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Document Cover Page
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Document Type	OECD Annex Point(s) Planned (Registration docs only)	PTR Number	Report / File Number(s)	Date Issued (dd-MMM-yy)
Regulatory		71582	081120	8-**May-09**

Document Title

Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a Single Generation

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Materials being tested:

Active Ingredient(s) / Structural Gene(s) / Protein(s), etc.	Formulation Nos.	Product Name(s) (DAS only)
Genomic DNA Extracted from Traits		

Lab Notebook(s)	Information Release No.	GLP Substance Nos (TSN, AGR)	Other Batch/Lot Number(s)

Key Terms including compound numbers (ex: XDE-123)

AAD-1

Trial Category (s) (Efficacy or Research docs only)		Bayer Codes (target crops and pests)

Data Requirement(s) (Guideline Numbers)

Not Applicable — Global Data Matrix? ☐

State or Province of Study	Country of Study	Protocol Number(s)	Internal Study/Trial Number(s)	External Study/Trial Number(s)	Study Completion Date (dd-MMM-yy)
Indiana	USA	081120	081120		8-May-09
					GLP Status
					GLP

Analytical Methods Only:

Method Type	Analyte(s)

Method Technique(s)	Matrix

SUMMARY

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

STUDY TITLE

Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a Single Generation

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

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STUDY COMPLETED ON

8-May-2009

PERFORMING LABORATORY

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LABORATORY STUDY ID

081120

Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a single Generation

SUMMARY

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, etc.) herbicides and is also used as a selectable marker during plant transformation. Whiskers transformation of corn with a DNA fragment released by *Fsp* 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. The initial transgenic event DAS-40278-9, carrying the insert from pDAS1740, has been bred into elite germplasm to stabilize the agronomic performance.

The purpose of this study was to characterize transgenic corn event DAS-40278-9 by Southern blot analysis to determine the genetic equivalence of the inserted DNA within a single generation (BC3S1) of transgenic plants.

The results from this characterization study indicate that the inheritance of the inserted DNA of event DAS-40278-9 is stable within a segregating generation (BC3S1). All 85 individual plants analyzed by Southern blot analysis show a segregation pattern with the *aad*-1 gene probe, which matches the lateral flow strip (LFS) assay testing for expression of the AAD-1 protein. The ratio of 65 positive to 20 null segregants in the BC3S1 generation fits the expected segregation ratio of 3:1 based on a single locus. The Southern blot results indicate an intact *aad*-1 gene has inserted into a single site of the corn genome and the results correlate with the Southern results from the across generation study (Zhuang, et al., 2009). The results of both studies indicate that the DNA insertion occurred at a single locus and was equivalent across five generations and within a single generation.

STUDY TITLE

Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a Single Generation

DATA REQUIREMENTS

Not Applicable

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: **Aryloxyalkanoate Dioxygenase-1**

Title: Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a Single Generation

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

Company: Dow AgroSciences LLC

Company Agent: Penny L. Hunst

Title: Regulatory Manager

Signature: Penny L. Hunst

Date: 3-Apr-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-1 Corn Event DAS-40278-9 within a Single Generation

Study Initiation Date: Nov-26-2008

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

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: 081120

Title: Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a Single Generation

Study Initiation Date: 26-Nov-2008 **Study Completion Date:** 8-May-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
26-Nov-2008	26-Nov-2008	Protocol Review
04-Dec-2008	05-Dec-2008	Planting
26 & 27-Feb-2009	02-Mar-2009	DNA digestion and extraction
21, 22-Apr-2009	22-Apr-2009	Report and Raw Data; Test Substance Container and Sample Verification

QUALITY ASSURANCE STATEMENT:

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

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Tracey Froggatt
Dow AgroSciences, Quality Assurance

8 May 2009

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Study Director: Meibao Zhuang

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Molecular Characterization of AAD-1 Corn Event DAS-40278-9 within a Single Generation

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, etc.) herbicides and is also used as a selectable marker during plant transformation. Whiskers transformation of corn with a DNA fragment released by *Fsp* 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. The initial transgenic event DAS-40278-9, carrying the insert from pDAS1740, has been bred into elite germplasm to stabilize the agronomic performance.

The purpose of this study was to characterize transgenic corn event DAS-40278-9 by Southern blot analysis to determine the genetic equivalence of the inserted DNA within a single generation (BC3S1) of transgenic plants.

The results from this characterization study indicate that the inheritance of the inserted DNA of event DAS-40278-9 is stable within a segregating generation (BC3S1). All 85 individual plants analyzed by Southern blot analysis show a segregation pattern with the *aad*-1 gene probe, which matches the lateral flow strip (LFS) assay testing for expression of the AAD-1 protein. The ratio of 65 positive to 20 null segregants in the BC3S1 generation fits the expected segregation ratio of 3:1 based on a single locus. The Southern blot results indicate an intact *aad*-1 gene has inserted into a single site of the corn genome and the results correlate with the Southern results from the across generation study (Zhuang, et al., 2009). The results of both studies indicate that the DNA insertion occurred at a single locus and was equivalent across five generations and within a single generation.

ABBREVIATIONS

AAD-1	aryloxyalkanoate dioxygenase-1
bp	base pair
°C	degrees Celcius
DNA	deoxyribonucleic acid
DIG	digoxigenin
EDTA	ethylenediaminetetraacetic acid
kb	kilobase
µg	microgram
µL	microliter
mL	milliliter
M	molar mass
OLP	overlapping probe
PCR	polymerase chain reaction
PTU	plant transcription unit
SDS	sodium dodecyl sulfate
SOP	standard operating procedure
SSC	a buffer solution containing a mixture of sodium chloride and sodium citrate, pH 7.0
TBE	a buffer solution containing a mixture of Tris base, boric acid and EDTA, pH 8.3
V	volts

INTRODUCTION

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, etc.) herbicides and is also used as a selectable marker during plant transformation. Whiskers transformation of corn with 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.

This report describes the molecular characterization of the inserted DNA in AAD-1 corn event DAS-40278-9. The event was produced via Whiskers transformation with the *Fsp* I fragment of plasmid pDAS1740. The integration of genetically modified material into a plant genome can occur at virtually any site in the plant genome, but the integration site is generally unique for each event and can be detected by Southern blot analysis using a gene specific probes in combination with restriction enzymes that have cleavage sites located within the plasmid to produce hybridizing fragments that span the junction of the plasmid with the corn genomic DNA (border fragments). In this report, restriction enzyme *Nco* I was chosen to release a border fragment containing the *aad*-1 gene from DAS-40278-9 DNA samples, Southern blot analysis using the *aad*-1 gene probe was used to determine the genetic equivalence of the inserted DNA within a single corn generation containing event DAS-40278-9. Southern blot data suggested that the DNA insertion was stable within a single segregating generation (BC3S1) of corn containing event DAS-40278-9.

MATERIALS AND METHODS

Test Substances/Test systems

The test substances and test systems were genomic DNA prepared from leaf of the individual plants of the AAD-1 corn event DAS-40278-9 (pDAS1740-278) generation BC3S1. Genomic DNA was extracted from leaf tissue harvested from individual plants carrying AAD-1 corn event DAS-40278-9. Transgenic corn seeds from the segregating generation (BC3S1) of event DAS-40278-9, 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, plant generation, and plant number.

Control Substances

The control substance was genomic DNA prepared from leaf of the individual conventional corn XHH13. The conventional control plant has the genetic background representative of the test substance line, but does not contain *aad*-1 gene. The conventional corn seeds were provided by the Department of TG&T, Dow AgroSciences (Table 1). Control substance was labeled with unique IDs associated with sample name and plant number.

Reference Materials

A 1 kb plus DNA ladder (Invitrogen, Cat #: 10787-018) and DIG DNA Marker II (Roche Diagnostics, Cat #: 11218590910), each contains a mixture of DNA fragments with different sizes, served as size references for agarose gel electrophoresis and Southern blot analysis.

The *Fsp* I fragment of plasmid pDAS1740 (pDAB3812) was used as the transformation DNA to generate AAD-1 corn event DAS-40278-9 (pDAS1740-278). Plasmid pDAS1740 (pDAB3812) therefore served as a positive control for the *aad*-1 transgene sequence in AAD-1 corn event

DAS-40278-9 (pDAS1740-278). The reference plasmid was added to DNA samples from conventional corn plants for Southern blot analysis.

Seed Planting

Test and control corn 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 corn. One hundred and ten pots, one seed per pot, were planted for the DAS-40278-9 generation BC3S1. Ten pots, one seed per pot, were planted for the conventional control XHH13. Emerged plants were labeled accordingly and were grown for at least 2 weeks prior to AAD-1 protein expression verification using lateral flow strips screening test.

Lateral Flow Strips Screening Test

Leaf punches were taken from each plant to test AAD-1 protein expression using a rapid test strip kit specifically for AAD-1 corn leaf (American Bionostica, Cat #: 701K100) according to the manufacturer's recommended procedure. Each leaf punch sample was given a score of + or – for the presence or absence of AAD-1, respectively.

Corn Leaf Sample Collection

Corn leaf samples were collected from each of the emerged individual plants of the AAD-1 corn event DAS-40278-9 and the conventional control XHH13. Leaf samples were quickly frozen in liquid nitrogen and stored at -80°C until usage. Detail source information was listed in Table 1.

Genomic DNA Extraction

Individual genomic DNA was extracted from frozen corn leaf tissue following the standard CTAB method. When necessary, some of the genomic DNA were further purified with Qiagen Genomic-Tip (Qiagen, Cat #: 10243, 10262) following procedures recommended by the manufacturer. Following extraction, the DNA was quantified spectrofluorometrically using Pico Green reagent (Invitrogen, Cat #: P7589). The DNA was then visualized on an agarose gel to confirm values from the Pico Green analysis and to determine the DNA quality.

DNA Probes

DNA probes specific to the *aad-1* gene were produced by polymerase chain reaction (PCR) amplification using pDAS1740 plasmid DNA (Figure 1) as template. The probes used for the study is described in Table 2.

DNA Digestion and Separation

For molecular characterization of the DNA, nine micrograms (9µg) of genomic DNA from the corn event DAS-40278-9 sample and the conventional control sample were digested by adding approximately eleven units of selected restriction enzyme per µg of DNA and the corresponding reaction buffer to each DNA sample. Each sample was incubated at approximately 37 °C overnight. The restriction enzyme *Nco* I was used for the digests (New England Biolabs, Cat #: R0193L). The positive hybridization control sample was prepared by combining plasmid DNA pDAS1740 (pDAB3812) with genomic DNA from the conventional control at a ratio of approximately equivalent to 1 copy of transgene per corn genome, and digested using the same procedures and restriction enzyme as the test samples. DNA from the conventional corn control (XHH13) was digested using the same procedures and restriction enzymes 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 (Invitrogen, Cat#: 10816-015) to achieve the desired volume for gel loading. The DNA samples and molecular size markers were then electrophoresed through 0.8% agarose gels with 1×TBE buffer (Fisher Scientific, Cat #: BP1333) at 50-60 volts for approximately 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide (Invitrogen, Cat #: 15585-011) and the DNA was visualized under ultraviolet (UV) light. A photographic record was made for each stained gel.

Southern Transfer and Membrane Treatment

Southern blot analysis was performed essentially as described by Memelink, et al (2). Briefly, following electrophoretic separation and visualization of the DNA fragments, the gels were depurinated with 0.25N HCl (Fisher Scientific, Cat #: 5A48) for approximately 15 minutes, and then exposed to a denaturing solution (AccuGENE, Cat #: 51228; Sigma, Cat #: N1531) for approximately 30 minutes followed by neutralizing solution (AccuGENE, Cat #: 51230) for at least 30 minutes. Southern transfer was performed overnight onto nylon membranes (Roche Diagnostics, Cat #: 1417240) using a wicking system with 10×SSC (Sigma, Cat #: S6639). After transfer the membranes were washed in a 2× SSC solution and the DNA was bound to the membrane by UV crosslinking. This process resulted in Southern blot membranes ready for hybridization.

DNA Probe Labeling and Hybridization

The DNA fragments bound to the nylon membrane were detected using a labeled probe. Probes used for the study were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, from fragments generated by primers specific to *aad*-1 gene element from plasmid pDAS1740. Generation of DNA probes by PCR synthesis was carried out using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Cat #: 11636090910) following the manufacturer's 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 with DNA fixed on were briefly washed in 2×SSC and prehybridized with 20-25 mL of prewarmed DIG Easy Hyb solution in hybridization bottles at approximately 50°C for a minimum of 30 minutes in a hybridization oven. The prehybridization solution were then decanted and replaced with 20 mL of prewarmed DIG Easy Hyb solution containing a desired amount of specific probes predenatured by boiling in water for 5 minutes. The hybridization step was then conducted at approximately 50-55°C overnight in the hybridization oven.

Detection

At the end of the probe hybridization, DIG Easy Hyb solutions containing the probes were decanted into clean tubes and stored at -20°C. These probes could be reused for 2-3 times according to the manufacturer's recommended 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 approximately 5 minutes at room temperature, followed by washing twice with high stringency wash buffer (0.1×SSC, 0.1% SDS) for 15 minutes each at approximately 65°C. The membrane blots were then transferred to other clean plastic containers and briefly washed with 1×washing buffer from the DIG Wash and Block Buffer Set (Roche Diagnostics, Cat #: 1585762) for approximately 2 minutes, proceeded to blocking in 1× blocking buffer for a minimum of 30 minutes, followed by incubation with anti-DIG-AP (alkaline phosphatase) 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 standards. Films were then developed with an All-Pro 100 Plus film developer (Konica SRX-101) and images were scanned for report. The number and sizes of detected bands were documented for each probe. DIG-labeled DNA Molecular Weight Marker II (MWM DIG II), visible after DIG detection as described, 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 obtained, and the membrane blots could be reused for hybridization with a different DNA probe according to the manufacturer's recommended procedures. To strip the DNA probes, after signal detection and film exposure, membrane blots were thoroughly rinsed with Milli-Q water and followed by washing twice in stripping buffer (0.2N NaOH, 0.1% SDS) for approximately 15 minutes at room temperature or at 37°C. The membrane blots were then briefly washed in 2×SSC and were ready for prehybridization and hybridization with another DNA probe. The membrane blots were exposed to a new chemiluminescent film to ensure all the DNA probes were stripped off before proceeded to the next hybridization. The re-exposed films were kept along with the previous hybridization data package in the study file for record.

RESULTS AND DISCUSSION

Confirmation of Test Individuals by AAD-1 Protein Expression

All test plants that germinated (85 plants germinated, 25 did not) were tested for the presence or the absence of the AAD-1 protein using an AAD-1 specific rapid test strip kit. Results from AAD-1 protein expression assays for test plants are presented in Table 4. The BC3S1 generation of DAS-40278-9 was expected to display a segregating ratio of 3 positive to 1 negative for the phenotype of AAD-1 protein expression. Of the 85 plants, 65 tested plants were positive for AAD-1 protein expression and 20 tested plants were negative (null segregants). All the plants were employed for Southern blot analysis to test the presence of *aad*-1 gene. Southern

hybridization using *aad*-1 gene probe confirmed that the *aad*-1 gene is present in corn plants tested positive for AAD-1 protein expression, and *aad*-1 gene is absent from the null segregants and the conventional control (Table 4).

Characterization of the stability of the insert in Transgenic Corn Event DAS-40278-9 BC3S1

A total of 85 individual genomic DNA samples from the DAS-40278-9 BC3S1 generation were digested with the *Nco* I restriction enzyme to characterize the event and determine equivalency of the insertion in all individuals within a single generation. Appropriate conventional controls and positive controls were included on the same Southern blot. Hybridization of the *aad-1* probe to *Nco* I digested DNA was expected to yield a hybridization band of > 2764 bp. A hybridization band of ~4000 bp was observed in all 65 plant samples that tested positive for AAD-1 protein expression. Each individual plant with the hybridizing band was also positive for AAD-1 protein expression and *vice versa* (Table 4). In addition, the probe hybridized to the same band in each individual plant analyzed, indicating that within the generation, all individual plants contained the same insertion and were equivalent to one another. The 20 null segregants did not hybridize to the *aad*-1 gene probe. The size of the observed band (~4000 bp) in all AAD-1 expression positive samples correlates with another report on Southern analysis on five distinct generations of DAS-40278-9 (Zhuang, et al., 2009). A summary map of the DNA insertion region with the location of *Nco* I restriction enzyme sites in the corn genome is presented in Figure 2.

Comparison of Observed Segregation Data to the Hypothesized Segregation Ratio

A chi-square (χ^2) test for specified proportions was used to compare the observed segregation data of 65 positive: 20 negative to the hypothesized segregation ratio of 3:1 based on a single locus. The analysis was carried out using the SAS FREQ procedure and did not indicate a statistically significant deviation from the hypothesized ratio (*p*-value = 0.75). The program output for these segregation data are given in Appendix 1.

CONCLUSIONS

The results from this characterization study indicate that the inheritance of the inserted DNA of event DAS-40278-9 is stable within a segregating generation (BC3S1). All 65 individual plants analyzed indicated the insertion is equivalent in all individuals within the generation. These results were consistent with assay testing for the expression of AAD-1 protein. The ratio of 65 positive to 20 null segregants in the BC3S1 generation fit the expected segregation ratio of 3:1 based on a single locus. The Southern blot results indicated an intact copy of the *aad*-1 gene has been inserted into the corn genome and the result correlates with the Southern results from the across generation study (Zhuang, et al., 2009). The results of both molecular characterization studies indicate that the DNA insertion was equivalent across five distinct generations and within a single segregating generation.

ARCHIVING

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

STATISTICAL TREATMENT OF DATA

A chi-square test for specified proportions was used to compare the observed segregation data to the hypothesized ratio of 3:1. The analysis was carried out using the FRET procedure in SAS Version 9.2.

REFERENCES

1. Perkin Elmer, Molecular Biology Assistant 2000 User's Guide, pages 17-23.
2. Memelink, J.; Swords, K.; Harry J.; Hoge, C.; (1994) Southern, Northern, and Western Blot Analysis. Plant Mol. Biol. Manual F1:1-23.
3. DIG Application Manual for Filter Hybridization, (2003). Roche Diagnostics.
4. Zhuang, M.; Mo, J.; Poorbaugh, J.; Richey, K.; Cruse, J.; Thomas. A. (2009). Molecular Characterization of AAD-1 Corn Event DAS-40278-9. Dow AgroScience unpublished report 081052.

Table 1. Description of Seed Sources for AAD-1 Corn Event DAS-40278-9 (pDAS1740-278) and Control Used in the Study.

Seed	Description	Source ID	Purpose
BC3S1	AAD-1 Corn Event DAS-40278-9, generation: BC3S1	ZQ07GQ546693.026	Test Substance
XHH13	Isoline control corn seed: XHH13	ZQ07LQ573115	Control Substance

Table 2. Location and Length of Probes used in Southern Analysis.

Probe Name	Genetic Element	Position on pDAS1740 (bp)	Length (bp)
OLP2	*aad*-1 gene	2103-3022	920

Table 3. Predicted and Observed Hybridizing Fragments in Southern Blot Analysis.

DNA Probe	Restriction Enzymes		Figures	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
aad-1	*Nco* I	pDAS1740	3-7	8512	8512
		XHH13	3-7	none	none
		BC3S1*	3-7	>2764 (border)*	~4000
		BC3S1**	3-7	None**	none

Note: * An asterisk after the sample name/ observed fragment size indicates expected size for DAS-40278-9 samples which are tested positive for AAD-1 protein expression. ** Two asterisks after the sample name/ observed fragment size indicates no specific hybridization band is expected for null segregants from BC3S1.

1. Expected fragment sizes are based on the plasmid map of the pDAS1740 (pDAB3812) as shown in Figure 1.
2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker II fragments. Due to the incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

Table 4. Results of AAD-1 Protein Expression Assay for Plants Grown from the Test Substance (BC3S1) and Control Substance (XHH13) Seeds.

Purpose	Plant ID	AAD-1 Expression	Southern (Expected size, bp)	Southern (Observed size, bp)	Southern (Figure)
Test Substance	DAS-40278-9-BC3S1-1	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-2	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-3	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-4	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-5	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-6	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-7	Negative	none	none	3
	DAS-40278-9-BC3S1-8	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-9	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-10	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-11	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-12	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-13	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-14	Negative	none	none	3
	DAS-40278-9-BC3S1-15	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-16	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-17	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-18	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-19	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-20	Positive	>2764 (border)	~4000	3
	DAS-40278-9-BC3S1-21	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-22	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-23	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-24	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-25	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-26	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-27	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-28	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-29	Negative	none	none	4
	DAS-40278-9-BC3S1-30	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-31	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-32	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-33	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-34	Negative	none	none	4
	DAS-40278-9-BC3S1-35	Negative	none	none	4
	DAS-40278-9-BC3S1-36	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-37	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-38	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-39	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-40	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-41	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-42	NG	N/A	N/A	N/A

Table 4. (Cont.) Results of AAD-1 Protein Expression Assay for Plants Grown from Test Substance (BC3S1) and Control Substance (XHH13) Seeds.

Purpose	Plant ID	AAD-1 Expression	Southern (Expected size, bp)	Southern (Observed size, bp)	Southern (Figure)
Test Substance	DAS-40278-9-BC3S1-43	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-44	Negative	none	none	4
	DAS-40278-9-BC3S1-45	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-46	Positive	>2764 (border)	~4000	4
	DAS-40278-9-BC3S1-47	Negative	none	none	5
	DAS-40278-9-BC3S1-48	Negative	none	none	5
	DAS-40278-9-BC3S1-49	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-50	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-51	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-52	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-53	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-54	Negative	none	none	5
	DAS-40278-9-BC3S1-55	Negative	none	none	5
	DAS-40278-9-BC3S1-56	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-57	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-58	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-59	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-60	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-61	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-62	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-63	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-64	Positive	>2764 (border)	~4000	5
	DAS-40278-9-BC3S1-65	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-66	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-67	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-68	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-69	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-70	Negative	none	none	6
	DAS-40278-9-BC3S1-71	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-72	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-73	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-74	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-75	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-76	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-77	Negative	none	none	6
	DAS-40278-9-BC3S1-78	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-79	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-80	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-81	Negative	none	none	6
	DAS-40278-9-BC3S1-82	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-83	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-84	Negative	none	none	6
	DAS-40278-9-BC3S1-85	Positive	>2764 (border)	~4000	6

Table 4.(Cont.) Results of AAD-1 Protein Expression Assay for Plants Grown from Test Substance (BC3S1) and Control Substance (XHH13) Seeds.

Purpose	Plant ID	AAD-1 Expression	Southern (Expected size, bp)	Southern (Observed size, bp)	Southern (Figure)
Test Substance	DAS-40278-9-BC3S1-86	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-87	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-88	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-89	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-90	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-91	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-92	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-93	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-94	Positive	>2764 (border)	~4000	6
	DAS-40278-9-BC3S1-95	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-96	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-97	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-98	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-99	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-100	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-101	Negative	none	none	7
	DAS-40278-9-BC3S1-102	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-103	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-104	Negative	none	none	7
	DAS-40278-9-BC3S1-105	Negative	none	none	7
	DAS-40278-9-BC3S1-106	Negative	none	none	7
	DAS-40278-9-BC3S1-107	NG	N/A	N/A	N/A
	DAS-40278-9-BC3S1-108	Negative	none	none	7
	DAS-40278-9-BC3S1-109	Positive	>2764 (border)	~4000	7
	DAS-40278-9-BC3S1-110	Negative	none	none	7
Control Substance	XHH13-2	Negative	none	none	3, 4, 6
	XHH13-3	Negative	none	none	4, 6
	XHH13-5	Negative	none	none	3
	XHH13-6	Negative	none	none	5, 7
	XHH13-7	Negative	none	none	5, 7

Note: *Positive 65, Negative 20, NG (not germinated) 25; Positive AAD-1 protein expression indicates detection of AAD-1 protein as determined by lateral flow strips specific for AAD-1 corn leaf, negative indicates no detection of AAD-1 protein; N/A: not applicable*

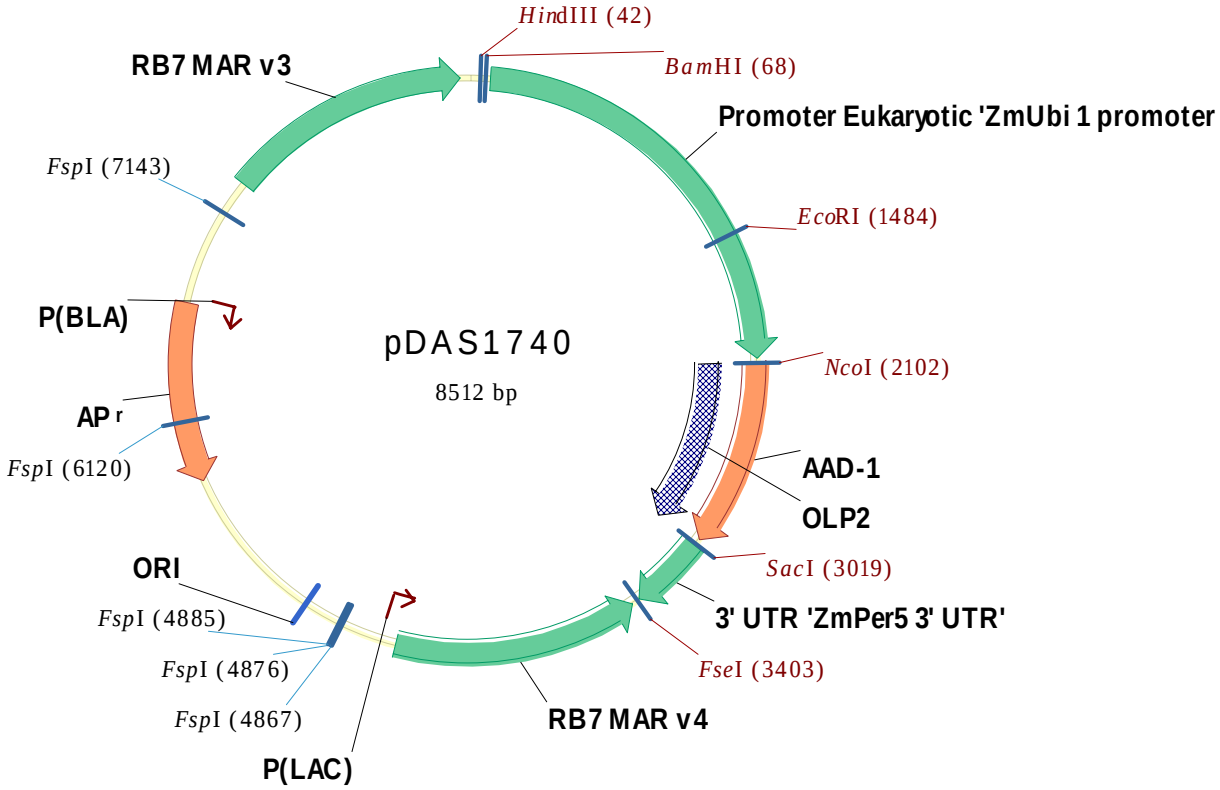


Figure 1. Plasmid Map of pDAS1740 (pDAB3812) with Restriction Enzyme Site and *aad*-1 gene probe (OLP2) location.

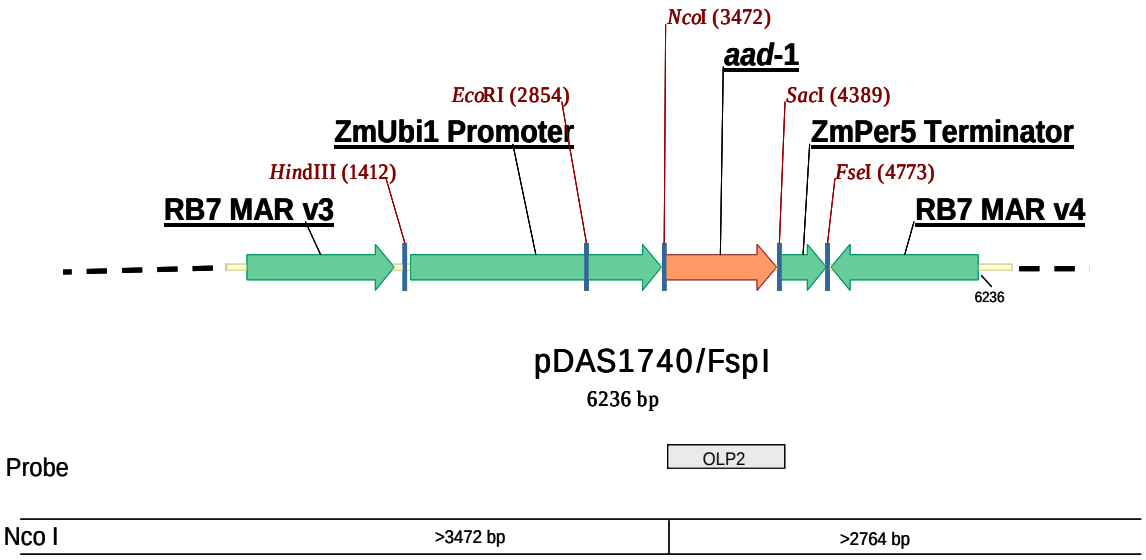
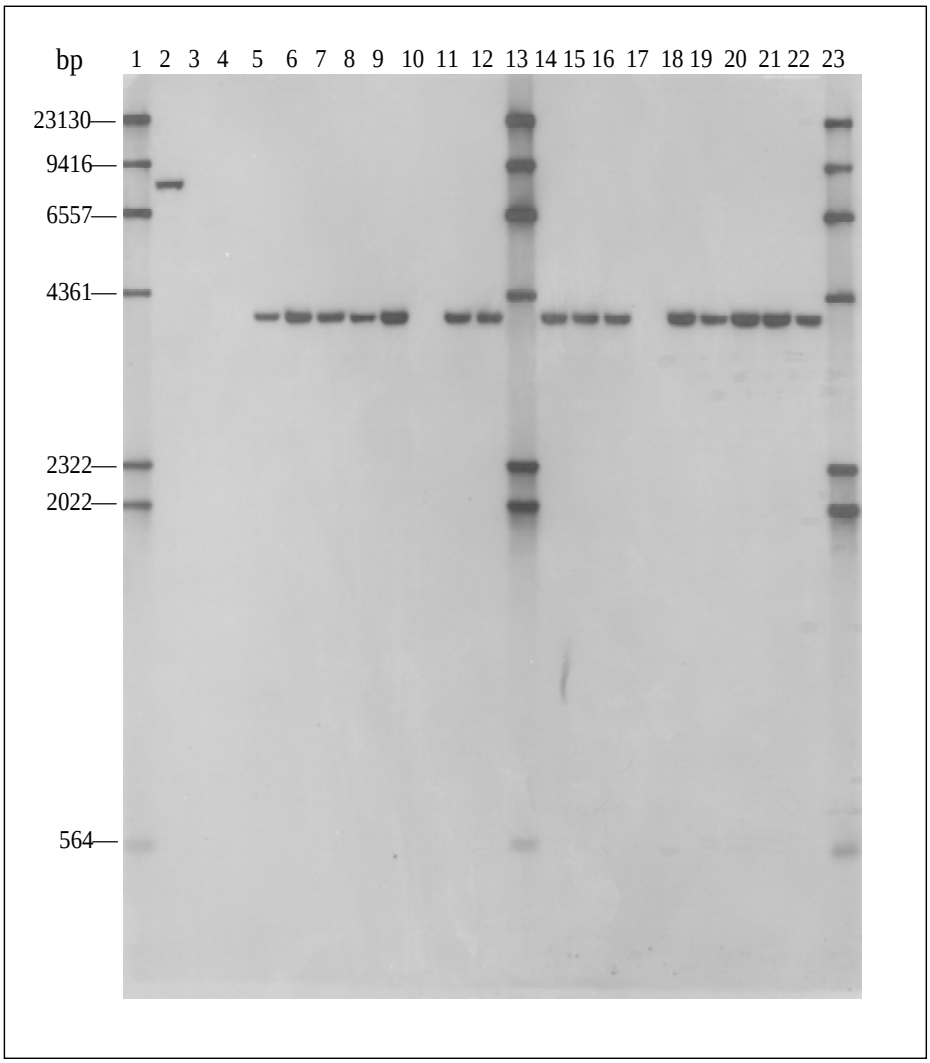


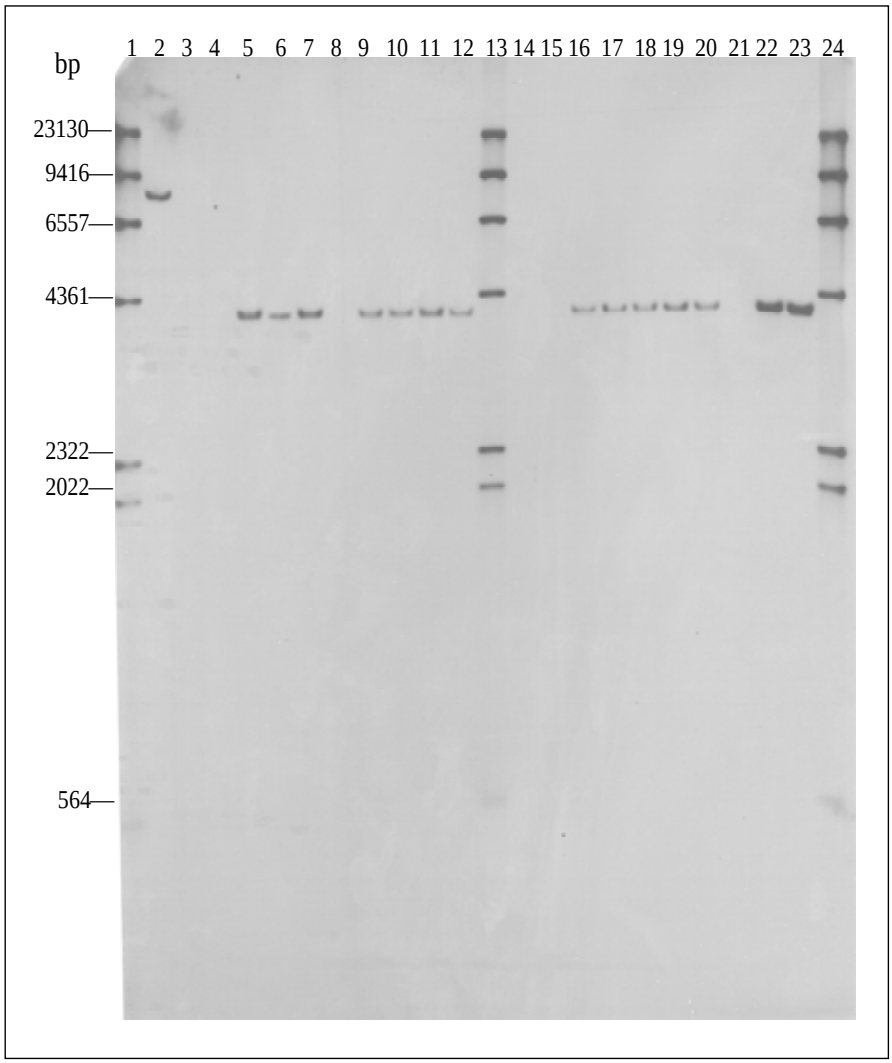
Figure 2. Restriction Map of the Single Insertion Site Present in the Genome of DAS-40278-9 Maize.



Genomic DNA isolated from corn event DAS-40278-9 and conventional corn XHH13 was digested with *Nco* I and probed with the *aad*-1 gene probe. Nine (9) µg of DNA were digested and loaded per lane. The plasmid control contained the approximate equivalent of 1 transgene copy per genome of plasmid pDAS1740 and 9 µg of genomic DNA isolated from the conventional control XHH13.

Lane	Sample	AAD-1 Protein		Lane	Sample	AAD-1 Protein
1	DIG MWM II	N/A		13	DIG MWM II	N/A
2	pDAS1740 + XHH13-2	N/A		14	DAS40278-9-BC3S1-11	Positive
3	XHH13-2	Negative		15	DAS40278-9-BC3S1-12	Positive
4	XHH13-5	Negative		16	DAS40278-9-BC3S1-13	Positive
5	DAS40278-9-BC3S1-1	Positive		17	DAS40278-9-BC3S1-14	Negative
6	DAS40278-9-BC3S1-2	Positive		18	DAS40278-9-BC3S1-15	Positive
7	DAS40278-9-BC3S1-3	Positive		19	DAS40278-9-BC3S1-16	Positive
8	DAS40278-9-BC3S1-5	Positive		20	DAS40278-9-BC3S1-18	Positive
9	DAS40278-9-BC3S1-6	Positive		21	DAS40278-9-BC3S1-19	Positive
10	DAS40278-9-BC3S1-7	Negative		22	DAS40278-9-BC3S1-20	Positive
11	DAS40278-9-BC3S1-8	Positive		23	DIG MWM II	N/A
12	DAS40278-9-BC3S1-10	Positive				

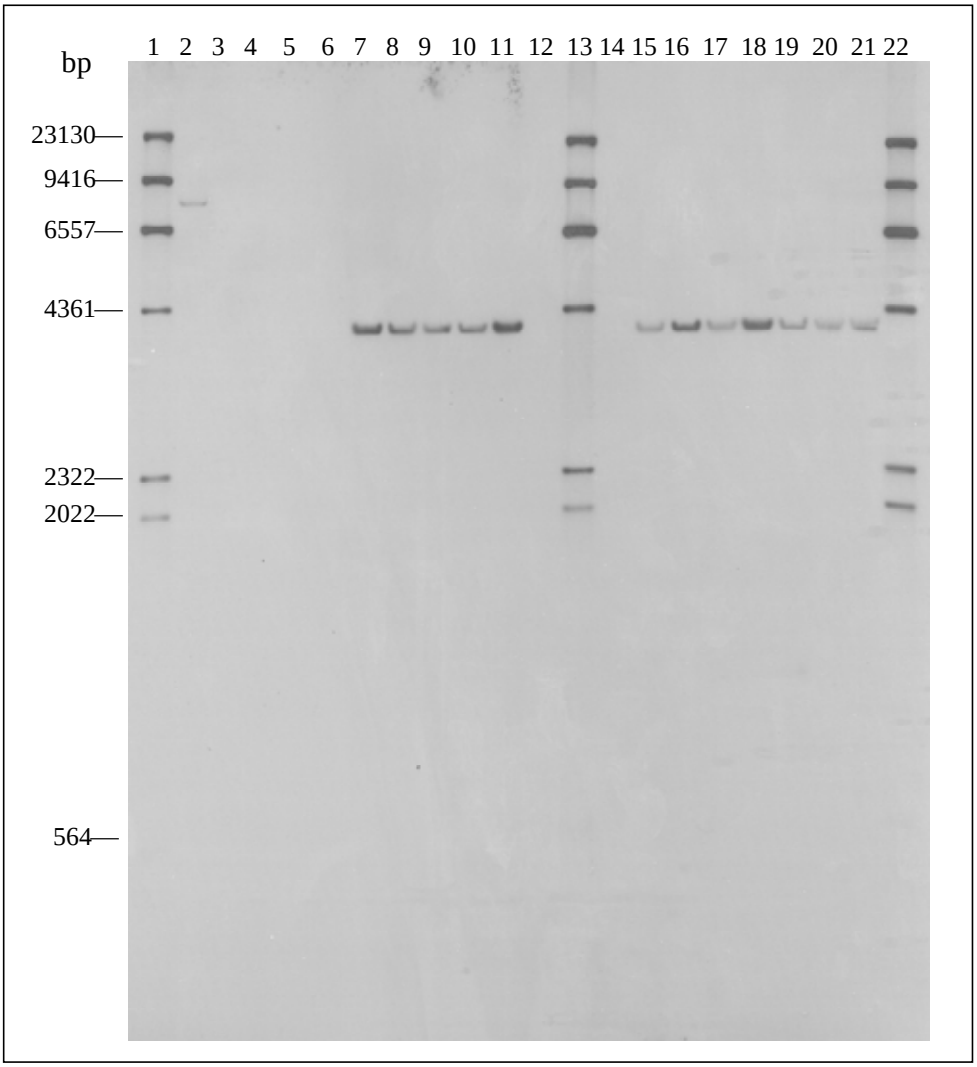
Figure 3. Southern blot analysis of DAS-40278-9; Gel A, *aad*-1 probe (OLP2), *Nco* I digest



Genomic DNA isolated from corn event DAS-40278-9 and conventional corn XHH13 was digested with *Nco* I and probed with the *aad*-1 gene probe. Nine (9) μg of DNA were digested and loaded per lane. The plasmid control contained the approximate equivalent of 1 transgene copy per genome of plasmid pDAS1740 and 9 μg of genomic DNA isolated from the conventional control XHH13.

Lane	Sample	AAD-1 Protein		Lane	Sample	AAD-1 Protein
1	DIG MWM II	N/A		13	DIG MWM II	N/A
2	pDAS1740 + XHH13-2	N/A		14	DAS40278-9-BC3S1-34	Negative
3	XHH13-2	Negative		15	DAS40278-9-BC3S1-35	Negative
4	XHH13-3	Negative		16	DAS40278-9-BC3S1-37	Positive
5	DAS40278-9-BC3S1-21	Positive		17	DAS40278-9-BC3S1-38	Positive
6	DAS40278-9-BC3S1-23	Positive		18	DAS40278-9-BC3S1-39	Positive
7	DAS40278-9-BC3S1-25	Positive		19	DAS40278-9-BC3S1-41	Positive
8	DAS40278-9-BC3S1-29	Negative		20	DAS40278-9-BC3S1-43	Positive
9	DAS40278-9-BC3S1-30	Positive		21	DAS40278-9-BC3S1-44	Negative
10	DAS40278-9-BC3S1-31	Positive		22	DAS40278-9-BC3S1-45	Positive
11	DAS40278-9-BC3S1-32	Positive		23	DAS40278-9-BC3S1-46	Positive
12	DAS40278-9-BC3S1-33	Positive		24	DIG MWM II	N/A

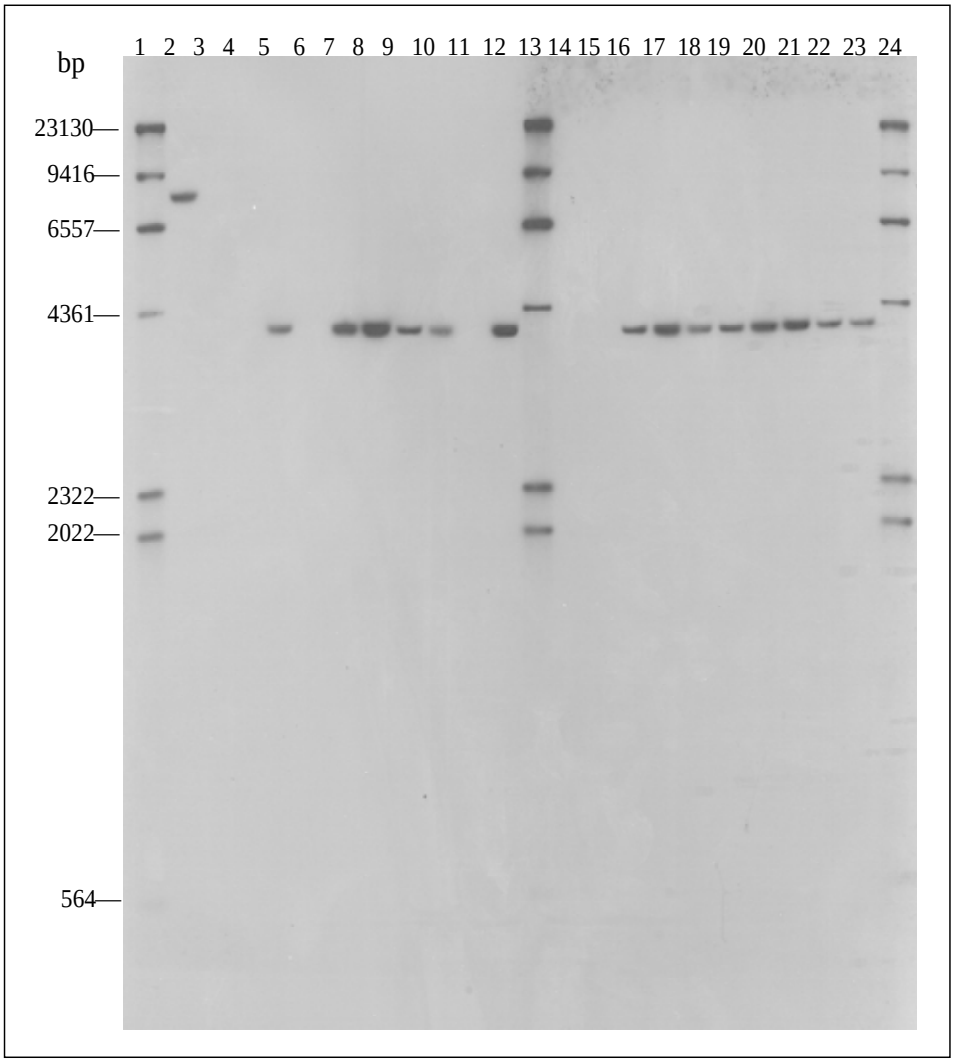
Figure 4. Southern blot analysis of DAS-40278-9; Gel B, *aad*-1 probe (OLP2), *Nco* I digest



Genomic DNA isolated from corn event DAS-40278-9 and conventional corn XHH13 was digested with *Nco* I and probed with the *aad*-1 gene probe. Nine (9) μg of DNA were digested and loaded per lane. The plasmid control contained the approximate equivalent of 1 transgene copy per genome of plasmid pDAS1740 and 9 μg of genomic DNA isolated from the conventional control XHH13.

Lane	Sample	AAD-1 Protein		Lane	Sample	AAD-1 Protein
1	DIG MWM II	N/A		12	DAS40278-9-BC3S1-54	Negative
2	pDAS1740 + XHH13-6	N/A		13	DIG MWM II	N/A
3	XHH13-6	Negative		14	DAS40278-9-BC3S1-55	Negative
4	XHH13-7	Negative		15	DAS40278-9-BC3S1-56	Positive
5	DAS40278-9-BC3S1-47	Negative		16	DAS40278-9-BC3S1-58	Positive
6	DAS40278-9-BC3S1-48	Negative		17	DAS40278-9-BC3S1-59	Positive
7	DAS40278-9-BC3S1-49	Positive		18	DAS40278-9-BC3S1-60	Positive
8	DAS40278-9-BC3S1-50	Positive		19	DAS40278-9-BC3S1-61	Positive
9	DAS40278-9-BC3S1-51	Positive		20	DAS40278-9-BC3S1-63	Positive
10	DAS40278-9-BC3S1-52	Positive		21	DAS40278-9-BC3S1-64	Positive
11	DAS40278-9-BC3S1-53	Positive		22	DIG MWM II	N/A

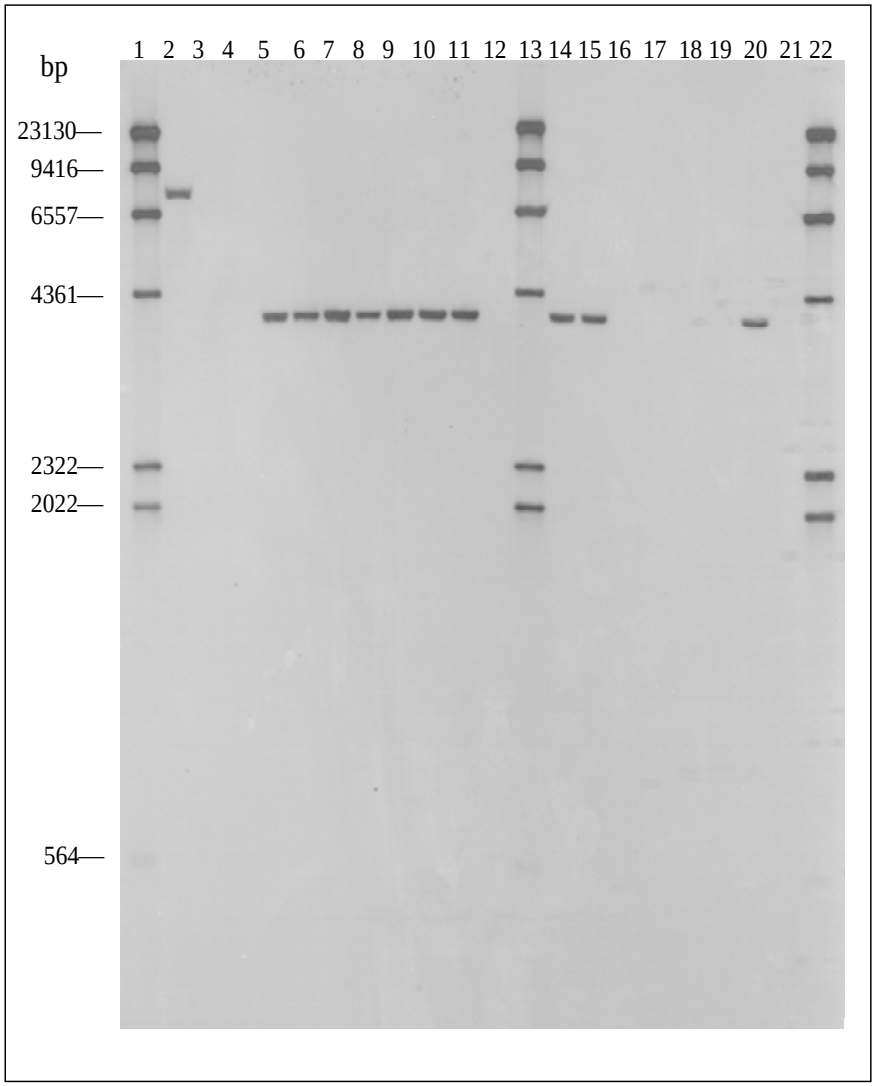
Figure 5. Southern blot analysis of DAS-40278-9; Gel C, *aad*-1 probe (OLP2), *Nco* I digest



Genomic DNA isolated from corn event DAS-40278-9 and conventional corn XHH13 was digested with *Nco* I and probed with the *aad*-1 gene probe. Nine (9) µg of DNA were digested and loaded per lane. The plasmid control contained the approximate equivalent of 1 transgene copy per genome of plasmid pDAS1740 and 9 µg of genomic DNA isolated from the conventional control XHH13.

Lane	Sample	AAD-1 Protein		Lane	Sample	AAD-1 Protein
1	DIG MWM II	N/A		13	DIG MWM II	N/A
2	pDAS1740 + XHH13-2	N/A		14	DAS40278-9-BC3S1-81	Negative
3	XHH13-2	Negative		15	DAS40278-9-BC3S1-84	Negative
4	XHH13-3	Negative		16	DAS40278-9-BC3S1-85	Positive
5	DAS40278-9-BC3S1-69	Positive		17	DAS40278-9-BC3S1-86	Positive
6	DAS40278-9-BC3S1-70	Negative		18	DAS40278-9-BC3S1-87	Positive
7	DAS40278-9-BC3S1-71	Positive		19	DAS40278-9-BC3S1-88	Positive
8	DAS40278-9-BC3S1-72	Positive		20	DAS40278-9-BC3S1-89	Positive
9	DAS40278-9-BC3S1-73	Positive		21	DAS40278-9-BC3S1-90	Positive
10	DAS40278-9-BC3S1-75	Positive		22	DAS40278-9-BC3S1-92	Positive
11	DAS40278-9-BC3S1-77	Negative		23	DAS40278-9-BC3S1-94	Positive
12	DAS40278-9-BC3S1-80	Positive		24	DIG MWM II	N/A

Figure 6. Southern blot analysis of DAS-40278-9; Gel D, *aad*-1 probe (OLP2), *Nco* I digest



Genomic DNA isolated from corn event DAS-40278-9 and conventional corn XHH13 was digested with *Nco* I and probed with the *aad*-1 gene probe. Nine (9) μg of DNA were digested and loaded per lane. The plasmid control contained the approximate equivalent of 1 transgene copy per genome of plasmid pDAS1740 and 9 μg of genomic DNA isolated from the conventional control XHH13.

Lane	Sample	AAD-1 Protein		Lane	Sample	AAD-1 Protein
1	DIG MWM II	N/A		12	DAS40278-9-BC3S1-101	Negative
2	pDAS1740 + XHH13-6	N/A		13	DIG MWM II	N/A
3	XHH13-6	Negative		14	DAS40278-9-BC3S1-102	Positive
4	XHH13-7	Negative		15	DAS40278-9-BC3S1-103	Positive
5	DAS40278-9-BC3S1-65	Positive		16	DAS40278-9-BC3S1-104	Negative
6	DAS40278-9-BC3S1-67	Positive		17	DAS40278-9-BC3S1-105	Negative
7	DAS40278-9-BC3S1-95	Positive		18	DAS40278-9-BC3S1-106	Negative
8	DAS40278-9-BC3S1-96	Positive		19	DAS40278-9-BC3S1-108	Negative
9	DAS40278-9-BC3S1-97	Positive		20	DAS40278-9-BC3S1-109	Positive
10	DAS40278-9-BC3S1-98	Positive		21	DAS40278-9-BC3S1-110	Negative
11	DAS40278-9-BC3S1-99	Positive		22	DIG MWM II	N/A

Figure 7. Southern blot analysis of DAS-40278-9; Gel E, *aad*-1 probe (OLP2), *Nco* I digest

Appendix 1: SAS Results

The SAS System 09:44 Tuesday, March 3, 2009

---------- event=A gen=A ----------
-

The FREQ Procedure

Status	Frequency	Percent	Test Percent	Cumulative Frequency	Cumulative Percent
null	20	23.53	25.00	20	23.53
trans	65	76.47	75.00	85	100.00

Chi-Square Test
for Specified Proportions

Chi-Square	0.0980
DF	1
Pr > ChiSq	0.7542

Sample Size = 85