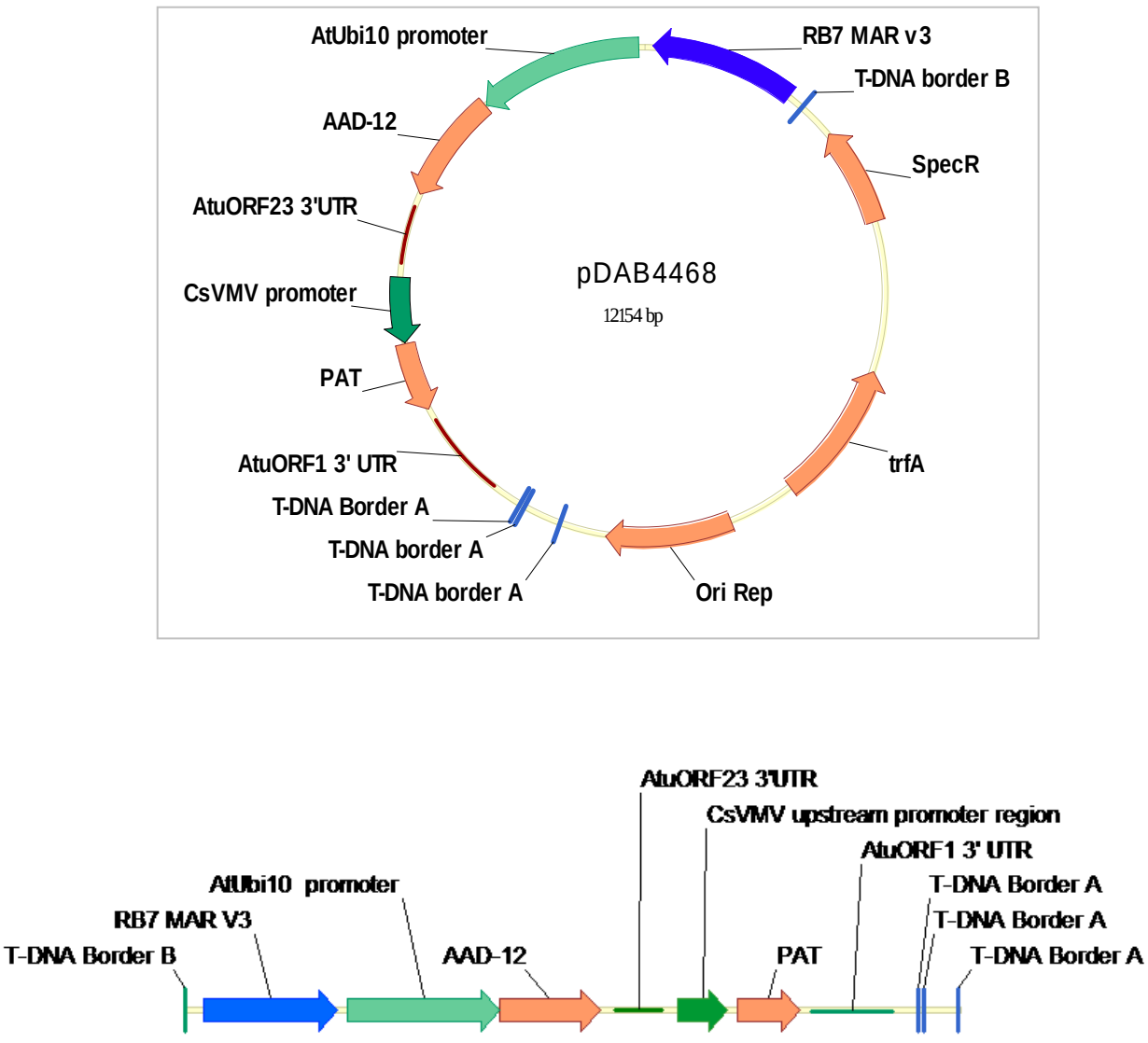


Figure 1. Plasmid Map of pDAB4468 containing the *aad*-12 and *pat* Expression Cassettes

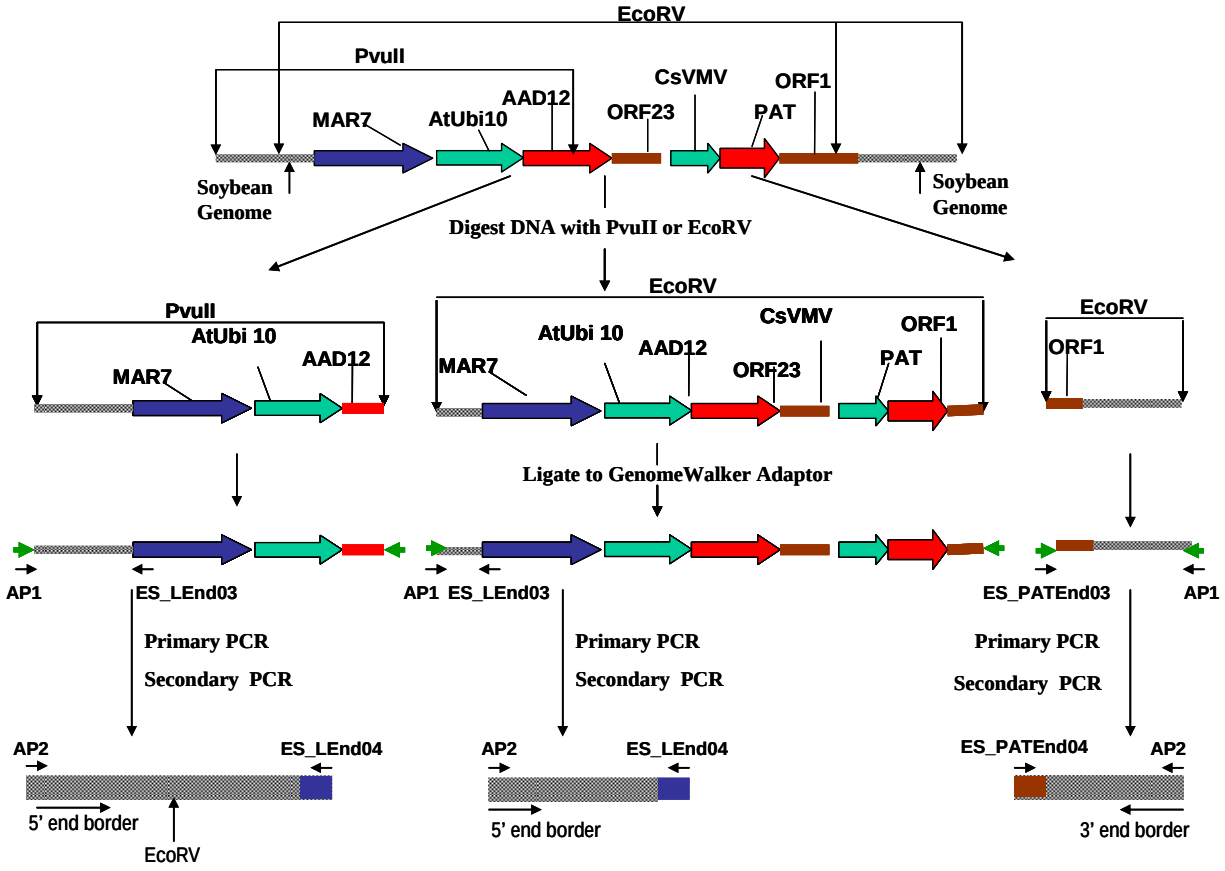


Figure 2. Diagram of Cloning the Flanking Border Sequences from AAD-12 Soybean Event DAS-68416-4

Genomic DNA of the AAD-12 soybean event DAS-68416-4 was digested with *Eco*RV, or *Pvu* II and used to generate corresponding GenomeWalker™ libraries, which were used as templates to amplify the target DNA sequences.

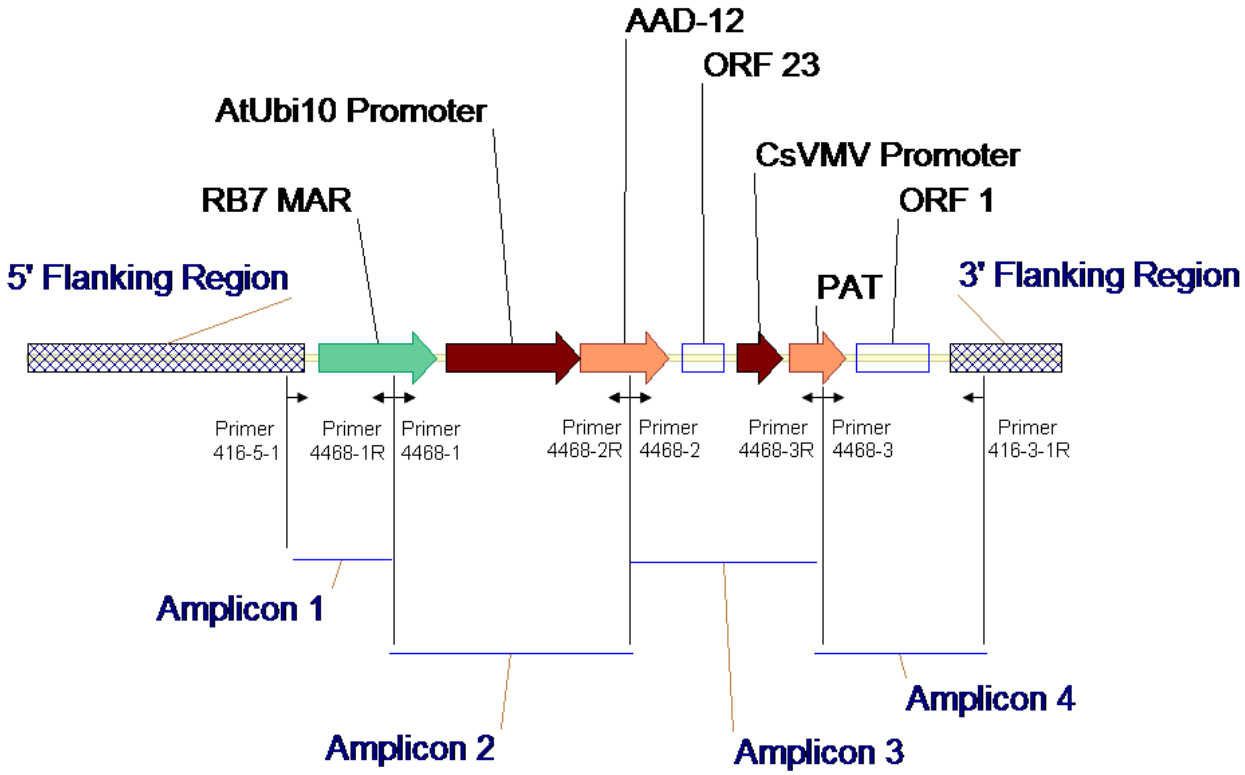


Figure 3. Diagram of Cloning the Flanking Border Sequences from the Insert in AAD-12 Soybean Event DAS-68416-4

The schematic diagram depicts the primer locations and cloning strategy for full length sequencing of the AAD-12 soybean event DAS-68416-4 from 5' to 3' borders.

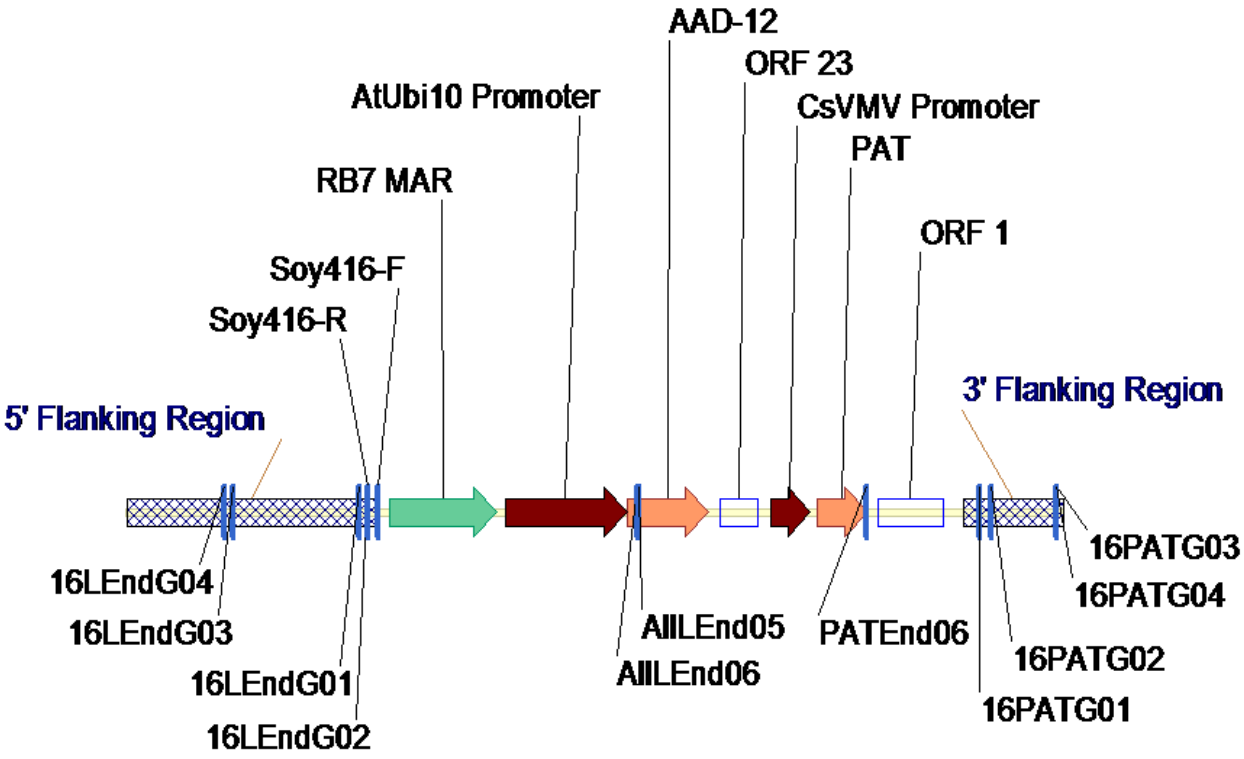


Figure 4. The Complete Insert and Flanking Border regions of AAD-12 Soybean Event DAS-68416-4

The schematic diagram depicts the primer locations for confirming the full length sequence of the AAD-12 soybean event DAS-68416-4 from 5' to 3' borders.

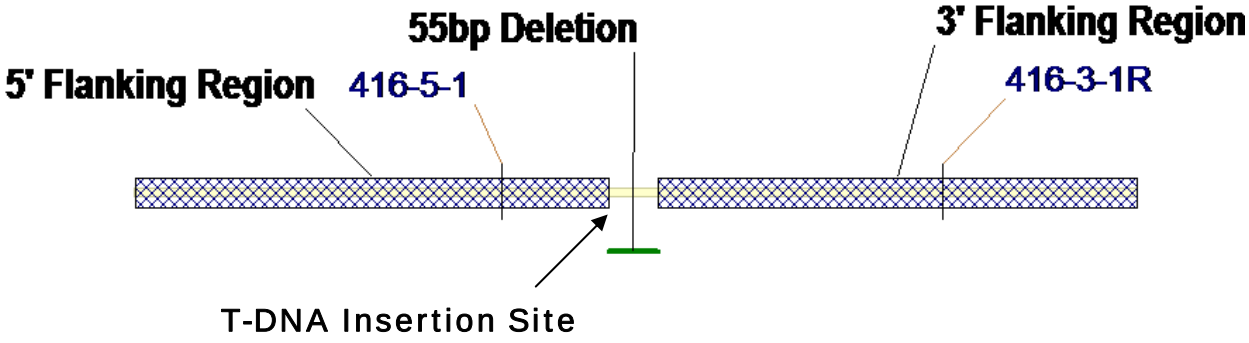
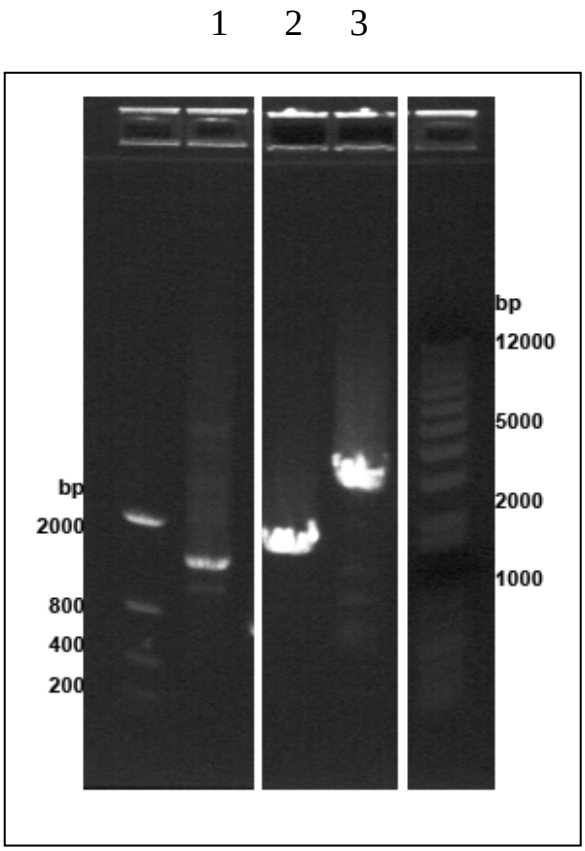


Figure 5. Insertion Site Characterization of the Parental Maverick line used to Generate the AAD-12 Soybean Event DAS-68416-4

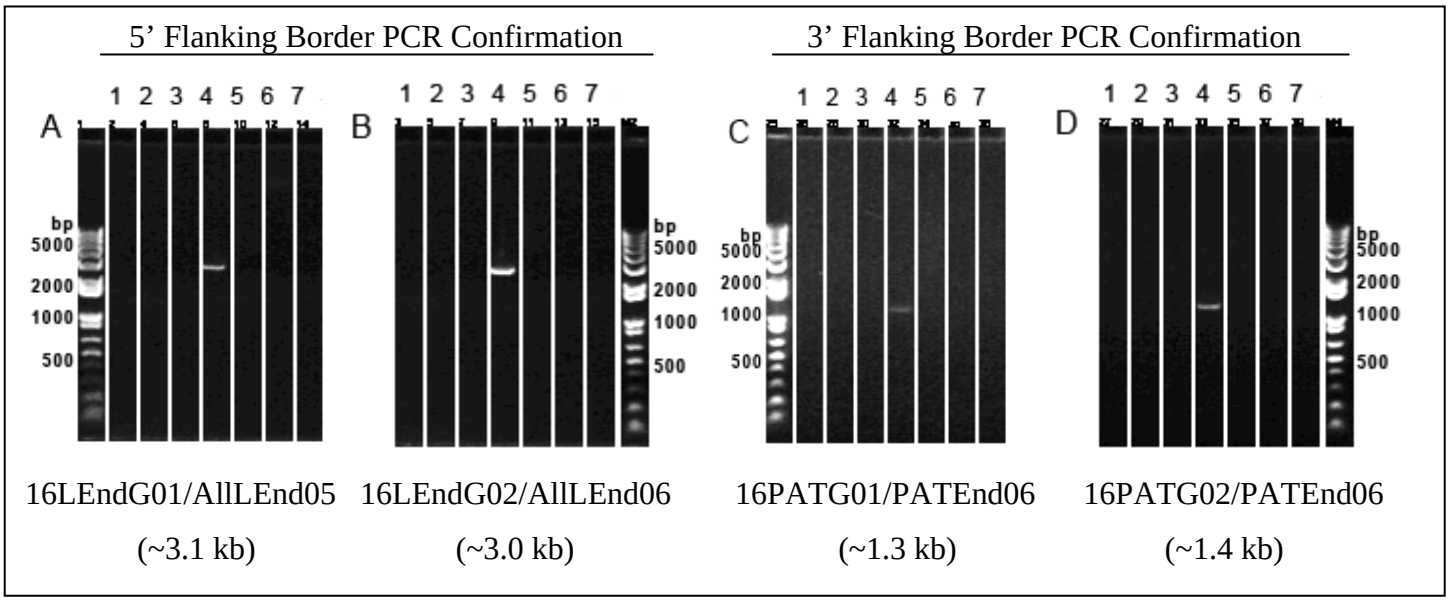
The schematic diagram depicts the primer locations for confirming the insertion site sequence of the AAD-12 soybean event DAS-68416-4.



Lane	Sample ID
1	3' End Secondary PCR *Eco*R V GenomeWalker library
2	5' End Secondary PCR *Eco*R V GenomeWalker library
3	5' End Secondary PCR *Pvu* II GenomeWalker library

Figure 6. PCR Amplified bands of the 5' and 3' end Border Sequence from the *Eco*R V and *Pvu* II Genome Walking Libraries for AAD-12 Soybean Event DAS-68416-4

DNA fragments were amplified from each AAD-12 soybean event DAS-68416-4 GenomeWalker™ library using the specific nested primer set for the 5' and 3'end of the transgene. The fragment is ~1.8 kb from *Eco*R V GenomeWalker™ library and ~ 3 kb from *Pvu* II GenomeWalker™ library, respectively for the 5' end and ~1.3 kb for the 3' end from *Eco*R V GenomeWalker™ library. These fragments were cloned into pCR®4-TOPO® vector and used to determine the border sequences.

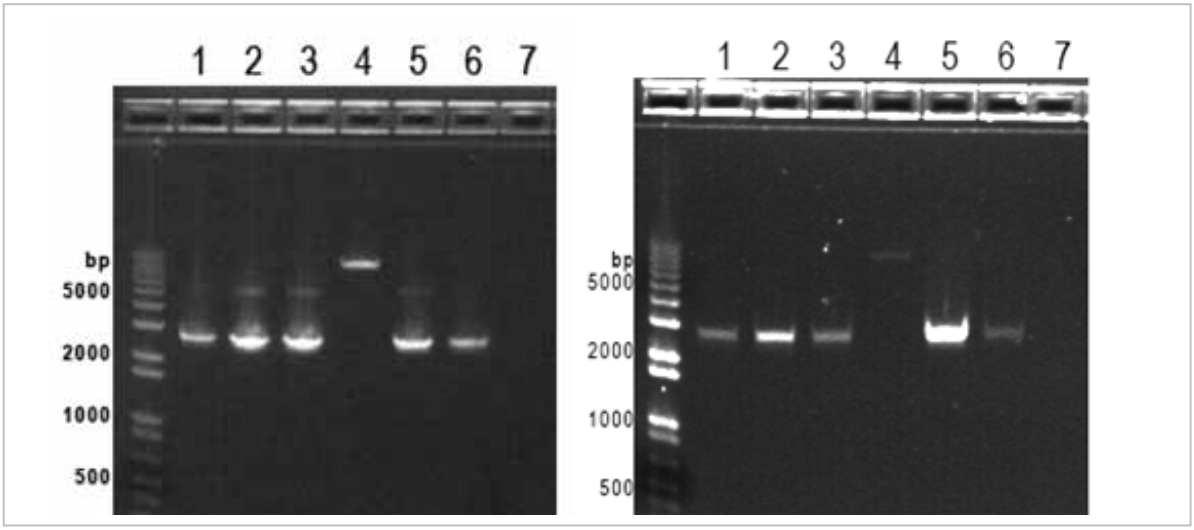


Lane	Sample ID
1	Soybean Event pDAB4464-1104
2	Soybean Event pDAB4464-0209
3	Soybean Event pDAB4468-0309
4	Soybean Event DAS-68416-4
5	Soybean Event pDAB4472-1606
6	Soybean non-transgenic line Maverick
7	Water

Figure 7. PCR Confirmation of 5' end and 3' end Border Sequence of AAD-12 Soybean Event DAS-68416-4

Specific primer pairs were used to confirm the Genome Walking-derived gDNA border regions for AAD-12 soybean event DAS-68416-4. Two primer pairs generated the predicted-sized bands for both the 5' flanking regions (A, B), as well as, the 3' flanking region (C, D) indicating that the amplified DNA is specific to AAD-12 soybean event DAS-68416-4.

A. 16LEndG03/16PATG03 B. 16LEndG04/16PATG04



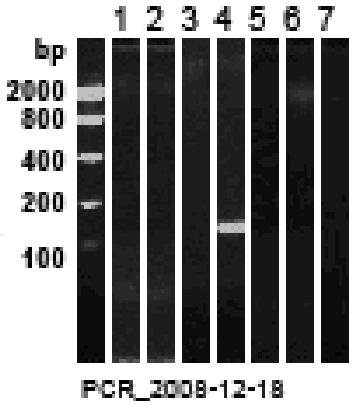
Lane	Sample ID
1	Soybean Event pDAB4464-1104
2	Soybean Event pDAB4464-0209
3	Soybean Event pDAB4468-0309
4	Soybean Event DAS-68416-4
5	Soybean Event pDAB4472-1606
6	Soybean non-transgenic line Maverick
7	Water

Figure 8. PCR Amplification of the Insertion Locus of AAD-12 Soybean Event DAS-68416-4 Transgene

A. Primers 16LEndG03/16PATG03 were used to amplify the entire transgene DNA region in an event-specific manner. The 16LEndG03 primer is located in the 5' flanking region upstream of the DNA insert while 16PATG03 is located in the 3' flanking region downstream of the DNA insert. Standard PCR was used to amplify an approximately 9 kb band in only the soybean event pDAB4468-0416 gDNA (Lane 4), while a 2.7 kb band amplifies, as expected, in the other event/control lanes. B. Similarly, primers 16LEndG04/16PATG04 were used to amplify the entire transgene DNA region in an event-specific manner. The 16LEndG04 primer is located in the 5' flanking region upstream of the DNA insert while 16PATG04 is located in the 3' flanking region downstream of the DNA insert. Standard PCR was used to amplify an approximately 9 kb band in only the soybean event pDAB4468-0416 gDNA (Lane 4), while a 2.8 kb band amplifies, as expected, in the other event/control lanes.

Soy416-F/Soy416-R

(128 bp)



Lane	Sample ID
1	Soybean Event pDAB4464-1104
2	Soybean Event pDAB4464-0209
3	Soybean Event pDAB4468-0309
4	Soybean Event DAS-68416-4
5	Soybean Event pDAB4472-1606
6	Water
7	Soybean non-transgenic line Maverick

Figure 9. PCR Amplification of AAD-12 Soybean Event DAS-68416-4

Event-specific PCR was carried out using the primer pair Soy416-F/Soy416-R indicated above the Figure. Primers were designed using the sequence available from the genome walking experiments and demonstrate an event-specific PCR band amplified at the predicted size. No band was amplified in the transgenic control soybean gDNA or in the non-transgenic control line Maverick.