## SUMMARY

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## STUDY TITLE

Cloning and Characterization of DNA Sequence in the Insert and the Flanking Border Regions of AAD-1 Corn Event DAS-40278-9

## DATA REQUIREMENTS

Not Applicable

## AUTHOR(S)

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## STUDY COMPLETED ON

05 June, 2009

## PERFORMING LABORATORY

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## LABORATORY STUDY ID

091023

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## SUMMARY

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To characterize the inserted DNA and describe the genomic insertion site, DNA sequences of the insert and the border regions of AAD-1 event DAS-40278-9 were determined. In total, 8557 bp of event DAS-40278-9 genomic sequence were confirmed, comprising 1873 bp of 5' flanking border sequence, 1868 bp of 3' flanking border sequence, and 4816 bp of DNA insert. The 4816 bp DNA insert contains an intact *aad*-1 expression cassette, a 249 bp partial MAR v3 on the 5' terminus, and a 1096 bp partial MAR v4 on the 3' terminus. Sequence analysis revealed a 21 bp insertion at 5'-integration junction and a two base pair deletion from the insertion locus of the corn genome. A one base pair insertion was found at 3'-integration junction between the corn genome and the DAS-40278-9 insert. Also, a single base change (T to C) was found in the insert at position 5212 in the non-coding region of the 3' UTR. None of these changes affect the open reading frame composition of the *aad*-1 expression cassette.

PCR amplification based on the AAD-1 corn event DAS-40278-9 insert and border sequences confirmed that the border regions were of corn origin and that the junction regions could be used for event-specific identification of DAS-40278-9. Analysis of the sequence spanning the

junction regions indicated that no novel open reading frames (ORF>= 200 codons) were resulted from the DNA insertion in event DAS-40278-9 and also no genomic open reading frames were interrupted by the DAS-40278-9 integration in the native corn genome. Overall, characterization of the insert and border sequences of the AAD-1 corn event DAS-40278-9 indicated that a single intact copy of the *aad*-1 expression cassette was integrated into the native corn genome.

## STUDY TITLE

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Not Applicable

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# STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Aryloxyalkanoate Dioxygenase-1 (AAD-1)

Title: Cloning and Characterization of DNA Sequence in the Insert and the Flanking Border Regions of AAD-1 Corn Event DAS-40278-9

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

Company: Dow AgroSciences LLC

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Signature: Laura Tagliani

Date: 27-May-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 DNA Sequence in the Insert and the Flanking Border Regions of AAD-1 Corn Event DAS-40278-9

Study Initiation Date: 11/03/2008

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

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

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

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

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## QUALITY ASSURANCE STATEMENT

Compound: Aryloxyalkanoate Dioxygenase-1

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# NON-GLP STUDY

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Principal Analyst: Jing Mo

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Cloning and Characterization of DNA Sequence in the Insert and the Flanking Border Regions of AAD-1 Corn Event DAS-40278-9

## ABSTRACT

Corn has been modified by the insertion of the *aad*-1 gene from *Sphingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dicholrophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (e.g. haloxyfop, cyhalofop, quizalofop) herbicides and is also used as a selectable marker during plant transformation. Whiskers transformation of corn with a DNA fragment released by *Esp* I from plasmid pDAS1740 (also known as pDAB3812) was carried forward, through breeding, to produce AAD-1 corn event DAS-40278-9 (pDAS1740-278) which was the focus of this study. Southern analysis indicated that AAD-1 corn event DAS-40278-9 contains one intact copy of the *aad*-1 expression cassette.

To characterize the inserted DNA and describe the genomic insertion site, DNA sequences of the insert and the border regions of AAD-1 event DAS-40278-9 were determined. In total, 8557 bp of event DAS-40278-9 genomic sequence were confirmed, comprising 1873 bp of 5' flanking border sequence, 1868 bp of 3' flanking border sequence, and 4816 bp of DNA insert. The 4816 bp DNA insert contains an intact *aad*-1 expression cassette, a 249 bp partial MAR v3 on the 5' terminus, and a 1096 bp partial MAR v4 on the 3' terminus. Sequence analysis revealed a 21 bp insertion at 5'-integration junction and a two base pair deletion from the insertion locus of the corn genome. A one base pair insertion was found at 3'-integration junction between the corn genome and the DAS-40278-9 insert. Also, a single base change (T to C) was found in the insert at position 5212 in the non-coding region of the 3' UTR. None of these changes affect the open reading frame composition of the *aad*-1 expression cassette.

PCR amplification based on the AAD-1 corn event DAS-40278-9 insert and border sequences confirmed that the border regions were of corn origin and that the junction regions could be used for event-specific identification of DAS-40278-9. Analysis of the sequence spanning the junction regions indicated that no novel open reading frames (ORF>= 200 codons) were resulted

from the DNA insertion in event DAS-40278-9 and also no genomic open reading frames were interrupted by the DAS-40278-9 integration in the native corn genome. Overall, characterization of the insert and border sequences of the AAD-1 corn event DAS-40278-9 indicated that a single intact copy of the *aad*-1 expression cassette was integrated into the native corn genome.

# INTRODUCTION

Corn has been modified by the insertion of the *aad*-1 gene from *Sphingobium herbicidovorans* which encodes the aryloxyalkanoate dioxygenase (AAD-1) protein. The resultant trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides and was also used as a selectable marker during plant transformation and in breeding nurseries. Whiskers transformation of corn with the *Fsp* I fragment of plasmid pDAS1740 (also known as pDAB3812) was carried forward, through breeding, to produce AAD-1 corn event DAS-40278-9, which is the focus of this study.

Southern analysis indicated that the AAD-1 corn event DAS-40278-9 contained one intact copy of the *aad*-1 expression cassette (Zhuang *et al.*, 2009). Cloning and sequence analysis of the transgene insertion and the corresponding flanking border regions allows verification of the DNA sequence and identification of the transgene location in maize genome. The border sequence can be utilized to develop an event specific assay to detect the presence of AAD-1 corn event DAS-40278-9. This report describes the cloning and characterization of the DNA sequence in the transgene insertion and the flanking border regions of AAD-1 corn event DAS-40278-9.

# LABORATORY PHASE

## Test Substance

The test substance was leaf genomic DNA extracted from AAD-1 corn event DAS-40278-9 plants. Individual plants of BC3S2 and BC3S3 (Table 1) were used in the study. The corn HiII derived from corn line A188 and corn line B73 was used for transformation. Transgenic HiII containing event DAS-40278-9 was crossed to corn line XHH13 and then subsequently backcrossed to XHH13 to generate the BC3S2 and BC3S3 generations used in the study.

## Control Substance

The control substance was the genomic DNA isolated from leaves of individual transgenic or non-transgenic corn lines listed in Table 1.

## Reference Substance

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

## Genomic DNA Extraction and Quantification

Genomic DNA was extracted from lyophilized or freshly ground leaf tissues using a modified CTAB method. DNA samples were dissolved in 1× TE (10mM Tris pH8.0, 1mM EDTA, Fluka, cat. # 93283) and quantified with the Pico Green method according to manufacturer's instructions (Molecular Probes, Eugene, OR, cat. # P7581). For PCR analysis, DNA samples were diluted with molecular biology grade water (5 PRIME, Gaithersburg, MD, cat. # 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 DNA insert and the flanking border regions of AAD-1 corn event DAS-40278-9, with positions and descriptions marked in Figure 3. Table 2 in the Confidential Appendix lists the primer sequences that were used to confirm the insert and border sequences. The primer positions are marked in Figure 3 and 4, respectively. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Primers were dissolved in water (5 PRIME, Gaithersburg, MD, cat. # 2500020) to a concentration of 100 µM for the stock solution and diluted with water to a concentration of 10 µM for the working solution.

Genome Walking

The GenomeWalker™ Universal Kit (Clontech Laboratories, Inc., Mountain View, CA, cat. # 638904) was used to clone the 5' and 3' flanking border sequences of AAD-1 corn event DAS-40278-9. According to the manufacturer's instruction, about 2.5 µg of genomic DNA from AAD-1 corn event DAS-40278-9 was digested overnight with *Eco*R V, *Stu* I (both provided by the kit) or *Sca* I (New England Biolabs, Ipswich, MA, cat. # R0122) (Figure 2). Digested DNA was purified using the DNA Clean & Concentrator™-25 (ZYMO Research,Orange, CA, cat. # D4006) followed by ligation to GenomeWalker™ adaptors to construct GenomeWalker™ libraries. Each GenomeWalker™ library was used as DNA template for primary PCR amplification with the adaptor primer AP1, provided in the kit, and each construct-specific primer 5End3812_A and 3End3812_C. One microliter of 1:25 dilution of primary PCR reaction was then used as template for secondary PCR amplification with the nested adaptor primer AP2 and each nested construct-specific primer 5End3812_B and 3End3812_D (Figure 2). TaKaRa LA Taq™ HS (Takara Bio Inc., Shiga, Japan, cat. # RR042A) was used in the PCR amplification. In a 50 µL PCR reaction, 1 µL of DNA template, 8 µL of 2.5 mM of dNTP mix, 0.2 µM of each primer, 2.5 units of TaKaRa LA Taq™ HS DNA Polymerase, 5 µl of 10 × LA

PCR Buffer II (Mg2+ plus), and 1.5 µL of 25 mM $MgCl_2$ were used. Specific PCR conditions are listed in Table 2.

Conventional PCR

Standard PCR was used to clone and confirm the DNA insert and border sequence in the AAD-1 corn event DAS-40278-9. TaKaRa LA Taq™ (Takara Bio Inc., Shiga, Japan, cat. # RR002M), HotStarTaq DNA Polymerase (Qiagen, Valencia, CA, cat. # 203207), Expand High Fidelity PCR System (Roche Diagnostics, Inc., cat. # 11732641001), or the Easy-A® High-Fidelity PCR Cloning Enzyme & Master Mix (Stratagene, CA, cat. # 600400) was used for conventional PCR amplification according to the manufacturer's recommended procedures. Specific PCR conditions and amplicon descriptions are listed in Table 3.

PCR Product Detection and Purification

PCR products were inspected by electrophoresis using 1.2 % or 2 % E-gel (Invitrogen, Carlsbad, CA, cat. # G402002) according to the product instruction. Fragment size was estimated by comparison with the DNA markers. If necessary, PCR fragments were purified by excising the fragments from 1% agarose gel in 1× TBE stained with ethidium bromide, using the QIAquick Gel Extraction Kit (Qiagen, cat. # 28704).

Sub-cloning of PCR Products

PCR fragments were sub-cloned into the pCR®4-TOPO® using TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, cat. # K4575-01) according to the product instruction. Specifically, two to five microliters of the TOPO® cloning reaction was transformed into the One Shot chemically competent TOP10 cells following the manufacturer's instruction. Cloned fragments were verified by minipreparation of the plasmid DNA (QIAprep Spin Miniprep Kit, Qiagen, CA, cat. # 27106) followed by restriction digestion with *Eco*R I or by direct colony PCR

using T3 and T7 primers, provided in the kit. Plasmid DNA or glycerol stocks of the selected colonies were then sent for sequencing.

DNA Sequencing

After sub-cloning, the putative target PCR products were sequenced initially to confirm that the expected DNA fragments had been cloned. The colonies containing appropriate DNA sequences were selected for primer walking to determine the complete DNA sequences. Sequencing was performed by Cogenics (Houston, TX).

DNA Sequencing Analysis

Final assembly of insert and border sequences was completed using Sequencher software (Version 4.8 Gene Codes Corporation, Ann Arbor, MI). Annotation of the insert and border sequences of AAD-1 corn event DAS-40278-9 was performed using the Vector NTI (Version 10 and 11, Invitrogen, Carlsbad, CA).

Homology searching was done using the BLAST program against the GenBank database. Open reading frame (ORF) analysis using Vector NTI (Version 11, Invitrogen) was performed to identify ORFs (>= 200 codons) in the full insert and flanking border sequences.

# RESULTS AND DISCUSSION

## 5' End Border Sequence

A DNA fragment was amplified from each AAD-1 corn event DAS-40278-9 GenomeWalker™ library using the specific nested primer set for 5' end of the transgene. An approximately 800 bp PCR product was observed from both the AAD-1 corn event DAS-40278-9 *Eco*R V and *Stu* I GenomeWalker™ libraries. The *Sca* I GenomeWalker™ library generated a product around 2 kb. The fragments were cloned into pCR®4-TOPO® and six colonies from each library were randomly picked for end sequencing to confirm the insert contained the expected sequences. Complete sequencing by primer walking of the inserts revealed that the fragments amplified from AAD-1 corn event DAS-40278-9 *Stu* I, *Eco*R V, and *Sca* I GenomeWalker™ libraries were 793, 822, and 2132 bp, respectively. The DNA fragments generated from the *Stu* I and *Eco*R V GenomeWalker™ libraries were a 100% match to the DNA fragment generated from *Sca* I GenomeWalker™ library, suggesting that these DNA fragments were amplified from the 5' region of the transgene insert. BLAST search of the resultant 1873 bp corn genomic sequence indicated a high similarity to the sequence of a corn BAC clone. Moreover, sequence analysis of the insertion junction indicated that 917 bp of the MAR v3 at its 5' end region was truncated compared to the plasmid pDAS1740/*Fsp* I fragment, leaving a 249 bp partial MAR v3 at the 5' region of the *aad*-1 expression cassette.

## 3' End Border Sequence

A DNA fragment with size of approximately 3 kb was amplified from AAD-1 corn event DAS-40278-9 *Stu* I GenomeWalker™ library using the specific nested primer set for the 3' end of the transgene. The DNA fragment was cloned into pCR®4-TOPO® and ten colonies were randomly picked for end sequencing to confirm the insertion of the expected sequences. Three clones with the expected inserts were completely sequenced, generating a 2997 bp DNA fragment. Sequence analysis of this DNA fragment revealed a partial MAR v4 element (missing 70 bp of its 5' region) and 1867 bp corn genomic sequence. BLAST search showed the 1867 bp

genomic DNA sequence was a 100% match to sequence in the same corn BAC clone as was identified with the 5' border sequence.

DNA Insert and Junction Sequence

The DNA insert and the junction regions were cloned from AAD-1 corn event DAS-40278-9 using PCR based methods as previously described. Five pairs of primers were designed based on the 5' and 3' flanking border sequences and the expected transgene sequence. In total, five overlapping DNA fragments (Amplicon 1 of 1767 bp, Amplicon 2 of 1703 bp, Amplicon 3 of 1700 bp, Amplicon 4 of 1984 bp, and Amplicon 5 of 1484 bp) were cloned and sequenced (Figure 3). The whole insert and flanking border sequences were assembled based on overlapping sequence among the five fragments. The final sequence confirms the presence of 4816 bp of the DNA insert derived from pDAS1740/*Fsp* I, 1873 bp of the 5' flanking border sequence, and 1868 bp of 3' flanking border sequence. The 4816 bp DNA insert contains an intact *aad*-1 expression cassette, a 249 bp partial MAR v3 on the 5' terminus, and a 1096 bp partial MAR v4 on the 3' terminus (Confidential Appendix, Figure 1).

MARs are natural and abundant regions found in genomic DNA. When positioned on the flanking ends of gene cassettes, MARs are thought to buffer transgenes from the influence of surrounding chromosomal sequences, and have been shown to increase transgene expression and reduce the incidence of gene silencing (Abranches *et al.*, 2005; Han *et al.*, 1997; Allen *et al.*, 2000). In contrast, some studies suggest that MARs have small and variable effects on transgene expression (Li *et al.*, 2008; Mankin *et al.*, 2003). MARs were included in the *aad*-1 expression cassette in the pDAS1740/*Fsp* I fragment to potentially increase the consistency of transgene expression in the events. However, with the described truncation of the MARs in DAS-40278-9, its unknown if the partial MAR regions contribute any effect on transgene expression. There is clearly no evidence that the truncations have any negative affect on the expression of *aad*-1 in DAS-40278-9 (Schafer *et al.*, 2008).

At least two clones for each primer pair were used for primer walking in order to obtain the complete sequence information on the DNA insert and its border sequences. Sequence analysis indicated a 21 bp insertion at 5'-integration junction between corn genome DNA and the integrated partial MAR v3 from the pDAS1740/*Fsp* I. BLAST search and Vector NTI analysis results indicated that the 21 bp insert DNA do not demonstrate homology to any plant species DNA or the pDAS1740 plasmid DNA. A single base pair insertion was found at the 3'-integration junction between corn genome DNA and the partial MAR v4 from the pDAS1740/*Fsp* I. DNA integration also resulted in a two base pair deletion at the insertion locus of the corn genome (Confidential Appendix, Figure 2). In addition, one nucleotide difference (T to C) at the position of 5212 bp was observed in the non-translated 3' UTR region of the DNA insert (Confidential Appendix, Figure 1). However, none of these changes seem to be critical to *aad*-1 expression or create any new ORFs (>= 200 codons) across the junctions in the insert of DAS-40278-9.

Confirmation of Corn Genomic Sequences

To confirm the insertion site of AAD-1 event DAS-40278-9 transgene in the corn genome, PCR amplification was carried out with different pairs of primers (Figure 3). Genomic DNA from AAD-1 corn event DAS-40278-9 and other transgenic or non-transgenic corn lines (Table 1) was used as a template. Two *aad*-1 specific primers, AI5End01 and AI5End02, and two primers designed according to the 5' end border sequence, 1F5End01 and 1F5End02, were used to amplify DNA fragments spanning the *aad*-1 gene to 5' end border sequence (Figure 5, Gel A, B). Similarly, to amplify a DNA fragment spanning the *aad*-1 to 3' end border sequence, 1F3End05 primer derived from the 3' end border sequence and *aad*-1 specific AI3End01 primer were used (Figure 5, Gel C). As shown in Figure 5, DNA fragments with expected sizes were amplified only from the genomic DNA of AAD-1 corn event DAS-40278-9, with each primer pair consisting of one primer located on the flanking border of AAD-1 corn event DAS-40278-9 and one *aad-1* specific primer. The control DNA samples did not yield PCR products with the same primer pairs indicating that the cloned 5' and 3' end border sequences are indeed the upstream and downstream sequence of the inserted *aad*-1 gene construct, respectively. It is

noted that a faint band with size of about 8 kb (Figure 5, Gel A) was observed in all the corn samples including AAD-1 corn event DAS-40278-9, AAD-1 corn event DAS-40474 and non transgenic corn line XHH13 when the primer pair of 1F5End01 and AI5End01 were used for PCR amplification. This faint band could be a result of nonspecific amplification in corn genome with this pair of primers.

To further confirm the DNA insertion in the corn genome, two primers located at the 5' end border sequence, 1F5End03 and 1F5End04, and two primers located at the 3' end border sequence, 1F3End03 and 1F3End04, were used to amplify DNA fragments spanning the insertion locus. As shown in Figure 6, PCR amplification with either the primer pair of 1F5End03/1F3End03 (Gel A) or the primer pair of 1F5End04/1F3End04 (Gel B) resulted in a fragment with expected size of approximately 8 kb from the genomic DNA of AAD-1 corn event DAS-40278-9. In contrast, no PCR products resulted from the genomic DNA of AAD-1 corn event DAS-40474-7 or the non-transgenic corn line XHH13. Given that AAD-1 corn event DAS-40278-9 and event DAS-40474-7 were generated by transformation of HiII, followed by backcrossing the original transgenic events with the corn line XHH13, the majority of genome in each of these two events is theoretically from the corn line XHH13. It is very likely that only the flanking border sequences close to the *aad*-1 transgene are carried over from the original genomic DNA and preserved during the AAD-1 event introgression process, while other regions of genome sequences might have been replaced by the genome sequences of XHH13. Therefore, it is not surprising that no fragments were amplified from the genomic DNA of AAD-1 corn event DAS-40474-7 and XHH13 with either the primer pair of 1F5End03/1F3End03 or the primer pair of 1F5End04/1F3End04. Approximately 3.1 and 3.3 kb fragments were amplified with the primer pair of 1F5End03/ 1F3End03 and 1F5End04/1F3End04 respectively in the genomic DNA of the corn lines HiII and B73 but not in the corn line A188 (Figure 6). The results indicate that the border sequences originated from the genome of the corn line B73.

Additional cloning of corn genomic DNA from B73/HiII was performed to ensure validity of the flanking border sequences. The PCR amplified fragments were sequenced in order to prove the insert DNA region integrated into the specific location of B73/HiII genomic DNA. Primers were designed based on the sequence obtained. Primer set Amp 1F/Amp 5R was used to amplify a

2212 bp fragment spanning the 5' to 3' junctions from native B73/HiII genome without insert DNA. Sequence analysis revealed that there was a two base pair deletion from the native B73 genome in the transgene insertion locus. Analysis of the DNA sequences from the cloned native B73 genomic fragment identified one ORF (>= 200 codons) located downstream of the 3'-integration junction region. Additionally, there are no other ORFs across the original locus where the AAD-1 corn event DAS-40278-9 integrated. BLAST search also confirmed that both 5' end and 3' end border sequences from the event DAS40278-9 are located side by side on the same corn BAC clone.

Given the uniqueness of the 5'-integration junction of the AAD-1 corn event DAS-40278-9, two pairs of specific PCR primers, 1F5EndT1F/1F5EndT1R and Corn278-F/Corn278-R, were designed to amplify this insert-to-plant genome junction. As predicted, the desired DNA fragment was only generated in the genomic DNA of the AAD-1 corn event DAS-40278-9 but not any other transgenic or non-transgenic corn lines (Figure 7). Therefore, those two primer pairs can be used as AAD-1 corn event DAS-40278-9 event-specific identifiers.

## CONCLUSIONS

The entire insert and flanking border regions in AAD-1 corn event DAS-40278-9 were cloned and sequenced. The insert contains an intact *aad*-1 expression cassette from pDAS1740/*Fsp* I flanked by two truncated MAR elements. DNA sequence of the intact *aad*-1 expression cassette matched the transgene sequence of pDAS1740/*Fsp* I except a single base pair change (T to C) in the non-coding region of 3' UTR. PCR analysis confirmed the cloned 5' and 3' border sequences were indeed from the corn genome and the original locus where the insert of event DAS-40278-9 was identified. Sequence analysis also revealed that a 21 bp insertion at 5'-integration junction and a two base pair deletion from the native corn genome at the insertion locus. A one base pair insertion was found at 3'-integration junction between corn genome DNA and DAS-40278-9 insert as well. None of these changes affect the open reading frame composition of the *aad*-1 expression cassette.

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Table 1. Test and Control Substance Used in the Study

| Genomic DNA | Description | Source ID |
|---|---|---|
| BC3S2 | Transgenic AAD-1 corn | ZQ07LQ570792.012 |
| BC3S3 | Transgenic AAD-1 corn | ZW08EW011463.009 |
| AAD-1 corn event DAS-40474-7 | Transgenic AAD-1 corn | ZQ07LQ571692.048 |
| XHH13 | Non transgenic corn inbred line | ZQ07LQ573115 |
| A188 | Non transgenic corn inbred line | N/A |
| B73 | Non transgenic corn inbred line | N/A |
| HiII | Non transgenic corn hybrid line | N/A |

Table 2. Conditions for Genome Walking of the AAD-1 Corn Event DAS-40278-9 to Amplify the Flanking Border Regions

| Target Sequence | Primer Set | 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 | 5End3812_A / AP1 | 95/3 | 95/30 | $68^{-0.5/cycle}\rightarrow 64$ /30 | 68/10:00 | 95/30 | 64/30 | 68/10:00 | 72/10 |
| | | | 8 cycles | | | 22 cycles | | | |
| 5' border (nested) | 5End3812_B / AP2 | 95/3 | 95/30 | $68^{-0.5/cycle}\rightarrow 64$ /30 | 68/10:00 | 95/30 | 64/30 | 68/10:00 | 72/10 |
| | | | 8 cycles | | | 22 cycles | | | |
| 3' border | 3End3812_C / AP1 | 95/3 | 95/30 | $68^{-0.5/cycle}\rightarrow 64$ /30 | 68/10:00 | 95/30 | 64/30 | 68/10:00 | 72/10 |
| | | | 8 cycles | | | 22 cycles | | | |
| 3'border (nested) | 3End3812_D / AP2 | 95/3 | 95/30 | $68^{-0.5/cycle}\rightarrow 64$ /30 | 68/10:00 | 95/30 | 64/30 | 68/10:00 | 72/10 |
| | | | 8 cycles | | | 22 cycles | | | |

Table 3. Conditions for Standard PCR Amplification of the Border Regions in the AAD-1 Corn Event DAS-40278-9

| Target Sequence | Primer Set | Pre-denature (°C/min) | Denature (°C/sec.) | Anneal (°C/sec.) | Extension (°C/min:sec) | Final Extension (°C/min) |
|---|---|---|---|---|---|---|
| 5' border | 1F5End01 / AI5End01 | 95/3 | 95/30 | 60/30 | 68/5:00 | 72/10 |
| | | | 35 cycles | | | |
| 5' border | 1F5End02 / AI5End02 | 95/3 | 95/30 | 60/30 | 68/5:00 | 72/10 |
| | | | 35 cycles | | | |
| Across the insert locus | 1F3End03 / 1F5End03 | 95/3 | 95/30 | 60/30 | 68/5:00 | 72/10 |
| | | | 35 cycles | | | |
| Across the insert locus | 1F3End04 / 1F5End04 | 95/3 | 95/30 | 60/30 | 68/5:00 | 72/10 |
| | | | 35 cycles | | | |
| 5' junction (Amplicon 1) | Amp 1F / Amp 1R | 95/2 | 94/60 | 55/60 | 72/2:00 | 72/10 |
| | | | 35 cycles | | | |
| Amplicon 2 | Amp 1F / Amp 1R | 95/2 | 94/60 | 55/60 | 72/2:00 | 72/10 |
| | | | 35 cycles | | | |
| Amplicon 3 | Amp 1F / Amp 1R | 95/2 | 94/60 | 55/60 | 72/2:00 | 72/10 |
| | | | 35 cycles | | | |
| Amplicon 4 | Amp 1F / Amp 1R | 95/2 | 94/60 | 55/60 | 72/2:00 | 72/10 |
| | | | 35 cycles | | | |
| 3' junction (Amplicon 5) | Amp 1F / Amp 1R | 95/2 | 94/60 | 55/60 | 72/2:00 | 72/10 |
| | | | 35 cycles | | | |
| 3' border | 1F3End05 / AI3End01 | 95/3 | 95/30 | 60/30 | 68/5:00 | 72/10 |
| | | | 35 cycles | | | |

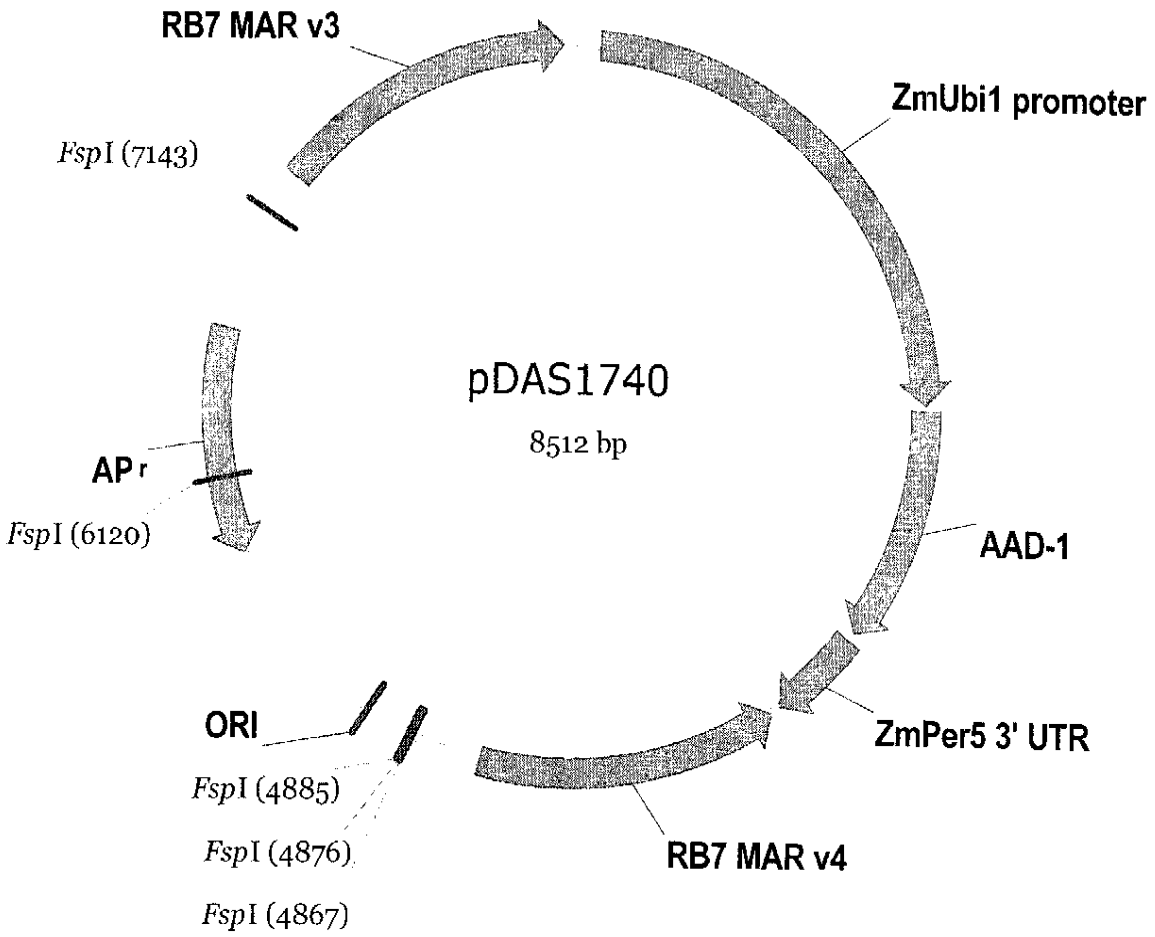



Figure 1. Plasmid Map of pDAS1740, the DNA Fragment Containing *aad*-1 Expression Cassette Generated with *Fsp* I Digestion was Used for Transformation

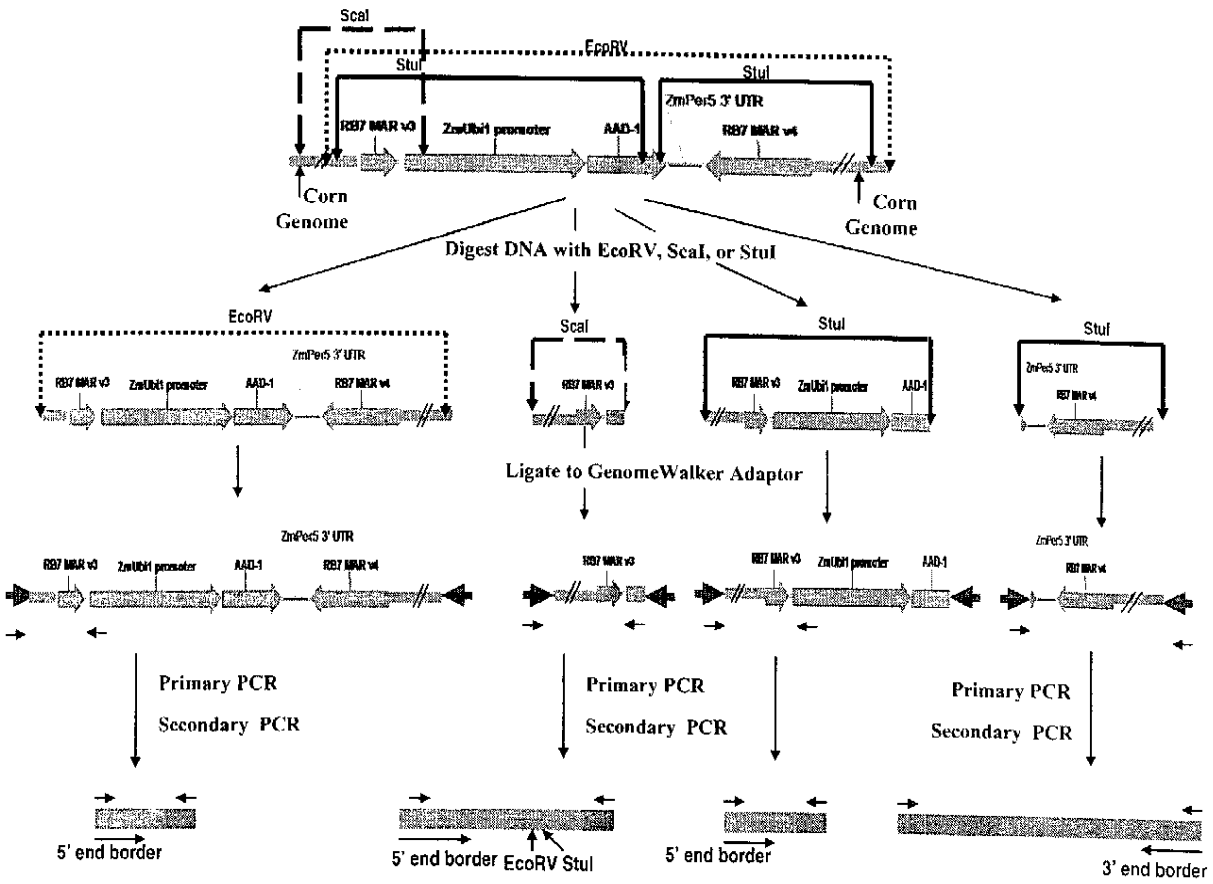



Figure 2. Cloning Strategy for the Flanking Border Sequences from the AAD-1 Corn Event DAS-40278-9

Genomic DNA of the AAD-1 corn event DAS-40278-9 was digested with *Eco*R V, *Stu* I, or *Sca* I and generated corresponding GenomeWalker™ libraries, which were used as templates to amplify the target DNA sequences.

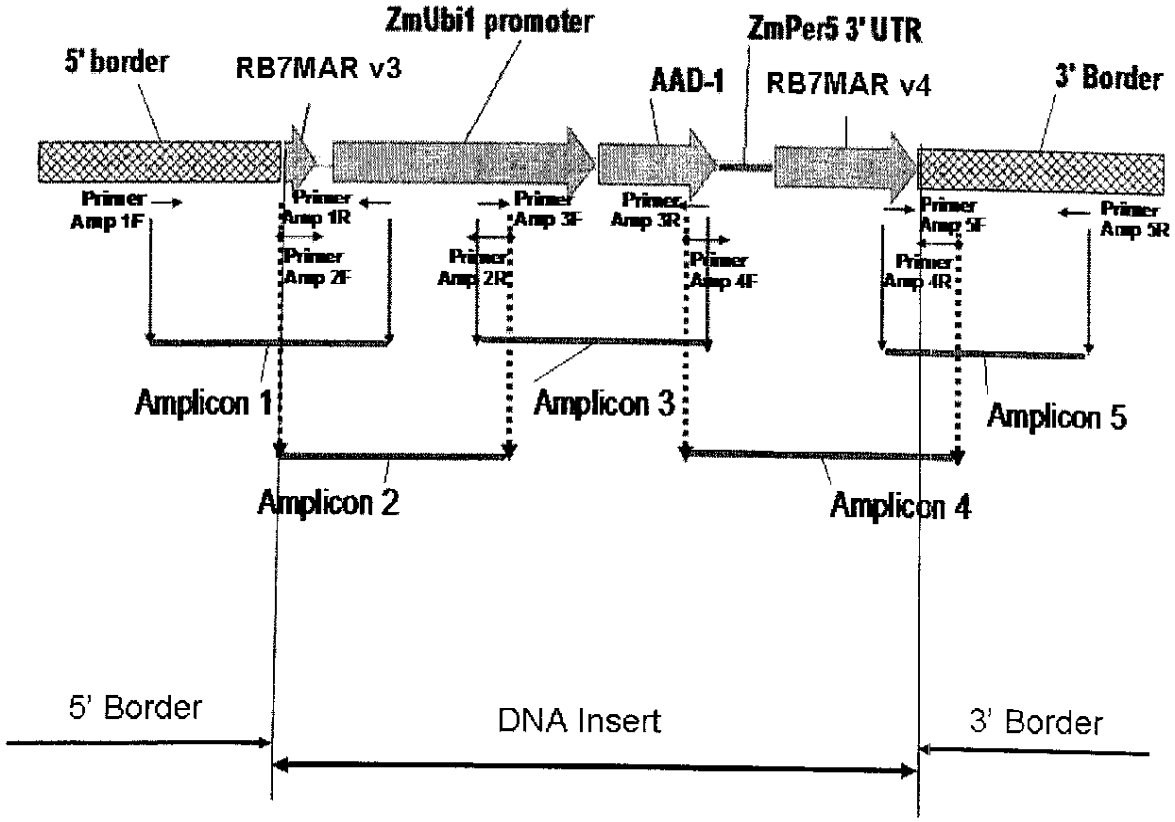



Figure 3. Cloning Strategy for the DNA Insert in the AAD-1 Corn Event DAS-40278-9

The schematic diagram depicts the primer locations and cloning strategy for full length sequencing of the AAD-1 corn event DAS-40278-9 from 5' to 3' borders.

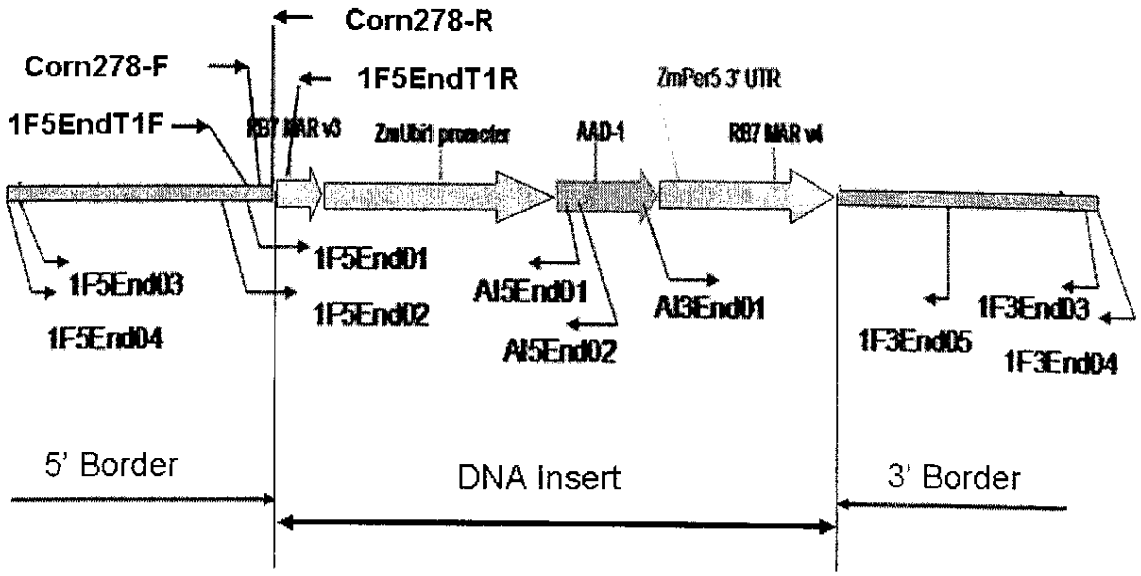
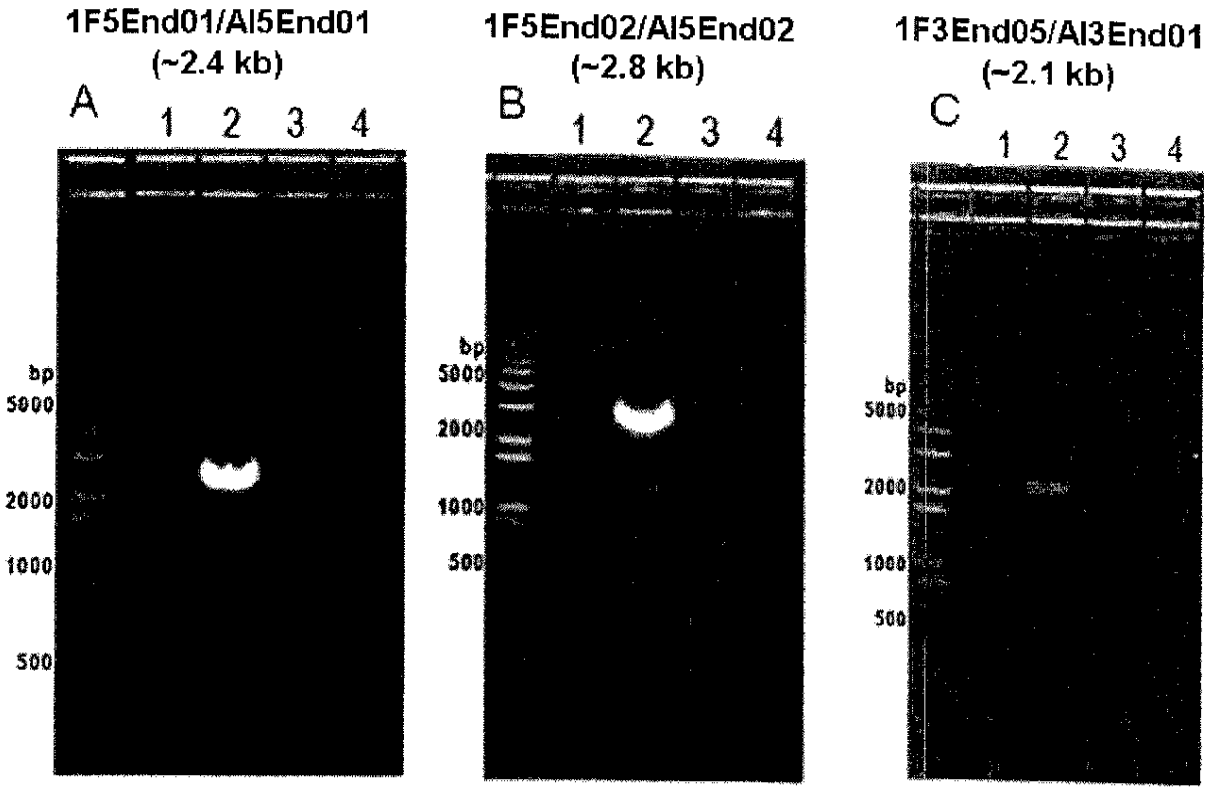



Figure 4. Diagram of the Primers Used in PCR Amplification for Confirmation of Flanking Border Regions of the AAD-1 Corn Event DAS-40278-9

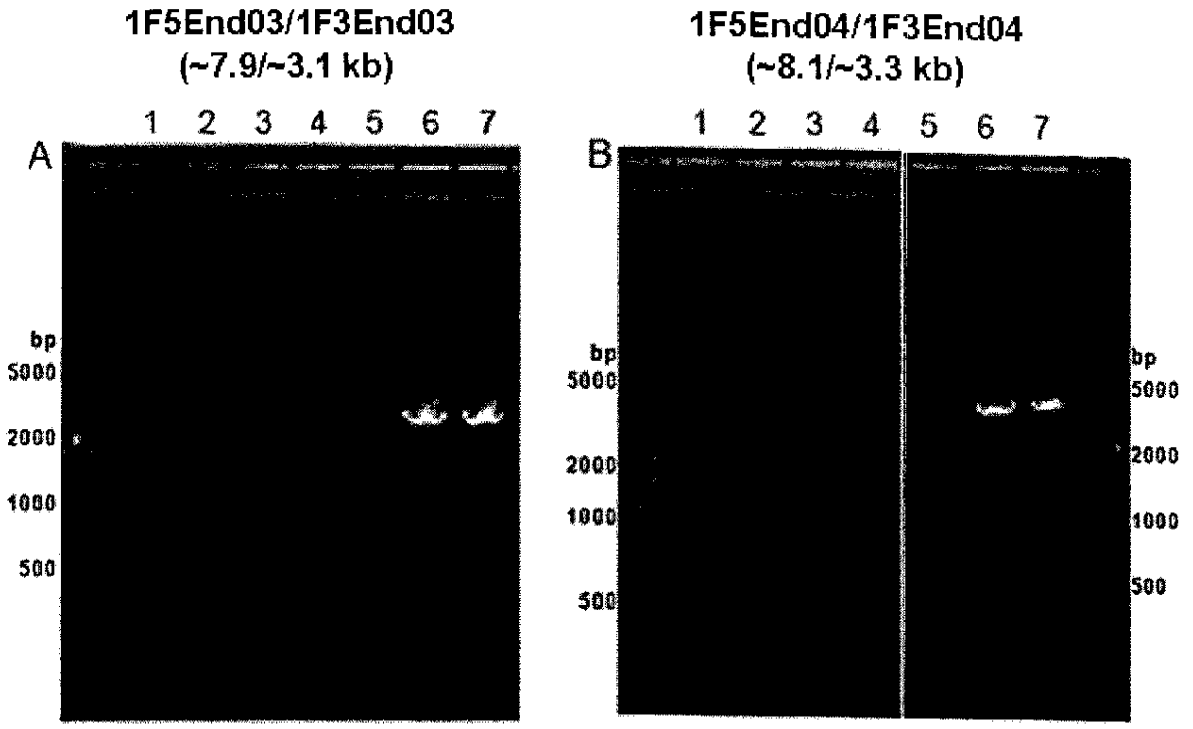
The schematic diagram depicts the primer locations for confirming the full length sequencing of the AAD-1 corn event DAS-40278-9 from 5' to 3' borders.




| Lane | Sample in Gel A, B, and C |
|---|---|
| 1 | Water |
| 2 | Event DAS-40278-9 |
| 3 | Event DAS-40474-7 |
| 4 | Non-transgenic corn line XHH13 |

Figure 5. PCR Confirmation of Corn Genomic Sequences from the Border Regions of the Insert in the AAD-1 Corn Event DAS-40278-9

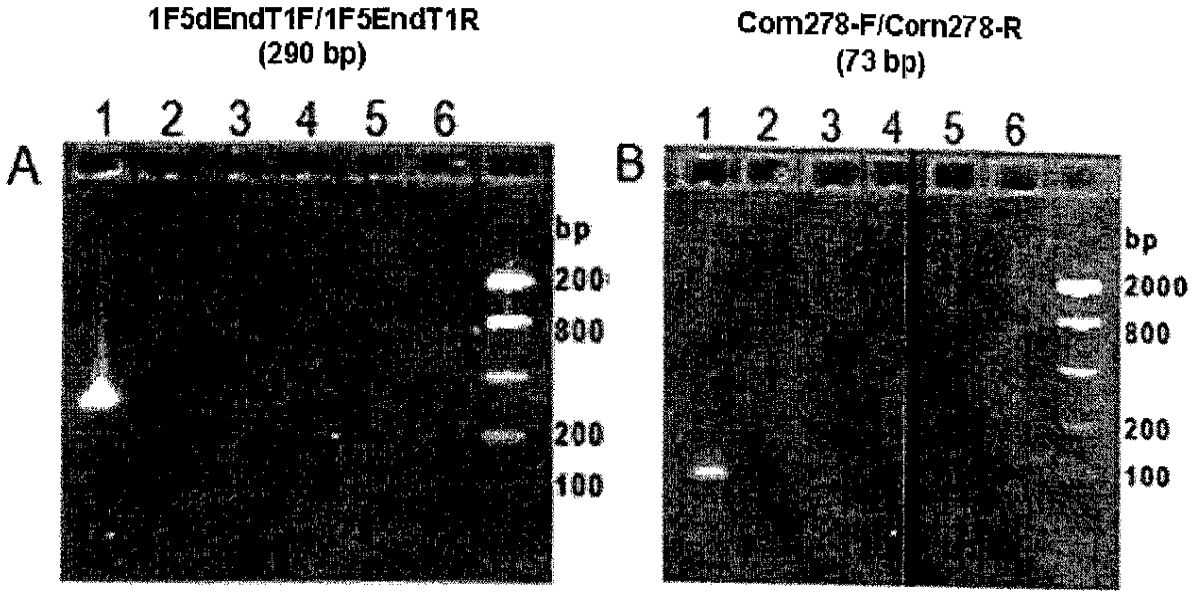
Primer pairs, 1F5End01/AI5End01, 1F5End02/AI5End02, or 1F3End05/AI3End01, were used to amplify border sequences from the 5' or 3' end of the AAD-1 corn event DAS-40278-9 genomic DNA. 10 µL out of 50 µL PCR reaction was loaded in each well on the gels.




| Lane | Sample in Gel A and B |
|---|---|
| 1 | Event DAS-40278-9 |
| 2 | Event DAS-40474-7 |
| 3 | Non-transgenic corn line XHH13 |
| 4 | Water |
| 5 | Non-transgenic corn line A188 |
| 6 | Non-transgenic corn line B73 |
| 7 | Non-transgenic corn line HiII |

Figure 6. PCR Amplification of the Original Locus Where the AAD-1 Corn Event DAS-40278-9 Transgene was integrated

Primer pairs, 1F5End03/1F3End03 or 1F5End03/1F3End03, were used to amplify an approximately 8 kb fragment from the genomic DNA of AAD-1 corn event DAS-40278-9 and a fragment without transgene insert from the genomic DNA of B73/HiII. 10 µL out of 50 µL PCR reaction was loaded in each well on the gels.




| Lane | Sample in Gel A and B |
|---|---|
| 1 | Event DAS-40278-9 |
| 2 | Event DAS-40474-7 |
| 3 | Non-transgenic corn line XHH13 |
| 4 | Water |
| 5 | Non-transgenic corn line A188 |
| 6 | Non-transgenic corn line B73 |

Figure 7. PCR Amplification of the AAD-1 Corn Event DAS-40278-9 Specific Sequences

Primer pairs, 1F5EndT1F/1F5EndT1R or Corn278-F/Corn278-R, were used to amplify fragments from the genomic DNA of AAD-1 corn event DAS-40278-9. 20 µL out of 50 µL PCR reaction was loaded in each well on the gels.