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Dow AgroSciences

Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-40278-9 Corn

OECD Unique Identifier: DAS-40278-9

The undersigned submits this petition under 7 CFR 340.6 to request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should not be regulated under 7 CFR 340.

Submitting Company:

Dow AgroSciences LLC

Submitted by:

Laura Tagliani
Regulatory Leader
Regulatory Sciences & Government Affairs
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
Telephone: (317)337-3504
FAX: (317)337-4649
Email: latagliani@dow.com

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Release of Information

Dow AgroSciences LLC (DAS) is submitting the information in this petition for deregulation to USDA APHIS as part of the regulatory process. By submitting this information, DAS does not authorize release of this information to any third party except to the extent the information is requested under the Freedom of Information Act (FOIA), 5 U.S.C., Section 522. In the event that USDA receives a FOIA request covering all or some of the information in this submission, DAS expects that, in advance of the release of the document(s), USDA will provide DAS with a copy of the material proposed to be released and the opportunity to object to the release of any information based upon appropriate legal grounds, e.g. responsiveness, confidentiality and/or competitive concerns. DAS expects that no information that has been identified as CBI (confidential business information) will be provided to any third party. DAS understands that a CBI-deleted copy of this information may be made available to the public in a reading room and by individual request, as part of the public comment period. Except in accordance with the foregoing, DAS does not authorize the release, publication or other distribution of this information (including website posting) without DAS' prior notice and consent.

Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes all relevant data and information known to the petitioner that are unfavorable to the petition.

Laura Tagliani
Regulatory Leader

Address:

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
Phone: (317) 337-3504
FAX: (317) 337-4649
E-mail: latagliani@dow.com

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Summary

Dow AgroSciences LLC (herein referred to as "DAS"), is submitting a Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-40278-9 Corn. DAS requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that corn transformation event DAS-40278-9 and any corn lines derived from crosses with DAS-40278-9 corn no longer be considered regulated articles under 7 CFR Part 340.

DAS-40278-9 corn is a transgenic corn product that provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (AOPP) acetyl coenzyme A carboxylase (ACCase) inhibitors ("fop" herbicides). DAS-40278-9 may be stacked with glyphosate and other herbicide tolerance traits to generate commercial hybrids with multiple herbicide tolerances. This trait will provide growers with greater flexibility in selection of herbicides for the improved control of key broadleaf weeds; allow an increased application window for effective weed control; provide an effective resistance management prevention solution to the increased incidence of glyphosate and acetolactate synthase (ALS) resistant weeds; and enable the use of a fop herbicide (such as quizalofop) for commercial production and as a selection agent in breeding nurseries.

DAS-40278-9 corn plants have been genetically modified to express the aryloxyalkanoate dioxygenase (AAD-1) protein. The AAD-1 protein is an enzyme with an alpha ketoglutarate-dependent dioxygenase activity which results in metabolic inactivation of the herbicides of the aryloxyalkanoate family. The *aad-1* gene, which expresses the AAD-1 protein, was derived from *Sphingobium herbicidovorans*, a gram-negative soil bacterium. *Sphingobium* spp. are widespread in the environment, therefore, animals and humans are regularly exposed to the organism and its components, without adverse consequences. *Sphingobium* spp. degrade a number of chemicals in the environment which include aromatic and chloroaromatic compounds, phenols, herbicides and polycyclic hydrocarbons.

The *aad-1* gene was introduced into DAS-40278-9 corn using *Whiskers*-mediated transformation. Molecular characterization of the DAS-40278-9 event by Southern analyses confirmed that a single, intact insert of the *aad-1* gene was stably integrated into the corn genome. A single copy of each of the genetic elements of the *aad-1* expression cassette is present and the integrity of the inserted DNA fragment was demonstrated in five different breeding generations, confirming the stability during traditional breeding procedures. Southern analyses also confirmed the absence of unwanted DNA such as the plasmid backbone DNA in DAS-40278-9 corn. Segregation data for six generations confirmed the predicted inheritance of the *aad-1* gene.

The AAD-1 protein in DAS-40278-9 corn was characterized biochemically and measured using an AAD-1 specific enzyme linked immunosorbent assay (ELISA). Protein

expression was analyzed in leaf, root, pollen, whole plant and grain tissues collected throughout the growing season from DAS-40278-9 plants treated with 2,4-D, quizalofop, both 2,4-D and quizalofop, or not treated with either herbicide. The results showed low level expression of the AAD-1 protein across herbicide treatments and environments, indicating a low exposure risk to humans and animals.

The AAD-1 protein was assessed for any potential adverse effects to humans or animals resulting from the environmental release of crops containing the AAD-1 protein. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-1 protein. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-1 amino acid sequence. The AAD-1 protein hydrolyzes rapidly in simulated gastric fluid and there was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-1 protein. Glycosylation analysis of the plant- and microbe-derived AAD-1 proteins revealed no detectable covalently linked carbohydrates. Results of the overall safety assessment of the AAD-1 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

DAS-40278-9 corn has been field tested in the major corn growing regions of the continental United States as well as Hawaii. All field tests were conducted under field permits granted by USDA APHIS. Agronomic performance assessments were conducted on DAS-40278-9 corn in multi-site field studies to measure characteristics such as emergence, seedling vigor, plant height, lodging, yield, and pollen parameters. All field trials were also observed for opportunistic disease or insect stressors as well as normal phenotypic characteristics. There were no meaningful differences observed between DAS-40278-9 corn and control lines for plant pest characteristics and no indication of a selective advantage that would result in increased weediness potential of DAS-40278-9 corn.

Nutrient composition analyses of forage and grain was conducted to compare the composition of DAS-40278-9 corn with the composition of a non-transgenic near-isoline. Compositional analyses were used to evaluate any changes in the levels of key nutrients and anti-nutrients in DAS-40278-9 corn which was sprayed with either 2,4-D, quizalofop, both 2,4-D and quizalofop, or which was not sprayed with either herbicide. Along with the agronomic data, the compositional analyses indicate that DAS-40278-9 corn is substantially equivalent to conventional corn and will not exhibit unexpected or unintended effects with respect to plant pest risk.

Since DAS-40278-9 corn is agronomically and nutritionally similar to conventional corn, and the safety of the AAD-1 protein has been demonstrated, no significant impact is expected on current crop production practices, non-target or endangered species, crop rotation, volunteer management, or commodity food and feed corn products. The availability of DAS-40278-9 corn is expected to have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The use of DAS-40278-9 corn will allow growers to proactively manage weed

populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance in weeds.

In summary, information collected during field trials and laboratory analyses presented herein demonstrate that DAS-40278-9 corn exhibits no plant pathogenic properties or weediness characteristics. DAS-40278-9 corn is no more likely to become a plant pest than conventional corn, and the AAD-1 protein is unlikely to increase the weediness potential of any other cultivated plant or wild species.

DAS hereby requests a determination from APHIS that herbicide-tolerant DAS-40278-9 corn and all progeny derived from the conventional breeding of this line no longer be considered regulated articles under 7 CFR Part 340.

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Acronyms and Scientific Terms

2,4-D	2,4-Dichlorophenoxyacetic acid
DAS-40278-9	Corn line containing event DAS-40278-9
AAD-1	Aryloxyalkanoate Dioxygenase-1 protein
aad-1	Gene from *Sphingobium herbicidovorans* which encodes the AAD-1 protein
ACCase	Acetyl coenzyme A carboxylase
ADF	Acid detergent fiber
ae	Acid equivalent
ai	Active ingredient
ALS	Acetolactate synthase
ANOVA	Analysis of variance
AOPP	Aryloxyphenoxypropionate
APHIS	Animal and Plant Health Inspection Service, USDA
bp	Base pair
bu	Bushel
CFIA	Canadian Food Inspection Agency
CFSAN	Center for Food Safety and Nutrition, US FDA
CHD	Cyclohexanedinone
DAS	Dow AgroSciences LLC
DCP	2,4-Dichlorophenol
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (US)
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ESA	Endangered Species Act
Event DAS-40278-9	OECD identifier for the corn event expressing the AAD-1 protein
FDA	Food and Drug Administration (US)
FDR	False Discovery Rate
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FWS	Fish and Wildlife Service
ha	Hectare
Hi-II	Publicly available corn line used in transformation to produce for event DAS-40278-9
IWM	Integrated weed management
Kb	Kilobase pair
kDa	Kilodalton, a measurement of protein molecular weight
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
NDF	Neutral detergent fiber
OECD	Organisation for Economic Co-operation and Development
PBN	US FDA Pre-market Biotechnology Notice

pDAS1740	DNA vector carrying the transgene (*aad-1*) for insertion into the plant genome; also known as pDAB3812
pDAS1740/*Fsp* I	DNA vector restriction fragment used for Whiskers transformation
Pf	*Pseudomonas fluorescens*
PTU	Plant transcriptional unit consisting of promoter, gene, and termination sequences
RB7 MAR v3	Matrix attachment region (MAR) from *Nicotiana tabacum*
RB7 MAR v4	Matrix attachment region (MAR) from *Nicotiana tabacum*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
spp	species
subsp	subspecies
USDA	United States Department of Agriculture
ZmPer5 3' UTR	3' untranslated region from *Zea mays* peroxidase gene
ZmUbi1	Ubiquitin promoter from *Zea mays*

I. Rationale for the Development of DAS-40278-9 Corn

I.A. Basis for the Request for Nonregulated Status

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the United States. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR 340.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Dow AgroSciences LLC is submitting data for the genetically engineered herbicide-tolerant DAS-40278-9 corn and requests a determination from APHIS that event DAS-40278-9 and all progeny derived thereof no longer be considered regulated articles under 7 CFR 340.

I.B. Benefits of DAS-40278-9 Corn

Dow AgroSciences LLC (herein referred to as "DAS") has developed transgenic corn plants that are tolerant to phenoxy auxin herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (AOPP) acetyl coenzyme A carboxylase (ACCase) inhibitors ("fop" herbicides). Event DAS-40278-9 is the unique identifier for DAS-40278-9 corn in accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2004).

DAS-40278-9 was developed using direct *Whiskers*-mediated transformation to stably incorporate the *aad-1* gene from *Sphingobium herbicidovorans* into corn. The *aad-1* gene encodes the aryloxyalkanoate dioxygenase (AAD-1) enzyme which, when expressed in plants, degrades 2,4-D into herbicidally-inactive 2,4-dichlorophenol (DCP). Additionally, plants expressing AAD-1 have been demonstrated to convert certain AOPP herbicides (quizalofop, cyhalofop, haloxyfop, etc.) into their corresponding inactive phenols (Wright *et al.*, 2009). The availability of DAS-40278-9 corn is expected to have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The availability of DAS-40278-9 corn will allow growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance in weeds.

With the introduction of genetically engineered, glyphosate-tolerant crops in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds that was unparalleled in agriculture. Consequently, producers were quick to adopt glyphosate-tolerant crops, and in many instances, abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, and incorporation of mechanical with chemical and cultural weed control. Currently glyphosate-tolerant soybean, cotton, corn, sugar beets, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More glyphosate-tolerant crops (*e.g.*, wheat, rice, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate-tolerant species are in experimental or development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias) (USDA APHIS, 2009). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate-tolerant crops systems (Wright *et al.*, 2009).

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts) (Heap, 2009). Although glyphosate has been widely used globally for more than 30 years, only a handful of weeds have been reported to have developed resistance to glyphosate; however, most of these have been identified in the past 5-8 years. Resistant weeds in the U.S. include both grass and broadleaf species—*Lolium rigidum* (Rigid ryegrass), *Lolium multiflorum* (Italian ryegrass), *Sorghum halapense* (Johnsongrass), *Amaranthus palmeri* (Palmer amaranth), *Amaranthus rudis* (Common waterhemp), *Ambrosia artemisiifolia* (Common ragweed), *Ambrosia trifida* (Giant ragweed), *Conyza canadensis* (Horseweed), and *Conyza bonariensis* (Hairy fleabane). Additionally, weeds that had previously not been an agronomic problem prior to the wide use of glyphosate-tolerant crops are now becoming more prevalent and difficult to control in the context of glyphosate-tolerant crops, which now comprise >90% of U.S. soybean acres and >60% of U.S. corn and cotton acres (USDA ERS 2009). These weed shifts are occurring predominantly, but not exclusively, with difficult-to-control broadleaf weeds. Some examples include *Ipomoea*, *Amaranthus*, *Chenopodium*, *Taraxacum*, and *Commelina* species. See Appendix 6 for more details on herbicide-resistant weeds.

In areas where growers are faced with glyphosate-resistant weeds or a shift to more difficult-to-control weed species, growers can compensate by tank mixing or alternating with other herbicides that will control the surviving weeds. One popular and efficacious tank mix active ingredient for controlling broadleaf escapes has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant weed species have been reported, but 2,4-D remains one of the most widely used herbicides globally. The development of 2,4-D-tolerant corn provides

an excellent option for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications, allowing the grower to focus applications at the critical weed control stages and extending the application window without the need for specialized sprayer equipment. Combining the 2,4-D-tolerance trait and a glyphosate-tolerance trait through conventional breeding ("stacking" traits) would give growers the ability to use tank mixes of glyphosate/2,4-D over-the-top of the tolerant plants to control the glyphosate-resistant broadleaf species.

Expression of the AAD-1 protein in corn plants also provides tolerance to AOPP ("fop") herbicides. This allows the use of these herbicides to control grasses in corn, some of which have recently been reported to be glyphosate-resistant (e.g., Johnsongrass). AOPP herbicides, such as quizalofop, are post-emergent herbicides used for the control of annual and perennial grass weeds in crops such as potatoes, soybeans, peanuts, vegetables, cotton, flax and others. In corn plants carrying the *aad-1* gene, fop herbicides can also be used as selection agents in breeding nurseries and hybrid production fields to select herbicide-tolerant plants to maintain seed trait purity.

In summary, the commercial introduction of transgenic corn exhibiting tolerance to aryloxyalkanoate herbicides will provide growers the ability to safely use a broad spectrum herbicide like 2,4-D in corn. This new weed management tool will allow for the improved control of key broadleaf weeds which can affect the vigor and yield of the crop, allow an increased herbicide application window for effective weed control, and provide an effective resistance management/prevention solution to the increased incidence of glyphosate- and acetolactate synthase (ALS)-tolerant weeds. Furthermore, the added tolerance to AOPP herbicides like quizalofop will enable corn breeders the use of quizalofop herbicide as a selection agent in breeding nurseries, and allow the use of a graminicide for the improved control of weedy grass species in corn.

I.C. Submissions to Other Regulatory Agencies

AAD-1 corn event DAS-40278-9 falls within the scope of the FDA policy statement, published in the Federal Register on May 29, 1992, concerning regulation of products derived from new plant varieties, including those developed via biotechnology. Dow AgroSciences LLC (DAS) will submit a pre-market biotechnology notification (PBN) to FDA.

As per EPA's authority over the use of pesticidal substances under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), a submission of 2,4-D metabolism and residue data, as well as proposed labeling for the use of 2,4-D over-the-top of DAS-40278-9 corn, will be made to EPA. A similar submission of metabolism and residue data accompanied with proposed labeling will be made for the use of quizalofop applied over-the-top in corn.

DAS intends to submit dossiers to the regulatory authorities of trade partners for import clearance and production approval which may include Canada, Japan, Korea, Taiwan, European Union, Australia/New Zealand, South Africa, Brazil, Argentina and Mexico in 2009-2010.

I.D. References

Heap, I. 2009. The International Survey of Herbicide Resistant Weeds. http://www.weedscience.com

OECD 2004. Organisation for Economic Co-operation and Development. Series on Harmonization of Regulatory Oversight in Biotechnology, No. 23, OECD Guidance for the Designation of a Unique Identifier for Transgenic Plants. ENV/JM/MONO(2002)7. 12pp. http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/env-jm-mono(2002)7

USDA APHIS 2009. Field Test Release Applications in the U.S. Accessed June 17, 2009. http://www.isb.vt.edu/cfdocs/fieldtests1.cfm

USDA ERS 2009. USDA Economic Research Service Data Sets – Adoption of Genetically Engineered Crops in the U.S. http://www.ers.usda.gov/Data/BiotechCrops/

Wright, T.R., Lira, J.M., Merlo, D.J. and Hopkins, N. 2009. Novel Herbicide Resistance Genes. U.S. Patent # 2009/0093366.

II. The Biology of Corn

II.A. Overview of Corn Biology

Refer to the OECD Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize), 2003, for the following aspects of corn biology:

- general description, including the uses of corn as a crop plant;
- taxonomy;
- methods for identification of *Zea mays* races and wild species;
- center of origin and diversity;
- reproductive biology;
- crosses and gene flow;
- cultivation, volunteers and weediness;
- soil ecology;
- interactions with insects.

II.B. Characterization of the Recipient Corn Line

The publicly available corn line, Hi-II, was used as the recipient line for the generation of event DAS-40278-9 corn (Armstrong *et al.*, 1991). Hi-II is a derivative of the A188 and B73 inbred corn lines, which are publicly available lines developed by the University of Minnesota and Iowa State University, respectively. Hi-II is approximately a 50:50 combination of the two lines and was developed to have a higher regeneration potential (from the combination of genes from A188 and B73).

Transformed Hi-II corn plants were subsequently crossed with elite proprietary inbred corn lines to derive the DAS-40278-9 corn lines used in the studies presented here.

II.C. References

Armstrong, C.L., Green, C.E. & Phillips, R.L. 1991. Development and availability of germplasm with High Type II culture formation response. Maize Genetics Cooperation Newsletter 65: 92-93.

OECD. 2003. Organisation for Economic Co-operation and Development. Series on Harmonisation of Regulatory Oversight in Biotechnology, No. 27, Consensus document on the biology of *Zea mays* subsp. *mays* (maize). ENV/JM/MONO(2003)11. 49 pp. www.olis.oecd.org/olis/2003doc.nsf/LinkTo/env-jm-mono(2003)11.

III. Development of DAS-40278-9 Corn

III.A. Description of Transformation Method

The recipient corn line Hi-II was transformed using direct insertion of the DNA fragment from plasmid pDAS1740 via W*hiskers*-mediated transformation (Petolino *et al*., 2003; Petolino and Arnold, 2009). The vector DNA fragment was isolated by digesting the whole plasmid pDAS1740 DNA with the restriction enzyme *Fsp* I which resulted in 5 fragments: a 6236 bp fragment containing the *aad-1* expression cassette, two fragments (1023 bp and 1235 bp respectively) each containing a portion of the ampicillin resistance gene sequence from the plasmid backbone, and two minor fragments (9 bp each) (Figure 1). The two smaller ampicillin resistance gene fragments and the two minor fragments were separated from the larger desired *aad-1* expression cassette fragment via column chromatography. The final transformation fragment was a 6236 bp linear DNA carrying the *aad-1* expression cassette for insertion into the plant genome. The isolated fragment, pDAS1740/*Fsp* I, contained the following elements: RB7 MAR, maize ZmUbi1 promoter, *aad-1* gene, maize ZmPer5 3' UTR, RB7 MAR (Figure 2).

Immature embryos of corn were aseptically removed from the developing caryopsis, callused on semi-solid media, initiated in liquid suspension cultures, cryopreserved, thawed, and re-established as embryogenic suspensions. The re-established suspensions were agitated with pDAS1740/*Fsp* I isolated fragment DNA and silicon carbide whisker fibers to introduce the DNA into the cells. Following three days of growth on non-selective, semi-solid media, the cells were transferred to a medium containing the herbicide *R*-haloxyfop (an AOPP herbicide). The culture medium was selective for those cells expressing the *aad-1* gene. The callus that survived on the herbicide-containing medium proliferated and produced embryogenic tissue which was presumably genetically transformed. Callus samples were taken for molecular analysis to verify the presence of the transgene and the absence of the ampicillin resistance gene from the vector backbone. The embryogenic tissue was then manipulated to regenerate whole transgenic plants which were then transferred to a greenhouse environment. The plants were sprayed with a commercial formulation of the AOPP herbicide quizalofop to confirm herbicide-tolerance. Surviving plants were crossed with proprietary inbred corn lines to obtain T1 seed from the initially transformed T0 plants.

Figure 1 is the plasmid map of pDAS1740 with all the elements identified. Figure 2 is a schematic diagram of the pDAS1740/*Fsp* I used in the *Whiskers*-meditated transformation. Figure 3 outlines the steps used to develop DAS-40278-9 corn. Figure 4 is a breeding diagram for DAS-40278-9 corn with identification of the generations that were used in the various safety assessment studies.

Figure 1. Schematic map of plasmid pDAS1740

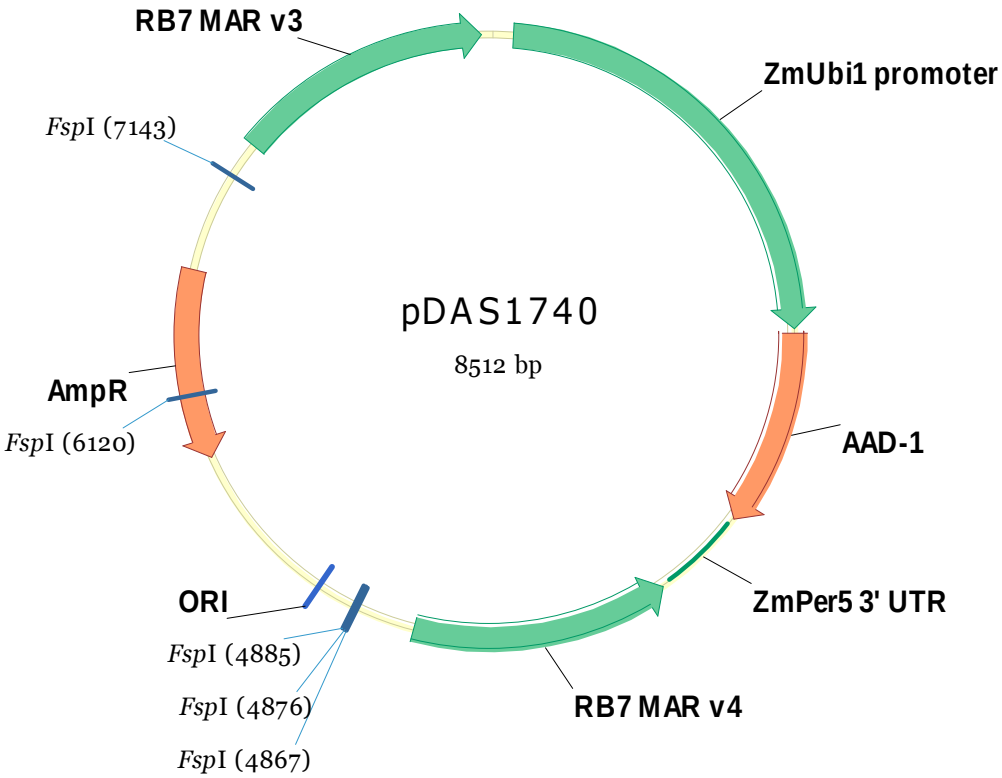


Figure 2. Schematic diagram of the linearized DNA fragment from pDAS1740

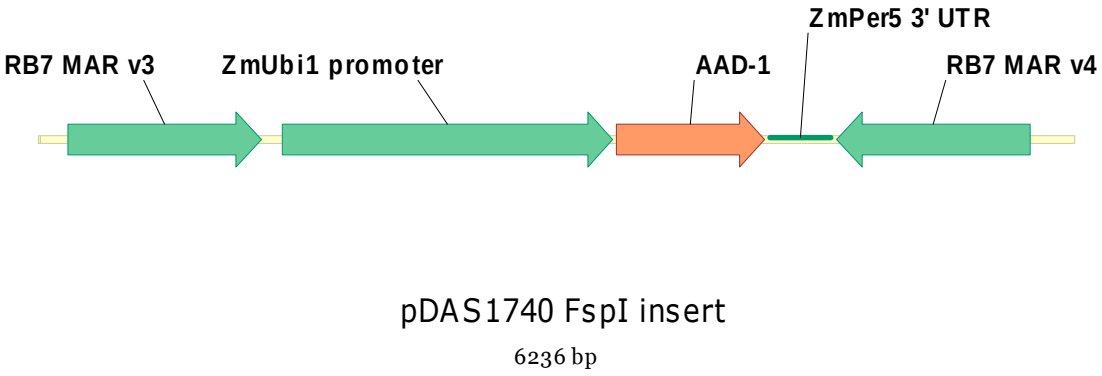


Figure 3. Schematic of the development of DAS-40278-9 corn

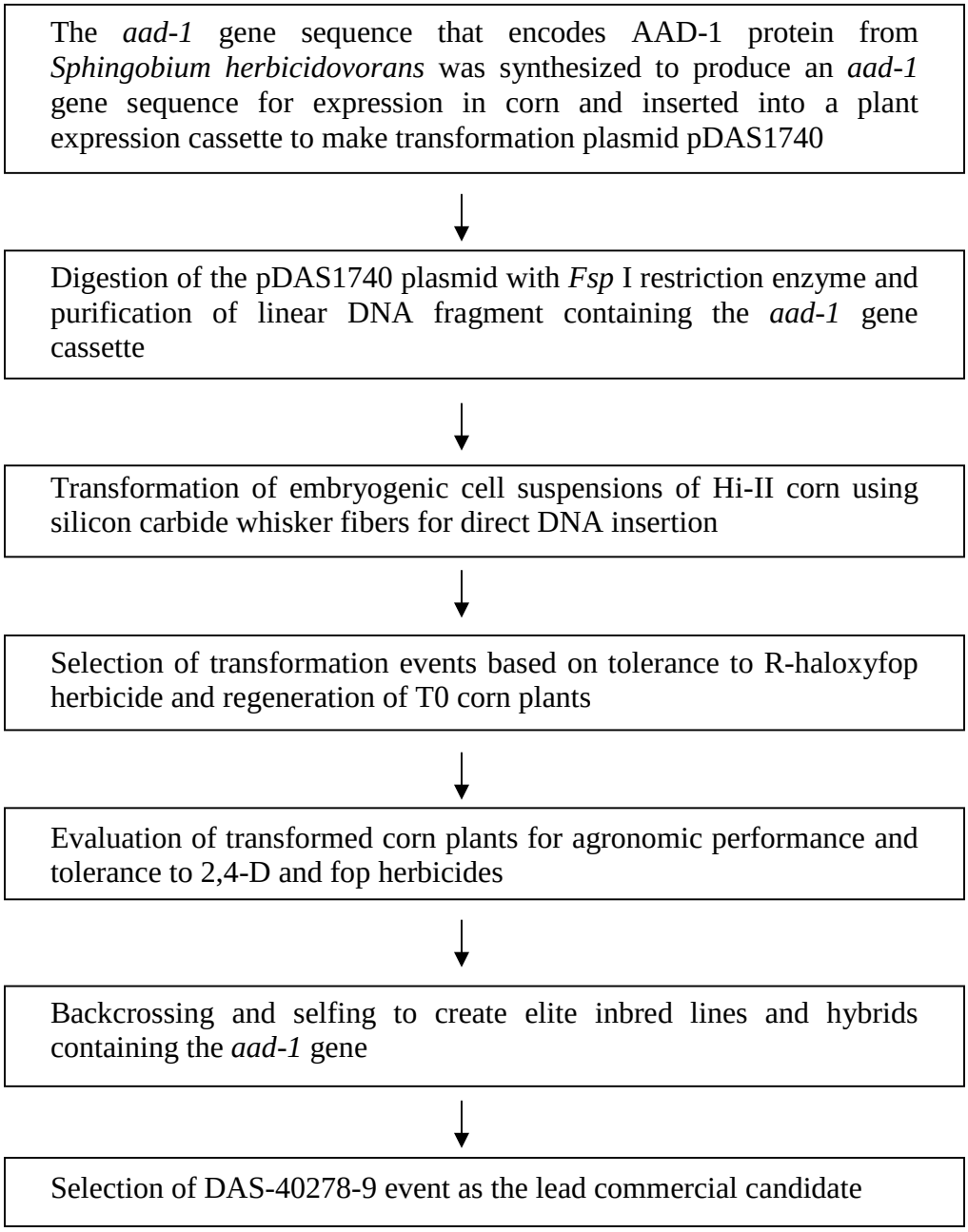
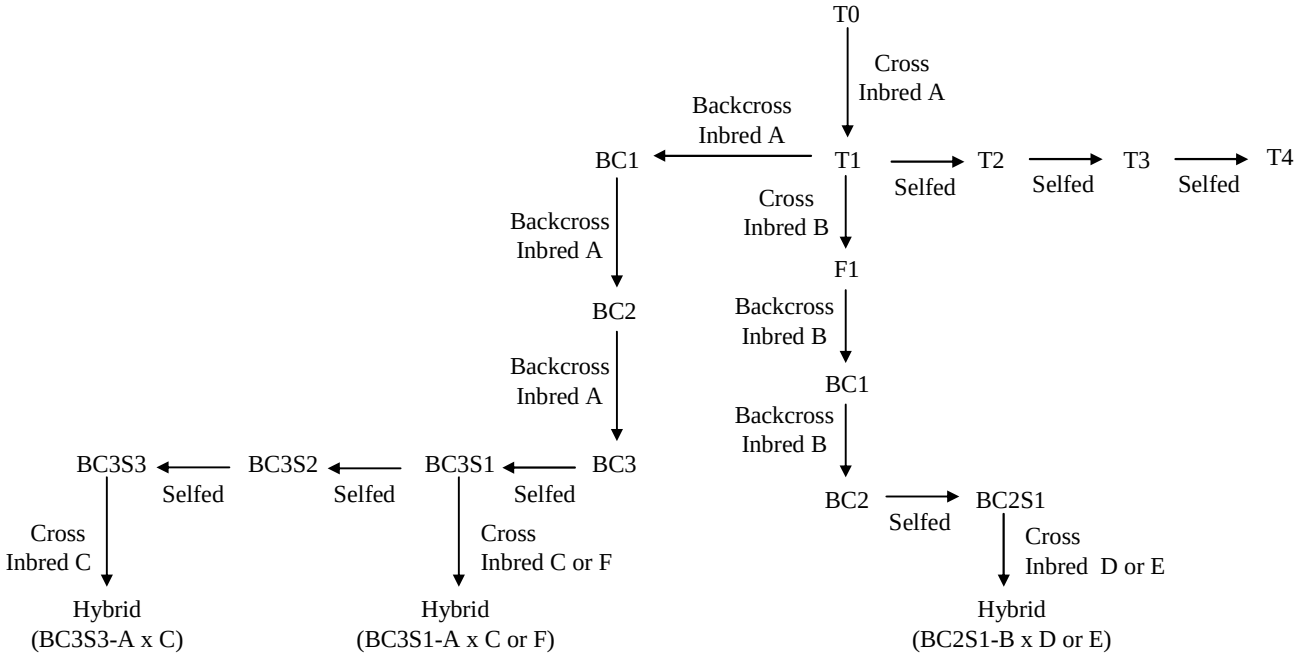


Figure 4. Breeding diagram for DAS-40278-9 corn and generations used for analyses



Inbred A = DAS elite inbred XHH13 Inbred B = DAS elite inbred SLB01 Inbred C, D, E, F = DAS elite inbreds used to make hybrids	BC1 = first backcross with elite parental inbred BC2 = second backcross with elite parental inbred BC3 = third backcross with elite parental inbred
T0 = original transformant T1 = first generation, derived from cross of T0 with elite inbred T2 (T1S1) = derived from self-pollination of T1 T3 (T1S2) = derived from self-pollination of T2 T4 (T1S3) = derived from self-pollination of T3	BC2S1 = derived from self-pollination of BC2 BC3S1 = derived from self-pollination of BC3 BC3S2 = derived from self-pollination of BC3S1 BC3S3 = derived from self-pollination of BC3S2 Hybrid = cross between two elite inbreds

Analysis	Petition Section	Inbred Lineage	DAS-40278-9 Corn Generation	Control
Molecular Analysis	V.A. – V.C.	A	T3, T4, BC3S1, BC3S2, BC3S3	Inbred A
Segregation Analysis	V.D.	A	T1, T2 BC1, BC2, BC3, BC3S1	--
Protein Characterization	VI.C.	A	BC3S1-A x C Hybrid	A x C Hybrid
Protein Expression	VI.D.	A	BC3S1-A x C Hybrid	A x C Hybrid
Agronomics	VII.A.1, VII.A.2	A	BC3S1-A x C Hybrid	A x C Hybrid
	VII.A.2	A	BC3S1-A x F Hybrid	A x F Hybrid
		B	BC2S1-B x D Hybrid	B x D Hybrid
		B	BC2S1-B x E Hybrid	B x E Hybrid
Germination/ Dormancy	VII.C.	A	BC3S3-A x C Hybrid	A x C Hybrid
Composition	VIII.	A	BC3S1-A x C Hybrid	A x C Hybrid
Efficacy	IX.A.1	A	BC3S1-A x C Hybrid	A x C Hybrid
		B	BC2S1-B x D Hybrid	B x D Hybrid

III.B. Selection of Comparators for DAS-40278-9 Corn

Appropriate comparator plants are important to ensure the accurate assessment of the impact of transgene insertion on various characteristics of DAS-40278-9 corn. Control plants should have a genetic background similar to that of DAS-40278-9 corn but lack the transgene insert. All of the studies presented here used near-isolines as the comparators to the specific DAS-40278-9 corn lines being tested. The near-isolines had the same genetic background as the DAS-40278-9 line being tested, but did not contain the DAS-40278-9 event. The breeding chart in Figure 4 shows two lineages of backcrossing of the DAS-40278-9 event to elite inbreds (Inbred A and Inbred B). The comparators used in the studies presented here were the elite inbred lines A or B, either in an inbred or hybrid state, as appropriate to the test material.

III.C. References

Petolino, J.F., Welter M., Cai, C.Q. 2003. Whiskers-mediated transformation of maize. In Molecular Methods of Plant Analysis, Vol. 23, Jackson, J.F. *et al* (eds.), Springer-Verlag, pp 147-158.

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IV. Donor Genes and Regulatory Sequences

IV.A. Identity and Source of Genetic Material in pDAS1740

Event DAS-40278-9 was generated using a linear *Fsp* I fragment from plasmid pDAS1740 (Fig. 2), containing the synthetic, plant-optimized *aad-1* gene from *Sphingobium herbicidovorans*. A summary of the genetic elements is given in Table 1.

Table 1. Genetic elements of the linear *Fsp* I fragment from plasmid pDAS1740

Location on pDAS1740 *Fsp*I fragment	Genetic Element	Size (base pairs)	Description
1-164	Intervening sequence	164 bp	Sequence from pUC19 (Yanisch-Perron *et al.*, 1985)
165-1330	RB7 MAR v3	1166 bp	Matrix attachment region (MAR) from *Nicotiana tobacum* (Hall *et al.*, 1991)
1331-1459	Intervening sequence	129 bp	Sequence used for DNA cloning and sequence from pUC19 (Yanisch-Perron *et al.*, 1985)
1460-3450	ZmUbi1 promoter	1991 bp	Ubiquitin promoter from *Zea mays* (Christensen *et al.*, 1992)
3451-3472	Intervening sequence	22 bp	Sequences used for DNA cloning
3473-4363	*aad-1*	891 bp	Synthetic, plant-optimized version of an aryloxyalkanoate dioxygenase gene from *Sphingobium herbicidovorans* (Wright *et al*., 2009)
4364-4397	Intervening sequence	34 bp	Sequence used for DNA cloning
4398-4762	ZmPer5 3' UTR	365 bp	3’ untranslated region from *Zea mays* peroxidase gene (Ainley *et al.*, 2002)
4763-4801	Intervening sequence	39 bp	Sequence used for DNA cloning
4802-5967	RB7 MAR v4	1166 bp	Matrix attachment region (MAR) from *Nicotiana tobacum* (Hall *et al.*, 1991)
5968-6236	Intervening sequence	269 bp	Sequence from pUC19 (Yanisch-Perron *et al.*, 1985)

The *aad-1* expression cassette contained in the pDAS1740/*Fsp* I fragment is designed to express the plant-optimized aryloxyalkanoate dioxygenase (*aad-1*) gene that encodes the AAD-1 protein. The *aad-1* gene was isolated from *Sphingobium herbicidovorans* and the synthetic version of the gene was optimized to modify the G+C codon bias to a level more typical for plant expression. The insertion of the *aad-1* gene into corn plants confers tolerance to 2,4-D and AOPP ("fop") herbicides. The *aad-1* gene encodes a protein of 296 amino acids that has a molecular weight of approximately 33 kDa.

Sphingobium herbicidovorans, the source organism for the *aad-1* gene, is a gram-negative soil bacterium. As with other soil dwelling bacteria, *Sphingobium herbicidovorans* has evolved over time the ability to use phenoxy auxin and AOPP herbicides as carbon sources for growth, thus affording the bacterium a competitive advantage in soil (Wright *et al*., 2009). *Sphingobium* spp. are commonly isolated from soil and were previously grouped with other sphinogmonads under the genus *Sphingomonas*. Sphingomonads are widely distributed in nature and have been isolated from land and water habitats, as well as from plant root systems, clinical specimens, etc. Due to their biodegradative and biosynthetic capabilities, the sphinogmonads have been used for a wide range of biotechnological applications, including bioremediation of environmental contaminants and production of extracellular polymers such as sphingans which are used extensively in the food industry (Bower *et al*., 2006; Lal *et al*., 2006).

Expression of the *aad-1* gene in the pDAS1740/*Fsp* I expression cassette is controlled by the ZmUbi1 promoter and ZmPer5 termination sequences both from *Zea mays*. The ZmUbi1 promoter has been used in previously deregulated products (USDA, 2001; USDA, 2005) and is known to drive constitutive expression of the genes it controls (Christensen and Quail, 1996).

Matrix attachment regions (MARs) from *Nicotiana tabacum* were included in the expression cassette on both flanking ends of the *aad-1* PTU (plant transcriptional unit; includes promoter, gene, and terminator sequences) to potentially increase expression of the *aad-1* gene in the plant. Matrix attachments regions are natural and abundant regions found in genomic DNA that are thought to attach to the matrix or scaffold of the nucleus. When positioned on the flanking ends of gene cassettes, some MARs have been shown to increase expression of transgenes and to reduce the incidence of gene silencing (Abranches *et al*., 2005; Han *et al.*, 1997; Verma *et al.*, 2005). It is hypothesized that MARs may act to buffer effects from neighboring chromosomal sequences that could destabilize the expression of genes (Allen *et al*., 2000). MARs were included in the pDAS1740 to potentially increase the consistency of *aad-1* expression in transgenic plants.

The genetic elements were assembled in pDAS1740 using standard cloning techniques. No novel open reading frames (>30 amino acids) were introduced in the expression cassette other than the *aad-1* gene sequence.

IV.B. References

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V. Genetic Characterization of Event DAS-40278-9

V.A. Overview of Molecular Analysis

Molecular characterization of event DAS-40278-9 was conducted by Southern blot analyses. The results demonstrate that the transgene insert in corn event DAS-40278-9 occurred as a simple integration of a single, intact copy of the *aad-1* expression cassette from plasmid pDAS1740. The event is stably integrated and inherited across and within breeding generations, and no plasmid backbone sequences are present in DAS-40278-9 corn.

Detailed Southern blot analysis was conducted using probes specific to gene, promoter, terminator, and other regulation elements contained in the pDAS1740 transformation plasmid. The probes used and locations of each on the pDAS1740 plasmid are described in Table 2 and shown in Figure 5. The expected and observed fragment sizes with particular digest and probe combinations, based on the known restriction enzyme sites of the pDAS1740 plasmid and pDAS1740/*Fsp* I fragment, are shown in Table 3 and Figures 6 and 7, respectively. The Southern blot analyses described here made use of two types of restriction fragments: a) internal fragments in which known enzyme restriction sites are completely contained within the pDAS1740/*Fsp* I insert and b) border fragments in which a known enzyme site is located within the pDAS1740/*Fsp* I insert and a second site is in the corn genome. Border fragment sizes vary by event because they rely on the DNA sequence of flanking genomic region. Since integration sites are unique for each event, border fragments provide a means to evaluate both copy number of the DNA insertion and to specifically identify the event.

Genomic DNA for Southern blot analysis was prepared from leaf material of individual DAS-40278-9 corn plants from five distinct breeding generations in genetic background XHH13 (see breeding diagram Figure 4). Genomic DNA from leaves of conventional XHH13 corn plants was used as the control material. Plasmid DNA of pDAS1740 added to genomic DNA from the conventional XHH13 control corn served as the positive control for the transgene sequences. Materials and methods used for the Southern analyses are further described in Appendix 1.

Southern blot analysis showed that event DAS-40278-9 contains a single intact copy of the *aad-1* expression cassette integrated at a single locus (Sections V.B.). A restriction map of the insertion has been hypothesized based on the Southern blot analyses of event DAS-40278-9 (Figure 7). The hybridization patterns across five generations of DAS-40278-9 corn (T3, T4, BC3S1, BC3S2, BC3S3) were identical, indicating that the insertion is stably integrated in the corn genome (Section V.C.). No vector backbone sequences were detected in event DAS-40278-9 (Section V.D.). Additionally, the inheritance of DAS-40278-9 corn in segregating generations was investigated using

Southern blot analysis, protein detection, and herbicide screening methods, and all results confirmed the predicted inheritance of the transgene (Section V.E.).

Table 2. Location and length of probes used in Southern blot analysis

Probe	Position on pDAS1740 (bp)	Length (bp)
ZmUbi1 promoter	28-2123	2096
aad-1 gene	2103-3022	920
ZmPer5 terminator	3002-3397	396
RB7 Mar v4	3375-4865	1491
Backbone (OLP4A)	4900-5848	949
Backbone Ap^r gene (OLP4B)	5828-6681	855
Backbone (OLP4C)	6660-7144	485
RB7 Mar v3	7124-8507	1384

Figure 5. Location of probes on pDAS1740 used in Southern blot analysis of DAS-40278-9 corn

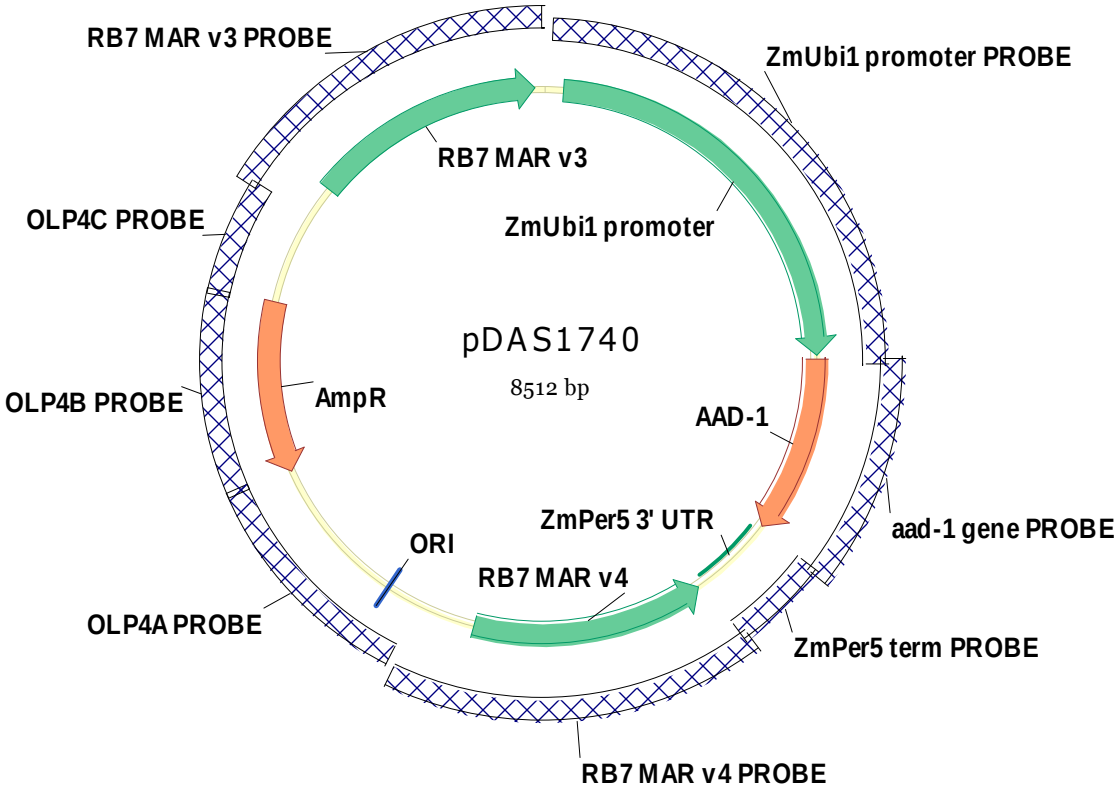


Table 3. Predicted and observed hybridizing fragments in Southern blot analysis

DNA Probe	Restriction Enzymes		Figure	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
aad-1	*Eco*R I	pDAS1740	8	8512	8512
		XHH13	8	none	none
		DAS-40278-9	8	>3382 (border)	~12000
	Nco I	pDAS1740	9	8512	8512
		XHH13	9	none	none
		DAS-40278-9	9	>2764 (border)	~4000
	Sac I	pDAS1740	10	8512	8512
		XHH13	10	none	none
		DAS-40278-9	10	>4389 (border)	~16000
	Fse I / *Hin*d III	pDAS1740	11	3361	3361
		XHH13	11	none	none
		DAS-40278-9	11	3361	3361
ZmUbi1 prom.	*Nco* I	pDAS1740	12	8512	8512, ~3600*
		XHH13	12	none	~3600*
		DAS-40278-9	12	>3472 (border)	~6300, ~3600*
	Sac I	pDAS1740	13	8512	8512, ~3800*
		XHH13	13	none	~3800*
		DAS-40278-9	13	>4389 (border)	~3800*, ~16000
	Fse I / *Hin*d III	pDAS1740	14	3361	3361, ~6400*
		XHH13	14	none	~6400*
		DAS-40278-9	14	3361	3361, ~6400*#
ZmPer5 term.	*Nco* I	pDAS1740	15	8512	8512, ~3900*
		XHH13	15	none	~3900*
		DAS-40278-9	15	>2764 (border)	~4000, ~3900*
	Sac I	pDAS1740	16	8512	8512, ~9000*
		XHH13	16	none	~9000*
		DAS-40278-9	16	>1847 (border)	~1900, ~9000*
	Fse I / *Hin*d III	pDAS1740	17	3361	3361, ~2100*
		XHH13	17	none	~2100*
		DAS-40278-9	17	3361	3361, ~2100*

Table 3. (cont.) Predicted and observed hybridizing fragments in Southern blot analysis

DNA Probe	Restriction Enzymes		Figures	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
RB7 MAR4	*Nco* I	pDAS1740	18	8512	8512
		XHH13	18	none	none
		DAS-40278-9	18	>2764 (border) >3472 (border)	~4000 ~6300
	Sac I	pDAS1740	19	8512	8512
		XHH13	19	none	none
		DAS-40278-9	19	>1847 (border) >4389 (border)	~1900 ~16000
RB7 MAR3	*Nco* I	pDAS1740	20	8512	8512
		XHH13	20	none	none
		DAS-40278-9	20	>2764 (border) >3472 (border)	~4000 ~6300
	Sac I	pDAS1740	21	8512	8512
		XHH13	21	none	none
		DAS-40278-9	21	>1847 (border) >4389 (border)	~1900 ~16000
backbone	*Nco* I	pDAS1740	22	8512	8512
		XHH13	22	none	none
		DAS-40278-9	22	none	none
	Sac I	pDAS1740	23	8512	8512
		XHH13	23	none	none
		DAS-40278-9	23	none	none

1. Expected fragment sizes are based on the plasmid map of the pDAS1740 as shown in Figure 6.
2. Observed fragment sizes are considered approximate 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.

* An asterisk after the observed fragment size indicates endogenous sequence hybridization that was detected across all samples (including negative controls).

\# Doublets in the conventional control, BC3S1, and some BC3S2 samples.

Figure 6. Plasmid map of pDAS1740 with restriction enzyme sites used for Southern blot analysis

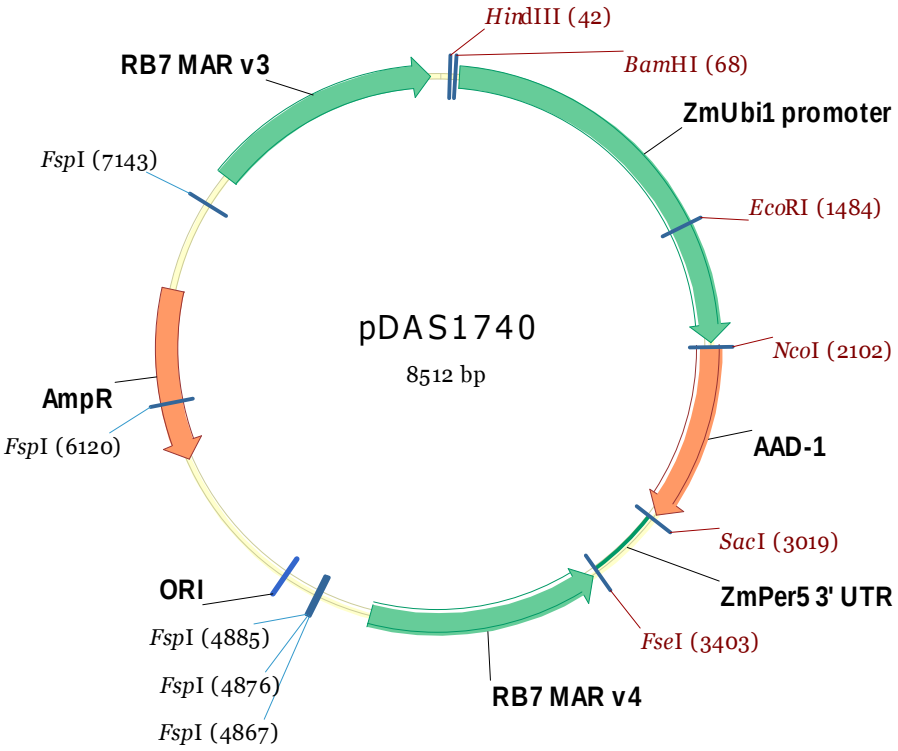
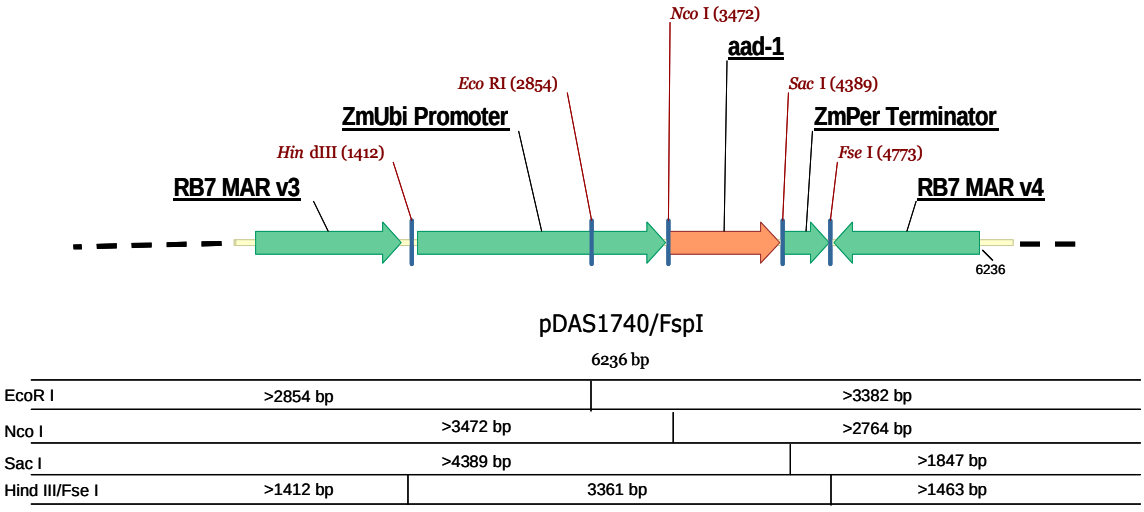


Figure 7. Restriction map of the DAS-40278-9 insertion site



V.B. Analysis of the Insert and Its Genetic Elements

V.B.1. Analysis of the *aad-1* Gene

To characterize the *aad-1* gene insert in event DAS-40278-9, restriction enzymes *Eco*R I, *Nco* I, *Sac* I, and *Fse* I/*Hin*d III were used. These enzymes possessed unique restriction sites in the pDAS1740/*Fsp* I insert. Border fragments of >3382 bp, >2764 bp, >4389 bp were predicted to hybridize with the *aad-1* gene probe following digestion with *Eco*R I, *Nco* I, and *Sac* I enzymes respectively (Table 3). The results showed single hybridization bands of ~12000 bp, ~4000 bp and ~16000 bp respectively when *Eco*R I , *Nco* I and *Sac* I enzymes were used, indicating a single site of *aad-1* gene insertion in the corn genome of event DAS-40278-9 (Figures 8, 9, 10). A double enzyme digestion with *Fse* I and *Hind* III was conducted to release a fragment of 3361 bp which contained the *aad-1* promoter, gene, and terminator sequences. The predicted 3361 bp fragment was observed following the double enzyme digestion (Figure 11). Results obtained from the individual and double enzyme digestions indicated that a single copy of an intact *aad-1* expression cassette from pDAS1740 was inserted into the corn genome of event DAS-40278-9 as shown in the restriction map in Figure 7.

V.B.2. Analysis of the ZmUbi1 Promoter

Restriction enzymes *Nco* I, *Sac* I and *Fse* I/*Hin*d III were used to characterize the ZmUbi1 promoter region for *aad*-1 in event DAS-40278-9. *Nco* I and *Sac* I digests were expected to generate border fragments of >3472 bp and >4389 bp, respectively, when hybridized to the ZmUbi1 promoter DNA probe (Table 3). Two hybridization bands of ~6300 bp and ~3600 bp were detected with ZmUbi1 promoter probe following *Nco* I digestion (Figure 12). The ~3600 bp band, however, was present across all sample lanes including the conventional controls, suggesting that the ~3600 bp band is a non-specific signal resulting from the homologous binding to the corn endogenous *ubi* gene. On the contrary, the ~6300 bp signal band was detected in DAS-40278-9 samples but not in the conventional controls, indicating that the ~6300 bp band is specific to the ZmUbi1 promoter probe from plasmid pDAS1740. Similarly, two hybridization bands of ~3800 bp and ~16000 bp were detected with ZmUbi1 promoter probe following *Sac* I digestion (Figure 13). The ~3800 bp band appeared in all sample lanes including conventional controls and thus is considered as non-specific hybridization to the corn endogenous *ubi* gene. The ~16000 bp hybridization band was only present in DAS-40278-9 samples indicating it is unique to the DAS-40278-9 insert. Double digestion with *Fse* I/ *Hin*d III releases the *aad-1* PTU fragment of 3361 bp. This 3361 bp band and a non-specific hybridization band of ~6400 bp were detected by ZmUbi1 promoter probe following *Fse* I/ *Hin*d III digestion (Figure 14). The ~6400 bp band is considered non-specific binding to the corn endogenous *ubi* gene because this band is present in all sample lanes including the conventional controls. Additionally, another band very close to ~6400 bp

was observed in the conventional control, BC3S1, and some of the BC3S2 samples. This additional band very close to ~6400 bp is also considered non-specific because it is present in the conventional control XHH13 sample lanes and is most likely associated with the genetic background of XHH13. Results obtained with these digestions of the DAS-40278-9 sample followed by ZmUbi1 promoter probe hybridization further confirmed that a single copy of an intact *aad-1* PTU from plasmid pDAS1740 was inserted into the corn genome of event DAS-40278-9.

V.B.3. Analysis of the ZmPer5 3'UTR

The termination sequence for *aad-1*, ZmPer5 3' UTR, was characterized using the restriction enzymes *Nco* I, *Sac* I and *Fse* I/*Hin*d III. The *Nco* I digest was expected to generate a border fragment of >2764 bp when hybridized to the ZmPer5 DNA probe (Table 3). Two hybridization bands of ~4000 bp and ~3900 bp were detected with ZmPer5 terminator probe following *Nco* I digestion (Figure 15). The ~3900 bp band was present across all sample lanes including the conventional controls, suggesting that the ~3900 bp band is a non-specific signal probably due to the homologous binding to the corn endogenous *per* gene. On the contrary, the ~4000 bp signal band was detected in DAS-40278-9 samples but not in the conventional controls, indicating that the ~4000 bp band is specific to the ZmPer5 terminator probe from plasmid pDAS1740. A >1847 bp border fragment was expected to hybridize to the ZmPer5 terminator probe following *Sac* I digestion. Two hybridization bands of ~1900 bp and ~9000 bp were detected (Figure 16), with the ~9000 bp band appearing in all sample lanes including conventional controls and thus considered as non-specific hybridization to the corn endogenous *per* gene. The ~1900 bp hybridization band that was only present in DAS-40278-9 samples is considered the expected *Sac* I ZmPer5 band. The expected 3361 bp band and an additional non-specific hybridization band of ~2100 bp were detected by ZmPer5 terminator probe following *Fse* I/ *Hin*d III digestion (Figure17). The additional ~2100 bp band is the non-specific binding of the ZmPer5 terminator probe to the corn endogenous gene since this band is present in all sample lanes including the negative controls. Results obtained with these digestions of the DAS-40278-9 sample followed by ZmPer5 terminator probe hybridization further confirmed that a single copy of an intact *aad-1* PTU from plasmid pDAS1740 was inserted into the corn genome of event DAS-40278-9.

V.B.4. Analysis of the RB7 MAR Elements

Restriction enzymes, *Nco* I and *Sac* I, were selected to characterize the RB7 MAR elements from the pDAS1740/*Fsp* I fragment in DAS-40278-9 (Table 3). DNA sequences of RB7 MAR v3 and RB7 MAR v4 have over 99.7% identity, therefore DNA probes specific for RB7 MAR v3 or RB7 MAR v4 hybridize to DNA fragments containing either version of the RB7 MAR. Two border fragments of >2764 bp and >3472 bp were expected to hybridize with RB7 MAR v4 and RB7 MAR v3 probes following *Nco* I digestion (Table 3). Two hybridization bands of ~4000 bp and ~6300 bp

were observed with either RB7 MAR v4 (Figure 18) or RB7 MAR v3 (Figure 20) probe after *Nco* I digestion. Similarly, two border fragments of >1847 bp and >4389 bp were predicted with RB7 MAR v4 and RB7 MAR v3 probes following *Sac* I digestion (Table 3). Hybridization bands of ~1900 bp and ~16000 bp were detected in DAS-40278-9 samples with RB7 MAR v4 (Figure 19) or RB7 MAR v3 (Figure 21) probe after *Sac* I digestion. Taken together, the results indicate that the DNA inserted in corn event DAS-40278-9 contains an intact *aad-1* PTU along with the matrix attachment regions RB7 MAR v3 and RB7 MAR v4 at the 5' and 3' ends of the insert, respectively. While Southern blot analysis confirms the presence of RB7 MAR sequences flanking the *aad-1* PTU, these data do not confirm that full-length MAR elements were inserted. The MAR elements were included in the expression cassette to potentially improve consistency of expression, but utility of the elements is unknown and not required for stable expression of the *aad-1* gene.

V.C. Absence of Vector Backbone Sequences

To assess the absence of vector backbone sequences, equal molar ratio combinations of three DNA fragments (Table 2) covering nearly the entire *Fsp* I backbone region of pDAS1740 were used as the backbone probes. The pDAS1740/*Fsp* I fragment was used to generate event DAS-40278-9, therefore, no specific hybridization signal was expected with the backbone probe combination (Table 3). Following digestions with *Nco* I and *Sac* I, no specific hybridization signals were seen in the DAS-40278-9 samples (Figures 22 & 23). The results indicated that the PTU insertion in event DAS-40278-9 did not include any vector backbone sequences.

V.D. Stability of the Insert Across Generations

Southern blot hybridizations were conducted with five distinct generations, T3, T4, BC3S1, BC3S2, and BC3S3, of event DAS-40278-9 (see breeding diagram Figure 4). In some cases the generation used was segregating for the DAS-40278-9 event and therefore, prior to initiation of Southern blot analysis, all plants were tested for AAD-1 protein expression using a rapid test strip kit to allow confirmation of AAD-1 positive plants. All of the genetic element probes; *aad-1* gene, ZmUbi1 promoter, ZmPer5 terminator, RB7 MAR v3, RB7 MAR v4, and the plasmid backbone, were hybridized with the five generations of DAS-40278-9 corn. As described in Sections V.B. and V.C, results across all DAS-40278-9 samples were as expected (Table 3, Figures 8-23), indicating stable inheritance of the intact, single copy insert across multiple generations of DAS-40278-9 corn.

Figure 8. Southern blot analysis of DAS-40278-9; *aad-1* probe, *Eco*R I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Eco*R I and probed with the *aad-1* gene probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

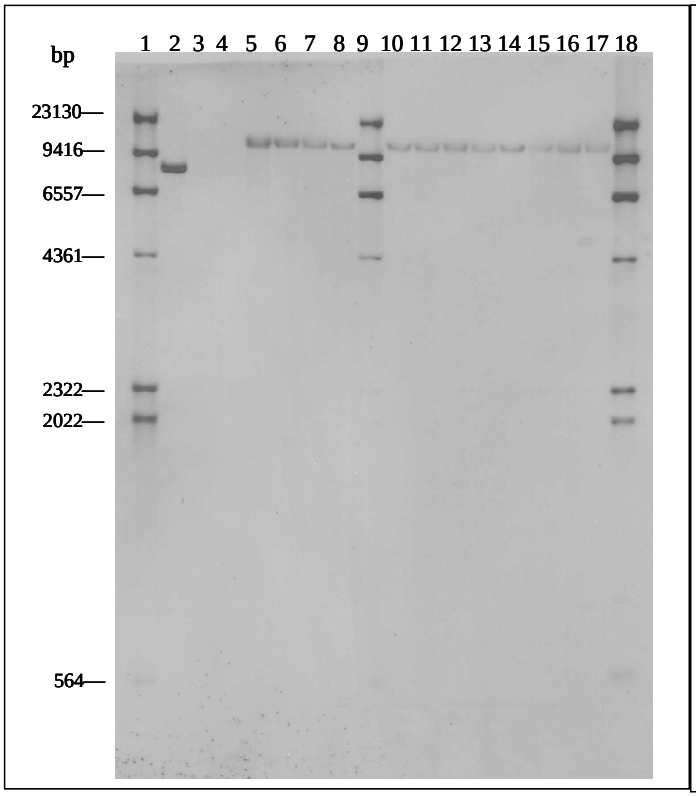
Panel A

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

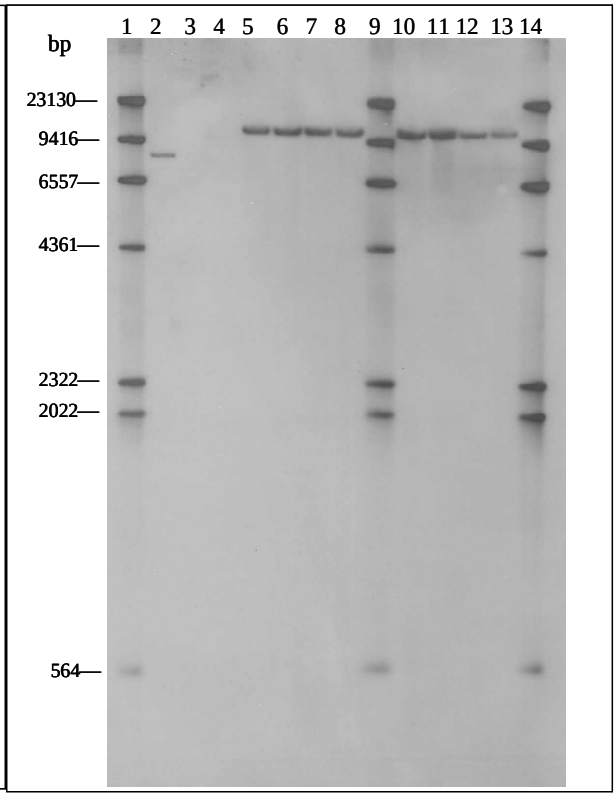


Figure 9. Southern blot analysis of DAS-40278-9; *aad-1* probe, *Nco* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Nco* I and probed with the *aad-1* gene probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

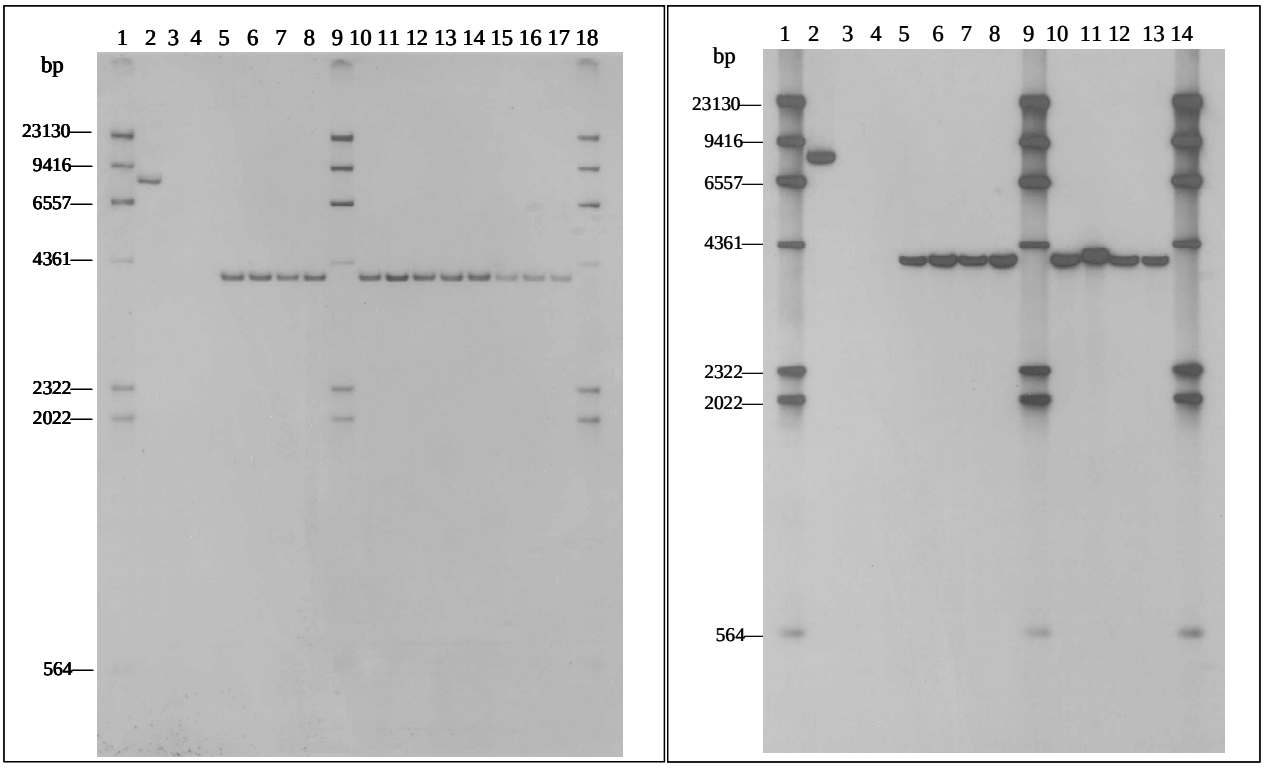


Figure 10. Southern blot analysis of DAS-40278-9; *aad-1* probe, *Sac* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Sac* I and probed with the *aad-1* gene probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

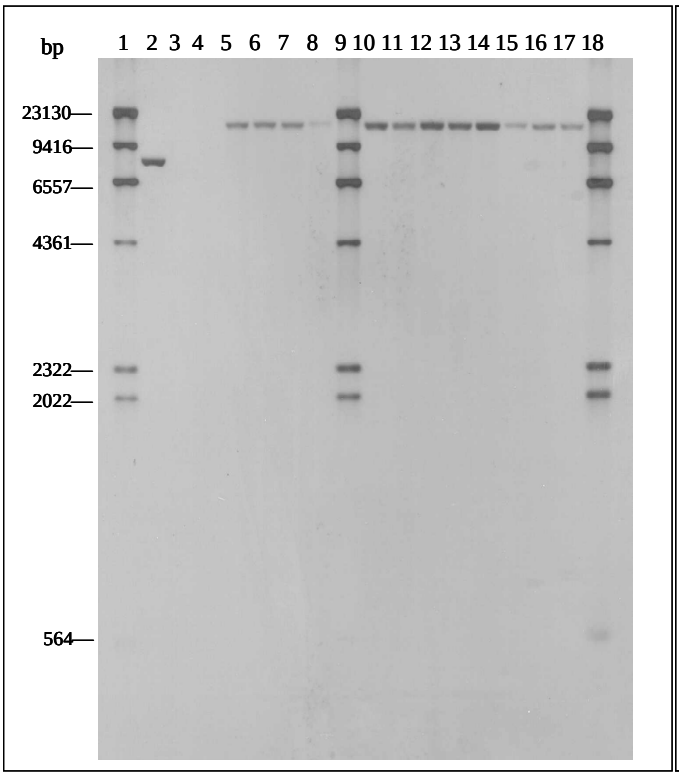
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

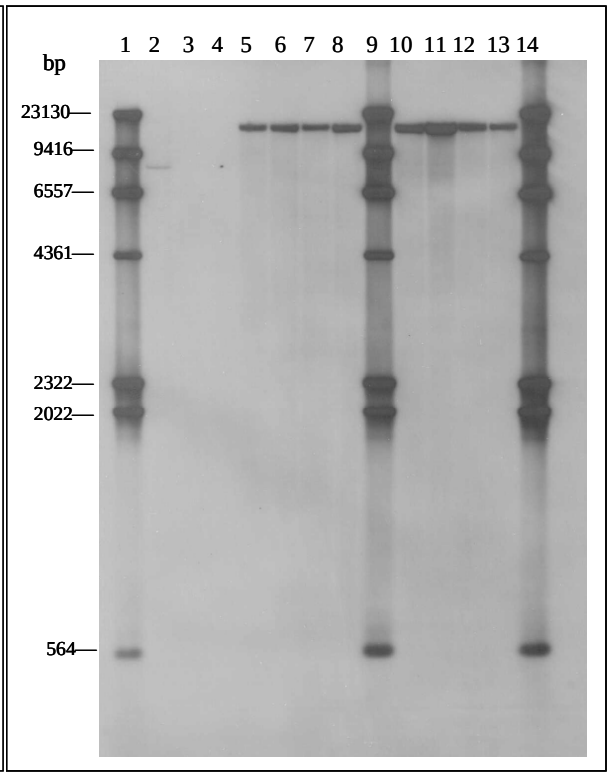


Figure 11. Southern blot analysis of DAS-40278-9; *aad-1* probe, *Fse* I / *Hind* III digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Fse* I / *Hin*d III and probed with the *aad-1* gene probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

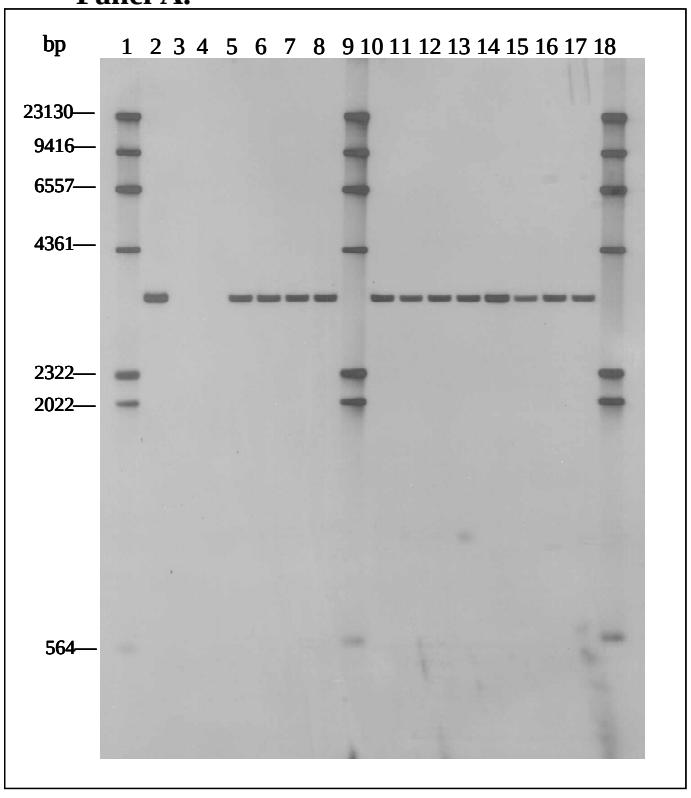
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

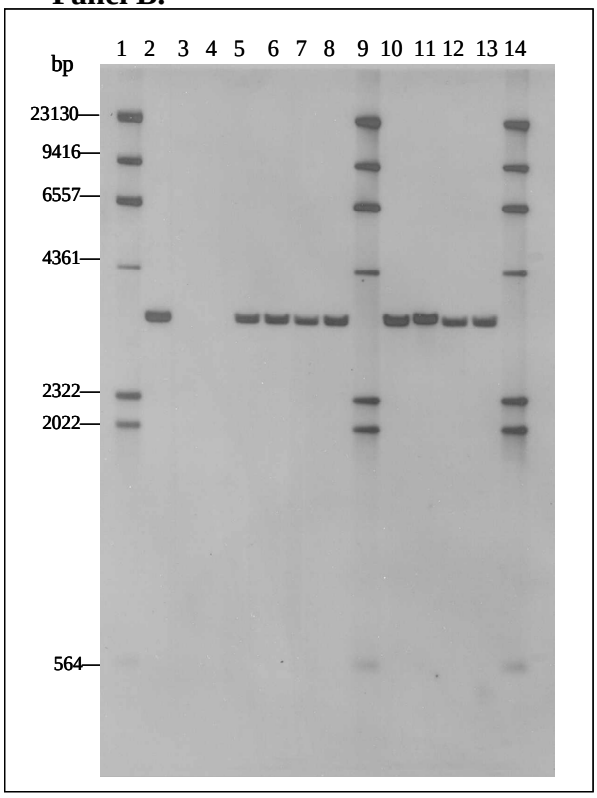


Figure 12. Southern blot analysis of DAS-40278-9; ZmUbi promoter probe, *Nco* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Nco* I and probed with the ZmUbi promoter probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

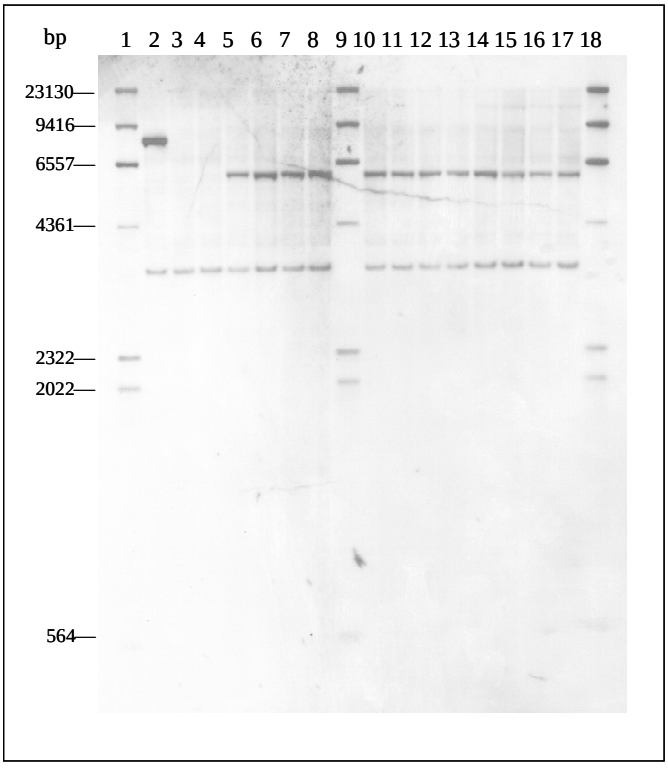
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

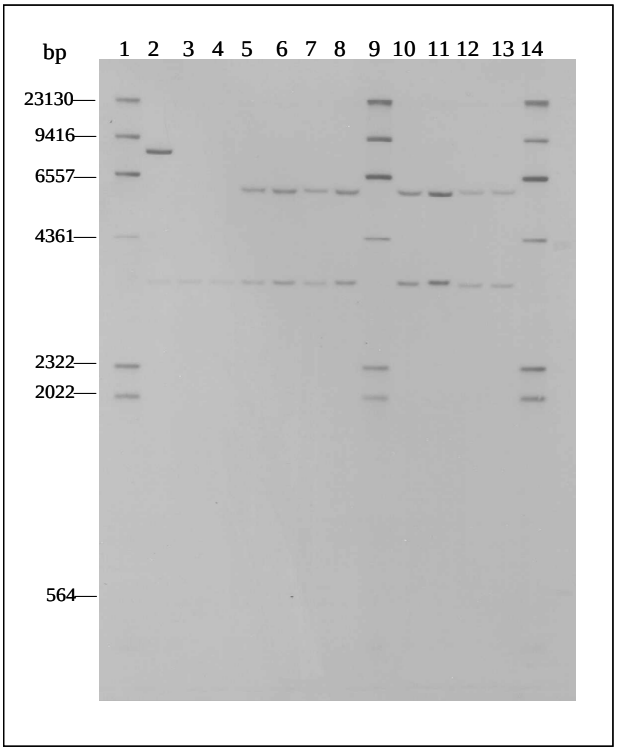


Figure 13. Southern blot analysis of DAS-40278-9; ZmUbi promoter probe, *Sac* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Sac* I and probed with the ZmUbi promoter probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

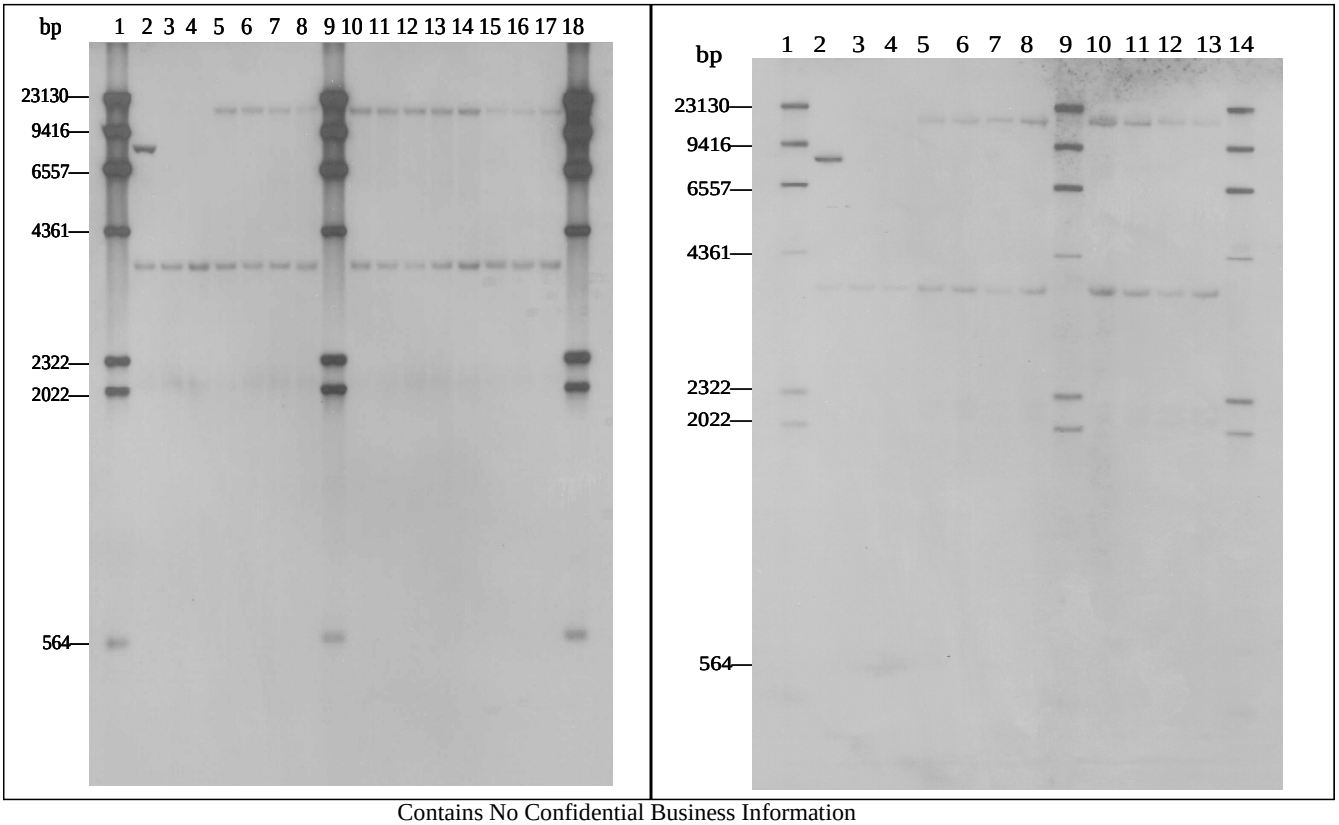


Figure 14. Southern blot analysis of DAS-40278-9; ZmUbi promoter probe, *Fse* I / *Hind* III digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Fse* I / *Hin*d III and probed with the ZmUbi promoter probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used. **Note:** The ZmUbiI probe hybridized to the endogenous *ubi* gene in the corn genome at ~6400bp and to another endogenous band very close to ~6400bp in lanes 2-4 and lanes 14-17 which is most likely from the XHH13 genome.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

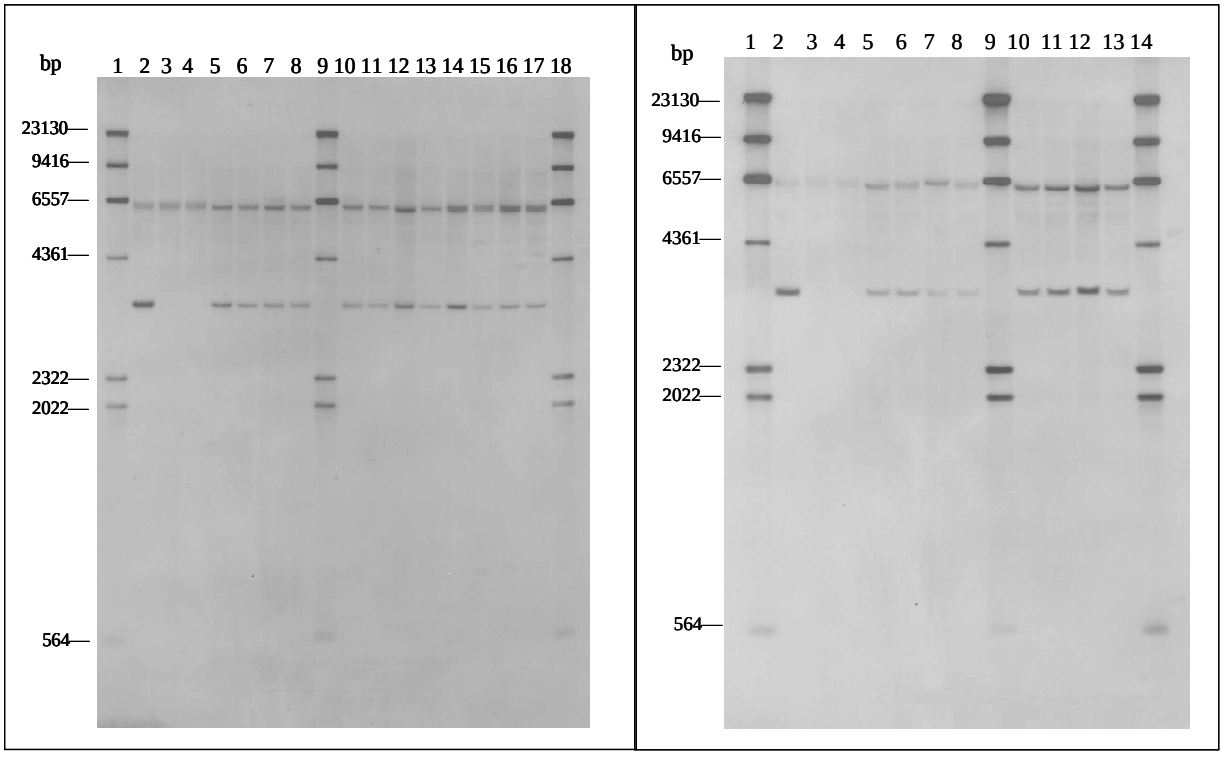


Figure 15. Southern blot analysis of DAS-40278-9; ZmPer terminator probe, *Nco* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Nco* I and probed with the ZmPer terminator probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

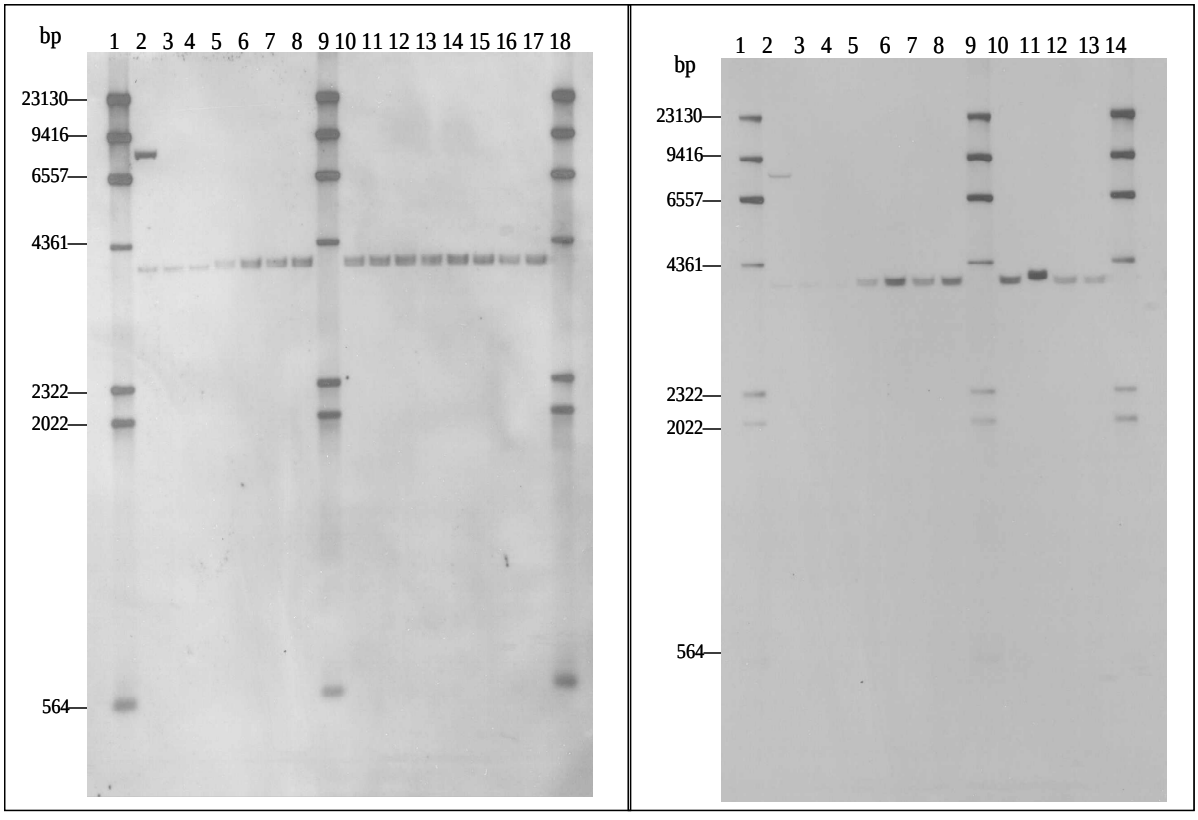


Figure 16. Southern blot analysis of DAS-40278-9; ZmPer terminator probe, *Sac* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Sac* I and probed with the ZmPer terminator probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

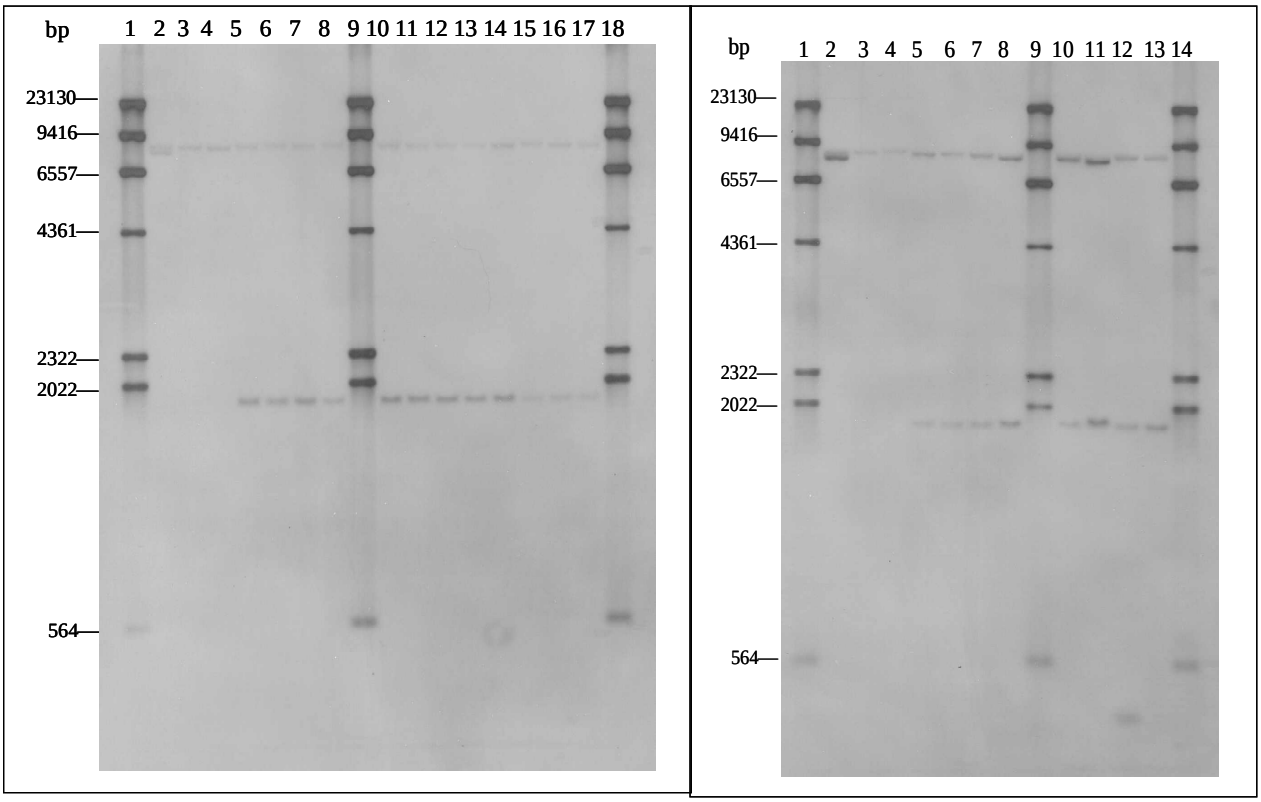


Figure 17. Southern blot analysis of DAS-40278-9; ZmPer terminator probe, *Fse* I / *Hind* III digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Fse* I / *Hin*d III and probed with the ZmPer terminator probe. Nine (9) μg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

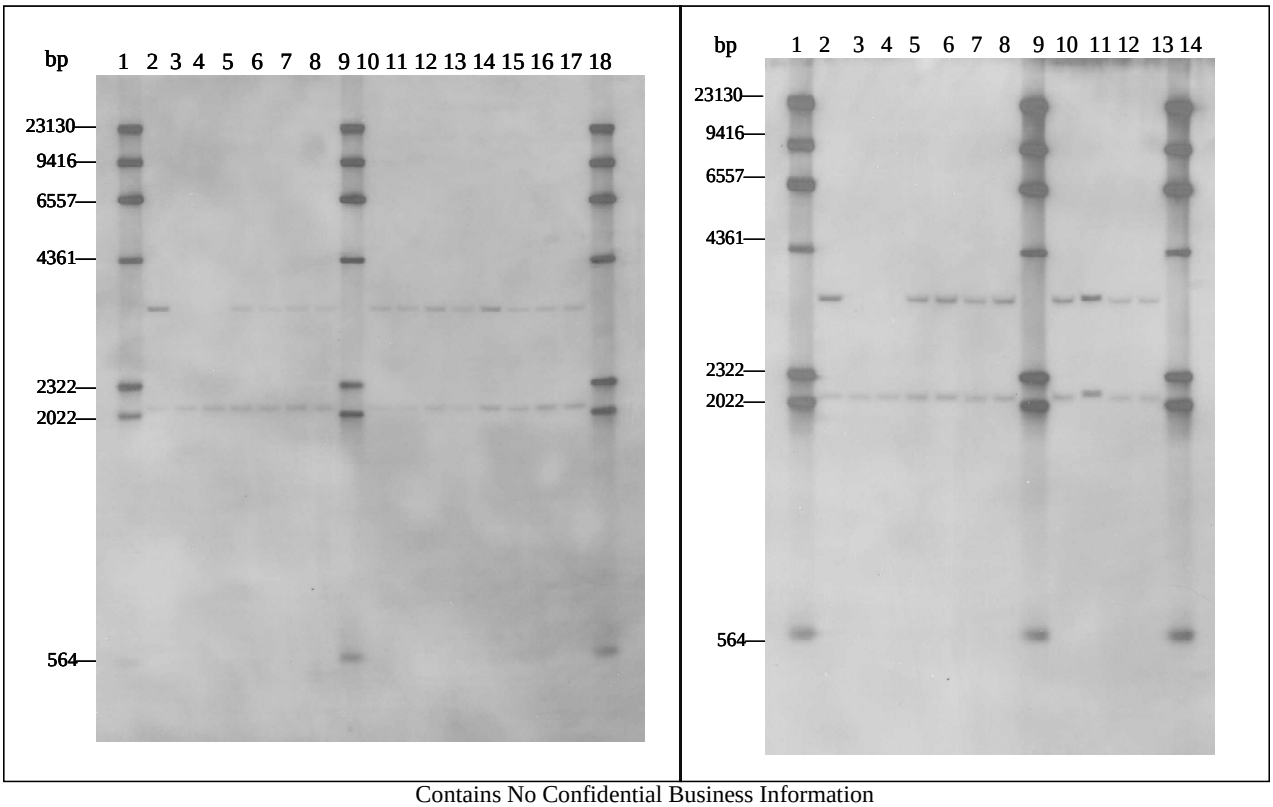


Figure 18. Southern blot analysis of DAS-40278-9; RB7 Mar v4 probe, *Nco* I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Nco* I and probed with the RB7 Mar v4 probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

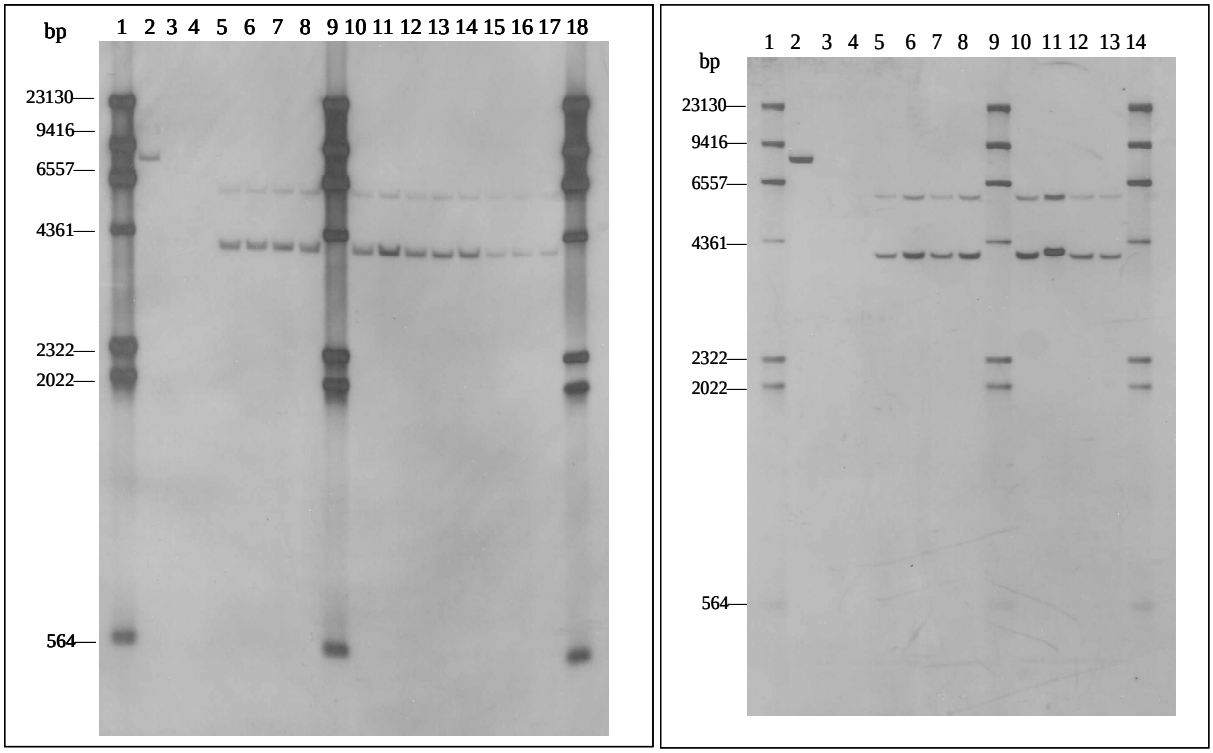


Figure 19. Southern blot analysis of DAS-40278-9; RB7 Mar v4 probe, *Sac* I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Sac* I and probed with the RB7 Mar v4 probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

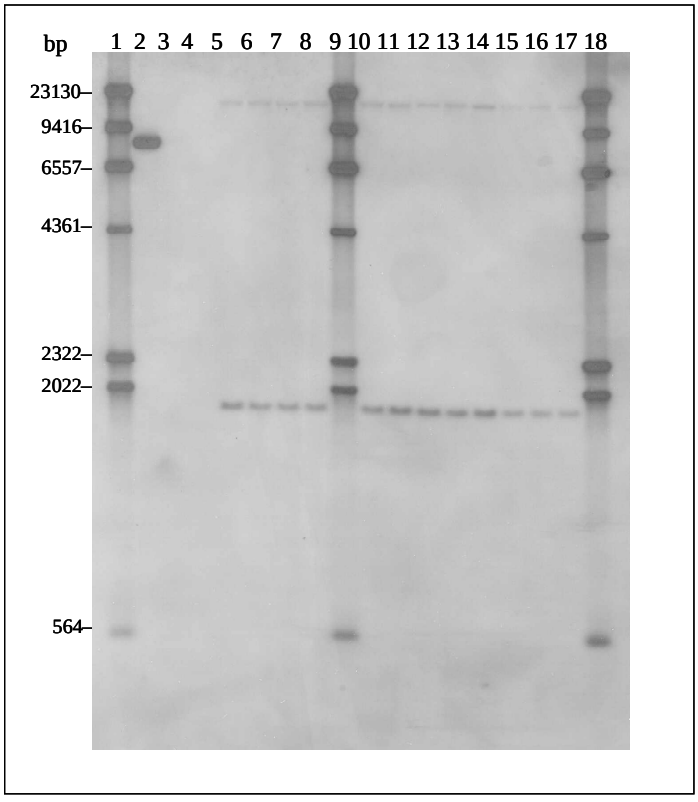
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

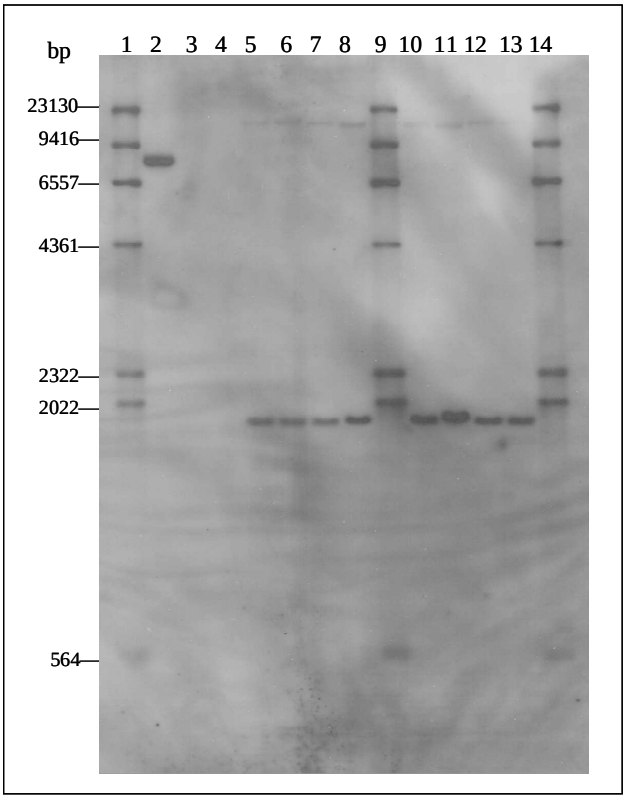


Figure 20. Southern blot analysis of DAS-40278-9; RB7 Mar v3 probe, *Nco* I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Nco* I and probed with the RB7 Mar v3 probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

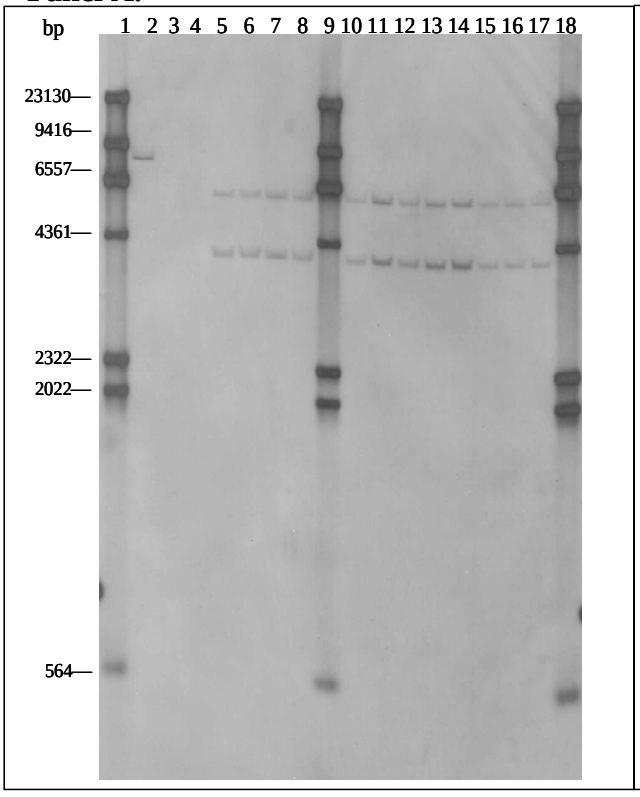
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

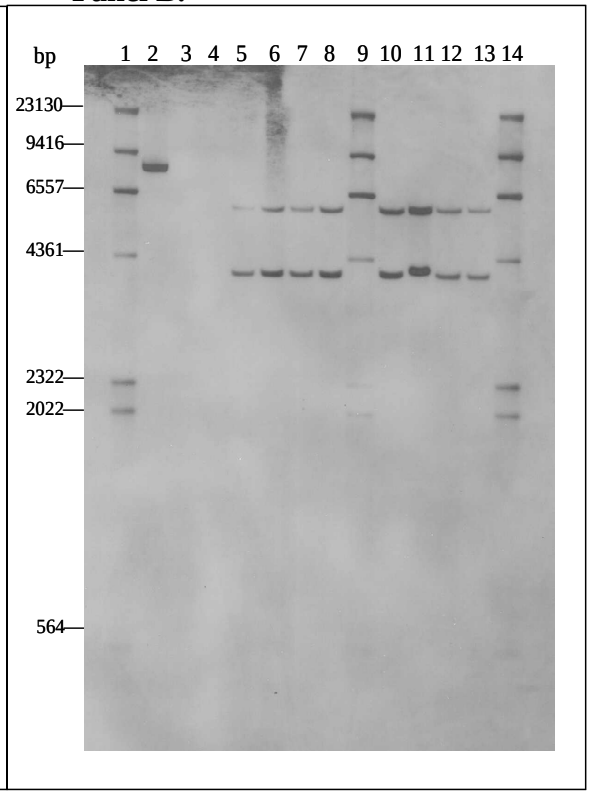


Figure 21. Southern blot analysis of DAS-40278-9; RB7 Mar v3 probe, *Sac* I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Sac* I and probed with the RB7 Mar v3 probe. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

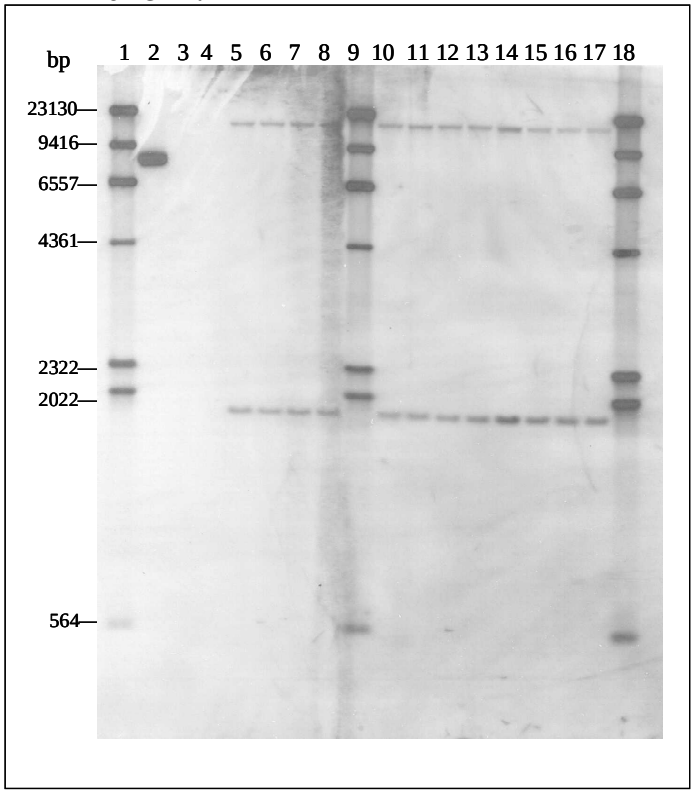
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A.



Panel B.

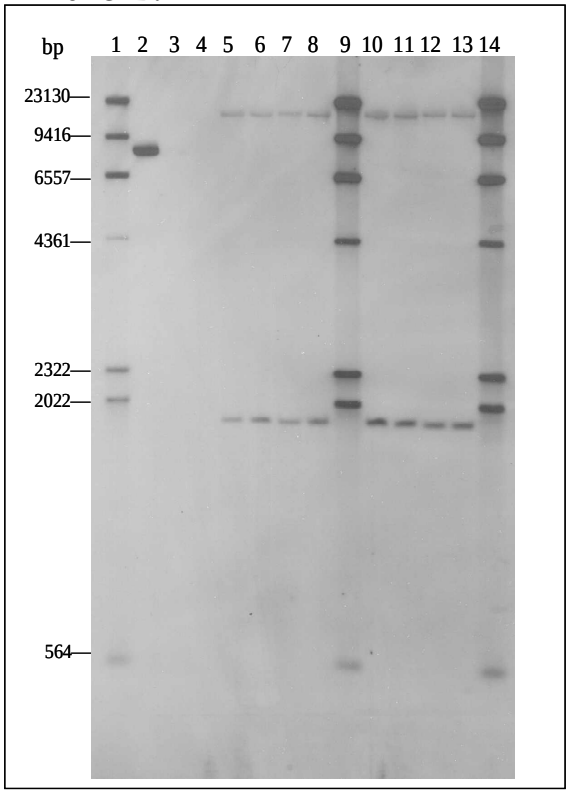


Figure 22. Southern blot analysis of DAS-40278-9; backbone probes, *Nco* I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Nco* I and probed with the backbone probes. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used.

Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-1		11	DAS-40278-9-T4-3
3	XHH13-1		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-4		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**

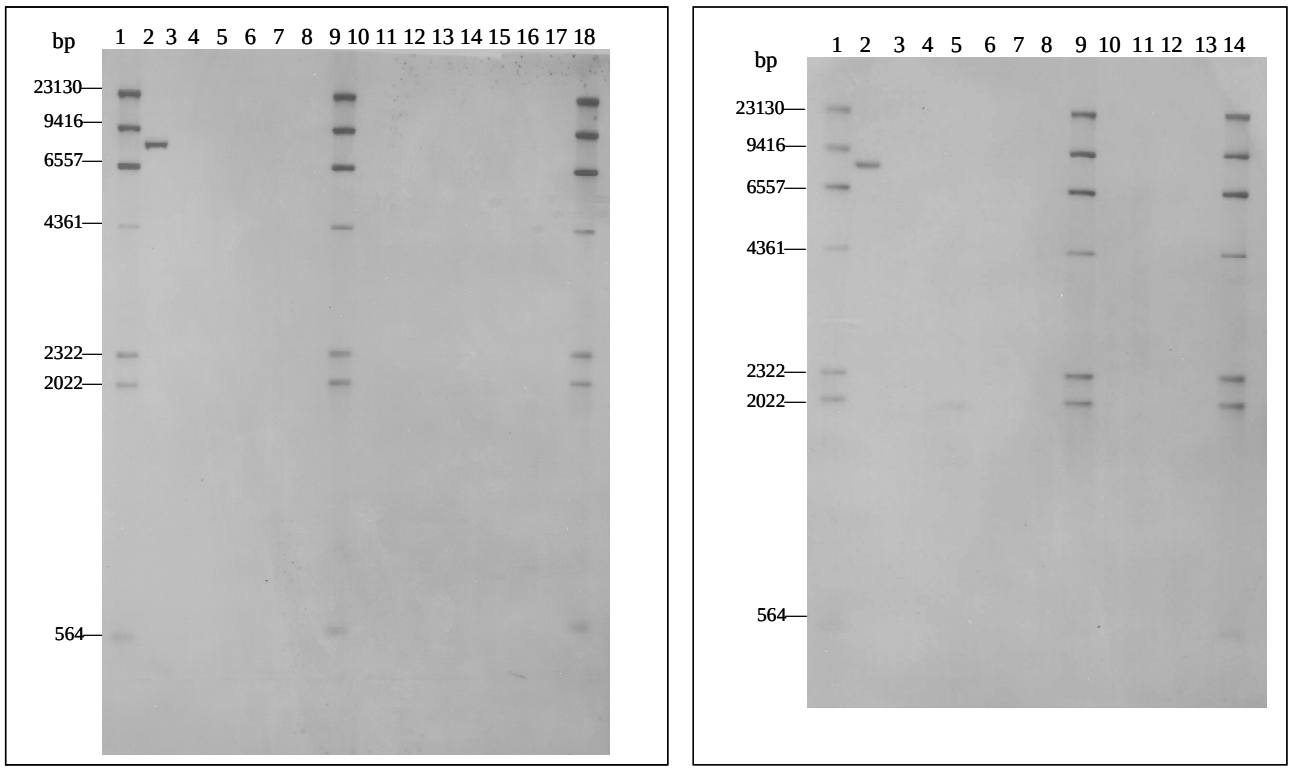


Figure 23. Southern blot analysis of DAS-40278-9; backbone probes, *Sac* I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional corn XHH13 was digested with *Sac* I and probed with the backbone probes. Nine (9) µg of DNA was 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. Sample name indicates test material, generation, and individual plant number used. ***Note**: Background splotches were visible below 2022bp marker between lanes 5 and 6 on Panel B.*

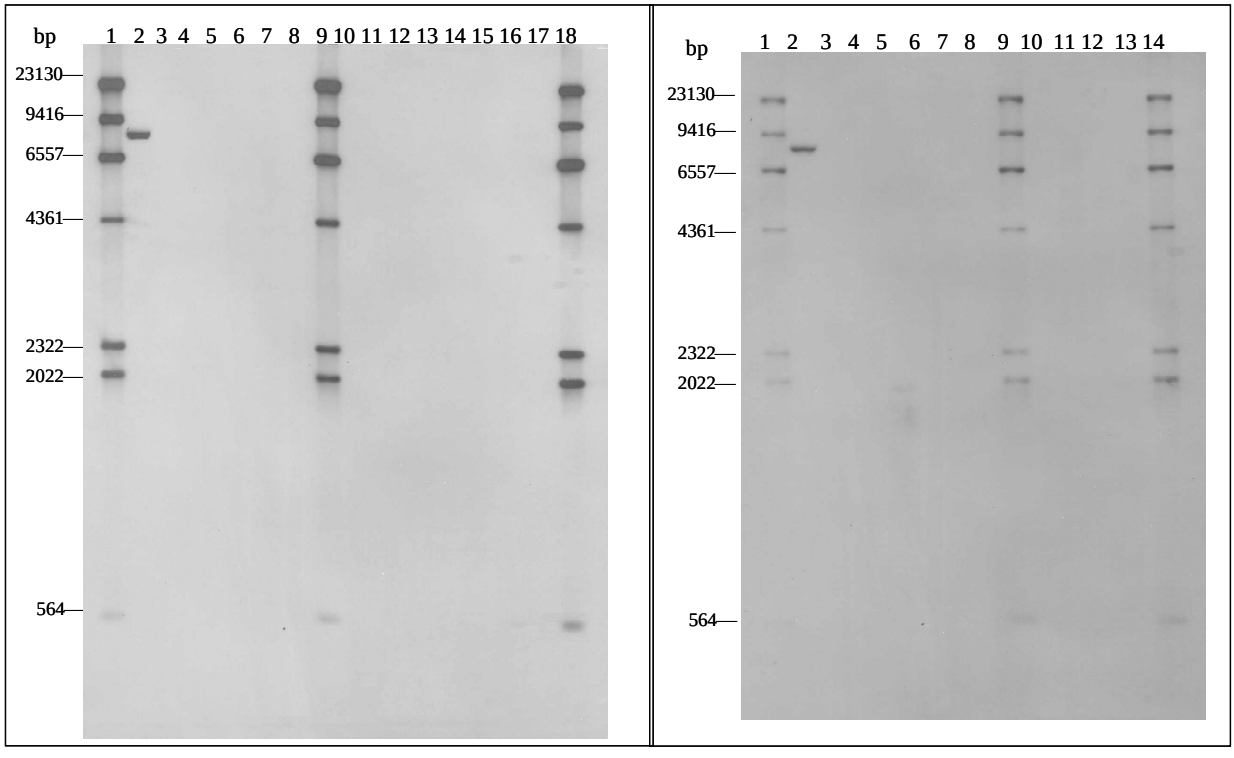
Panel A.

Lane	Sample		Lane	Sample
1	DIG MWM II		10	DAS-40278-9-T4-1
2	pDAS1740 + XHH13-4		11	DAS-40278-9-T4-3
3	XHH13-4		12	DAS-40278-9-T4-5
4	XHH13-2		13	DAS-40278-9-T4-6
5	DAS-40278-9-T3-1		14	DAS-40278-9-BC3S1-6
6	DAS-40278-9-T3-4		15	DAS-40278-9-BC3S1-8
7	DAS-40278-9-T3-13		16	DAS-40278-9-BC3S1-11
8	DAS-40278-9-T3-14		17	DAS-40278-9-BC3S1-12
9	DIG MWM II		18	DIG MWM II

Panel B.

Lane	Sample		Lane	Sample
1	DIG MWM II		8	DAS-40278-9-BC3S2-5
2	pDAS1740 + XHH13-4		9	DIG MWM II
3	XHH13-5		10	DAS-40278-9-BC3S3-2
4	XHH13-2		11	DAS-40278-9-BC3S3-3
5	DAS-40278-9-BC3S2-1		12	DAS-40278-9-BC3S3-5
6	DAS-40278-9-BC3S2-2		13	DAS-40278-9-BC3S3-7
7	DAS-40278-9-BC3S2-3		14	DIG MWM II

Panel A. **Panel B.**



V.E. Segregation Analysis of DAS-40278-9 Corn

V.E.1. Molecular Characterization of a Segregating Generation

The stability of inheritance of the gene insert within a segregating generation was demonstrated with Southern analysis and protein detection of individual plants from a BC3S1 line of DAS-40278-9 corn (Figure 4). Eighty five (85) BC3S1 seeds germinated in the greenhouse were leaf tested for the presence or absence of the AAD-1 protein using an AAD-1 specific lateral flow strip test kit. Of the 85 plants tested, 65 were positive for AAD-1 protein expression and 20 plants were negative.

Similarly, Southern blot analysis was used to determine the genetic equivalence of the inserted DNA among the same 85 BC3S1 individuals. DNA from leaf tissue of individual plants was digested with *Nco* I and hybridized with the *aad-1* probe (Table 2). An ~4000 bp hybridization band was observed in the 65 plant samples that had tested positive for AAD-1 protein (Table 4). A representative Southern blot is presented in Figure 24. The probe hybridized to the same band in each individual plant which indicated that all individual plants contained the same insertion and were equivalent to one another. The 20 null segregant samples did not hybridize with the *aad-1* probe. The Southern analyses confirmed that the *aad-1* gene was present in those corn plants testing positive for the AAD-1 protein and that the gene was absent from the null segregants and the conventional control.

The expected segregation ratio for a BC3S1 generation is 3:1. 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 (Table 5).

Table 4. Predicted and observed hybridizing fragments in BC3S1 Southern blot analysis.

DNA Probe	Restriction Enzymes		Figure	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
aad-1	*Nco* I	pDAS1740	24	8512	8512
		XHH13	24	none	none
		BC3S1*	24	>2764 (border)*	~4000
		BC3S1**	24	None**	none

1. Expected fragment sizes are based on the plasmid map of the pDAS1740 as shown in Figure 6.
2. Observed fragment sizes are considered approximate 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.

* 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.

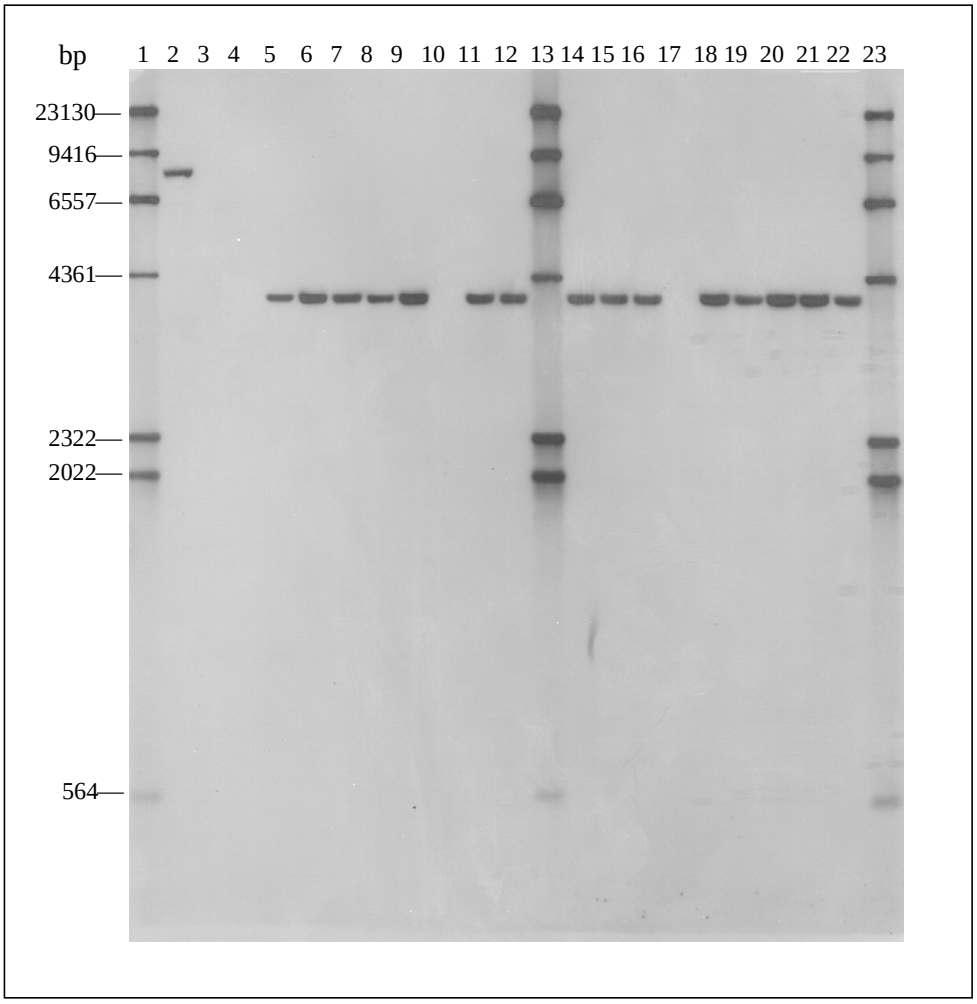
Table 5. Results of BC3S1 individual plant testing for segregation within a generation

Gen	Total plants tested	AAD-1 protein positive*	AAD-1 protein negative	*aad-1* gene positive*	*aad-1* gene negative	Expected ratio	Chi square P-value
BC3S1	85	65	20	65	20	3:1	0.7542

*All plants that tested positive for AAD-1 protein expression were also positive for the presence of the *aad-1* gene insert. All negative plants were negative for both the protein and the gene.

Figure 24. Southern blot analysis of DAS-40278-9; *aad-1* probe, *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. Sample name indicates individual plant number. AAD-1 protein was analyzed by lateral flow strip testing of leaf tissue.

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	DAS-40278-9-BC3S1-11	Positive
3	XHH13-2	Negative		15	DAS-40278-9-BC3S1-12	Positive
4	XHH13-5	Negative		16	DAS-40278-9-BC3S1-13	Positive
5	DAS-40278-9-BC3S1-1	Positive		17	DAS-40278-9-BC3S1-14	Negative
6	DAS-40278-9-BC3S1-2	Positive		18	DAS-40278-9-BC3S1-15	Positive
7	DAS-40278-9-BC3S1-3	Positive		19	DAS-40278-9-BC3S1-16	Positive
8	DAS-40278-9-BC3S1-5	Positive		20	DAS-40278-9-BC3S1-18	Positive
9	DAS-40278-9-BC3S1-6	Positive		21	DAS-40278-9-BC3S1-19	Positive
10	DAS-40278-9-BC3S1-7	Negative		22	DAS-40278-9-BC3S1-20	Positive
11	DAS-40278-9-BC3S1-8	Positive		23	DIG MWM II	N/A
12	DAS-40278-9-BC3S1-10	Positive				



V.E.2. Segregation Analysis of Breeding Generations

The segregation ratios of six distinct breeding generations of DAS-40278-9 (Figure 4) were recorded and analyzed using Chi-square analysis (Table 6). Since DAS-40278-9 should segregate as a single dominate gene, each generation was sprayed with the herbicide quizalofop (560 g ai/ha) to identify herbicide-susceptible plants and allow analysis of the inheritance of the event based on the expected and observed segregation ratios.

Table 6. Segregation ratios of six breeding generations of DAS-40278-9

Generation	Expected Segregation	Number Resistant*	Number Susceptible*	Chi- Square P-Value
T1	1:1	34	28	0.4461
T2	3:1	61	27	0.2184
BC1	1:1	23	21	0.7630
BC2	1:1	80	91	0.4002
BC3	1:1	181	177	0.8326
BC3S1	3:1	761	269	0.4079

*Data expressed as [number of plants expected to be tolerant to quizalofop]:[number of plants expected to be susceptible to quizalofop].

V.F. Summary of the Genetic Characterization

AAD-1 corn event DAS-40278-9 was produced using *Whiskers*-mediated transformation with a linear DNA fragment from the plasmid pDAS1740. The pDAS1740/*Fsp* I fragment consisted of the *aad-1* gene, controlled by the ZmUbi1 promoter and ZmPer5 3' UTR regulatory sequences, flanked on both ends by RB7 MAR elements. Various breeding generations were developed and used to examine the integrity, stability, and inheritance of the *aad-1* transgenic insert in DAS-40278-9.

Molecular characterization of corn event DAS-40278-9 by Southern blot analysis confirmed the insertion of a single intact copy of the *aad-1* expression cassette from the pDAS1740/*Fsp* I transformation fragment. No additional DNA fragments from the *aad-1* expression cassette were identified in DAS-40278-9, and no plasmid backbone sequences were present. DAS-40278-9 was also shown to be stably integrated across five distinct breeding generations (T3, T4, BC3S1, BC3S2, BC3S3) and displayed the expected inheritance patterns in six generations (T1, T2, BC1, BC2, BC3, BC3S1) that were segregating for the DAS-40278-9 event.

VI. Characterization of the AAD-1 Protein

VI.A. Identity of the AAD-1 Protein

The aryloxyalkanoate dioxygenase (AAD-1) protein was derived from *Sphingobium herbicidovorans*, a gram-negative soil bacterium. The *aad-1* transgene in DAS-40278-9 encodes a protein sequence that is identical to the native AAD-1 protein, except for the addition of an alanine residue at the second position. AAD-1 is comprised of 296 amino acids and has a molecular weight of 33 kDa (Figure 25).

Figure 25. Amino acid sequence of the AAD-1 protein

```
001 MAHAALSPLS QRFERIAVQP LTGVLGAEIT GVDLREPLDD STWNEILDAF
051 HTYQVIYFPG QAITNEQHIA FSRRFGPVDP VPLLKSIEGY PEVQMIRREA
101 NESGRVIGDD WHTDSTFLDA PPAAVVMRAI DVPEHGGDTG FLSMYTAWET
151 LSPTMQATIE GLNVVHSATR VFGSLYQAQN RRFSNTSVKV MDVDAGDRET
201 VHPLVVTHPG SGRKGLYVNQ VYCQRIEGMT DAESKPLLQF LYEHATRFDF
251 TCRVRWKKDQ VLVWDNLCTM HRAVPDYAGK FRYLTRTTVG GVRPAR
```

VI.B. Biochemical Characterization of the AAD-1 Protein

Large quantities of purified AAD-1 protein are required to perform safety assessment studies. Because it is technically infeasible to extract and purify sufficient amounts of AAD-1 protein from transgenic plants, the protein was microbially produced using *Pseudomonas fluorescens (Pf)*. Characterization tests were done to confirm the equivalency of the AAD-1 protein expressed *in planta* in DAS-40278-9 corn with the *Pf* microbe-derived AAD-1 protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycoprotein detection, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and protein sequencing analysis by tandem MS were used to characterize the biochemical properties of the protein. Using these methods, the AAD-1 protein from *Pf* and the transgenic corn event DAS-40278-9 were shown to be biochemically equivalent, thereby supporting the use of the microbial protein in safety assessment studies.

The methods and results of the biochemical characterization of the DAS-40278-9 plant- and microbe-derived AAD-1 proteins are described in detail in Appendix 2. Briefly, both the plant and *Pf*-derived AAD-1 proteins showed the expected molecular weight of ~33 kDa by SDS-PAGE and were immunoreactive to AAD-1 protein specific antibodies by western blot analysis. There was no evidence of glycosylation of the DAS-40278-9 corn-derived AAD-1 protein. Amino acid sequence was confirmed by enzymatic peptide mass fingerprinting using MALDI-TOF MS and peptide sequence obtained from tandem mass spectrometry. The N-terminal methionine was found to be cleaved from both protein

sources and a small portion (~3%) of the N-terminal peptide of the plant AAD-1 was acetylated after the N-terminal methionine was cleaved. These two co-translational processes, cleavage of the N-terminal methionine residue and N-terminal acetylation, are common modifications that have been found to occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman, 2003).

VI.C. Expression of AAD-1 Protein in Plant Tissues

A field expression study was conducted at locations in the U.S. (USDA notification number 08-021-104n) and Canada in 2008. Six sites (Iowa, Illinois (2 sites), Indiana, Nebraska and Ontario, Canada) were planted with DAS-40278-9 hybrid corn and a near-isoline control (Figure 4). The test sites represented regions of diverse agronomic practices and environmental conditions for corn. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop. Four treatments of the DAS-40278-9 corn (unsprayed, sprayed with 2,4-D, sprayed with quizalofop, or sprayed with both 2,4-D and quizalofop herbicides) were tested. Plant tissues sampled included leaf, root, whole plant, pollen, and grain. Tissues were collected from across the growing season at plant stages V2-4, V9, R1, R4, and R6 (plant stages as described by Ritchie *et al.* (2008) in Table 7). The soluble, extractable AAD-1 protein was measured using a quantitative enzyme-linked immunosorbent assay (ELISA) method. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed in Appendix 3.

Table 7. Vegetative and reproductive stages of a corn plant.
From Ritchie *et al*., 2008

Vegetative Stages		Reproductive Stages	
VE	Emergence	R1	Silking
V1	First leaf	R2	Blister
V2	Second leaf	R3	Milk
V3	Third leaf	R4	Dough
V6	Sixth leaf	R5	Dent
V9	Ninth leaf	R6	Physiological maturity

*This system accurately identifies the stages of a corn plant. However, all plants in a given field will not be in the same stage at the same time. When staging a field of corn, each specific V or R stage is defined only when 50 percent or more of the plants in the field are in or beyond that stage.

A summary of the AAD-1 protein concentrations (averaged across sites) in the various corn matrices is shown in Table 8. Average expression values ranged from 2.87 ng/mg dry weight in R1 stage root to 127 ng/mg in pollen tissue. Expression values were similar for the sprayed treatments as well as for the plots sprayed and unsprayed with 2,4-D and quizalofop herbicides. Expression in pollen was found to be higher than other plant tissues, however the levels of AAD-1 in pollen are in a similar range as the transgenic herbicide tolerance protein in another commercial trait (http://www.aphis.usda.gov/brs/aphisdocs/04_12501p.pdf). No AAD-1 protein was

detected in the control tissues across the six locations, with the exception of one root sample from the IN site. This control sample was strongly positive and was likely the result of a sampling error.

Table 8. Levels of AAD-1 protein measured in DAS-40278-9 Corn across locations

Corn Tissue	Treatment	AAD-1 ng/mg Tissue Dry Weight		
		Mean	Std. Dev.	Range
V2-V4 Leaf	AAD-1 Unsprayed	13.4	8.00	1.98-29.9
	AAD-1 + Quizalofop	13.3	6.89	4.75-24.5
	AAD-1 + 2,4-D	14.2	7.16	4.98-26.7
	AAD-1 + Quizalofop and 2,4-D	12.3	7.09	4.07-22.5
V9 Leaf	AAD-1 Unsprayed	5.96	2.50	2.67-10.9
	AAD-1 + Quizalofop	5.38	1.84	2.52-9.15
	AAD-1 + 2,4-D	6.37	2.41	3.03-10.9
	AAD-1 + Quizalofop and 2,4-D	6.52	2.38	3.11-11.1
R1 Leaf	AAD-1 Unsprayed	5.57	1.66	3.47-9.34
	AAD-1 + Quizalofop	5.70	1.63	2.70-7.78
	AAD-1 + 2,4-D	5.99	1.90	2.40-9.42
	AAD-1 + Quizalofop and 2,4-D	6.06	2.27	1.55-10.2
Pollen	AAD-1 Unsprayed	127	36.2	56.3-210
	AAD-1 + Quizalofop	108	29.9	52.2-146
	AAD-1 + 2,4-D	113	30.2	37.5-137
	AAD-1 + Quizalofop and 2,4-D	112	32.6	45.4-162
R1 Root	AAD-1 Unsprayed	2.92	1.87	0.42-6.10
	AAD-1 + Quizalofop	3.09	1.80	0.56-6.06
	AAD-1 + 2,4-D	3.92	2.03	0.91-7.62
	AAD-1 + Quizalofop and 2,4-D	2.87	1.23	1.09-5.56
R4 Forage	AAD-1 Unsprayed	6.87	2.79	2.37-12.1
	AAD-1 + Quizalofop	7.16	2.84	3.05-11.6
	AAD-1 + 2,4-D	7.32	2.46	2.36-10.6
	AAD-1 + Quizalofop and 2,4-D	6.84	2.31	2.25-10.3
R6 Whole plant	AAD-1 Unsprayed	4.53	2.55	0.78-8.88
	AAD-1 + Quizalofop	4.61	2.22	0.75-8.77
	AAD-1 + 2,4-D	5.16	2.53	0.83-10.2
	AAD-1 + Quizalofop and 2,4-D	4.55	1.77	1.30-8.21
Grain	AAD-1 Unsprayed	5.00	1.53	2.66-8.36
	AAD-1 + Quizalofop	4.63	1.51	1.07-6.84
	AAD-1 + 2,4-D	4.98	1.78	2.94-9.10
	AAD-1 + Quizalofop and 2,4-D	4.61	1.62	1.81-7.49

VI.D. Food and Feed Safety Assessment for the AAD-1 Protein

Dow AgroSciences conducted a detailed safety assessment of the AAD-1 protein to assess any potential adverse effects to humans or animals resulting from the environmental release of crops containing the AAD-1 protein. The conclusion from that assessment is that the AAD-1 protein is unlikely to cause allergic or toxic reactions in humans or animals. A detailed assessment was presented to US FDA on September 6, 2007 as part of the New Protein Consultation process and further information will be submitted to US FDA as part of the consultation process for bioengineered foods.

History of safe use

- The donor organism, *Sphingobium herbicidovorans* (formerly designated *Sphingomonas herbicidovorans*) is a soil dwelling bacterium carrying genes which encode enzymes that facilitate the breakdown of phenoxy auxin and AOPP herbicides to compounds that can be used as carbon sources for the bacterium (Wright *et al*., 2009). *Sphingobium herbicidovorans* is a member of the sphingomonads, a widely distributed bacterial group in nature which has been isolated from land and water habitats, as well as from plant root systems. Due to their biodegradative and biosynthetic capabilities, the sphingomonads have been used for a wide range of biotechnological applications such as bioremediation of environmental contaminants and production of extracellular polymers such as sphingans which are used extensively in the food industry (Bower *et al*., 2006; Pollock and Armentrout, 1999; Lal *et al*., 2006; Johnsen *et al*., 2005).

Lack of allergenic potential

- A step-wise, weight-of-evidence approach (Codex, 2003) was used to assess the allergenic potential of the AAD-1 protein.
- The AAD-1 protein does not share meaningful amino acid sequence similarities with known allergens. No significant homology was identified when the AAD-1 protein sequence was compared to known allergens in the FARRP (Food Allergy Research and Resource Program) version 7.00 allergen database, using the search criteria of either a match of eight or more contiguous identical amino acids, or 35% identity over 80 amino acid residues.
- The AAD-1 protein is rapidly and completely degraded in simulated gastric fluid (SGF). The AAD-1 protein was readily digested, i.e., not detectable after 30 seconds, under *in vitro* SGF conditions (0.32% pepsin, pH 1.2; 37 ^{o}C) as demonstrated by both SDS-PAGE and western blot analyses.
- The AAD-1 protein is not present in a glycosylated state. No glycosylation of the AAD-1 protein was detected using SDS-PAGE and a glycosylation detection system.

Lack of toxic potential

- The AAD-1 protein does not share meaningful amino acid sequence similarities with known toxins. Amino acid homologies were evaluated using a global sequence similarity search against the GenBank non-redundant protein dataset (posted on February 10, 2007 containing 4,554,902 sequences with 1,568,234,006 amino acids). The only significant homologies identified were with other alpha-ketoglutarate-dependent dioxygenases, the same class of enzymes as AAD-1. None of the similar proteins returned by the search identified any safety concerns that might arise from the expression of AAD-1 protein in plants.
- In acute mouse toxicity testing, there were no mortalities or clinical signs in CD-1 mice after oral administration by gavage of AAD-1 protein at 2,000 mg protein/kg body weight.

Results of the overall safety assessment of the AAD-1 protein indicated that it is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals.

VI.E. Summary of AAD-1 Protein Characterization

The aryloxyalkanoate dioxygenase (AAD-1) protein was derived from *Sphingobium herbicidovorans*, a gram-negative soil bacterium. AAD-1 is comprised of 296 amino acids and has a molecular weight of 33 kDa. Detailed biochemical characterization of the AAD-1 protein derived from plant and microbial sources was conducted. Additionally, characterization of AAD-1 protein expression in DAS-40278-9 plants over the growing season was determined by analyzing leaf, root, pollen, whole plant and grain tissues from DAS-40278-9 plants sprayed with 2,4-D, quizalofop, both 2,4-D and quizalofop, and unsprayed.

A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-1 protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the AAD-1 amino acid sequence. The AAD-1 protein hydrolyzes rapidly in simulated gastric fluid and there was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-1 protein. Glycosylation analysis revealed no detectable covalently linked carbohydrates in AAD-1 protein expressed in DAS-40278-9 corn plants. Therefore, the low level expression of the AAD-1 protein presents a low exposure risk to humans and animals and the results of the overall safety assessment of the AAD-1 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

VI.F. References

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VII. Agronomic, Disease and Pest Characteristics

VII.A. Phenotypic and Agronomic Characteristics

Agronomic trials, encompassing 27 locations in the U.S. and Canada, were conducted by DAS in 2008 on hybrids containing event DAS-40278-9. All trials were planted under notifications from USDA APHIS (USDA notification numbers 08-021-104n and 08-021-110n). The agronomic characterization data collected were representative of the type of data used by commercial corn seed companies (Table 9).

Two different experiments were conducted to evaluate the agronomic characteristics of DAS-40278-9 hybrids compared to the near-isoline control hybrids. Experiment 1 used the same plots that were used for the 2008 protein expression and nutrient composition studies. In that study, the DAS-40278-9 corn was evaluated at six locations, with and without 2,4-D and quizalofop herbicide spray treatments. Experiment 2 included four different hybrids to allow for testing in a broader range of environmental conditions. A total of twenty-one (21) locations were used in various combinations for testing of the four hybrids (Table 11). The results across both experiments showed that the overall range of values for the measured agronomic parameters were all within the range of values obtained for conventional corn hybrids.

Table 9. Agronomic parameters evaluated in DAS-40278-9 field trials

Parameter	Evaluation Timing[a]	Description of Data	Scale
Early Population	V1, V4	Number of plants emerged per plot.	Actual count per plot
Seedling Vigor	V4	Visual estimate of average vigor of emerged plants per plot	(1 – 9); 1 = short plants with small, thin leaves; 9 = tall plants with large, robust leaves.
Plant Vigor /Injury	1-2 weeks after applications	Injury from herbicide applications	0-100 % with 0 = no injury and 100 = dead plant.
Time to Silking	50% silking	The number of accumulated heat units[a] from the time of planting until approximately 50% of the plants have emerged silks.	Number of days
Time to Pollen Shed	50% pollen shed	The number of accumulated heat units[a] from the time of planting until approximately 50% of the plants are shedding pollen	Number of days
Plant Height	R6	Height to the tip of the tassel	Height in centimeters (cm) or inches (in)
Ear Height	R6	Height to the base of the primary ear	Height in centimeters (cm) or inches (in)
Stalk Lodging	R6	Visual estimate of percent of plants in the plot with stalks broken below the primary ear	(0 – 100%)
Root Lodging	R6	Visual estimate of percent of plants in the plot leaning approximately 30º or more in the first ~1/2 meter above the soil surface	(0 – 100%)
Final Population	R6	The number of plants remaining per plot	Actual count per plot, including plants removed during previous sampling
Days to Maturity	R6	The number of accumulated heat units[a] from the time of planting until approximately 50% of the plants have reached physiological maturity.	Number of days
Stay Green	R6	Overall plant health	(1 – 9); 1 = no visible green tissue; 9 = 90% or greater green tissue
Grain Moisture	R6	Moisture in the grain at maturity	Percent moisture
Test Weight	R6	Grain density at maturity adjusted to 15% grain moisture	Pounds per bushel
Yield	R6	Harvest weight adjusted to 15.5% grain moisture	Bushels per acre

[a]Corn tissue stages as shown in Table 7

VII.A.1. Experiment 1

The purpose of this study was to evaluate agronomic characteristics of DAS-40278-9 corn compared to the near-isoline hybrid. Treatments included DAS-40278-9 hybrid corn sprayed with 2,4-D, quizalofop, 2,4-D and quizalofop, and neither herbicide, as well as the near-isoline control hybrid.

The experimental design included six (6) field sites; Richland, IA; Carlyle, IL; Wyoming, IL; Rockville, IN; York, NE; and Branchton, Ontario, Canada. At each site, four replicate plots of each treatment were established, with each plot consisting of two 25 ft rows. Plots were arranged in a randomized complete block design, with a unique randomization at each site. Each corn plot was bordered by two rows of a non-transgenic corn hybrid of similar maturity. The entire trial site was surrounded by a minimum of 12 rows (or 30 ft) of a non-transgenic corn hybrid of similar relative maturity.

Herbicide treatments were designed to replicate maximum label rate commercial practices. 2,4-D (Weedar 64) was applied as 3 broadcast applications at a total seasonal rate of 3360 g acid equivalent/hectare (ae/ha). Individual applications (1120 g ae/ha) were at pre-emergence, and approximately V4 and V8 –V8.5 stages. Quizalofop (Assure II) was applied as a single broadcast over-the-top application. Application timing was at approximately V6 growth stage. The target application rate was 92 g active ingredient /hectare (ai/ha) for Assure II.

Analysis of variance was conducted across the field sites using a mixed model. Entry was considered a fixed effect, and location, block within location, and location-by-entry were designated as random effects. Significant differences were declared at the 95% confidence level. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between unsprayed DAS-40278-9, DAS-40278-9 sprayed with quizalofop, DAS-40278-9 sprayed with 2,4-D, and DAS-40278-9 sprayed with both quizalofop and 2,4-D, and the near-isoline control entry using T-tests. P-values were adjusted using False Discovery Rate (FDR) procedures to improve discrimination of true differences among treatments from random effects (Benjamini and Hochberg, 1995).

An analysis of the agronomic data collected from the DAS-40278-9 unsprayed, DAS-40278-9 sprayed with 2,4-D, quizalofop, or both 2,4-D and quizalofop, and the control was conducted. For the across-site analysis, no statistically significant differences were observed for early population (V1 and V4), vigor, crop injury, time to silking, time to pollen shed, plant height, stalk lodging, root lodging, final population, and days to maturity in the across location summary analysis (Table 10). For stay green and ear height, significant paired t-tests were observed between the control and the DAS-40278-9 sprayed with quizalofop entries, and between the control and the DAS-40278-9 sprayed with both 2,4-D and quizalofop (ear height only), but they were not accompanied by significant overall treatment effects or significant FDR adjusted p-values.

Table 10. Analysis of agronomic characteristics from Experiment 1

Parameter	Overall Trt. Effect (Pr>F)[a]	Control	Unsprayed (P-value[b], Adj. P[c])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Early population V1 (no. of plants)	(0.351)	42.8	41.3 (0.303, 0.819)	41.7 (0.443, 0.819)	41.9 (0.556, 0.819)	44.1 (0.393, 0.819)
Early population V4 (no. of plants)	(0.768)	43.1	43.3 (0.883, 0.984)	43.7 (0.687, 0.863)	44.3 (0.423, 0.819)	44.8 (0.263, 0.819)
Seedling Vigor[d]	(0.308)	7.69	7.39 (0.197, 0.819)	7.36 (0.161, 0.819)	7.58 (0.633, 0.819)	7.78 (0.729, 0.889)
Crop Injury – 1st app.[e]	NA[h]	0	0	0	0	0
Crop Injury – 2nd app.[e]	(0.431)	0	0 (1.00, 1.00)	0 (1.00, 1.00)	0 (1.00, 1.00)	0.28 (0.130, 0.819)
Crop Injury – 3rd app.[e]	NA	0	0	0	0	0
Crop Injury – 4th app.[e]	NA	0	0	0	0	0
Time to Silking (heat units)[f]	(0.294)	1291	1291 (0.996, 1.00)	1293 (0.781, 0.917)	1304 (0.088, 0.819)	1300 (0.224, 0.819)
Time to Pollen Shed (heat units)[f]	(0.331)	1336	1331 (0.564, 0.819)	1342 (0.480, 0.819)	1347 (0.245, 0.819)	1347 (0.245, 0.819)
Plant Height (cm)	(0.676)	294	292 (0.206, 0.819)	290 (0.209, 0.819)	291 (0.350, 0.819)	291 (0.286, 0.819)
Ear Height (cm)	(0.089)	124	121 (0.089, 0.819)	118 (**0.018**[i], 0.786)	121 (0.214, 0.819)	118 (**0.016**[i], 0.786)
Stalk Lodging (%)	(0.261)	5.11	5.22 (0.356, 0.819)	5.00 (0.356, 0.819)	5.00 (0.356, 0.819)	5.00 (0.356, 0.819)
Root Lodging (%)	(0.431)	0.44	0.17 (0.457, 0.819)	0.72 (0.457, 0.819)	0.17 (0.457, 0.819)	0.11 (0.373, 0.819)
Final population (number of plants)	(0.873)	40.1	39.6 (0.747, 0.889)	39.7 (0.802, 0.924)	39.9 (0.943, 1.00)	41.1 (0.521, 0.819)
Days to Maturity (heat units)[f]	(0.487)	2411	2413 (0.558, 0.819)	2415 (0.302, 0.819)	2416 (0.185, 0.819)	2417 (0.104, 0.819)
Stay Green[g]	(0.260)	4.67	4.28 (0.250, 0.819)	3.92 (**0.034**[i], 0.819)	4.17 (0.144, 0.819)	4.11 (0.106, 0.819)

[a] Overall treatment effect estimated using an F-test.
[b] Comparison of the sprayed and unsprayed treatments to the control using a t-test.
[c] P-values adjusted using a False Discovery Rate (FDR) procedure.
[d] Visual estimate on 1-9 scale; 9 = tall plants with large robust leaves.
[e] 0-100% scale; with 0 = no injury and 100 = dead plant.
[f] The number of heat units that have accumulated from the time of planting.
[g] Visual estimate on 1-9 scale with 1 no visible green tissue.
[h] NA = statistical analysis not performed, no variability across replicates or treatments.
[i] Statistical difference indicated by P-Value <0.05.

VII.A.2. Experiment 2

The purpose of this study was to evaluate agronomic characteristics of DAS-40278-9 corn compared to a near-isoline corn line across diverse environments. Treatments included four hybrids and their appropriate near-isoline control hybrids tested across a total of 21 locations.

The four test hybrids were medium to late maturity hybrids ranging from 99 to 113 day relative maturity. Hybrid 2A contained event DAS-40278-9 in the genetic background Inbred A x Inbred C (Figure 4). This hybrid has a relative maturity of 109 days and was tested at 16 locations (Table 11). Hybrid 2B, a cross of Inbred A x Inbred F, is a 113 day relative maturity hybrid. This hybrid was tested at 14 locations, using a somewhat different set of locations than Experiment 2A (Table 11). Hybrids 2C and 2D contain the genetic backgrounds Inbred B x Inbred D and Inbred B x Inbred E, respectively. Both of these hybrids have a 99 day relative maturity and were tested at the same 10 locations.

Table 11. Locations of Experiment 2 agronomic trials

Location	Hybrids			
	2A	2B	2C	2D
Atlantic, IA	X	X		
Fort Dodge, IA	X	X	X	X
Huxley, IA	X	X	X	X
Nora Springs, IA	X			
Wyman, IA	X	X		
Lincoln, IL		X		
Pontiac, IL	X	X	X	X
Princeton, IL	X	X		
Seymour, IL	X			
Shannon, IL	X		X	X
Viola, IL	X	X		
Bremen, IN	X	X	X	X
Evansville, IN		X		
Fowler, IN	X	X	X	X
Mt. Vernon, IN		X		
Olivia, MN			X	X
Wayne, NE	X	X		
York, NE	X	X		
Arlington, WI	X		X	X
Patteville, WI	X		X	X
Watertown, WI			X	X

For each trial, a randomized complete block design was used with two replications per location with two rows per plot. Row length was 20 feet and each row was seeded at 34 seeds per row. Standard regional agronomic practices were used in the management of the trials.

Data were collected and analyzed for eight agronomic characteristics; plant height, ear height, stalk lodging, root lodging, final population, grain moisture, test weight, and yield (Table 9). The parameters plant height and ear height provide information about the appearance of the hybrids. The agronomic characteristics of percent stalk lodging and root lodging determine the harvestability of a hybrid. Final population count measures seed quality and seasonal growing conditions that affect yield. Percent grain moisture at harvest defines the maturity of the hybrid, and yield and test weight describe the reproductive capability of the hybrid.

All statistical analyses were done using JMP version 8. All agronomic traits, with the exception of plant height and ear height, were analyzed using mixed model equations as:

$$y_{ijkl} = \mu + transgene_i + line_j + location_k + line*transgene_{ij} + e_{ijkl} \quad [1]$$

where y_{ijkl} is the replicate of the agronomic response variable measured in line *j* at location *k* and under the influence of transgene *i*; μ is the model intercept; $transgene_i$ is the fixed effect associated with the presence or absence of the AAD-1 event; $line_j$ is the fixed effect of line *j*; $location_k$ is the random effect of location *k*; $line*transgene_{ij}$ is the interaction effect between line *j* and transgene *i*; and e_{ijkl} is the random residual. Ear height and plant height were analyzed using a reduced model due to the fact that measurements were only collected at one location:

$$y_{ijk} = \mu + transgene_i + line_j + e_{ijk} \quad [2]$$

with the model terms being the same as those described for [1]. Significance of the transgene effect was calculated for each agronomic trait using an F-ratio test. To satisfy the normality assumption required for the F-ratio test, a power transformation using Log_{10} was applied to percent stalk and root lodging. For purposes of reporting, least square means (LSM) for percent stalk and root lodging were transformed back to the original scale after analysis was complete.

Results from the agronomic characterization trials can be found in Table 12. No statistically significant differences were found for the four DAS-40278-9 hybrids compared with their isoline controls ($p>0.05$) for the parameters of ear height, plant height, final population, stalk lodging, root lodging, grain moisture, test weight, and yield. Plant height was close to significant ($p=.0525$), but event DAS-40278-9 was only 3% taller than controls which is not biologically significant. The overall range of values

for the measured parameters are all within the range of values typically obtained for traditional corn hybrids and would not lead to a conclusion of increased weediness. In summary, agronomic characterization data indicate that DAS-40278-9 corn is biologically equivalent to conventional corn.

Table 12. Analysis of agronomic characteristics from Experiment 2

Parameter (units)	Treatment	Least Square Mean	Range		P-value
			Min	Max	
Plant Height (inches)*	DAS-40278-9	96.51	90.00	108.00	0.0525
	Control	93.39	89.00	104.00	
Ear Height (inches)*	DAS-40278-9	42.42	30.00	50.00	0.8081
	Control	41.73	37.00	50.00	
Stalk Lodging (%)	DAS-40278-9	5.252	0.00	27.00	0.1475
	Control	4.290	0.00	39.06	
Root Lodging (%)	DAS-40278-9	2.481	0.00	9.68	0.7383
	Control	2.563	0.00	62.07	
Final Population (plants/acre in 1000's)	DAS-40278-9	31.18	26.00	37.00	0.2762
	Control	31.55	24.00	36.00	
Grain Moisture (%)	DAS-40278-9	20.97	13.33	31.00	0.6607
	Control	21.08	13.35	31.10	
Test Weight (lb/bushel)	DAS-40278-9	55.75	42.1	59.5	0.2353
	Control	55.47	51.00	61.00	
Yield (bushels/acre)	DAS-40278-9	197.07	102.32	272.50	0.4225
	Control	200.12	95.35	285.58	

*****One location at Fowler IN only

VII.B. Ecological Observations

The DAS-40278-9 corn field trials were monitored and observed by personnel familiar with corn cultivation practices (breeders, field station managers, field agronomists, field associates). The personnel conducting the field tests visually monitored the incidence of plant disease and pests on DAS-40278-9 corn compared to the conventional corn lines in the same trials. As part of Experiment 1 in VII.A.1, disease and insect damage was rated on a numerical scale of 1-9, with 1 being poor disease or insect resistance and 9 being excellent. Results shown are the average across six locations as described in Section VII.A.1. No significant differences were found between the DAS-40278-9 plants and the near-isoline control plants (Table 13).

Table 13. Analysis of disease and insect characteristics from Experiment 1

Ecological Observation	Overall Trt. Effect (Pr>F)[a]	Control	Unsprayed (P-value,[b] Adj. P)[c]	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Disease Incidence[d]	(0.741)	6.42	6.22 (0.383, 0.819)	6.17 (0.265, 0.819)	6.17 (0.265, 0.819)	6.17 (0.265, 0.819)
Insect Damage[e]	(0.627)	7.67	7.78 (0.500, 0.819)	7.78 (0.500, 0.819)	7.72 (0.736, 0.889)	7.56 (0.500, 0.819)

[a] Overall treatment effect estimated using an F-test.
[b] Comparison of the sprayed and unsprayed treatments to the control using a t-test.
[c] P-values adjusted using a False Discovery Rate (FDR) procedure.
[d] Visual estimate on 1-9 scale with 1 being poor disease resistance.
[e] Visual estimate on 1-9 scale with 1 being poor insect resistance.

Ecological observations were also made for all USDA APHIS notified field trials in 2007 and 2008 (Appendix 4). Incidence of disease and insect presence in trials of DAS-40278-9 corn were recorded and differences in incidence or response of DAS-40278-9 corn compared to the conventional control was examined. In all cases, no differences were seen in any of the trials of DAS-40278-9 corn compared to the conventional controls. The disease and insect stressors observed in trials of DAS-40278-9 and conventional corn are described in Table 14. The results support the conclusion that the response of DAS-40278-9 corn to ecological stressors does not differ from that of conventional corn.

Table 14. Disease and insect stressors observed in trials of DAS-40278-9 and conventional corn

Year	Notification	State	County	Emergence/Vegetative		Post flowering	
				Diseases	Insects	Diseases	Insects
2006	05-308-03n	Hawaii	Maui		leaf hoppers, thrips	ear rots	
2007	06-338-101n	Hawaii	Maui	leaf blight	leaf hoppers, thrips		aphids
		Hawaii	Maui		aphids		mites
		Hawaii	Maui		aphids	leaf blights	aphids
2007/08	07-242-103n	Hawaii	Maui			leaf blights	lacewings, aphids
		Hawaii	Maui			leaf blights	aphids
		Hawaii	Maui			leaf blights	lacewings, aphids
		Hawaii	Maui			leaf blights	aphids
		Hawaii	Maui			leaf blights, ear rots	aphids
2008	08-021-104n	Illinois	Carroll			rust	
		Illinois	Bureau			grey leaf spot	
		Indiana	Benton			leaf blights, rust	aphids, lady bugs
		Indiana	Benton			leaf blights, rust	lady bugs
		Indiana	Parke	leaf blight	Japanese beetles, grasshoppers, ground beetles	leaf blight, grey leaf spot, rust	grasshoppers, corn earworm
		Indiana	St. Joseph			leaf blights	aphids, lady bugs
		Indiana	Benton		aphids, lady bugs, grasshoppers	rust	aphids, lady bugs, grasshoppers
		Indiana	Benton		corn borer	leaf blights, rust	lady bugs
		Minnesota	Renville				aphids
		Minnesota	Sibley				aphids
		Minnesota	Stearns				aphids
		Minnesota	Dakota				aphids
		Mississippi	Washington		cutworm, army worm		cornborers
		Nebraska	York				aphids
		Nebraska	York		grasshoppers		aphids
2008	08-133-107n	Illinois	Stark			leaf blights, rust	
2008/09	08-259-103n	Hawaii	Maui		lacewings, aphids	leaf blights	aphids

VII.C. Germination and Dormancy

Changes in seed dormancy characteristics were evaluated by looking at the germination of DAS-40278-9 hybrid seed compared with near-isoline hybrid under warm and cold conditions. For the warm germination test, DAS-40278-9 and control corn seeds were placed 25/plate on two stacked moist germination pads and held at 25 °C for 7 days. Sixteen plates (400 seeds) were set up per line. After seven days, the number of non-germinated seed was recorded. For the cold germination test, seeds were planted at 100 seeds per half-flat filled with potting soil. Four half flats (400 seeds) were planted per line. Flats were sub-watered and held at 10 °C for 7 days followed by exposure to 25 °C for 5 days, after which the number of germinated seeds was recorded. Data from each test were analyzed by ANOVA using a completely randomized design with four replicates of 100 seeds per replicate. Data were transformed using the arcsine of the square root of the number of germinated seeds divided by 100 for statistical analysis. Percent germination is summarized in Table 15.

Table 15. Germination of DAS-40278-9 seeds under warm and cold conditions

Test	Line	Replicate				Mean
		1	2	3	4	
warm	DAS-40278-9	97	95	97	99	97.0
warm	control	97	100	98	98	98.3
cold	DAS-40278-9	86	91	97	94	92.0
cold	control	98	96	100	94	97.0

In the warm and cold germination tests, there were no significant line effects on germination ($Pr > F = 0.06$ and 0.16, respectively). Therefore, no statistically significant germination differences were seen between DAS-40278-9 corn and the non-transgenic control. Given the high percent germination in the DAS-40278-9 corn and no significant differences between the transgenic and control, the results indicate that seed dormancy characteristics have not been changed in DAS-40278-9 corn.

VII.D. Pollen Parameters

Morphology and viability characteristics of pollen from DAS-40278-9 corn were determined using shape and color measurements, and pollen staining for viability. Pollen collected from plants containing event DAS-40278-9 was compared with pollen from control plants of a similar genetic background to look for differences in pollen morphology or viability that could be attributed to the DAS-40278-9 event. Statistically significant differences are declared when the P-value ≤ 0.05.

Pollen viability was determined by analysis of pollen collected from 10-16 greenhouse grown plants at approximately the R1 growth stage (Table 7). The pollen from individual plants was combined and frozen prior to analysis. Approximately 16 mg of pollen was diluted with Lugol staining solution (Wang *et al.*, 2004) and viewed at 5X magnification with a laboratory microscope. Viable pollen was stained black and non-viable pollen was not stained. Results are listed in Table 16. The proportion of viable and non-viable pollen grains was not statistically different between the DAS-40278-9 corn and the control.

Pollen morphology determinations were made on pollen freshly harvested from 4 U.S. field trial sites in 2008 (IA, IL, IN and NE). Pollen was collected from individual plants in full anthesis (approximately 50% of the plants actively shedding pollen). Pollen color and shape was evaluated immediately after collection (0 minutes), and then at 30, 60, and 120 minutes post collection, using a 25X magnification hand lens. Pollen shape was evaluated as the proportion of pollen grains with collapsed walls and pollen color was scored as the proportion of pollen grains with intense yellow color (fresh pollen has a pearl white color that becomes intensely yellow over time). Results from pollen color and shape determinations summarized across field locations are found in Table 16. Like pollen viability, no statistically significant differences were found in pollen color or shape between DAS-40278-9 corn and the control.

Table 16. Viability and morphology of DAS-40278-9 pollen

Measurement	Control	DAS-40278-9 Corn	P-value[a]
Pollen Viability (% viable)	74.8	77.1	0.571
Pollen Color (% intense yellow)			
0 minutes	17.1	17.4	0.621
30 minutes	52.2	53.3	0.740
60 minutes	85.3	87.1	0.504
120 minutes	99.3	99.3	ND[b]
Pollen Shape (% collapsed walls)			
0 minutes	16.3	16.1	0.752
30 minutes	48.8	51.3	0.309
60 minutes	86.7	87.1	0.777
120 minutes	98.9	98.9	ND[b]

[a] P-value for Pollen Viability based on Chi-Square analysis, P-value for Pollen Color and Pollen Shape based on ANOVA (Pr>F)

[b] ND = No difference between treatment means

VII.E. Summary of Agronomic, Disease, and Pest Characteristics

Agronomic data evaluating plant growth characteristics throughout the growing season demonstrate the equivalence of DAS-40278-9 corn with conventional non-transgenic corn. Plant growth and phenotypic characteristics, response to ecological stressors as indicated by susceptibility to disease and insect pressure, pollen morphology and viability, and germination and dormancy characteristics were unchanged across diverse environments between DAS-40278-9 corn and conventional corn. Therefore, these data support the conclusion that agronomic, disease, and pest characteristics of DAS-40278-9 are not significantly different from that of conventional corn, and there is no indication that DAS-40278-9 corn will pose an increased plant pest risk.

VII.F. References

Benjamini, Y., Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of Royal Statistical Society, Series B, 57: 289-300.

Wang, Z., Ge, Y., Scott, M., Spangenberg, G. 2004. Viability and longevity of pollen from transgenic and nontransgenic tall fescue (*Festuca arundinacea*) (Paeceae) plants. American Journal of Botany 91(4):523-530.

VIII. Grain and Forage Composition

Compositional analysis was performed on corn forage and grain to investigate the equivalency between DAS-40278-9 corn (unsprayed, sprayed with 2,4-D, sprayed with quizalofop, and sprayed with 2,4-D and quizalofop) and the near-isoline control corn. Trials were conducted at six test sites located within the major corn-producing regions of the U.S and Canada using hybrid corn seed lines with and without event DAS-40278-9 (Figure 4). The test sites represent regions of diverse agronomic practices and environmental conditions and were the same sites used for protein expression analysis. The trials were located in Iowa, Illinois (2 sites), Indiana, Nebraska and Ontario, Canada.

Samples of corn forage and grain were analyzed for nutrient content with a variety of tests (OECD, 2002). The analyses performed for forage included ash, total fat, moisture, protein, carbohydrate, acid detergent fiber, neutral detergent fiber, calcium and phosphorus. The analyses performed for grain included proximates (ash, total fat, moisture, protein, carbohydrate), fiber (total dietary fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF)), minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients.

The results of the nutritional analysis for corn forage and grain were compared with values reported in literature. A summarization of the compositional data used for comparison can be found in Appendix 5, Tables 5.1-5.7. Analysis of variance was also conducted across the field sites using a mixed model. Entry was considered a fixed effect, and location, block within location, and location-by-entry were designated as random effects. Analysis at individual locations was done in an analogous manner with entry as a fixed effect and block as a random effect. Significant differences were declared at the 95% confidence level. Data were not rounded off for statistical analysis. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between unsprayed DAS-40278-9 (unsprayed), DAS-40278-9 sprayed with quizalofop (AAD-1 + quizalofop), DAS-40278-9 sprayed with 2,4-D (AAD-1 + 2,4-D) and DAS-40278-9 sprayed with both quizalofop and 2,4-D (AAD-1 + both) transgenic entries and the control entry using T-tests.

Due to the large number of contrasts made in this study, multiplicity was an issue. Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise p-values is very high ($1-0.95^{\text{number of comparisons}}$). In this study there were four comparisons per analyte and 66 quantitated analytes, resulting in 264 comparisons made in the across-site composition analysis. Therefore, the probability of declaring one or more false differences based on unadjusted p-values was >99.99% ($1-0.95^{264}$).

One method to account for multiplicity is to adjust p-values to control the experiment-wise error rate (probability that all declared differences are significant), but when many

comparisons are made in a study, the power for detecting specific effects can be reduced significantly. An alternative with much greater power is to adjust p-values to control the probability that each declared difference is significant. This can be accomplished using False Discovery Rate (FDR) procedures (Benjamini and Hochberg, 1995). Therefore the p-values were adjusted using FDR to improve discrimination of true differences among treatments from random effects (false positives).

VIII.A. Compositional Analyses of Corn Forage

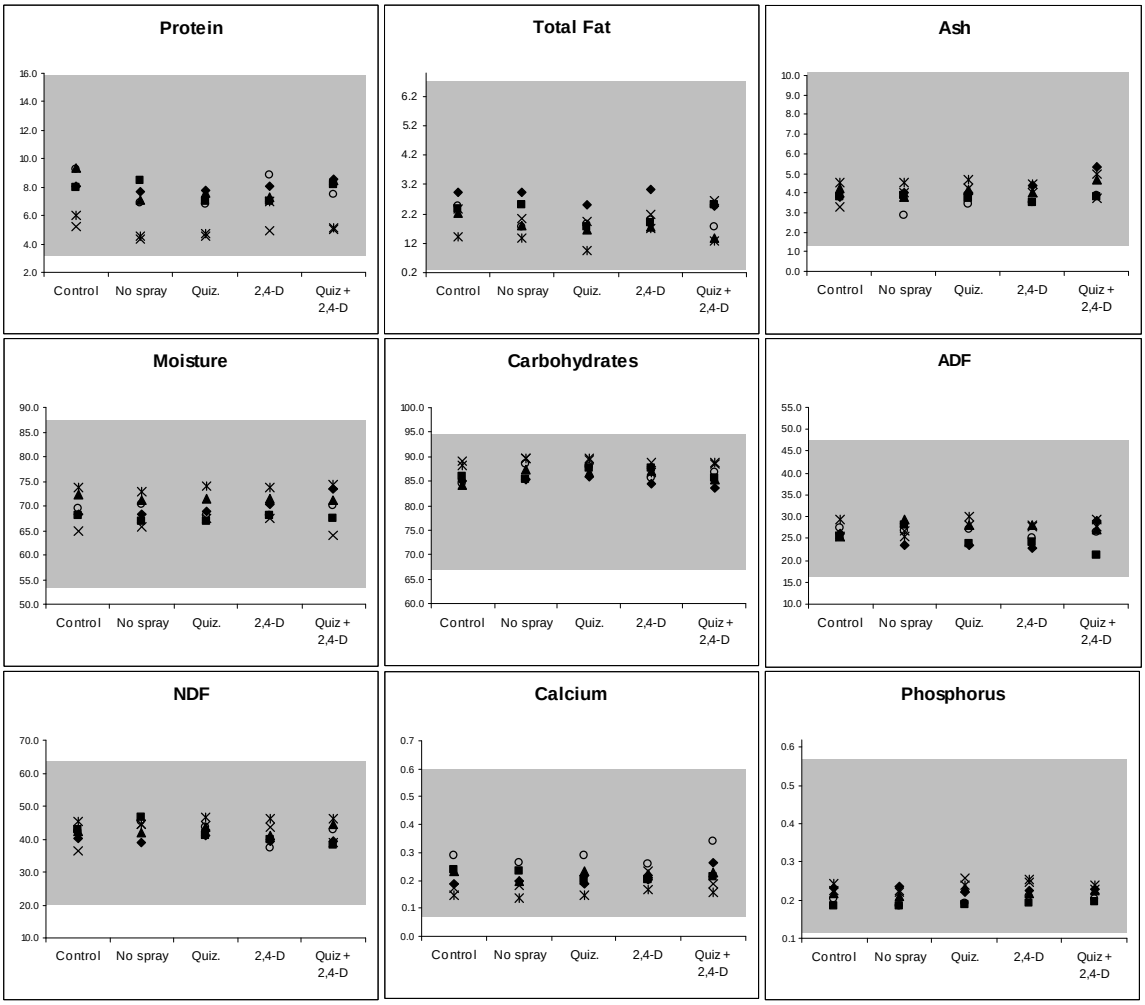
An analysis of the protein, fat, ash, moisture, carbohydrate, ADF, NDF, calcium and phosphorus in corn forage samples from the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both entries was performed. A summary of the results across all locations is shown in Table 17. For the across-site and individual-site analysis, all proximate, fiber and mineral mean values were within literature ranges (Figure 26). No statistical differences were observed in the across-site analysis between the control and transgenic entries for moisture, ADF, NDF, calcium and phosphorus. For protein and ash, significant paired t-tests were observed for the unsprayed AAD-1 (protein), the AAD-1 + quizalofop (protein), and AAD-1 + both (ash), but were not accompanied by significant overall treatment effects or FDR adjusted p-values. For fat, both a significant paired t-test and adjusted p-value was observed for AAD-1 + quizalofop compared with the control, but a significant overall treatment effect was not observed. For carbohydrates, a statistically significant overall treatment effect, paired t-test and FDR adjusted p-value was observed between the AAD-1 + quizalofop and the control. Also for carbohydrates, a significant paired t-test for the unsprayed AAD-1 entry was observed, but without a significant FDR adjusted p-value. These differences are not biologically meaningful since all across-site results for these analytes were within the reported literature ranges for corn, and differences from the control were small (<23 %).

Table 17. Summary of the proximate, fiber and mineral analysis of corn forage

Proximate (% dry weight)	**Literature Values[a]**	**Overall Treatment Effect (Pr>F)[b]**	**Control**	**Unsprayed (P-value[c], Adj. P[d])**	**Sprayed Quizalofop (P-value, Adj. P)**	**Sprayed 2,4-D (P-value, Adj. P)**	**Sprayed Both (P-value, Adj. P)**
Protein	3.14-15.9	(0.054)	7.65	6.51 (**0.016[e]**, 0.066)	6.41 (**0.010[e]**, 0.051)	7.17 (0.285, 0.450)	7.13 (0.245, 0.402)
Fat	0.296-6.7	(0.068)	2.29	2.08 (0.202, 0.357)	1.78 (**0.005[e]**, **0.028[e]**)	2.10 (0.233, 0.391)	2.01 (0.093, 0.213)
Ash	1.3-10.5	(0.072)	3.90	3.84 (0.742, 0.859)	4.03 (0.525, 0.708)	3.99 (0.673, 0.799)	4.40 (**0.019[e]**, 0.069)
Moisture	53.3-87.5	(0.819)	69.5	69.2 (0.651, 0.782)	69.5 (0.988, 0.988)	69.8 (0.699, 0.820)	70.0 (0.501, 0.687)
Carbohydrates	66.9-94.5	(**0.026[e]**)	86.1	87.6 (**0.015[e]**, 0.061)	87.8 (**0.006[e]**, **0.034[e]**)	86.8 (0.262, 0.424)	86.5 (0.538, 0.708)
Fiber (% dry weight)							
Acid Detergent Fiber (ADF)	16.1-47.4	(0.968)	26.5	26.6 (0.925, 0.970)	26.8 (0.833, 0.925)	26.0 (0.677, 0.800)	26.8 (0.851. 0.937)
Neutral Detergent Fiber (NDF)	20.3-63.7	(0.345)	41.6	43.6 (0.169, 0.322)	43.3 (0.242, 0.402)	41.3 (0.809, 0.911)	41.6 (0.978, 0.985)
Minerals (% dry weight)							
Calcium	0.071-0.6	(0.321)	0.212	0.203 (0.532, 0.708)	0.210 (0.930, 0.970)	0.215 (0.815, 0.911)	0.231 (0.150, 0.296)
Phosphorus	0.094-0.55	(0.163)	0.197	0.189 (0.198, 0.354)	0.202 (0.427, 0.615)	0.203 (0.288, 0.450)	0.200 (0.608, 0.762)

[a] Combined range from Appendix 5.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
[e] Statistical difference indicated by P-Value <0.05.

Figure 26. Proximate, fiber, and mineral analysis of corn forage
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Table 5.1) are shaded.



VIII.B. Compositional Analyses of Corn Grain

VIII.B.1. Proximate and Fiber Analysis of Grain

A summary of the results for proximates (protein, fat, ash, moisture, and carbohydrates) and fiber (ADF, NDF and total dietary fiber) in corn grain across all locations is shown in Table 18. All results for proximates and fiber were within literature ranges (Figure 27), and no significant differences in the across-site analysis were observed between the control and DAS-40278-9 corn entries for fat, ash, NDF and total dietary fiber. For moisture, a significant overall treatment effect was observed, but not accompanied by significant paired t-tests or FDR adjusted p-values. For ADF, a significant paired t-test was observed for AAD-1 + both, but no significant overall treatment effect or FDR adjusted p-value was seen. For both protein and carbohydrates, significant pair-tests, adjusted p-values and overall treatment effects were found for the unsprayed AAD-1, AAD-1 + quizalofop, and AAD-1 + both. Since these differences were small (< 12%) and all values were within literature ranges, the differences are not biologically meaningful.

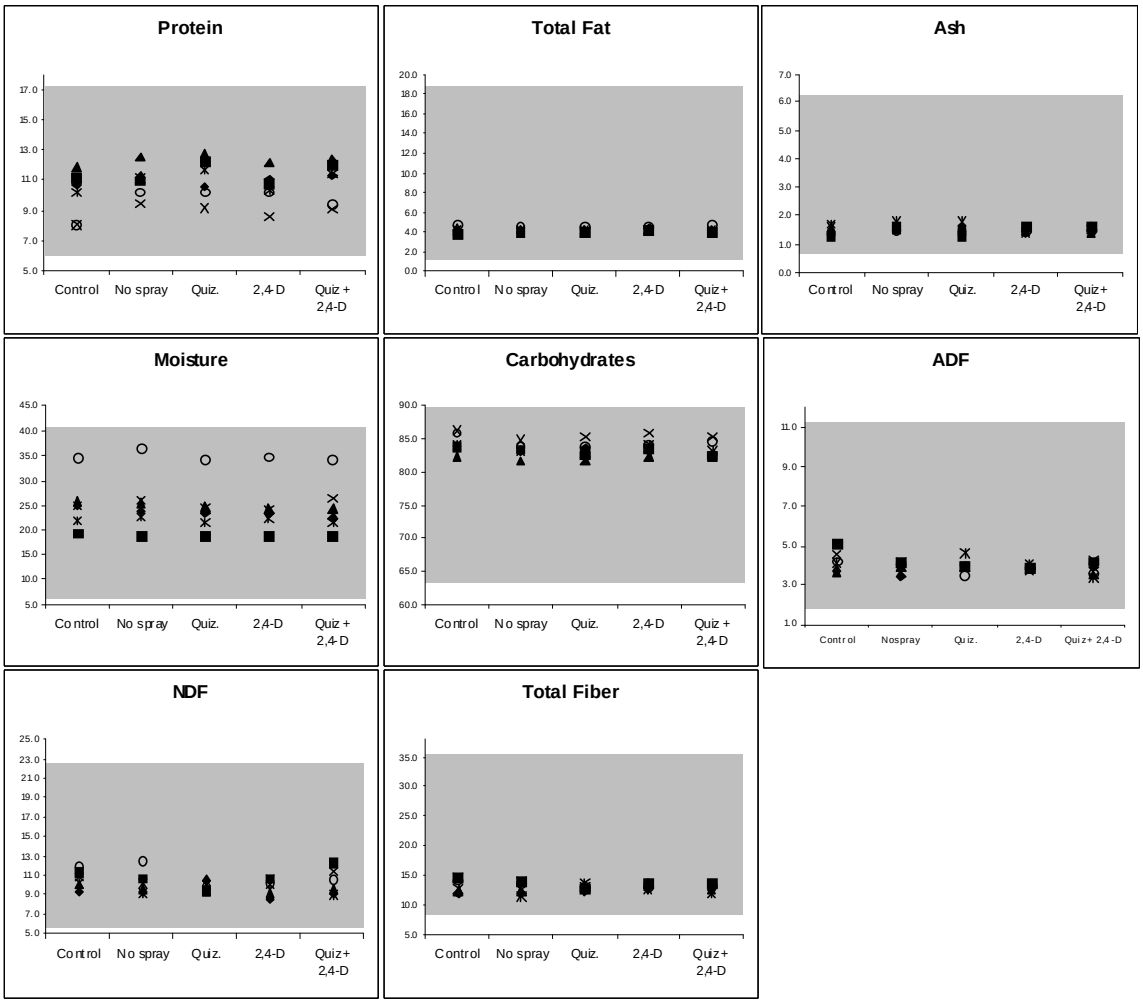
Table 18. Summary of the proximate and fiber analysis of corn grain

Proximate (% dry weight)	**Literature Values[a]**	**Overall Treatment Effect (Pr>F)[b]**	**Control**	**Unsprayed (P-value[c], Adj. P[d])**	**Sprayed Quizalofop (P-value, Adj. P)**	**Sprayed 2,4-D (P-value, Adj. P)**	**Sprayed Both (P-value, Adj. P)**
Protein	6-17.3	(**0.003**[e])	9.97	10.9 (**0.002**[e], **0.016**[e])	11.1 (**0.0004**[e], **0.013**[e])	10.5 (0.061, 0.161)	10.9 (**0.002**[e], **0.015**[e])
Fat	1.2-18.8	(0.369)	4.26	4.19 (0.238, 0.397)	4.16 (0.095, 0.215)	4.26 (0.955, 0.977)	4.22 (0.427, 0.615)
Ash	0.62-6.28	(0.553)	1.45	1.55 (0.178, 0.330)	1.52 (0.364, 0.557)	1.45 (0.982, 0.985)	1.51 (0.397, 0.587)
Moisture	6.1-40.5	(**0.038**[e])	25.1	25.5 (0.406, 0.594)	24.4 (0.056, 0.152)	24.5 (0.117, 0.254)	24.5 (0.114, 0.250)
Carbohydrate	63.3-89.8	(**0.005**[e])	84.3	83.3 (**0.002**[e], **0.015**[e])	83.2 (**0.001**[e], **0.013**[e])	83.8 (0.074, 0.185)	83.4 (**0.003**[e], **0.019**[e])
Fiber (% dry weight)							
Acid Detergent Fiber (ADF)	1.82-11.3	(0.247)	4.23	3.94 (0.130, 0.269)	3.99 (0.197, 0.354)	3.89 (0.078, 0.193)	3.82 (**0.035**[e], 0.106)
Neutral Detergent Fiber (NDF)	5.59-22.6	(0.442)	10.6	10.3 (0.455, 0.638)	9.89 (0.120, 0.254)	9.90 (0.121, 0.254)	10.3 (0.552, 0.708)
Total Dietary Fiber	8.3-35.3	(0.579)	13.4	12.8 (0.164, 0.313)	12.9 (0.195, 0.353)	13.1 (0.487, 0.679)	12.9 (0.215, 0.370)

[a] Combined range from Appendix 5.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
[e] Statistical difference indicated by P-Value <0.05.

Figure 27. Proximate and fiber analysis of corn grain

Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Table 5.2) are shaded.



VIII.B.2. Mineral Analysis of Grain

An analysis of corn grain samples for the minerals calcium, chromium, copper, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium, and zinc was performed. A summary of the results across all locations is shown in Table 19. All results were within the reported literature ranges (Figure 28). For the across-site analysis, no significant differences were observed for calcium, copper, iron, and potassium. Mean results for chromium, iodine, selenium and sodium were below the limit of quantitation of the method. For magnesium and phosphorus, significant paired t-tests were observed for the unsprayed AAD-1 and the AAD-1 + quizalofop entries, but were not accompanied by significant overall treatment effects or FDR adjusted p-values. For manganese and molybdenum, a significant paired t-test was observed for the unsprayed AAD-1, but a significant FDR adjusted p-value and overall treatment effect was not found. For the AAD-1 + both entry, a significant paired t-test was observed for zinc, but a significant FDR adjusted p-value or overall treatment effect was not present. Additionally, these differences from the control were small (< 13%), and all values were within literature ranges, when available.

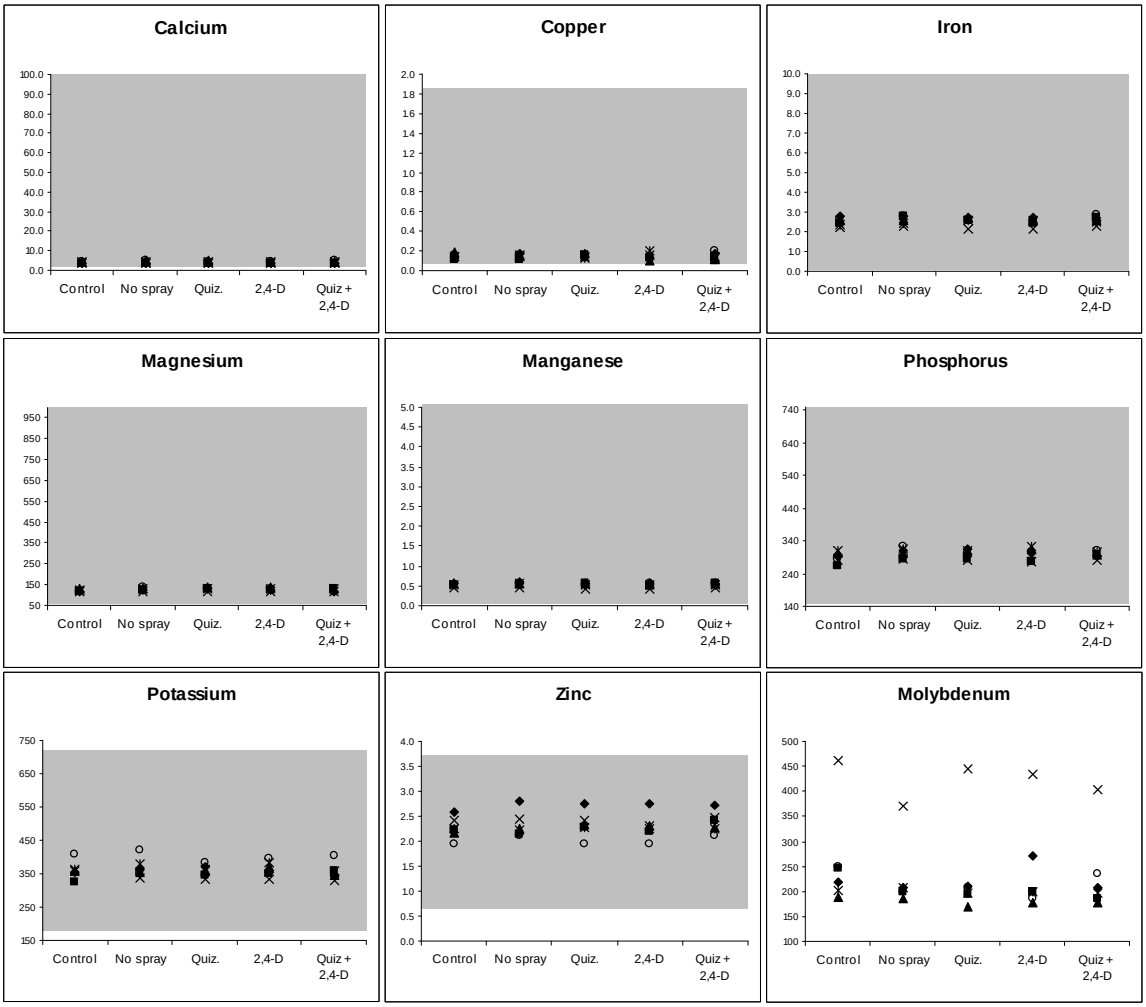
Table 19. Summary of the mineral analysis of corn grain

Minerals (mg/100g dry wt.)	**Literature Values[a]**	**Overall Treatment Effect (Pr>F)[b]**	**Control**	**Unsprayed (P-value[c], Adj. P[d])**	**Sprayed Quizalofop (P-value, Adj. P)**	**Sprayed 2,4-D (P-value, Adj. P)**	**Sprayed Both (P-value, Adj. P)**
Calcium	1.27-100	(0.493)	4.05	4.21 (0.146, 0.289)	4.12 (0.505, 0.687)	4.04 (0.944, 0.977)	4.06 (0.898, 0.957)
Chromium	0.006-0.016	NA[e]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Copper	0.073-1.85	(0.963)	0.144	0.151 (0.655, 0.782)	0.146 (0.890, 0.957)	0.141 (0.817, 0.911)	0.149 (0.749, 0.863)
Iodine	7.3-81	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Iron	0.1-10	(0.333)	2.49	2.60 (0.086, 0.206)	2.56 (0.310, 0.482)	2.51 (0.801, 0.911)	2.59 (0.145, 0.289)
Magnesium	59.4- 1000	(0.072)	122	129 (**0.010**[f], 0.051)	128 (**0.017**[f], 0.066)	126 (0.145, 0.289)	127 (0.070, 0.177)
Manganese	0.07-5.4	(0.099)	0.525	0.551 (**0.025**[f], 0.082)	0.524 (0.884, 0.957)	0.526 (0.942, 0.977)	0.532 (0.505, 0.687)
Molybdenum	NR	(0.143)	261	229 (**0.020**[f], 0.072)	236 (0.067, 0.173)	244 (0.206, 0.362)	234 (0.046, 0.132)
Phosphorus	147-750	(0.102)	289	303 (**0.012**[f], 0.057)	300 (**0.035**[f], 0.106)	299 (0.055, 0.150)	298 (0.085, 0.206)
Potassium	181-720	(0.453)	362	368 (0.330, 0.510)	359 (0.655, 0.782)	364 (0.722, 0.839)	357 (0.454, 0.638)
Selenium	0.001-0.1	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sodium	0-150	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Zinc	0.65-3.72	(0.166)	2.26	2.32 (0.183, 0.336)	2.34 (0.108, 0.238)	2.29 (0.627, 0.768)	2.37 (**0.027**[f], 0.085)

[a] Combined range from Appendix 5.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
[e] NA= statistical analysis was not performed since a majority of the data was < LOQ.
[f] Statistical difference indicated by P-Value <0.05.

Figure 28. Mineral analysis of corn grain

Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Table 5.3) are shaded. Grain was also analyzed for chromium, iodine, selenium and sodium, but results were less than the limit of quantitation.



VIII.B.3. Amino Acid Analysis of Grain

Corn samples were analyzed for amino acid content in the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both corn, and a summary of the results over all locations are shown in Table 20. Levels of all amino acids were within the reported literature ranges (Figure 29), and no significant differences in the across-site analysis were observed for arginine, lysine, and tyrosine. Significant differences were observed for several of the amino acids in the across-site analysis. In these instances, the amino acid content of the control was lower than the AAD-1 transgenic lines, which may be related to the overall lower protein content in the control grain compared with the AAD-1 lines. For the unsprayed AAD-1 entry, significant overall treatment effects along with significant paired t-tests and FDR adjusted p-values were found for all amino acids except arginine, glycine, lysine, tryptophan and tyrosine. For the AAD-1 + quizalofop entry, significant overall treatment effects along with significant paired t-tests and FDR adjusted p-values were found for all amino acids except arginine, cysteine, glycine, lysine, tryptophan and tyrosine. For the AAD-1 + 2,4-D entry, significant overall treatment effects along with significant paired t-tests (with significant FDR adjusted p-values) were found for all amino acids except arginine, aspartic acid, glycine, histidine, lysine, tyrosine and valine. For the AAD-1 + both entry, significant overall treatment effects along with significant paired t-tests and FDR adjusted p-values were found for all amino acids except arginine, glycine, lysine, serine, tryptophan and tyrosine. Although there were many differences observed for amino acids, the differences were small (< 15%), not observed across all sites, and all mean values were within reported literature ranges.

Table 20. Summary of the amino acid analysis of corn grain

Amino Acids (% dry weight)	Literature Values[a]	Overall Treatment Effect (Pr>F)[b]	Control	Unsprayed (P-value[c], Adj. P[d])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Alanine	0.44-1.39	(**0.002**[e])	0.806	0.901 (**0.0005**[e], **0.013**[e])	0.900 (**0.0005**[e], **0.013**[e])	0.863 (**0.021**[e], 0.074)	0.894 (**0.001**[e], **0.013**[e])
Arginine	0.12-0.64	(0.371)	0.486	0.499 (0.286, 0.450)	0.505 (0.139, 0.283)	0.487 (0.929, 0.970)	0.484 (0.897, 0.957)
Aspartic Acid	0.34-1.21	(**0.010**[e])	0.712	0.768 (**0.002**[e], **0.015**[e])	0.764 (**0.003**[e], **0.021**[e])	0.743 (0.060, 0.160)	0.762 (**0.004**[e], **0.027**[e])
Cysteine	0.08-0.51	(**0.033**[e])	0.213	0.225 (**0.009**[e], **0.050**[e])	0.223 (**0.020**[e], 0.072)	0.223 (**0.018**[e], 0.067)	0.226 (**0.005**[e], **0.028**[e])
Glutamic Acid	0.97-3.54	(**0.001**[e])	1.97	2.22 (**0.0003**[e], **0.013**[e])	2.21 (**0.0004**[e], **0.013**[e])	2.12 (**0.017**[e], 0.067)	2.20 (**0.001**[e], **0.013**[e])
Glycine	0.18-0.54	(0.052)	0.383	0.397 (**0.018**[e], 0.067)	0.398 (**0.013**[e], 0.059)	0.390 (0.217, 0.371)	0.397 (**0.016**[e], 0.066)
Histidine	0.14-0.43	(**0.005**[e])	0.283	0.303 (**0.001**[e], **0.013**[e])	0.302 (**0.002**[e], **0.014**[e])	0.295 (0.036, 0.109)	0.302 (**0.002**[e], **0.014**[e])
Isoleucine	0.18-0.71	(**0.003**[e])	0.386	0.427 (**0.001**[e], **0.014**[e])	0.427 (**0.001**[e], **0.014**[e])	0.410 (**0.044**[e], 0.127)	0.431 (**0.001**[e], **0.013**[e])
Leucine	0.64-2.49	(**0.001**[e])	1.35	1.54 (**0.0003**[e], **0.013**[e])	1.54 (**0.0003**[e], **0.013**[e])	1.47 (**0.013**[e], 0.059)	1.53 (**0.001**[e], **0.013**[e])

Table 20. (cont.) Summary of the amino acid analysis of corn grain

Amino Acids (% dry weight)	Literature Values[a]	Overall Treatment Effect (Pr>F)[b]	Control	Unsprayed (P-value[c], Adj. P[d])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Lysine	0.05-0.56	(0.211)	0.310	0.315 (0.210, 0.367)	0.316 (0.128, 0.265)	0.309 (0.879, 0.956)	0.316 (0.102, 0.226)
Methionine	0.10-0.47	(**0.003**[e])	0.195	0.209 (**0.001**[e], **0.013**[e])	0.209 (**0.001**[e], **0.013**[e])	0.205 (**0.014**[e], 0.061)	0.208 (**0.001**[e], **0.014**[e])
Phenylalanine	0.24-0.93	(**0.002**[e])	0.551	0.617 (**0.001**[e], **0.013**[e])	0.619 (**0.001**[e], **0.013**[e])	0.592 (**0.023**[e], 0.077)	0.615 (**0.001**[e], **0.013**[e])
Proline	0.46-1.63	(**0.002**[e])	0.910	1.01 (**0.0004**[e], **0.013**[e])	1.01 (**0.001**[e], **0.013**[e])	0.975 (**0.012**[e], 0.059)	0.997 (**0.001**[e], **0.014**[e])
Serine	0.24-0.91	(**0.009**[e])	0.498	0.550 (**0.002**[e], **0.014**[e])	0.550 (**0.001**[e], **0.014**[e])	0.529 (**0.042**[e], 0.122)	0.536 (**0.015**[e], 0.061)
Threonine	0.22-0.67	(**0.005**[e])	0.364	0.394 (**0.001**[e], **0.014**[e])	0.394 (**0.001**[e], **0.013**[e])	0.384 (**0.023**[e], 0.077)	0.390 (**0.003**[e], **0.020**[e])
Tryptophan	0.03-0.22	(0.088)	0.052	0.055 (0.067, 0.173)	0.056 (**0.025**[e], 0.082)	0.056 (**0.014**[e], 0.060)	0.056 (**0.029**[e], 0.092)
Tyrosine	0.10-0.79	(0.390)	0.336	0.355 (0.535, 0.708)	0.375 (0.214, 0.370)	0.339 (0.907, 0.964)	0.314 (0.500, 0.687)
Valine	0.21-0.86	(**0.005**[e])	0.495	0.537 (**0.002**[e], **0.014**[e])	0.538 (**0.002**[e], **0.014**[e])	0.519 (0.054, 0.148)	0.538 (**0.001**[e], **0.014**[e])

[a] Combined range from Appendix 5.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
[e] Statistical difference indicated by P-Value <0.05.

Figure 29. Amino acid analysis of corn grain

Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Table 5.4) are shaded.

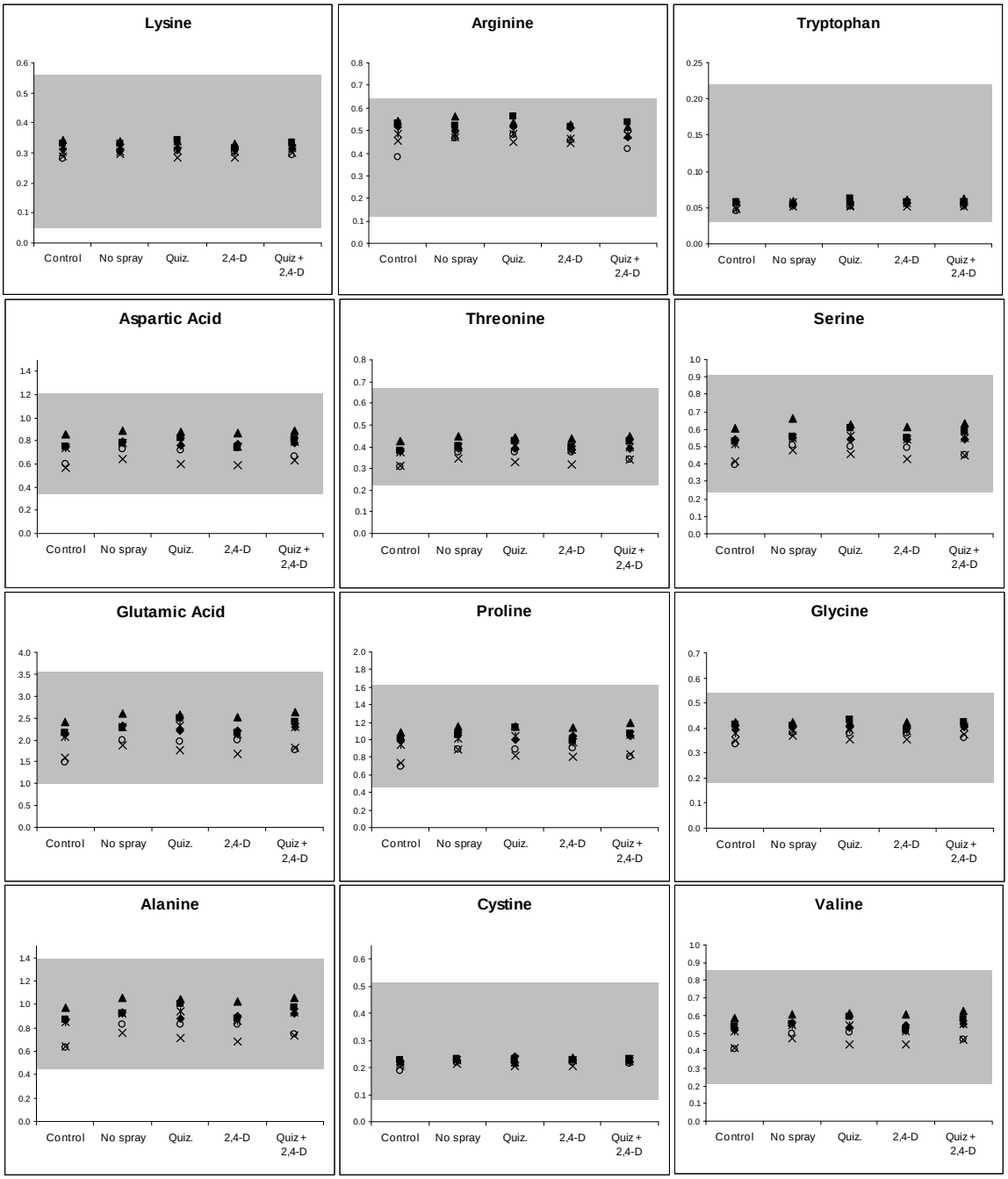
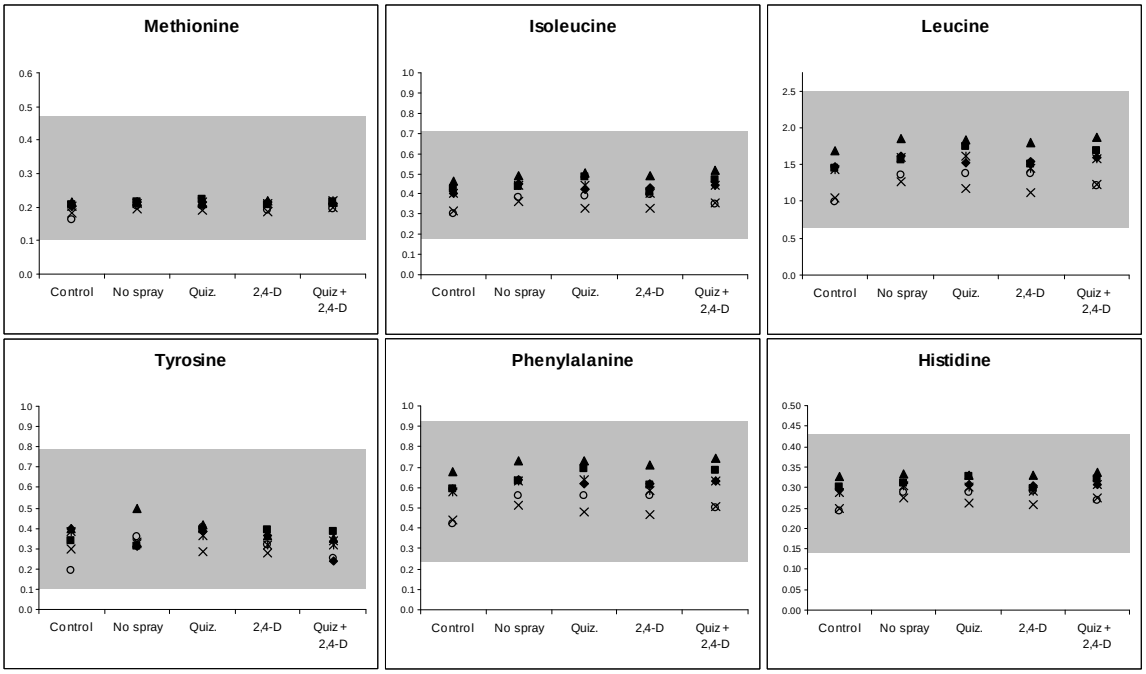


Figure 29. (cont.). Amino acid analysis of corn grain



VIII.B.4. Fatty Acid Analysis of Grain

An analysis of corn grain samples for fatty acids was performed. A summary of the results across all locations is shown in Table 21. All results for the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both corn grain samples analyzed for these fatty acids were within the published literature ranges (Figure 30). Results for caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), myristoleic (14:1), pentadecanoic (15:0), pentadecenoic (15:1), palmitoleic (16:1), heptadecanoic (17:0), heptadecenoic (17:1), gamma linolenic (18:3), eicosadienoic (20:2), eicosatrienoic (20:3), and arachidonic (20:4) were below the method LOQ. In the across-site analysis, no significant differences were observed for 16:0 palmitic, 18:0 stearic, 18:2 linoleic, 18:3 linolenic, and 20:0 arachidic. For 18:1 oleic and 20:1 eicosenoic, significant paired t-tests were observed for the unsprayed AAD-1 (18:1) and the AAD-1 + 2,4-D (18:1 and 20:1) entries, but were not accompanied by significant overall treatment effects or FDR adjusted p-values. For 22:0 behenic, a significant overall treatment effect and significant paired t-tests for AAD-1 + 2,4-D and AAD-1 + both were found, but significant FDR adjusted p-values were not present.

Table 21. Summary of the fatty acid analysis of corn grain

Fatty Acids (% total fatty acids)[a]	Literature Values[b]	Overall Treatment Effect (Pr>F)[c]	Control	Unsprayed (P-value[d], Adj. P[e])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
8:0 Caprylic	0.13–0.34	NA[f]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
10:0 Capric	ND	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
12:0 Lauric	ND–0.687	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
14:0 Myristic	ND-0.3	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
14:1 Myristoleic	NR[h]	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
15:0 Pentadecanoic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
15:1 Pentadecenoic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
16:0 Palmitic	7–20.7	(0.559)	9.83	9.89 (0.618, 0.763)	9.95 (0.280, 0.445)	9.78 (0.617, 0.763)	9.90 (0.544, 0.708)
16:1 Palmitoleic	ND–1.0	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
17:0 Heptadecanoic	ND–0.11	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
17:1 Heptadecenoic	ND– 0.1	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
18:0 Stearic	ND-3.4	(0.561)	2.04	1.98 (0.119, 0.254)	2.01 (0.437, 0.626)	2.00 (0.259, 0.421)	2.02 (0.598, 0.756)

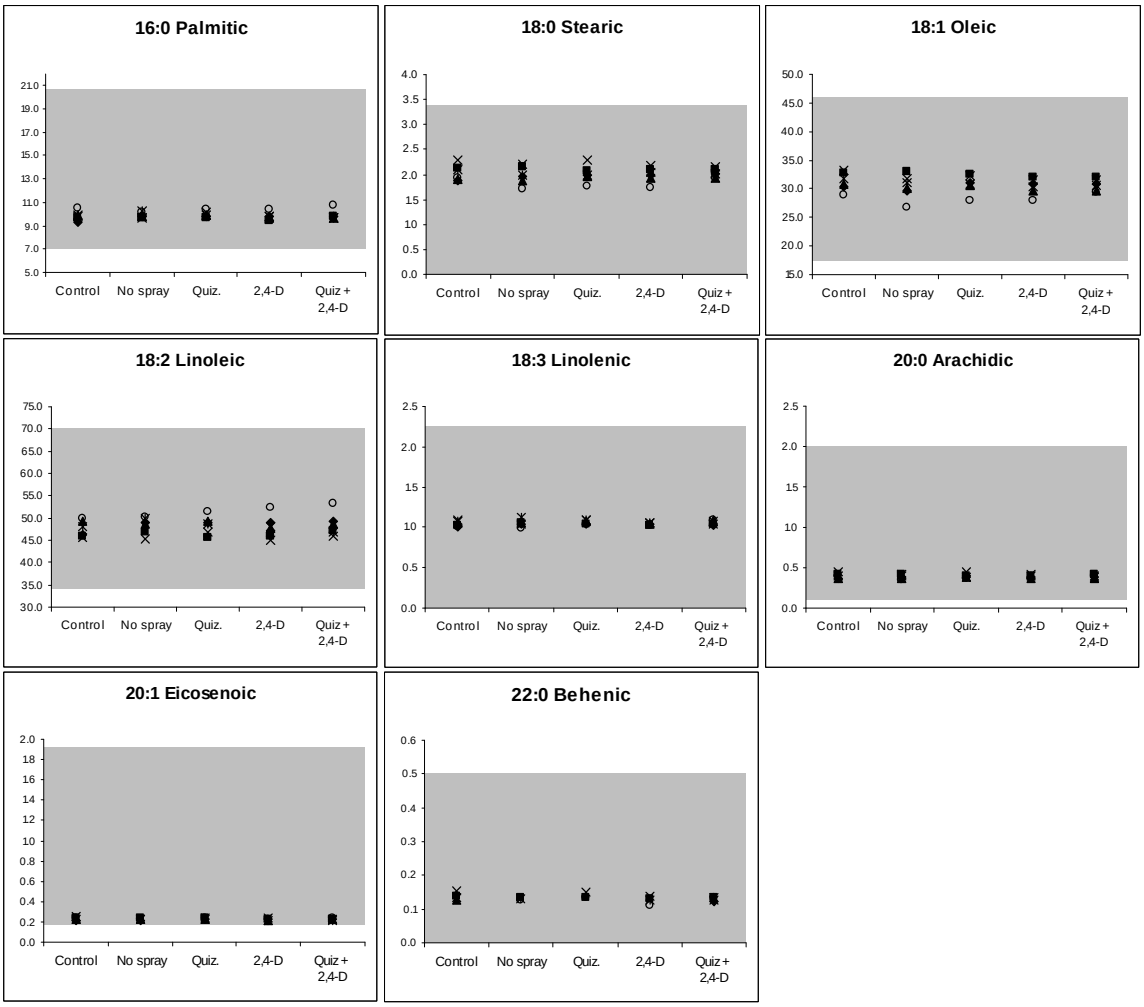
Table 21. (cont.). Summary of the fatty acid analysis of corn grain

Fatty Acids (% total fatty acids)[a]	Literature Values[b]	Overall Treatment Effect (Pr>F)[c]	Control	Unsprayed (P-value[d], Adj. P[e])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
18:1 Oleic	17.4 - 46	(0.076)	31.3	30.4 (**0.013**[g], 0.059)	30.8 (0.178, 0.329)	30.4 (**0.015**[g], 0.061)	30.7 (0.092, 0.213)
18:2 Linoleic	34.0-70	(0.474)	47.5	48.3 (0.189, 0.345)	48.4 (0.144, 0.289)	48.0 (0.453, 0.638)	48.5 (0.119, 0.254)
18:3 Gamma Linolenic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
18:3 Linolenic	ND-2.25	(0.479)	1.04	1.05 (0.537, 0.708)	1.06 (0.202, 0.357)	1.04 (0.842, 0.932)	1.06 (0.266, 0.428)
20:0 Arachidic	0.1-2	(0.379)	0.400	0.386 (0.061, 0.161)	0.393 (0.341, 0.525)	0.390 (0.153, 0.297)	0.390 (0.175, 0.328)
20:1 Eicosenoic	0.17–1.92	(0.107)	0.232	0.226 (0.089, 0.210)	0.230 (0.497, 0.687)	0.223 (**0.013**[g], 0.059)	0.227 (0.121, 0.254)
20:2 Eicosadienoic	ND–0.53	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
20:3 Eicosatrienoic	0.275	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
20:4 Arachidonic	0.465	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
22:0 Behenic	ND–0.5	(**0.044**[g])	0.136	0.088 (0.093, 0.213)	0.076 (0.887, 0.957)	0.086 (**0.011**[g], 0.054)	0.108 (**0.023**[g], 0.077)

[a] Results converted from units of % dry weight to % fatty acids.
[b] Combined range from Appendix 5.
[c] Overall treatment effect estimated using an F-test.
[d] Comparison of the transgenic treatments to the control using t-tests.
[e] P-values adjusted using a False Discovery Rate (FDR) procedure.
[f] NA= statistical analysis was not performed since a majority of the data was < LOQ.
[g] Statistical difference indicated by P-Value <0.05.
[h] NR = not reported.

Figure 30. Fatty acid analysis of corn grain

Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Table 5.5) are shaded. Grain was also analyzed for C8:0, C10:0, C12:0, C14:0, C14:1, C15:0, C15:1, C16:1, C17:0, C17:1, C18:3 gamma, C20:2, C20:3 and C20:4, but levels were below level of quantitation at some or all of the sites.



VIII.B.5. Vitamin Analysis of Grain

The levels of vitamin A, B1, B2, B5, B6, B12, C, D, E, niacin, and folic acid in corn grain samples from the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both corn entries were determined. A summary of the results across all locations is shown in Table 22. Mean results for vitamins B12, D and E were not quantifiable by the analytical methods used. All mean results reported for vitamins were similar to reported literature values, when available (Figure 31). Results for the vitamins without reported literature ranges (vitamins B5 and C) were similar to control values obtained (< 22% difference from control). For the across-site analysis, no statistical differences were observed, with the exception of vitamins B1, C and niacin. Significant paired t-tests for vitamin B1 were observed between the control and unsprayed AAD-1, AAD-1 + quizalofop, and AAD-1 + both, but were not accompanied by significant overall treatment effects or FDR adjusted p-values. For vitamin C, a significant overall treatment effect was observed along with significant paired t-tests and FDR adjusted p-values for AAD-1 + quizalofop and AAD-1 + 2,4-D. Similarly for niacin, a significant overall treatment effect was observed along with significant paired t-tests and FDR adjusted p-values for AAD-1 + quizalofop and AAD-1 + both. A significant paired t-test for the AAD-1 + 2,4-D was also found for niacin for the AAD-1 + 2,4-D entry, but was not accompanied by a significant overall treatment effect or FDR adjusted p-value. Since the differences were not observed across sites and values were within literature ranges (when available), the differences are not biologically meaningful.

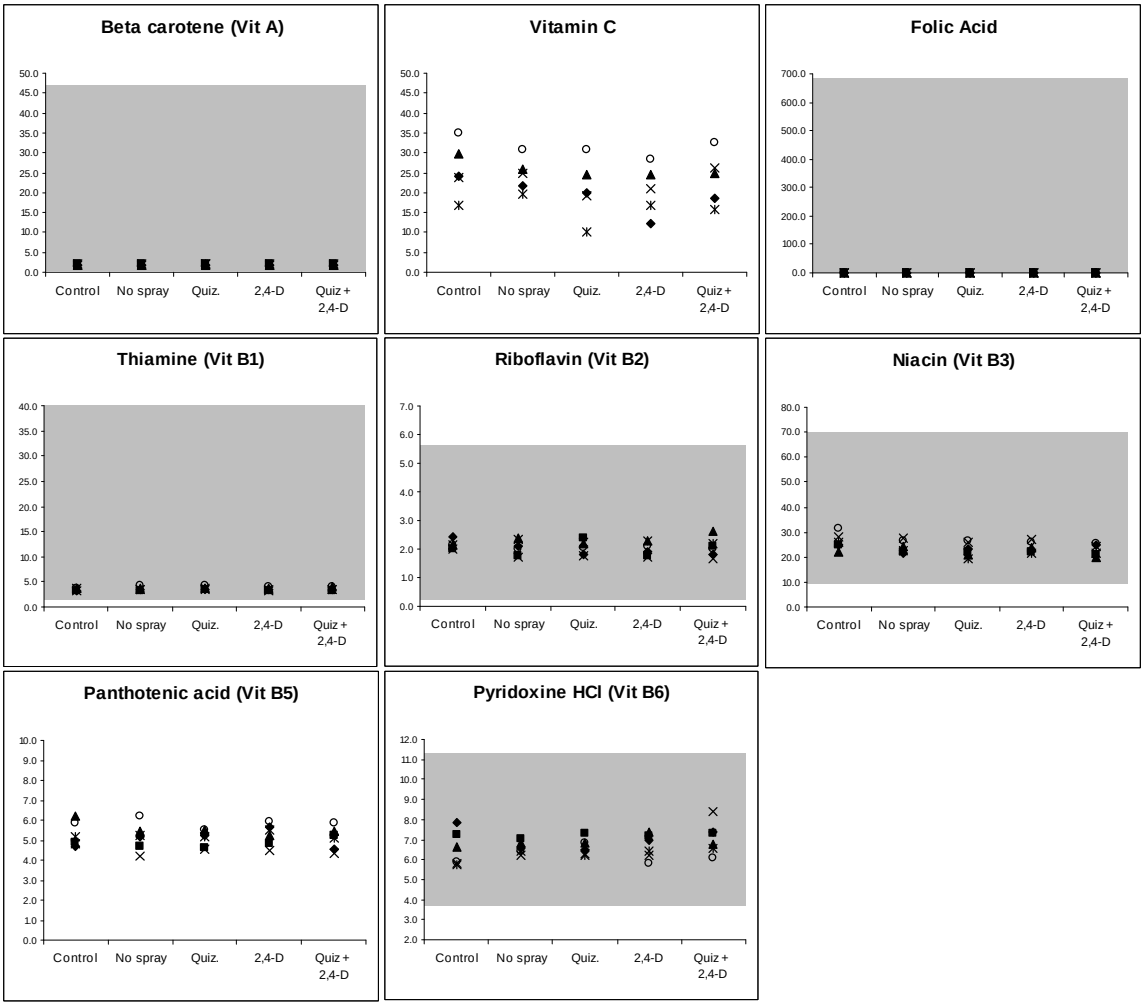
Table 22. Summary of the vitamin analysis of corn grain

Vitamins (mg/kg dry weight)	Literature Values[a]	Overall Treatment Effect (Pr>F)[b]	Control	Unsprayed (P-value[c], Adj. P[d])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Beta Carotene (Vitamin A)	0.19 - 46.8	(0.649)	1.80	1.85 (0.372, 0.566)	1.80 (0.967, 0.983)	1.82 (0.770, 0.883)	1.87 (0.221, 0.376)
Vitamin B1 (Thiamin)	1.3 - 40	(0.068)	3.47	3.63 (**0.041**[e], 0.121)	3.67 (**0.013**[e], 0.059)	3.54 (0.375, 0.567)	3.64 (**0.032**[e], 0.100)
Vitamin B2 (Riboflavin)	0.25 - 5.6	(0.803)	2.15	2.05 (0.443, 0.631)	2.08 (0.600, 0.756)	1.99 (0.227, 0.383)	2.07 (0.543, 0.708)
Vitamin B5 (Pantothenic acid)	NR[f]	(0.820)	5.28	5.17 (0.623, 0.766)	5.09 (0.391, 0.582)	5.29 (0.968, 0.983)	5.10 (0.424, 0.615)
Vitamin B6 (Pyridoxine)	3.68 – 11.3	(0.431)	6.52	6.57 (0.859, 0.938)	6.66 (0.652, 0.782)	6.66 (0.652, 0.782)	7.08 (0.088, 0.210)
Vitamin B12	NR	NA[g]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Vitamin C	NR	(**0.018**[e])	22.4	21.2 (0.268, 0.429)	17.5 (**0.005**[e], **0.028**[e])	18.0 (**0.004**[e], **0.026**[e])	20.4 (0.068, 0.173)
Vitamin D	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Vitamin E (alpha Tocopherol)	1.5 - 68.7	(0.558)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Niacin (Nicotinic acid,Vit. B3)	9.3 - 70	(**0.013**[e])	26.1	24.2 (0.050, 0.140)	22.9 (**0.002**[e], **0.017**[e])	23.7 (**0.018**[e], 0.067)	22.9 (**0.002**[e], **0.016**[e])
Folic Acid	0.15 - 683	(0.881)	0.594	0.588 (0.779, 0.890)	0.574 (0.403, 0.592)	0.592 (0.931, 0.970)	0.597 (0.916, 0.970)

[a] Combined range from Appendix 5.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
[e] Statistical difference indicated by P-Value <0.05.
[f] NR = not reported.
[g] NA= statistical analysis was not performed since a majority of the data was < LOQ.

Figure 31. Vitamin analysis of corn grain

Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Table 5.6) are shaded. Grain was also analyzed for Vitamin E, Vitamin B12 and Vitamin D, but results were less than the limit of quantitation.



VIII.B.6. Secondary Metabolite and Anti-Nutrient Analysis of Grain

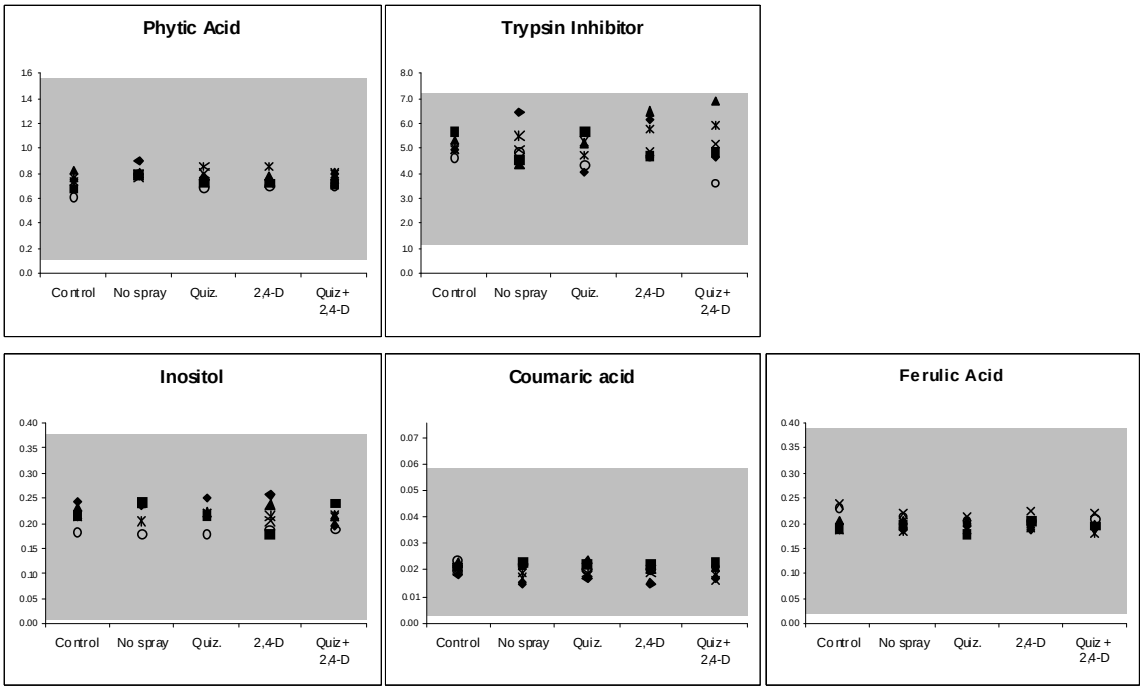
The secondary metabolite (coumaric acid, ferulic acid, furfural and inositol) and anti-nutrient (phytic acid, raffinose, and trypsin inhibitor) levels in corn grain samples from the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both corn entries were determined. A summary of the results across all locations is shown in Table 23. For the across-site analysis, all values were within literature ranges (Figure 32). No significant differences between the AAD-1 entries and the control entry results were observed in the across-site analysis for inositol and trypsin inhibitor. Results for furfural and raffinose were below the method's limit of quantitation. Significant paired t-tests were observed for coumaric acid (unsprayed AAD-1, AAD-1 + 2,4-D and AAD-1 + both), and ferulic acid (AAD-1 + quizalofop and AAD-1 + both). These differences were not accompanied by significant overall treatment effects or FDR adjusted p-values and were similar to the control (< 10% difference). A significant overall treatment effect, paired t-test, and FDR adjusted p-value was found for phytic acid (unsprayed AAD-1). Since all results were within literature ranges and similar to the control (<11% difference), these differences are not biologically meaningful.

Table 23. Summary of the secondary metabolite and anti-nutrient analysis of corn grain

Analyte	Literature Values[a]	Overall Treatment Effect (Pr>F)[b]	Control	Unsprayed (P-value[c], Adj. P[d])	Sprayed Quizalofop (P-value, Adj. P)	Sprayed 2,4-D (P-value, Adj. P)	Sprayed Both (P-value, Adj. P)
Secondary Metabolite (% dry weight)							
Coumaric Acid	0.003-0.058	(0.119)	0.021	0.020 (**0.038**[e], 0.113)	0.020 (0.090, 0.211)	0.019 (**0.022**[e], 0.074)	0.020 (**0.029**[e], 0.091)
Ferulic Acid	0.02-0.389	(0.077)	0.208	0.199 (0.051, 0.141)	0.196 (**0.010**[e], 0.051)	0.200 (0.080, 0.196)	0.197 (**0.019**[e], 0.069)
Furfural	0.0003-0.0006	NA[f]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Inositol	0.0089-0.377	(0.734)	0.218	0.224 (0.548, 0.708)	0.218 (0.973, 0.984)	0.213 (0.612, 0.763)	0.211 (0.526, 0.708)
Anti-Nutrient (% dry weight)							
Phytic Acid	0.11-1.57	(**0.046**[e])	0.727	0.806 (**0.003**[e], **0.020**[e])	0.767 (0.099, 0.224)	0.755 (0.245, 0.402)	0.761 (0.158, 0.304)
Raffinose	0.02-0.32	NA[f]	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Tryspin Inhibitor (TIU/mg)	1.09-7.18	(0.742)	5.08	5.10 (0.954, 0.977)	4.87 (0.631, 0.770)	5.45 (0.387, 0.582)	5.18 (0.813, 0.911)

[a] Combined range from Appendix 5.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
[e] Statistical difference indicated by P-Value <0.05.
[f] NA= statistical analysis was not performed since a majority of the data was < LOQ.

Figure 32. Secondary metabolite and anti-nutrient analysis of corn grain
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix 5, Tables 5.7) are shaded. Grain was also analyzed for furfural and raffinose, but results were less than the limit of quantitation.



VIII.C. Summary of Grain and Forage Composition

All mean values for the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both entry samples were within literature ranges for corn. A limited number of significant differences between unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D or AAD-1 + both corn and the control were observed, but the differences were not biologically meaningful because they were small and/or results were within ranges found for commercial corn. Plots of the composition results do not indicate any biologically-meaningful treatment-related compositional differences among unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D or AAD-1 + both corn and the control corn line (Figures 26-32). In conclusion, unsprayed DAS-40278-9, DAS-40278-9 sprayed with quizalofop, DAS-40278-9 sprayed with 2,4-D, and DAS-40278-9 sprayed with both quizalofop and 2,4-D composition results confirm the equivalence of DAS-40278-9 corn to conventional corn.

VIII.D. References

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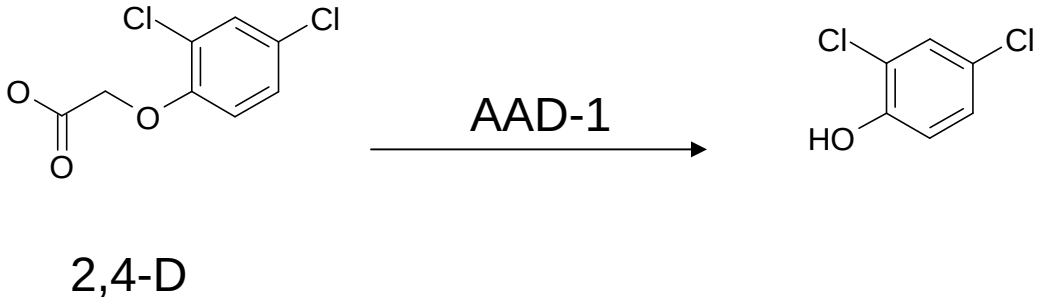
IX. Environmental Evaluation and Impact on Agronomic Practices

IX.A. Mode of Action of the AAD-1 Protein

The aryloxyalkanoate dioxygenase (AAD-1) gene and expressed protein are present in nature in the soil bacterium *Sphingobium herbicidovorans. S. hercidovorans*, like other soil dwelling bacteria, has evolved over time the ability to use herbicides as a carbon source for growth, affording the bacteria a competitive advantage in soil (Wright *et al.*, 2009). *Sphingobium* spp. are gram-negative bacteria commonly isolated from soil and were previously grouped with other sphinogmonads under the genus *Sphingomonas*. Sphingomonads are widely distributed in nature and have been isolated from land and water habitats, as well as from plant root systems, clinical specimens, etc. Due to their biodegradative and biosynthetic capabilities, the sphinogmonads have been used for a wide range of biotechnological applications, including bioremediation of environmental contaminants and production of extracellular polymers such as sphingans which are used extensively in the food industry (Bower *et al*., 2006; Pollock and Armentrout, 1999; Lal *et al*., 2006; Johnsen *et al*., 2005).

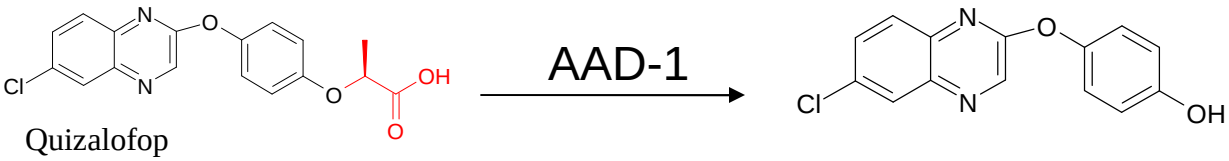
Sphingobium herbicidovorans carries genes which encode enzymes which facilitate the breakdown of 2,4-D and AOPP herbicides to allow them to be used as carbon sources for the bacterium (Kohler, 1999). The *aad-1* gene from *S. herbicidovorans* encodes one such enzyme, aryloxyalkanoate dioxygenase or AAD-1. This alpha-ketoglutarate-dependent dioxygenase enzyme has been shown to facilitate a one-step metabolic detoxification of 2,4-D to the herbicidally-inactive compound, dichlorophenol (DCP) (Figure 33) (Wright *et al.*, 2009). AAD-1 is able to degrade the *R*-enantiomers (herbicidally active isomers) of the chiral phenoxy auxins (*e.g.*, dichlorprop and mecoprop) in addition to achiral phenoxy auxins (*e.g.*, 2,4-D, MCPA, 4-chlorophenoxyacetic acid). AAD-1 also catalyzes the degradation reaction of the general class of herbicides known as aryloxyphenoxypropionates (AOPPs), such as quizalofop, to their corresponding inactive phenols (Figure 34) (Wright *et al.*, 2009).

Figure 33. Metabolic detoxification of 2,4-D by AAD-1



2,4-D

Figure 34. Metabolic detoxification of quizalofop by AAD-1



Quizalofop

IX.A.1. Field Efficacy

The efficacy of DAS-40278-9 corn for the detoxification of 2,4-D and aryloxyphenoxypropionate herbicides, and subsequent protection of corn from injury caused by these compounds, was characterized in field studies during 2007 and 2008. 2,4-D tolerance testing of initial DAS-40278-9 introgressions was conducted in 2007 under USDA notifications 06-338-101n and 07-242-103n. 2,4-D is expected to be used at or below 1120 g ae/ha, but tolerance assessments were conducted at highly exaggerated rates to identify any potential tolerance issues. Plant injury of 5% or less (0-100 visual scale) was observed on DAS-40278-9 plants 14 days after application of 4480 g ae/ha of 2,4-D amine at the V4 stage (see Table 7 for plant growth stages). The same treatment applied to a conventional hybrid resulted in approximately 35% injury.

Detailed field evaluations were conducted in 2008 at locations in Mississippi, Indiana, and Minnesota under USDA notification number 08-021-104n. The response of DAS-40278-9 hybrids to applications of 2,4-D amine and quizalofop (with 1% v/v crop oil concentrate) at the V4 and V7-V8 growth stages was compared to conventional control hybrids. The trials were a split plot design with three replications at each site consisting of single row plots approximately 6 m in length. Two test hybrids (see breeding diagram, Figure 4) were evaluated to investigate differences in auxin-herbicide sensitivity across genotypes. An application of quizalofop at 50 g ai/ha was applied at the V1-V2 growth

stage to all test plots to remove unintended nulls prior to application of herbicide treatments.
The results from evaluation of plant injury are presented in Table 24. The data were analyzed as a split-plot design where application timing served as the whole plot factor and the different hybrids served as the sub plot factor. Statistical analysis involved the use of mixed model methods using restricted maximum likelihood with the location and replication factors considered random. All injury ratings are relative to the corresponding non-treated controls.

Application of 2,4-D amine at the V4 growth stage resulted in 11 to 61% injury to the conventional control hybrids depending on rate and hybrid. The same treatments caused 1 to 8% injury to hybrids containing DAS-40278-9. When applications were made at the V7-V8 stage, injury to the conventional control hybrids ranged from 10 to 38% and injury to DAS-40278-9 hybrids ranged from 0-5%. The most evident symptom from 2,4-D treatments was plant leaning. Some leaf necrosis was also observed at the 2240 and 4480 g ae/ha rates, presumably due to formulation effects at the very high rates being applied to the leaves. Quizalofop at any rate or stage of application resulted in complete necrosis and death of all conventional control plants and caused 0 to 2% injury of DAS-40278-9 hybrids.

Table 24. Plant injury from applications of 2,4-D and quizalofop to DAS-40278-9 corn

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]			
			40278 Hybrid 1	Control Hybrid 1	40278 Hybrid 2	Control Hybrid 2
2,4-D amine	1120 g ae/ha	V4	1 ns	11 b	1 ns	13 c
2,4-D amine	2240 g ae/ha	V4	1 ns	26 b	6 ns	31 bc
2,4-D amine	4480 g ae/ha	V4	3 ns	39 a	8 ns	61 a
2,4-D amine	1120 g ae/ha	V7-V8	0 ns	10 b	0 ns	15 c
2,4-D amine	2240 g ae/ha	V7-V8	2 ns	16 b	0 ns	21 bc
2,4-D amine	4480 g ae/ha	V7-V8	2 ns	31 a	5 ns	38 b
Herbicide	**Rate**	**Application Stage**	**Percent Plant Injury[d]**			
			40278 Hybrid 1	**Control Hybrid 1**	**40278 Hybrid 2**	**Control Hybrid 2**
quizalofop	92 g ai/ha	V4	2	100	2	100
quizalofop	184 g ai/ha	V4	1	100	0	100
quizalofop	92 g ai/ha	V7-V8	0	100	0	100
quizalofop	184 g ai/ha	V7-V8	2	100	0	100

[a] ae/ha = acid equivalent/hectare, ai/ha = active ingredient/hectare
[b] Application stage in terms of corn plant growth development (see Table 7).
[c] Means within each hybrid-column followed by the same letter are not significantly different as determined by restricted maximum likelihood methods for mixed models and Tukey, or for unbalanced data, Tukey-Kramer HSD test (0.05). ns indicates no significant differences.

[d] No significant differences within hybrids were detected using restricted maximum likelihood methods appropriate for analysis of data from multiple random locations.

Additional testing of post-emergence herbicide applications to DAS-40278-9 corn and conventional corn hybrids gave similar results. Sequential applications of 2,4-D amine or 2,4-D amine followed by quizalofop were tested, resulting in 0-1% injury for DAS-40278-9 hybrids. Also, a single rate of 2240 g ae/ha of 2,4-D amine applied at the V6-V7 stage to 2 row by 6 m plots across six locations in five states (Nebraska, Iowa, Minnesota, Illinois, and Indiana) resulted in average injury scores of 21% for conventional control hybrids and 2% for DAS-40278-9 hybrids.

In addition to post-emergence tolerance, DAS-40278-9 corn was evaluated for its ability to provide tolerance to pre-emergence applications of 2,4-D. Trials were conducted at locations in Mississippi, Indiana, and Minnesota using a randomized complete block design with three replications of two row plots, approximately 6 m in length, at each site. Herbicide-treated plots were paired with untreated plots to provide accurate evaluation of emergence and early season growth. Herbicide treatments of 1120, 2240, and 4480 g ae/ha of 2,4-D amine were applied shortly after planting but before crop emergence. Approximately 16-21 days after planting and application, injury averaged from 18 to 34% for the conventional control hybrid as rate increased from 1120 to 4480 g ae/ha of 2,4-D amine. Injury to corn containing DAS-40278-9 ranged from 2 to 10% across the same rate range.

The current proposed target application rates for DAS-40278-9 corn are at or below 1120 g ae/ha for 2,4-D and 92 g ai/ha for quizalofop. Tolerance was tested at exaggerated rates with minimal damage. Results of field testing indicate that DAS-40278-9 provides robust tolerance for both 2,4-D and quizalofop herbicide treatments at rates more than two to four times the proposed target use rates.

IX.B. Weediness Potential of DAS-40278-9 Corn

Weediness characteristics have been generally described as (1) the ability for weed seed to germinate in many different environments; (2) discontinuous germination and great longevity of seed; (3) rapid growth through vegetative phase to flowering; (4) continuous seed production for as long as growing conditions permit; (5) self-compatibility but partially autogamous and apomictic; (6) ability to be cross-pollinated by unspecialized visitors or wind-pollinated; (7) high seed output in favorable environments and some seed production in a wide range of environments; (8) adaptation for short and long-distance dispersal; (9) vegetative production or regeneration from fragments and brittleness (hard to remove from the ground); and (10) ability to compete interspecifically by special means (Baker, 1974; GEO-PIE, 2009).

Corn does not exhibit any of the foregoing significant weedy tendencies and is non-invasive in natural environments (CFIA, 1994). As with other crop plants, corn hybrids

have been domesticated for such a long period of time that the seeds cannot be disseminated without human intervention, nor can corn readily survive in the U.S. from one growing season to the next due to the lack of seed dormancy. Volunteer corn plants are, in any case, easily identified and controlled through manual or chemical means.

The introduction of the trait for aryloxyalkanoate-tolerance should not confer weediness characteristics to corn since herbicide-tolerance will not change the seed dispersal and growth habits that prevent corn plants from spreading into the wild. Agronomic characteristics, including weediness traits like germination, seedling vigor, and response to environmental stressors, have been shown to be equivalent for DAS-40278-9 corn and conventional corn (Section VII). The survival of volunteer DAS-40278-9 corn plants in agricultural fields can be easily controlled by manual or chemical means (chemicals other than 2,4-D and aryloxyphenoxypropionate herbicides).

There are no wild, weedy relatives of *Zea mays* known to exist in the United States. Therefore, outcrossing of the *aad-1* gene does not pose a plant pest risk leading to the enhancement of weediness of wild relatives of corn.

IX.C. Gene Flow Assessment

IX.C.1. Vertical Gene Flow Assessment

Vertical gene flow is the transfer of genetic information to a species that can interbreed with corn, either a wild relative of corn or other commercial corn crops. Wild relatives of corn with the potential for interbreeding include some *Zea* spp. and the closely related *Tripsacum* spp.

Non-cultivated *Zea mays* species are not found in the United States. Teosinte is the common name for all the wild relatives of domesticated corn. The teosintes are a large group of grasses of the genus *Zea* found in Mexico, Guatemala and Nicaragua. There are five recognized species: *Zea diploperennis, Zea perennis, Zea luxurians, Zea nicaraguensis* and *Zea mays* (Buckler and Stevens, 2005). *Zea mays* subsp. *parviglumis* is the closest living relative of corn, however up to 12% of the genetic material in modern corn may have been obtained from *Zea mays* subsp. *mexicana* through introgression. Cultivated corn and the wild members of diploid and tetraploid *Zea* can be crossed to produce fertile F1 hybrids. However, in the wild, introgressive hybridization does not occur because of differences in flowering time, geographic separation, block inheritance, developmental morphology and timing of reproductive structures, dissemination and dormancy (Galinat, 1988).

The genus most closely related to *Zea mays* is *Tripsacum*, a genus of eleven species. *Tripsacum* spp. are found throughout North and South America and have almost twice as many chromosomes (N=18) as does *Zea* (N=10) (Buckler and Stevens, 2005). The three species of *Tripsacum* that are native to North America are: *T. floridanum, T.*

lanceolatium, and *T. dactyloides. T. floridanum* is found in south Florida and is used as an ornamental grass for landscaping. *T. lanceolatim* is found in the Mule Mountains of Arizona and possibly in southern New Mexico. *T. dactyloides* (Eastern gamma grass) is indigenous to most of the southern, central and northeastern U.S. It is commonly grown as a forage grass and is the only species that overlaps with corn production areas. Cultivated corn and all species of *Tripsacum* will hybridize, though F1 hybrids are highly male sterile. It has been suggested that *Tripsacum* and corn share a teosinte-like ancestor (Galinat, 1988). In 1999, research at USDA resulted in fertile *Tripsacum* x *Zea mays* F1 hybrid plants, but the resulting plants carried a *Tripsacum* cytoplasm with a *Zea mays* nuclear background (DeWald and Sims, 2000). The possibility of corn contributing genetic material to *Tripsacum* through random pollen flow in agricultural situations is considered to be extremely remote (CFIA, 1994). Even if this were to occur, the presence of the *aad-1* gene in progeny resulting from crossing with DAS-40278-9 corn would not be expected to have altered viability or plant-pest status compared with progeny resulting from crossing with conventional corn.

Outcrossing is known to occur in cultivated corn. Corn is monoecious with male (tassel) and female (ear) structures located on different parts of the plant. It is wind pollinated, with factors such as air movement, synchronization of flowering, and density of the pollen clouds affecting the extent of pollen movement (Henry *et al*. 2003). The prevalence of outcrossing is strongly distance dependent, with the amount of gene flow dropping off rapidly within the first 20 meters from the donor crop and a slower rate of decrease beyond that point (Henry *et al.* 2003). Overall, the frequency of outcrossing with neighboring fields is thought to be fairly low, due to the short distances corn pollen will travel and the limited window of viability (Luna *et al*., 2001). Additionally, outcrossing potential is minimized in seed production fields due to traditional containment practices to ensure seed genetic purity. Seed production fields are located in isolation to prevent introgression of genetic material from unwanted sources of corn pollen.

DAS-40278-9 corn pollen characteristics, such as shape and viability, are the same as for conventional corn (Section VII.D). There are no other morphological or phenotypic changes associated with DAS-40278-9 corn, or the herbicide trait it carries, that would lead to an increased risk of gene flow with DAS-40278-9 corn over that present today for cultivated corn. Any progeny that may arise from outcrossing with neighboring fields would not be any more persistent or weedy in the environment than seed arising from crosses with conventional corn.

IX.C.2. Horizontal Gene Flow Assessment

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Connor *et al.*, 2003). Even if such a transfer were to take place, transfer of the *aad-1* gene from line DAS-40278-9 would not present a human health or plant pest risk, based on the safety data presented in this petition. The gene encoding the AAD-1

protein is from a naturally occurring soil bacterium, *Sphingobium herbicidovorans*, and is already present in nature. Transfer recipients would, therefore, not pose a greater plant pest risk than the environmentally prevalent wild type microbes from which the genes originated.

IX.D. Current Agronomic Practices for U.S. Corn

IX.D.1. Corn Production

In the United States, corn is the most widely produced feed grain, accounting for more than 90% of total value and production of feed grains. Approximately 80 million acres of land are planted to corn annually in the U.S. Although corn is primarily the main energy ingredient in livestock feed, it is also processed into a multitude of food and industrial products (starch, sweeteners, corn oil, beverage and industrial alcohol, fuel ethanol). Approximately 20% of the U.S. corn crop is exported to other countries (USDA ERS, 2008a).

A total of 86.0 million acres were planted to corn in the United States in 2008 (USDA NASS, 2008). Of these planted acres, about 78.6 million acres were harvested for grain, valued at $47.4 billion. The majority of this corn was grown within the North Central states. About 80% of the planted corn acres and 82% of the corn harvested for grain was concentrated in 11 states – Iowa, Illinois, Nebraska, Minnesota, Indiana, South Dakota, Kansas, Wisconsin, Ohio, Missouri, and Michigan. An additional 6.0 million acres of the 86.0 million planted acres in 2008 was harvested for silage to feed livestock.

IX.D.2. Weeds in Corn

Weeds in corn compete with the crop for water, nutrients and light, especially during the first 3-5 weeks following emergence of the crop (U. of Calif., 2008). Hartzler and Pringnitz (2000) define the critical period of competition in corn as the point of time when weeds that emerge with the crop begin to impact yields. Most studies have found that corn yields are protected if weeds are controlled before they reach a height of 4 to 5 inches. The critical period varies widely, depending upon weed species and densities, environmental conditions, and cultural practices.

Corn yields are often reduced if weeds are not removed or controlled before the corn reaches 6-8 inches in height. Corn yield losses of up to 20% have been caused by 6-9 inch grasses or 16 inch ragweed in corn, mainly due to the weeds' ability to compete with corn for nitrogen (Univ. of IL, 2006). Gower *et al*. (1999) reported data collected from 37 experiments conducted across the U.S. in 1998 and 1999 that showed average corn yields were not affected if weeds were controlled by the time they reached a height of 2 inches. Delaying herbicide application in these trials until weeds were 4, 6, 9, and 12 inches tall resulted in a 2, 6, 8, and 22 percent yield loss, respectively.

Presence of weeds in the corn crop may also raise grain moisture, reduce silage feed quality and provide a seed source to infest subsequent crops. Late season infestations do not reduce corn yield as much as early season infestations, however, weeds at this time may harbor destructive insect pests such as thrips and armyworms.

The most common weed problems in corn are the annual grass and broadleaf weeds (see Table 25). Some fields are also infested with perennial weeds (quackgrass, Johnsongrass, Canada thistle, others) which are more difficult to control since they can reproduce from seed or underground rhizomes.

Table 25. Common troublesome weeds in corn in 2006-2008

Weed Species	Total Corn Acres Treated[1]		
	2006	2007	2008
Annual Broadleaf Weeds			
Velvetleaf	32,639,656	38,067,417	38,348,588
Lambsquarters, Common	27,729,773	38,799,304	35,411,846
Pigweed, Redroot	24,397,379	30,720,276	31,142,588
Cocklebur, Common	26,670,703	31,979,217	29,269,518
Waterhemp, Common	17,769,320	21,999,427	22,122,385
Ragweed, Giant	15,198,219	23,000,540	18,902,934
Kochia	8,113,965	11,360,340	12,209,017
Ragweed, Common	7,813,228	10,453,697	11,570,295
Morningglory Spp.	5,576,339	8,816,185	8,172,085
Sunflower, Wild	9,022,276	10,120,430	7,813,610
Marestail	1,691,196	4,652,221	7,634,113
Waterhmep, Tall	2,691,830	4,217,315	4,626,981
Horseweed	2,468,981	3,691,440	4,380,850
Smartweed Pennsylvania	2,220,680	3,598,403	3,125,235
Dandelion	1,549,611	1,850,465	2,954,936
Sunflower, Volunteer	2,107,552	2,242,900	2,446,062
Chickweed	1,203,086	1,492,489	2,284,681
Nighshade, Black	1,117,249	1,877,962	1,968,864
Annual Grass Weeds			
Foxtail Spp.	65,296,257	91,517,492	81,556,481
Crabgrass	5,616,575	7,348,358	7,271,287
Panicum, Fall	3,561,337	5,189,220	5,893,464
Shattercane	2,649,688	2,902,197	3,469,087
Barnyardgrass	2,127,617	3,432,993	3,750,535
Cupgrass, Woolly	2,301,147	4,402,913	3,255,254
Wheat, Volunteer	873,275	994,485	1,923,184
Perennial Weeds			
Johnsongrass	6,242,647	10,924,150	10,061,245
Thistle, Canada	3,507,840	4,642,652	5,456,394
Quackgrass	4,998,154	6,599,202	4,661,797

[1]Total corn acres in 2006, 2007, and 2008 was 78.3, 93.5, and 86.0 million acres, respectively. However, the total corn herbicide-treated acreage is much more, due to multiple sprays on each acre. Data from DMR-Kynetec.

The economic threshold for weeds is the density of a weed population at which control is economically justified due to the potential for yield reduction, quality loss, harvesting difficulties, or other problems that weeds may cause (Penn State Agronomy Guide, 2009-2010). Broadleaf and grass weeds compete at different levels of intensity depending upon the competitiveness of the crop, tillage system, environmental conditions and other weeds present. Generally, broadleaf weeds are more damaging to a broadleaf crop, while grass weeds are more competitive in a grass crop. Crop yield loss information is available for certain single weed species growing with corn in the U.S. Midwest (Table 26).

Table 26. Yield reduction from specific weed species in corn
(from Penn State Agronomy Guide, 2009-2010).

	Percent corn yield reduction[1]					
	1	2	4	6	8	10
Weed Species	**Weeds per 100 feet of row**					
Cocklebur	4	8	16	28	34	40
Pigweed or Lambsquarters	12	25	50	100	125	150
Shattercane (5-8/clump)	6	12	25	50	75	100
Giant foxtail	15	30	60	85	175	400
Velvetleaf	10	15	20	30	40	50
Yellow nutsedge[2]	400	800	--	--	--	--

[1] Interference data are from Stoller *et al*., 1985, *Reviews of Weed Science*; E. L. Knake and F. W. Slife, 1962, *Weeds* 10:26; and E. L. Werner and W. S. Curran, 1995, Proc. NEWSS 49:23.
[2] Dash (--) signifies that no data were available.

IX.D.3. Weed Management in Corn

In 2005, USDA NASS surveyed 19 states (CO, GA, IL, IN, IA, KS, KY, MI, MN, MO, NE, NY, NC, ND, OH, PA, SD, TX, WI; 93% of the total U.S. corn acreage) and found that 97% of the planted corn acreage was treated with herbicides, an indication of the intensive weed management that is used in U.S. corn (USDA NASS, 2006). Use of herbicides is especially important when weed populations are high and contain difficult-to-control weeds (U. of Calif., 2008). Use of herbicides to control weeds in corn results in reduction of early competition of weed infestation, reduction in the weed seed bank in the soil, and reduction of the potential for weed competition in the following crop. Many herbicides are registered for pre-plant, pre-emergent and/or post-emergent application in

corn to selectively control most weed species commonly found in corn. In general, corn receives a soil applied herbicide application followed by a post-emergence application. Atrazine (66% of acres), glyphosate (33% of planted acres—up from 19% in 2003), S-metolachlor (23% of acres) and acetochlor (23% of acres) were the most widely used herbicides in corn.

Integrated weed management (IWM) programs advocate the use of a combination of preventive, cultural, mechanical and chemical tools to keep weed pressure below threshold levels that reduce yield and profits (Knezevic, 2002). Herbicides are only one of several tools available for growers to consider using in an IWM approach. Herbicide-tolerant corn represents another tool that can be used in an IWM program.

As with any technology, some concerns have been raised about using herbicides and herbicide-tolerant crops as part of an IWM program (Knezevic, 2002). Some of these concerns include selection for herbicide-resistant weeds, shifts in weed species, drift to sensitive non-target crops or non-agricultural habitats, herbicide persistence in the soil resulting in carryover to the next crop, herbicide misapplications, and over-reliance on herbicides to control weeds.

Weed management decisions are difficult decisions for growers since no one tool will effectively control all possible weed problems. In general, growers need to implement management practices that limit the introduction and spread of weeds, help the crop to compete with weeds, and not allow weeds to adapt. The combination of weed control practices that a grower chooses is dependent upon the weed spectrum, level of infestation, soil type, cropping system, weather, and time and labor available for the treatment option.

IX.D.4. Crop Rotation Practices

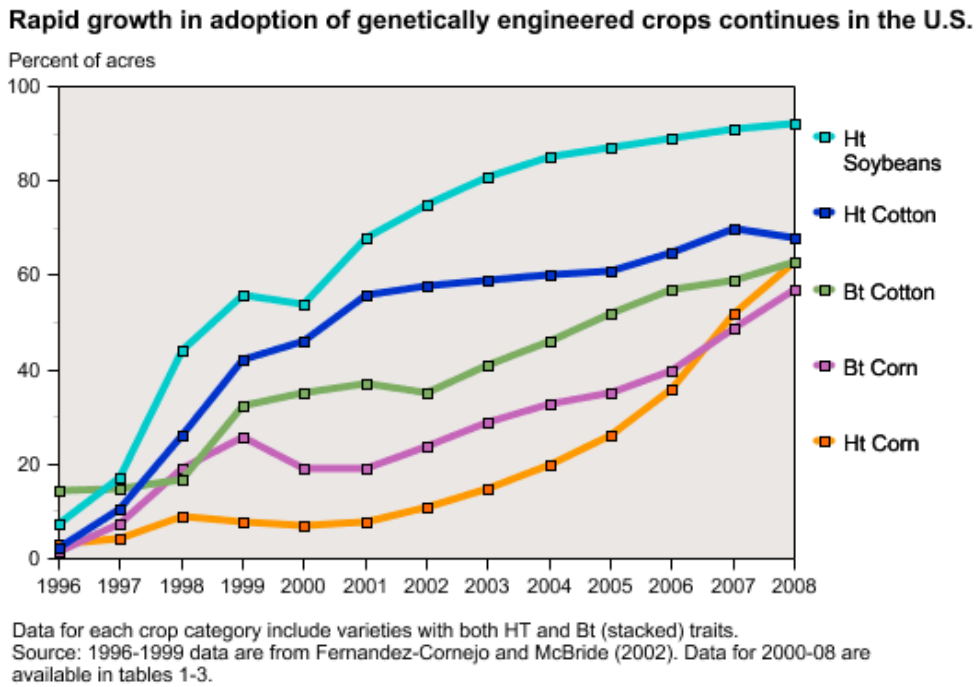
In 2005 in the U.S., 60% of corn acres were grown in rotation after soybeans, 26% were grown after corn, and 14% were grown after cotton, small grains, fallow or other crops (USDA ERS ARMS, 2005). Crop rotation is a widespread management practice that has been recognized and exploited for centuries to increase crop yields (Lauer, 2007). In the Midwestern U.S., a corn-soybean rotation produces at least 10% greater yields in both crops, and sometimes as much as 19% higher corn yields. The exact mechanism for the rotation effect is unknown, but may be influenced by increases in organic matter and soil fertility, as well as management of diseases and weeds. Midwest corn growers have been more inclined to plant corn-on-corn in recent years for several reasons: the traditional corn to soybean rotation is not solving corn rootworm problems in an expanding region centered on eastern Illinois and northern Indiana; soybeans have suffered an array of pest problems (soybean cyst nematode, sudden death syndrome, leaf aphids); soybean yield increases have generally not kept up with corn yield increases (Erickson and Lowenberg-DeBoer, 2005); the expansion in use of corn as a biofuel; and the relative economic benefits have favored production of corn over soybean.

IX.E. Potential Impact of the Introduction of DAS-40278-9 on Agronomic Practices

IX.E.1. Potential Impact on Cultivation and Management Practices

Corn lines that contain herbicide-tolerance traits have been on the market since 1996 (glufosinate) and have experienced broad adoption (Figure 35). These products have provided simple, inexpensive yet highly effective means of controlling weeds and have resulted in an increase in no-till corn production (Sankula and Blumenthal, 2004); a practice that is now accepted as improving soil health and agricultural sustainability. With the 63% of corn acres in the U.S. in 2008 planted to herbicide-tolerant corn (USDA ERS, 2009), typical cultivation and management practices used by growers today already take into account the management of herbicide-tolerant traits. DAS-40278-9 corn is comparable to conventional corn phenotypically and agronomically (Section VII.A.) and is not expected to alter the geographic range or seasonality of corn cultivation. Furthermore, ecological observations during field testing have shown no changes in insect susceptibility of DAS-40278-9 corn (Section VII.B.) and therefore, no impacts are expected on insect control practices for DAS-40278-9 corn. It is anticipated that the same management practices used today for corn with other herbicide-tolerance traits, will also be appropriate for DAS-40278-9 corn.

Figure 35. Adoption of genetically engineered crops in the U.S.
From USDA ERS, 2009 (http://www.ers.usda.gov/Data/BiotechCrops/)



IX.E.2. Potential Impact on Weed Control Practices

DAS-40278-9 corn confers tolerance to phenoxy auxin herbicides (such as 2,4-D) and aryloxyphenoxypropionate (AOPP or "fop") herbicides, which will provide expanded weed management options in corn. Post-emergence applications of 2,4-D control a broad spectrum of broadleaf weeds. 2,4-D also has some short-lived soil residual activity (4-10 day soil half life) which provides limited residual control of later germinating broadleaf weeds. DAS-40278-9 corn will provide alternatives to glyphosate in weed management systems. 2,4-D would control the already glyphosate-resistant and hard to control broadleaf weeds, plus slow down the selection for more glyphosate-resistant broadleaf weeds (Powles, 2008a).

The maximum seasonal use rate for 2,4-D today in corn without the *aad-1* gene is 3360 g acid equivalent per hectare (ae/ha). The maximum seasonal use rate for 2,4-D in DAS-40278-9 corn will remain unchanged at 3360 g ae/ha, however the pattern of use of 2,4-D on DAS-40278-9 corn will be different (Figure 36). Currently, for corn without the *aad-1* gene, 2,4-D can be applied pre-emergence (up to 1120 g ae/ha), post-emergent on plants 8 inches or less in height (560 g ae/ha), post-emergent on corn >8 inches in height using drop nozzles (560 g ae/ha), and pre-harvest at the dent stage of corn development (1680 g ae/ha). In DAS-40278-9 corn, 2,4-D may be applied pre-emergence (1120 g ae/ha) followed by one or two post-emergence (560-1120 g ae/ha) applications at least 12 days apart over-the-top of the corn up to the V8 stage (or 48 inches in height) of development. Thus, the maximum seasonal rate of 2,4-D on corn has not been increased. However, DAS-40278-9 corn will allow the grower to apply 2,4-D from pre-emergence up through 48 inch corn (without using drop nozzles) without risk of crop injury. This will provide improved weed control during the corn development period when weeds have the greatest potential yield impact (see Section IX.D.2). 2,4-D currently cannot be applied to conventional corn from 7-14 days prior to planting thru 3-5 days after planting, due to potential for crop injury. Growers using corn with *aad-1* gene will not have this planting restriction.

DAS-40278-9 corn is also tolerant to the aryloxyphenoxypropionate (AOPP) herbicides such as quizalofop. These herbicides are not currently used on corn, since they lack selectivity in corn and would cause severe crop injury. However, quizalofop (up to 92 g/ha) can be safely applied post-emergent on DAS-40278-9 corn through the V6 development stage without risk of crop injury. This provides another option for growers to control grass weeds in corn, help reduce selection for glyphosate-resistance in these weeds, and manage any such weeds that may become resistant to glyphosate in the future. The AOPP-tolerance of DAS-40278-9 also allows the use of quizalofop for selection during hybrid corn breeding.

While both 2,4-D and quizalofop are currently registered pesticides, supporting information on proposed label changes for the use of these products with DAS-40278-9 corn is being provided by Dow AgroSciences to U.S. EPA for review. Dow AgroSciences is also developing an extensive stewardship program that will include

technological advancements in application and off-target movement as well as utilizing several media venues to educate and facilitate adoption of the technology and decision management tools to ensure the proper use and stewardship of both the trait and chemical technologies.

DAS-40278-9 corn may also be stacked with glufosinate- or glyphosate-tolerant traits. Such combined trait products (stacks) would have the potential to improve weed control by allowing use of herbicide combinations or mixtures which can provide more consistent performance in post-emergence weed control programs, and counteract glyphosate "rate-creep" (steady increase in rates needed to obtain effective weed control; Figure 37) on hard-to-control weeds (Jaehnig, 2005). Planting DAS-40278-9 corn, and thus enabling the use of 2,4-D, will provide a low cost, high performance solution to reduce the escalation of glyphosate- and ALS-resistance in weed populations, in addition to supplying a different herbicide-tolerant selectable marker for combining traits.

Figure 36. 2,4-D and Fop herbicide application timing and rates for conventional and DAS-40278-9 corn

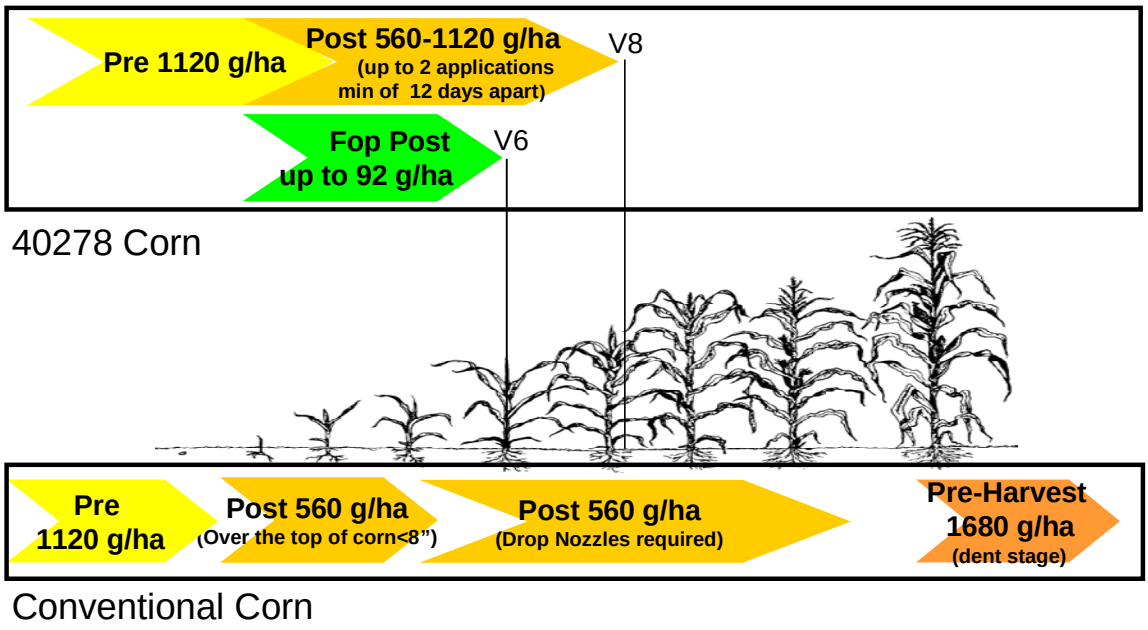
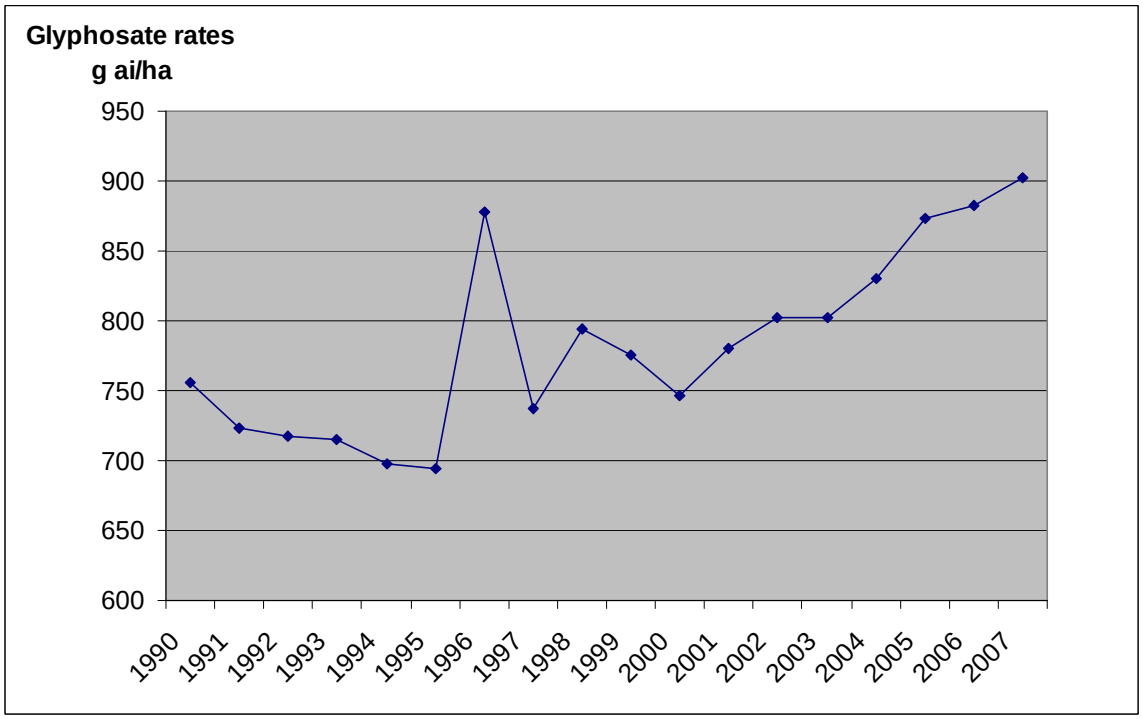


Figure 37. Glyphosate application rates in U.S. corn and soybeans from 1990 thru 2007. (Data from DMR-Kynetec)



IX.E.3. Potential Impact on Volunteer Management

Although DAS-40278-9 corn is tolerant to the AOPP "fop" herbicides such as quizalofop, it can still be effectively controlled with herbicides when present as unwanted volunteer plants in soybeans or other crops the following year. DAS-40278-9 corn is susceptible to the "dim" (cyclohexanedione) herbicides like clethodim and sethoxydim, plus some ALS inhibiting herbicides such as imazamox.

Approximately 63% of all corn planted in the U.S. in 2008 was herbicide-tolerant corn. This includes other glufosinate- and glyphosate-tolerant corn developed through recombinant technology, as well as imidazolinone-tolerant corn developed through selected mutagenesis and traditional plant breeding (USDA ERS, 2008b). If DAS-40278-9 corn crosses with corn lines expressing tolerance to herbicides with different modes of action to produce corn volunteers with multiple herbicide tolerances, they can still be effectively controlled mechanically or with post-emergent applications of "dim" herbicides. However, the competition from the pollen load within a given field would keep the incidence of this very low. Additionally, agronomic practices such as appropriate variety selections, crop rotation, and rotation of herbicides with different modes of action can be used to avoid or manage volunteer corn tolerant to one or a few herbicides.

IX.E.4. Potential Impact on Non-Target Organisms and Endangered Species

Based on substrate specificity of the aryloxyalkanoate dioxygenase (AAD-1) enzyme activity, no effect on non-target organisms or endangered species is anticipated for DAS-40278-9 corn. The AAD-1 gene and expressed protein are present in nature in the soil bacterium *Sphingobium herbicidovorans.* AAD-1 is not a potential food allergen or toxin (Section VI.E) and DAS-40278-9 corn has been shown to be substantially equivalent to conventional corn based on the compositional analysis of grain and forage (Section VIII). Observations made during field testing of DAS-40278-9 corn revealed no effects on invertebrate populations (Section VII.B) and agronomic characteristics equivalent to conventional corn (Section VII.A).

The Endangered Species Act (ESA) (16 USC 1531) is administered by the U.S. Fish & Wildlife Service (FWS). Under ESA, Section 6 requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact. It is not anticipated that DAS-40278-9 corn will impact any currently listed species of concern (U.S. FWS, 2009) since it is not anticipated that DAS-40278-9 corn will cause new corn acres to be planted in areas that are not already in agricultural use. Commercial cropping systems routinely disturb the ground in which crops are grown. Habitat disruption within DAS-40278-9 corn fields would be comparable to other no-till or herbicide-tolerant cropping systems.

Corn is not an invasive or weedy species, there are no invasive or weedy sexually compatible relatives of corn in the US, and these properties are not anticipated to be altered by the insertion of the *aad-1* gene conferring tolerance to a narrow set of herbicides. It is therefore reasonable to conclude that DAS-40278-9 corn will not affect threatened or endangered species or adversely affect or change designated critical habitats as compared to current commercial agricultural practices.

The ecological fate, and ecotoxicity effects on non-target organisms for the use of 2,4-D and quizalofop will be addressed by the EPA as part of their review process. 2,4-D is currently used in corn production as both a pre-plant and post-emergent herbicide treatment. In soybean, 2,4-D is used as a pre-plant burn down application prior to planting. Since corn and soybean are typically planted in rotation, no significant new geography will be treated. Further, the maximum seasonal rate and maximum single application rate for 2,4-D will not be increased. In fact, the current maximum application rate of 1680 g ae/ha will be reduced to 1120 g ae/ha for DAS-40278-9 corn (Figure 36). Similarly for quizalofop, which is currently registered for use in soybeans, the new post-emergent uses in corn will adhere to the same maximum application rate, maximum seasonal application, and general application timing as is currently registered for soybean. By maintaining the same, or reduced, application rates and maximum seasonal use rate there should be no change in the ecological risk assessments or endangered species assessments for 2,4-D or quizalofop uses with DAS-40278-9 corn

IX.F. Herbicide Resistance Management

IX.F.1. Herbicide Resistance

Herbicides have revolutionized weed control in most countries around the world. Herbicides are often the most reliable and least expensive method of weed control available, but reliance upon herbicides as the primary method of weed control can have unintended consequences. The widespread use of herbicides can lead to weed populations that are no longer susceptible to the herbicide being used.

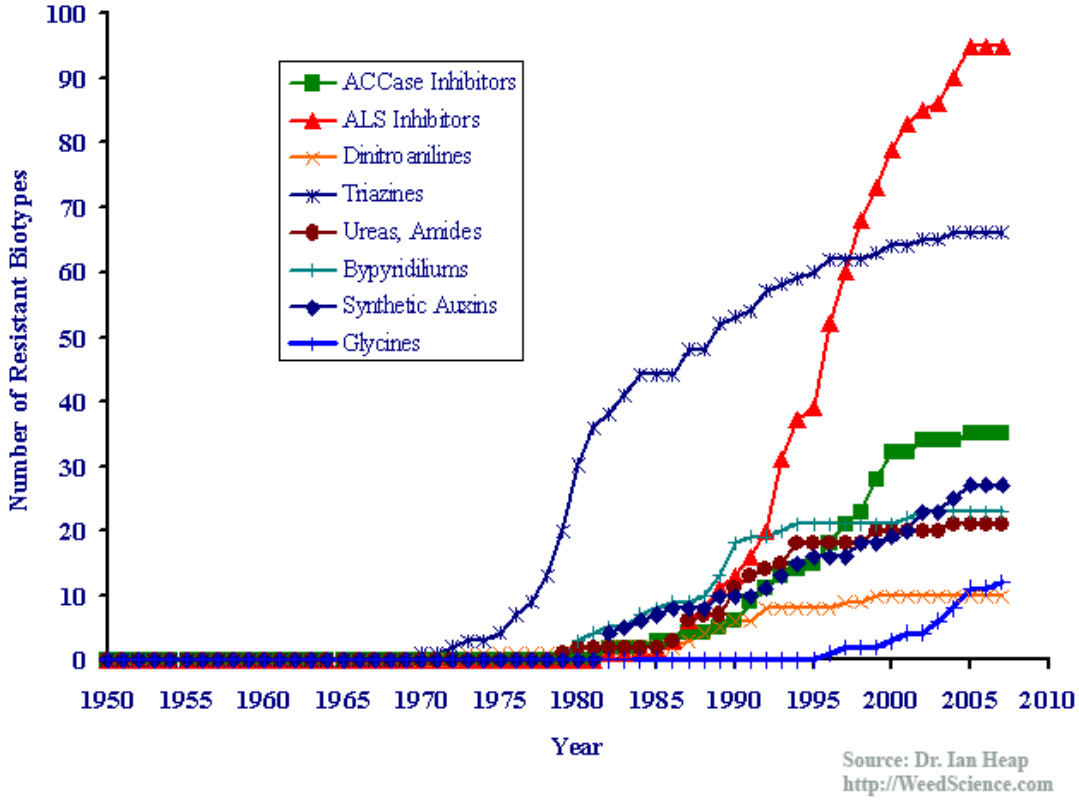
The Weed Science Society of America defines herbicide resistance as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type" (WSSA, 1998). Target plants with genes conferring resistance to a given herbicide can occur naturally within a population but in extremely small numbers. Such plants remain reproductively compatible with the wild-type and can pass resistance genes on to their progeny (Mallory-Smith, 2008). Repeated use of the herbicide may allow these resistant plants to survive and reproduce. The number of resistant plants then increases in the population until the herbicide no longer effectively controls the weed. Thus, this is an evolutionary process (Moss, 2002), whereby a population changes from being susceptible to being resistant. Individual plants do not change from being susceptible to being resistant; rather, the proportion of resistant individuals within the population increases over time.

Herbicide-resistant weeds have been a problem for growers for decades (Heap, 1997). The earliest documented reports of herbicide-resistant weeds were resistance to 2,4-D in wild carrot (*Daucus carota*) in 1952 and spreading dayflower (*Commelina diffusa*) in 1957 (Heap, 2009). During, the 1970s, up to 30 different weed species were reported to be resistant to the triazine herbicides (Bandeen *et al*., 1982). Today, more than 330 weed biotypes around the world have been reported to have some populations that are resistant to one or more herbicides (Heap, 2009). A weed biotype is a sub-type or sub-population of a weed species; in this case one that has developed resistance to one or more herbicides. Report of a resistant biotype for a given weed species does not mean that weed resistance is common, widespread, or persistent in that species. There are generally many other options available to control these resistant biotypes. Table 27 shows a tabular summary of the total number of resistant species for each herbicide mode of action. Figure 38 shows the number of resistant weed biotypes that have been reported over time for each herbicide mode of action. Additional information on glyphosate, 2,4-D, quizalofop, and the evolution of herbicide resistant weeds can be found in Appendix 6.

Table 27. Herbicide-resistant weeds
(Heap, 2009)

HERBICIDE RESISTANT WEEDS SUMMARY TABLE (June, 2009)					
Herbicide Group	**Mode of Action**	**HRAC Group**	**WSSA Group**	**Example Herbicide**	**Total Count**
ALS inhibitors	Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	B	2	Chlorsulfuron	101
Photosystem II inhibitors	Inhibition of photosynthesis at photosystem II	C1	5	Atrazine	68
ACCase inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	A	1	Diclofop-methyl	36
Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	O	4	2,4-D	27
Bipyridiliums	Photosystem-I-electron diversion	D	24	Paraquat	24
Ureas and amides	Inhibition of photosynthesis at photosystem II	C2	7	Chlorotoluron	21
Glycines	Inhibition of EPSP synthase	G	9	Glyphosate	16
Dinitroanilines and others	Microtubule assembly inhibition	K1	10	Trifluralin	10
Thiocarbamates and others	Inhibition of lipid synthesis - not ACCase inhibition	N	8	Triallate	8
Triazoles, ureas, isoxazolidiones	Bleaching: Inhibition of carotenoid biosynthesis (unknown target)	F3	11	Amitrole	4
PPO inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	E	14	Oxyfluorfen	3
Chloroacetamides and others	Inhibition of cell division (Inhibition of very long chain fatty acids)	K3	15	Butachlor	3
Carotenoid biosynthesis inhibitors	Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase	F1	12	Flurtamone	2
Arylaminopropionic acids	Unknown	Z	25	Flamprop-methyl	2
Nitriles and others	Inhibition of photosynthesis at photosystem II	C3	6	Bromoxynil	1
Mitosis inhibitors	Inhibition of mitosis / microtubule polymerization inhibitor	K2	23	Propham	1
Cellulose inhibitors	Inhibition of cell wall (cellulose) synthesis	L	27	Dichlobenil	1
Unknown	Unknown	Z	8	(chloro) - flurenol	1
Organoarsenicals	Unknown	Z	17	MSMA	1
Total Number of Unique Herbicide Resistant Biotypes					**330**

Figure 38. Resistant weed biotypes per herbicide mode of action.



IX.F.2. Factors Impacting Development of Resistance

There are several factors to consider when assessing the risk for herbicide resistance in a weed species. Some of these relate to the biology of the weed species in question, others relate to particular farming practices. The key factors influencing a plant's potential to develop resistance have been outlined by the Herbicide Resistance Action Committee (HRAC), an industry initiative that fosters cooperation between plant protection manufacturers, government, researchers, advisors and farmers. These key factors include the number or density of weeds, natural frequency of resistant plants in the population, seed soil dormancy potential, frequent use of herbicides with a similar mode of action, cropping rotations with reliance primarily on herbicides for weed control, and lack of non-chemical weed control practices (HRAC, 1998).

A matrix that can be used to evaluate the risk of selection for herbicide-resistant weeds based on cropping system practices in shown in Table 28. This table assesses the risk of herbicide-resistance development for each management practice as either "low", "medium", or "high". The greatest chance for resistance development occurs when several of these management practices fall into the "high" category.

Table 28. Assessment of resistance risk by evaluation of cropping systems
(Nevill, *et al*., 1998)

Management Option	Risk of Resistance		
	Low	**Moderate**	**High**
Herbicide mix or rotation in cropping system	>2 modes of action	2 modes of action	1 mode of action
Weed control in cropping system	Cultural, mechanical and chemical	Cultural* and chemical	Chemical only
Use of same MOA per season	Once	More than once	Many times
Cropping system	Full rotation	Limited rotation	No rotation
Resistance status to MOA	Unknown	Limited	Common
Weed infestation	Low	Moderate	High
Control in last 3 years	Good	Declining	Poor

*Cultural control can be by using cultivation, stubble burning, competitive crops, stale seedbeds, etc. See HRAC guidelines for more details.

Development of herbicide-resistance is often thought of as a problem caused by the herbicide itself. However, it is well documented that resistance results from management practices that have relied too heavily on a particular herbicide as the sole method of weed control. Under these conditions, the risk of weeds developing herbicide-resistance is greatest and the best defense is diversity in weed management practices.

IX.F.3. Herbicide Resistance Management

Although no cases of glyphosate-resistant weeds were documented for 20 years after the launch of glyphosate (Dyer, 1994), glyphosate-resistant biotypes of several weed species have now been reported in the United States (Powles, 2008b). This may be attributed to increased reliance on glyphosate for weed control after the launch of glyphosate-tolerant soybeans (1996), cotton (1997), and corn (1998). This evolution of glyphosate-resistant weed populations threatens the ongoing sustainability of glyphosate and its contributions to world food production (Duke and Powles, 2008).

As the number of glyphosate-resistant weed species increases, it becomes increasingly important for growers to introduce greater diversity into their weed management programs (Powles, 2008a). This diversity could be achieved with herbicide rotations/sequences, mixtures of robust herbicides with different modes of action, and use of non-herbicide weed control tools. Glyphosate is increasingly being mixed with effective doses of other herbicides to manage these hard-to-control and resistant weed species. New herbicide-tolerant traits are needed that are tolerant to at least one other class of herbicides that can be used to control the glyphosate-resistant weed populations and to reduce selection pressure for additional glyphosate-resistant weed species. Table 29 shows that several common weeds in U.S. corn and soybeans which are resistant to or difficult to control with glyphosate and ALS herbicides can be effectively controlled with 2,4-D.

Introduction of DAS-40278-9 corn will give farmers one more tool for use in their weed management programs which will help insure the long term sustainability of weed management programs, including the use of glyphosate. DAS-40278-9 corn will allow use of 2,4-D to control glyphosate-resistant weeds and significantly delay the selection for glyphosate-resistance in other weed species. The same benefits of DAS-40278-9 corn can be extended to other herbicide-tolerant cropping systems, such as those with tolerance to glufosinate or ALS-inhibiting herbicides. Furthermore, DAS-40278-9 corn will allow use of "fop" herbicides to significantly delay the selection for glyphosate-resistance in grass weed species.

Table 29. Glyphosate and ALS resistant weeds controlled by 2,4-D
(Heap, 2009).

Weed Species	Glyphosate	ALS Herbicides	2,4-D
Common lambsquarters	Difficult: Suspected Resistant (2004)	Resistant (2001)	Susceptible
Common ragweed	Confirmed Resistant (2004)	Resistant (1998)	Susceptible
Eastern black nightshade	Difficult (2004)	Resistant (1999)	Susceptible
Giant ragweed	Confirmed Resistant (2004)	Resistant (1998)	Susceptible
Marestail (horseweed)	Confirmed Resistant (2000)	Resistant (2000)	Susceptible
Palmer amaranth	Confirmed Resistant (2005)	Resistant (1991)	Susceptible
Waterhemp spp.	Difficult: Confirmed Resistant (2005)	Resistant (1993)	Susceptible
Prickly sida	Difficult (2004)	Resistant (1993)	Susceptible

In summary, DAS-40278-9 corn will expand the range of herbicides that can be used in herbicide-tolerant corn production systems, improving the ease and effectiveness of managing resistant and hard-to-control weeds and delaying the evolution of resistance to glyphosate and other herbicides.

IX.G. Summary of Environmental Evaluation

The AAD-1 protein is an enzyme with alpha-ketoglutarate-dependent dioxygenase activity which results in metabolic inactivation of the herbicides of the aryloxyalkanoate family. Field testing results confirm that AAD-1 protein expressed in DAS-40278-9 corn provides robust tolerance for both 2,4-D and quizalofop herbicide applications. There are no new phenotypic characteristics in DAS-40278-9 corn to indicate it is any different from conventional corn in weediness potential, and like conventional corn, the risk of gene flow from AAD-1 to wild relatives in the U.S. is negligible.

No significant impact is expected on current crop management practices, non-target or endangered species, crop rotation, or volunteer management with the use of DAS-40278-9 corn. The availability of DAS-40278-9 corn will have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The availability of DAS-40278-9 corn will allow growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance in weeds.

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X. Adverse Consequences of Introduction

Field and laboratory testing of DAS-40278-9 corn demonstrated no significant differences from non-transgenic conventional corn apart from the intended change of herbicide-tolerance. DAS knows of no study results or other observations associated with the DAS-40278-9 corn event that would be anticipated to result in adverse environmental consequences from introduction. Hybrids derived from event DAS-40278-9 will be the first corn hybrids to express tolerance to 2,4-D. As such, they will be an important tool to growers in areas where weeds have become resistant to other broad spectrum herbicides, such as glyphosate.

XI. Appendices

Appendix 1. Methods for Molecular Characterization of DAS-40278-9 Corn

1.1. DAS-40278-9 Corn Material

Transgenic corn seeds from five distinct generations of corn containing event DAS-40278-9 were planted in the greenhouse. After at least two weeks of growth, leaf punches were taken from each plant and were tested for AAD-1 protein expression using a rapid lateral flow test strip according to the manufacturer's instructions. Each plant was given a "+" or "-" for the presence or absence of the AAD-1 protein.

1.2. Control Corn Material

Seeds from the unmodified XHH13 were planted in the greenhouse. The XHH13 seeds had a genetic background representative of the transgenic seeds but did not contain the *aad-1* gene.

1.3. Reference Materials

DNA of the plasmid pDAS1740 was added to samples of the XHH13 control genomic DNA and used as the positive control to verify probe hybridization and sizes of internal fragments.

1.4. DNA Probe Preparation

DNA probes specific to the genetic elements of pDAS1740 were produced via polymerase chain reaction (PCR) amplification using pDAS1740 plasmid DNA as a template.

1.5. Sample Collection and DNA Extraction

Leaf samples collected from greenhouse-grown corn plants of event DAS-40278-9 and the XHH13 control were frozen in liquid nitrogen and stored at -80 ^{o}C. Genomic DNA was extracted from the frozen corn leaf tissue using the CTAB method. Briefly, approximately 15 mL of CTAB buffer (2.0% CTAB, 20 mM EDTA, 100 mM Tris-HCl, 1.4 M NaCl, pH8.0, autoclaved) and over 10 µl of RNase-A were added to individual tubes of ground leaf tissue. The samples were mixed and then incubated at 65 ^{o}C in an incubator-shaker (~50 RPM) for ~2 hours. After incubation, an equal volume of 24:1 chloroform:octanol was added to each sample tube and mixed by gentle rocking of the tubes for 5 minutes. The samples were then centrifuged for 20 minutes at 3500 RPM and the supernatants were subsequently transferred to individual tubes. The chloroform:octanol extraction step was repeated twice. After the second extraction step, an equal volume of isopropanol (~15 mL) was added to each tube and the sample tubes were centrifuged at 3000 RPM for ~10 minutes. The supernatant was decanted and

discarded. The pellets were dried at room temperature and then resuspended in ~1 mL 1× TE (10 mM Tris, 1 mM EDTA, pH 7.5) buffer which had been pre-warmed to ~65 ^{o}C. Following extraction, the DNA was allowed to completely dissolve in TE buffer before being quantified spectrofluorometrically using the Pico Green reagent (Invitrogen). The DNA was visualized by electrophoresis on an agarose gel to determine the DNA quality and confirm the Pico Green quantification analysis.

1.6. DNA Digestion and Electrophoretic Separation of the DNA Fragments

Genomic DNA extracted from the corn leaf tissue was digested with restriction enzymes by combining approximately 9 μg of genomic DNA with approximately 5-11 units of the selected restriction enzyme per μg of DNA in the corresponding reaction buffer. Each sample was incubated at 37 ^{o}C overnight. The positive control sample was prepared by combining pDAS1740 plasmid DNA with genomic DNA from the XHH13 control (at a ratio approximately equivalent to 1 copy of the transgene per corn genome) and was digested using the same procedures and restriction enzymes as the transgenic DNA samples. DNA from the XHH13 control was digested using the same procedures and restriction enzymes as the test samples to serve as the negative control.

The digested DNA samples were precipitated with Quick-Precip (Edge BioSystems) 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 (89mM Tris, 89mM Boric acid, 2mM EDTA) at 55-65 V for 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide and the DNA was visualized under UV light. A photographic record was made of each stained gel.

1.7. Southern Transfer

The DNA fragments on the agarose gels were transferred to nylon membranes via Southern transfer, essentially as described by Memelink, *et al*., 1994. The agarose gels were depurinated, denatured, neutralized *in situ* and transferred to a nylon membrane in 10× SSC buffer (3M NaCl, 0.3M Na citrate) using a wicking system. Following transfer to the membrane, the DNA was bound to the membrane by UV crosslinking.

1.8. Probe Synthesis and Hybridization

The hybridization probes were generated using a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, from DNA fragments generated by primers specific to the gene elements and other regions from plasmid pDAS1740. The PCR synthesis of the probes was performed using PCR DIG Probe Synthesis Kit (Roche Diagnostics) and following the manufacturer's recommended procedures.

Labeled probes were hybridized to the target DNA on the nylon membranes using the DIG Easy Hyb Solution according to manufacturer's instructions (Roche Diagnostics).

DIG-labeled DNA molecular weight marker II was used to determine the hybridizing fragment size on the Southern blots.

1.9 Detection

DIG-labeled probes bound to the nylon membranes after stringent washing, were incubated with AP (Alkaline Phosphatase)-conjugated anti-Digoxigenin antibody for ~ 1 hr in room temperature. The anti-DIG antibody specifically bound to the probes was then visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System (Roche Diagnostics). Blots were exposed to chemiluminescent film for one or more time points to detect the hybridizing fragments and to visualize the molecular weight standards. The images were then scanned and stored. The number and size of each of the detected bands were documented for each digest and for each probe.

Once the data was recorded, membranes were rinsed with milli-Q water and then stripped of the probe in a solution of 0.2M NaOH and 1.0% SDS. The alkali-based stripping procedure successfully removes the labeled probes from the membranes, allowing them to be re-probed with a different gene probe. After stripping, the membranes were exposed to chemiluminescent film to ensure all the previous DNA probes had been removed.

1.10. References

Memelink, J., Swords, K., Harry, J., Hoge, C. 1994. Southern, Northern, and Western Blot Analysis. Plant Molecular Biology Manual F1:1-23.

Appendix 2. Methods and Results for the Characterization of the AAD-1 Protein

2.1. DAS-40278-9 Corn Material

Greenhouse-grown DAS-40278-9 F1 hybrid plants were used as the plant source of the AAD-1 protein. Prior to use, individual plants were leaf tested to confirm expression of the AAD-1 protein using a rapid lateral flow test strip according to the manufacturer's instructions. Stalks from AAD-1 expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

2.2. Control Corn Material

Control corn line XHH13 had a genetic background representative of the DAS-40278-9 corn plants but did not contain the *aad-1* gene. Absence of AAD-1 expression in the control plants was confirmed by leaf testing using the AAD-1 specific rapid lateral flow test strip. Stalks of control plants were harvested, lyophilized, ground and stored under the same conditions as the DAS-40278-9 corn.

2.3. Reference Material

Recombinant AAD-1 microbial protein was produced in *Pseudomonas fluorescens* (*Pf*) and purified to a lyophilized powder. The microbe-derived AAD-1 protein preparation was stored dry and resuspended in buffer immediately prior to use.

2.4. Protein Purification from DAS-40278-9 Corn Plant Tissue

The AAD-1 protein was extracted from lyophilized stalk tissue in PBST (Phosphate Buffered Saline with 0.5% Tween 20, pH 7.4) buffer with added stabilizers, and the soluble proteins were collected by centrifugation. The supernatant was filtered and loaded onto an anti-AAD-1 immunoaffinity column which had been conjugated with an AAD-1 specific monoclonal antibody. The non-bound proteins were collected from the column and the column was washed extensively with 20 mM ammonium bicarbonate buffer, pH 8.0. The bound proteins were eluted from the column with a 3.5 M NaSCN, 50 mM Tris, pH 8.0 buffer and examined by SDS-PAGE and western blotting.

2.5. SDS-PAGE and Western Blot Analysis of Crude Extracts

Lyophilized stalk tissue from event DAS-40278-9 and XHH13 were mixed with PBST buffer containing 10% protease inhibitor cocktail (Sigma) and the protein was extracted by grinding with ball bearings in a Geno-Grinder. The samples were centrifuged and the supernatants were mixed with Laemmli sample buffer, heated and centrifuged briefly. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived AAD-1, was also mixed with sample buffer and loaded on to the gel. Electrophoresis was conducted with Tris/glycine/SDS buffer

(Bio-Rad). Following electrophoresis, the gel was cut in half, with one half stained with Pierce GelCode Blue protein stain and the other gel half was electro-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was then probed with an AAD-1 specific polyclonal rabbit antibody. A chemiluminescent substrate was used to visualize the immunoreactive bands.

2.6. Detection of Post-Translational Glycosylation

The immunoaffinity-purified, plant-derived AAD-1 protein was analyzed for evidence of glycosylation by electrophoresis with microbe-derived AAD-1 protein, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase as controls. The control protein samples were adjusted to concentrations approximately equal with the plant-derived AAD-1 protein and mixed with Laemmli buffer. The proteins were heated, centrifuged, and applied directly to a Bio-Rad Criterion SDS-PAGE gel. Following electrophoresis, the gel was cut in half. One gel half was stained with Pierce GelCode Blue stain for total protein. The remaining half of the gel was stained with GelCode Glycoprotein Stain to visualize the glycoproteins. The glycoproteins present on the gel were visualized as magenta bands on a light pink background.

2.7. Mass Spectrometry Peptide Mass Fingerprinting and Sequencing of Plant- and Microbe-Derived AAD-1 Protein

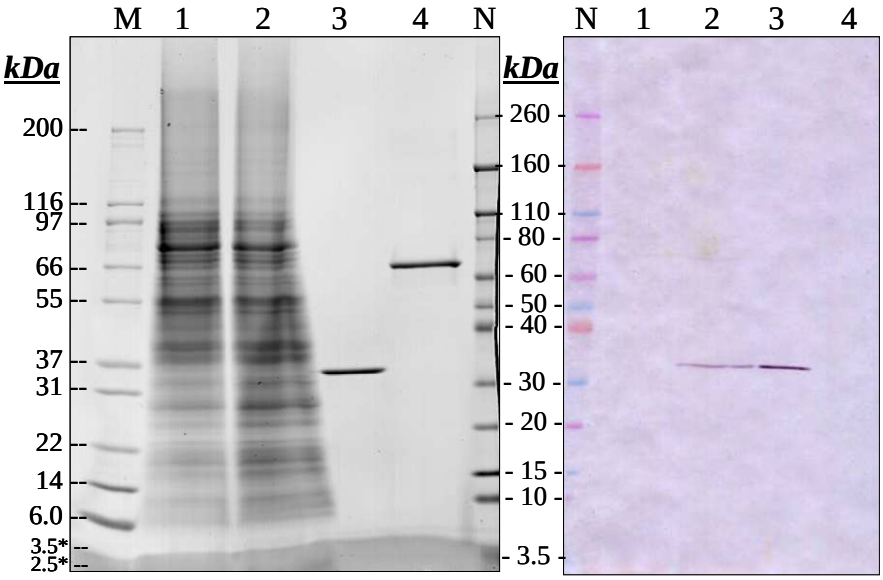
Mass spectrometry analysis of the plant- and microbe-derived AAD-1 proteins was conducted at the Analytical Sciences Laboratory of the Dow Chemical Company. The immunoaffinity purified AAD-1 plant-derived protein was subjected to in-solution digestion by trypsin followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray-ionization liquid chromatography/mass spectrometry (ESI-LC/MS). The amino acid residues at the N- and C-termini of the plant-derived AAD-1 protein were sequenced using tandem mass spectrometry and compared to the sequence of the microbe-derived protein.

2.8. Results of the SDS-PAGE and Western Blot Analysis

The plant extracts from the non-transgenic control and AAD-1 DAS-40278-9 are seen in lanes 1 and 2 on the Coomassie stained SDS-PAGE gel (Figure 2.1, Panel A). The microbe-derived AAD-1 is seen in lane 3 at the expected size of approximately 33 kDa. When probed with anti-AAD-1 polyclonal antibody in western blot analysis, the microbe-derived AAD-1 and DAS-40278-9 plant tissue extract showed a positive signal of the expected size (Figure 2.1, Panel B). No immunoreactive proteins were observed in the non-transgenic control plant extract and no alternate size proteins (aggregates or degradation products) were seen in the samples from the transgenic plant.

Figure 2.1. SDS-PAGE and western blot of plant- and microbe-derived AAD-1 protein extracts

Lyophilized stalk tissue from event DAS-40278-9 and XHH13 was extracted with PBST containing 10% plant protease inhibitor cocktail and loaded on the Bio-Rad Criterion gels with the positive reference standard, microbe-derived AAD-1. Panel A was stained with Pierce GelCode Blue protein stain and Panel B was electro-blotted to a nitrocellulose membrane, probed with an AAD-1 specific polyclonal rabbit antibody and detected by chemiluminescense.



Panel A: SDS-PAGE gel Panel B: Western blot membrane

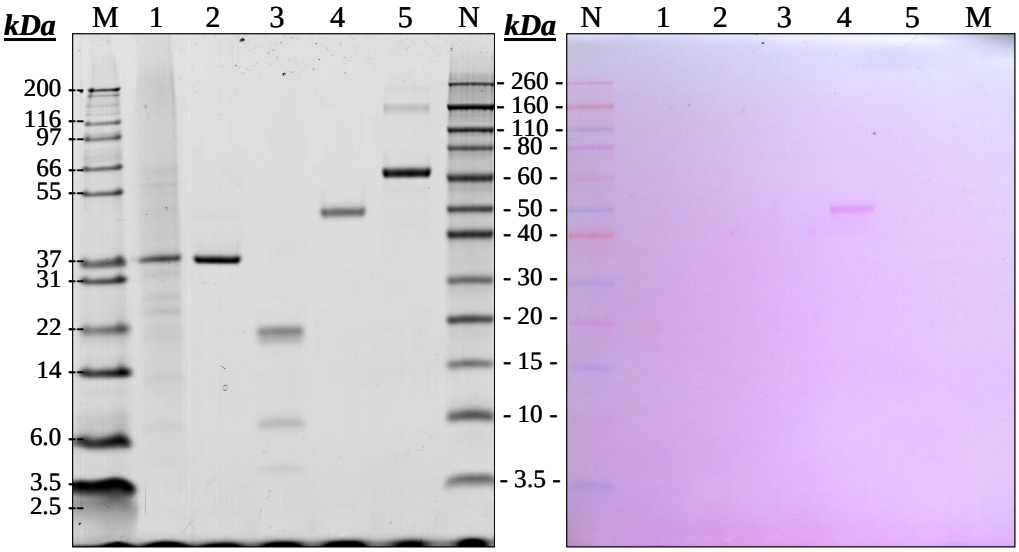
Lane	*Sample*	*Loaded*
M	Invitrogen Mark12 MW markers	10 µL
1	Nontransgenic stalk extract	36 µL
2	Event DAS-40278-9 stalk extract	36 µL
3_{gel}	Microbe-derived AAD-1	~ 1.0 µg
3_{blot}	Microbe-derived AAD-1	~ 10 ng
4	Bovine serum albumin (BSA)	1.0 µg
N	Novex (Invitrogen) prestained MW markers	5.0 µL

2.9. Results of Detection of Glycosylation

No covalently-linked carbohydrates were detectable on the plant-derived or the microbe-derived AAD-1 proteins (Figure 2.2). Horseradish peroxidase, a glycoprotein, was used as a positive indicator for glycosylation. Soybean trypsin inhibitor and bovine serum albumin, both non-glycoproteins, served as negative controls.

Figure 2.2. Glycosylation analysis of plant- and microbe-derived AAD-1 protein

The immunoaffinity-purified, plant-derived AAD-1 protein, microbe-derived AAD-1, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted to a similar concentration prior to loading on the gel. After electrophoresis, the gel was cut in half and one half was stained with GelCode Blue stain for total protein, the other half of the gel was stained with a GelCode Glycoprotein Staining Kit to visualize the glycoproteins.



Panel A: SDS-PAGE gel stained for total protein

Panel B: SDS-PAGE gel stained for glycoproteins

Lane	***Sample***	***Loaded***
M	Invitrogen Mark12 MW markers	10 µL
1	Maize-derived AAD-1 (Event DAS-40278-9)	32 µL
2	Microbe-derived AAD-1 (TSN105930)	1.0 µg
3	Soybean Trypsin Inhibitor (negative control)	1.0 µg
4	Horseradish Peroxidase (positive control)	1.0 µg
5	Bovine serum albumin (negative control)	1.0 µg
N	Novex (Invitrogen) prestained MW markers	5.0 µL

2.10. Results of MALDI-TOF MS Tryptic Peptide Mass Fingerprints

Following digestion of the AAD-1 protein by trypsin, the masses of the detected peptides were compared to those deduced based on potential trypsin cleavage sites in the sequence of the AAD-1 protein. Figure 2.3 illustrates the theoretical cleavage which was generated *in silico* (Proteometrics LLC).

The trypsin digestion of plant-derived AAD-1 protein yielded an extremely high detection of the expected peptides, resulting in 96.6% coverage of the AAD-1 protein sequence. The analysis confirmed the plant-derived protein amino acid sequence matched that of the microbe-derived AAD-1 protein. Results of these analyses indicated that the amino acid sequence of the plant-derived AAD-1 protein was equivalent to the *P. fluorescens*-expressed protein.

Figure 2.3. Theoretical trypsin cleavage of the AAD-1 protein.

Alternating blocks of upper and lower case letters within the amino acid sequence are used to differentiate the potential peptides after trypsin digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

```
  1  M A H A A L S P L S Q R f e r I A V Q P L T G V L G A E I T  30
 31  G V D L R e p l d d s t w n e i l d a f h t y q v i y f p g  60
 61  q a i t n e q h i a f s r R f g p v d p v p l l k S I E G Y  90
 91  P E V Q M I R r E A N E S G R v i g d d w h t d s t f l d a 120
121  p p a a v v m r A I D V P E H G G D T G F L S M Y T A W E T 150
151  L S P T M Q A T I E G L N V V H S A T R v f g s l y q a q n 180
181  r R f s n t s v k V M D V D A G D R e t v h p l v v t h p g 210
211  s g r K g l y v n q v y c q r I E G M T D A E S K P L L Q F 240
241  L Y E H A T R f d f t c r V R w k K d q v l v w d n l c t m 270
271  h r A V P D Y A G K f r Y L T R t t v g g v r p a r       296
```

2.11. Results of Tryptic Peptide Fragment Sequencing

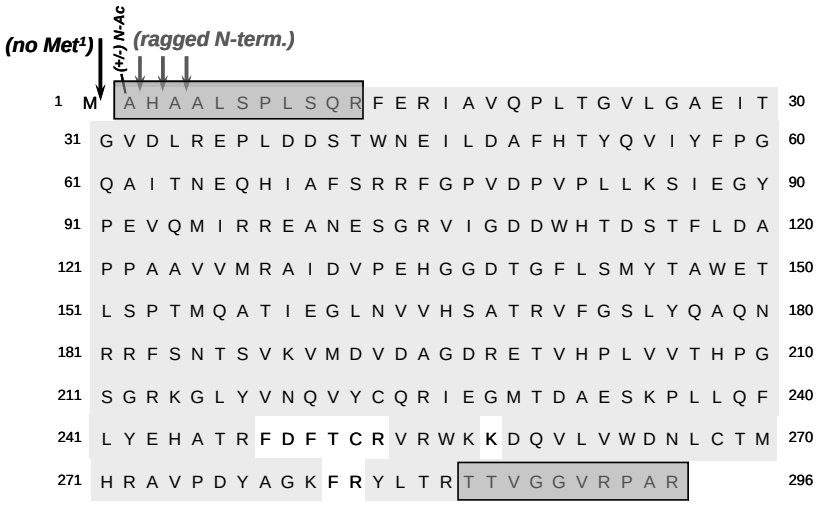
The sequences of the first 11 residues of the plant- and microbe-derived AAD-1 proteins were obtained by tandem mass spectrometry. The amino acid sequences for both proteins were A^{1} H A A L S P L S Q R^{11}, indicating the N-terminal methionine had been removed (Table 2.1). These results suggest that during or after translation in the plant and *P. fluorescens*, the N-terminal methionine is cleaved by a methionine aminopeptidase. In addition to the methionine being removed, a small portion of the N-terminal peptide of the AAD-1 protein was shown to be acetylated after the N-terminal methionine was cleaved. These two co-translational processes, cleavage of N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman, 2002). Furthermore, examples demonstrating biological significance associated with N-acetylation are rare (Polevoda and Sherman, 2000).

In addition to N-acetylation, there was a short N-terminal truncation (loss of amino acids A^{2}, H^{3}, A^{4}) that appeared during the purification of the plant-derived AAD-1 protein (Table 2.1 and Figure 2.3). This truncation is thought to have occurred during the purification of the AAD-1 protein since in the western blot probe of crude extracts, only a single, crisp band at the same molecular weight as the microbe-derived AAD-1 protein was visualized. The C-terminal sequences of the plant- and microbe-derived AAD-1 proteins were determined to be identical to the expected sequences (Table 2.2 and Figure 2.4).

Figure 2.4. Sequence coverage of plant- and microbe-derived AAD-1 protein based on enzymatic peptide mass fingerprinting and MS/MS sequencing.

The numbers on the left and right sides of the protein sequence indicate the amino acid residue numbers. Letters in the light gray area represent peptide fragments detected by enzymatic peptide mass fingerprinting. The letters in dark gray blocks indicate the peptide sequence confirmed by tandem MS sequencing. The dark gray arrow indicates the N-terminal methionine was removed by an aminopeptidase. Panel A: The "(+) N-Ac" on the N-terminal residue indicates the protein was partially acetylated *in planta*. The dark gray arrows indicate that trace amounts of the N-terminal peptide were found to have various additional truncations.

A. Plant-derived AAD-1 protein



Sequence coverage = 96.6%

B. Microbe-derived AAD-1 protein

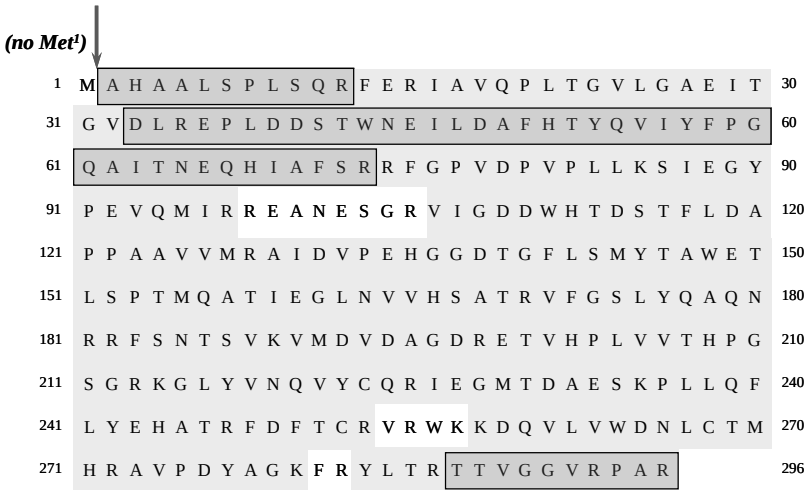


Table 2.1. Summary of N-terminal sequence data of plant- and microbe-derived AAD-1 proteins

Source	Expected N-terminal Sequence[1]
P. fluorescens	M^{1} A H A A L S P L S Q R^{12}
Maize Event DAS-40278-9	M^{1} A H A A L S P L S Q R^{12}

Source	Detected N-terminal Sequence[2]	Relative[3] Abundance
P. fluorescens	A H A A L S P L S Q R^{12}	100%
Maize Event DAS-40278-9	A H A A L S P L S Q R^{12}	31%
Maize Event DAS-40278-9	$^{N\text{-}Ac}$A H A A L S P L S Q R^{12}	3%
Maize Event DAS-40278-9	H A A L S P L S Q R^{12}	50%
Maize Event DAS-40278-9	A A L S P L S Q R^{12}	6%
Maize Event DAS-40278-9	A L S P L S Q R^{12}	12%

[1]Expected N-terminal sequence of the first 12 amino acid residues of *P. fluorescens*- and plant-derived AAD-1.
[2]Detected N-terminal sequences of *P. fluorescens*- and plant-derived AAD-1.
[3]The tandem MS data for the N-terminal peptides revealed a mixture of AHAALSPLSQR (non-acetylated) and *N-Acetyl*-AHAALSPLSQR (acetylated). "Ragged N-terminal ends" were also detected (peptides corresponding to amino acid sequences HAALSPLSQR, AALSPLSQR, and ALSPLSQR). The relative abundance, an estimate of relative peptide fragment quantity, was made based on the corresponding LC peak areas measured at 214 nm.

Notes:
Numbers in superscript (R^{x}) indicate amino acid residue numbers in the sequence.
Amino acid residue abbreviations:

A:	alanine	H:	histidine
L:	leucine	M:	methionine
P:	proline	Q:	glutamine
R:	arginine	S:	serine

Table 2.2. Summary of C-terminal sequence data of plant- and microbe-derived AAD-1 proteins

Source	Expected C-terminal Sequence[1]
P. fluorescens	^{287}T T V G G V R P A R^{296}
Maize Event DAS-40278-9	^{287}T T V G G V R P A R^{296}

Source	Detected C-terminal Sequence[2]
P. fluorescens	^{287}T T V G G V R P A R^{296}
Maize Event DAS-40278-9	^{287}T T V G G V R P A R^{296}

[1]Expected C-terminal sequence of the last 10 amino acid residues of *P. fluorescens*- and plant-derived AAD-1.
[2]Detected C-terminal sequences of *P. fluorescens*- and plant-derived AAD-1.

Notes:
Numbers in superscript (R^x) indicate amino acid residue numbers in the sequence.
Amino acid residue abbreviations:

A:	alanine	G:	glycine
P:	proline	R:	arginine
T:	threonine	V:	valine

2.12. Conclusions

The biochemical identity of microbe-derived AAD-1 protein was equivalent to the protein purified from stalk tissue of event DAS-40278-9. The plant and microbe derived AAD-1 proteins showed the expected molecular weight of ~33 kDa by SDS-PAGE and were immunoreactive to AAD-1 protein specific antibodies by western blot analysis. The amino acid sequence of both proteins was confirmed by enzymatic peptide mass fingerprinting by MALDI-TOF MS and peptide sequence obtained from tandem mass spectrometry. In addition, the lack of glycosylation of the plant-derived AAD-1 protein provided additional evidence that the AAD-1 protein produced by *P. fluorescens* and DAS-40278-9 corn are biochemically equivalent molecules.

2.13. References

Polevoda, B. and Sherman, F. 2000. N^{α}-terminal Acetylation of Eukaryotic Proteins. Journal of Biological Chemistry 275:47, 36479–36482.

Polevoda, B. and Sherman, F. 2002. The Diversity of Acetylated Proteins. Genome Biology, 3(5):0006.1–0006.6

Appendix 3. Methods for AAD-1 Expression Analysis

3.1 Experimental Design

The experimental design included six (6) field sites; Richland, IA; Carlyle, IL; Wyoming, IL; Rockville, IN; York, NE; and Branchton, Ontario, Canada (referred to as IA, IL1, IL2, IN, NE and ON). At each site, 4 replicate plots of each treatment were established, with each plot consisting of 2-25 ft rows. Plots were arranged in a randomized complete block design, with a unique randomization at each site. Each corn plot was bordered by 2 rows of a non-transgenic corn hybrid of similar maturity. The entire trial site was surrounded by a minimum of 12 rows (or 30 ft) of a non-transgenic corn hybrid of similar relative maturity. At each location, block 1 was designated for collection of samples for protein determination. Blocks 2, 3, and 4 were designated for the collection of samples for nutrient composition analysis.

Herbicide treatments were designed to replicate maximum label rate commercial practices. 2,4-D (Weedar 64) was applied as 3 broadcast applications at a total seasonal rate of 3360 g acid equivalent/hectare (ae/ha). Individual applications were at pre-emergence and approximately V4 and V8 –V8.5 stages. Individual target application rates were 1120 ae/ha for Weedar 64. Quizalofop (Assure II) was applied as a single broadcast over-the-top application. Application timing was at approximately V6 growth stage. The target application rate was 92 g active ingredient (ai)/ha for Assure II.

3.2. Sample Collection

Leaf (V2-4, V9 and R1)
Three leaf samples, each sample containing 1-3 leaves collected from separate plants (depending on the plant stage) were collected for each test and control entry. Each leaf sample was the youngest whorl leaf that had emerged at least 4 inches from the whorl.

Pollen (R1)
Each pollen sample contained available pollen collected from a single plant. Three samples were collected from each test and control entry.

Root (R1)
Three root samples were collected for each test and control entry at the R1 stage by cutting a circle approximately 7 to 9 inch in diameter around the base of the plant to a depth of approximately 7 to 9 inch (18 to 24 cm) cm. The root ball was removed and cleaned.

Forage (R4)
Three forage samples each consisting of the aerial portion (no roots) of 2 whole plants were collected from each test and control entry. Plants used for sampling also contained self-pollinated ears.

Grain (R6 – Maturity)
Three individual, self-pollinated ears were collected from each test and control entry.

Whole Plant (R6)
Three whole plant samples each consisting of the aerial portion (no roots) of 2 whole plants were collected from each test and control entry. Plants used for sampling also contained self-pollinated ears.

Samples were shipped to Dow AgroSciences RSGA laboratories and maintained frozen until use. Samples of corn tissues were prepared for expression analysis by coarse grinding, lyophilizing and/or fine-grinding with a Geno/Grinder (Certiprep, Metuchen, New Jersey). Pollen samples were lyophilized but no further grinding was required.

3.3. Determination of AAD-1 Protein Concentration

Samples were analyzed for the amount of AAD-1 protein using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Beacon Analytical Systems, Inc. The AAD-1 protein was extracted from corn tissues with a phosphate buffered saline solution with Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). For pollen, the protein was extracted with a 0.5% BSA/PBST buffer containing 1 mg/mL of sodium ascorbate and 2% protease inhibitor cocktail. The plant tissue and pollen extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analyzed using a AAD-1 ELISA kit in a sandwich format. Briefly, an aliquot of the diluted sample and a biotinylated anti-AAD-1 monoclonal antibody are incubated in the wells of a microtiter plate coated with an immobilized anti-AAD-1 monoclonal antibody. These antibodies bind with AAD-1 protein in the wells and form a "sandwich" with AAD-1 protein bound between soluble and the immobilized antibodies. The unbound samples and conjugate are then removed from the plate by washing with PBST. An excess amount of streptavidin-enzyme (alkaline phosphatase) conjugate is added to the wells for incubation. At the end of the incubation period, the unbound reagents were removed from the plate by washing. Subsequent addition of an enzyme substrate generated a colored product. Since the AAD-1 was bound in the antibody sandwich, the level of color development was related to the concentration of AAD-1 in the sample (i.e., lower residue concentrations result in lower color development). The absorbance at 405 nm was measured using a Molecular Devices V-max or Spectra Max 190 plate reader. A calibration curve was generated and the AAD-1 concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

3.4 Limit of Detection/Quantitation for Corn Samples

The limit of detection (LOD) and limit of quantitation (LOQ) for corn tissues were determined during the method validation for the method described above. Samples were reported as not detectable (ND) if the response was less than the method LOD. Reported sample concentrations that are less than the method LOQ values (Table 3.1) have lower precision than results reported above the LOQ values.

Table 3.1. Limits of Detection and Limits of Quantitation for AAD-1 in corn tissues

Matrix	LOD[a]	LOQ[a]
Leaf	0.2	0.4
Root	0.2	0.4
Pollen	0.2	0.4
Forage	0.2	0.4
Grain	0.2	0.4
Whole Plant	0.2	0.4

[a]Units of ng protein/mg sample weight.

Appendix 4. USDA Notifications for DAS-40278-9 Corn

USDA Notification Number	Notification Authorization Date	Notification Expiration Date	State(s)	Total Number of Trials Planted[1]	Status of Trial[2]
09-086-105n	4/20/2009	4/20/2010	IL, IN, IA, MN, MO, NE, WI	TBD	Pending
09-090-107n	4/21/2009	4/21/2010	CA, GA, IL, IA, IN, KS, MI, MN, MO, OH, NE, NJ, OK, PA, TX	TBD	Pending
09-075-106n	3/26/2009	3/26/2010	HI, IA, IL, IN, MN, NE, NE, SD, WI	TBD	Pending
09-061-005n	4/6/2009	4/6/2010	IA, MN, MS, NY, OH	TBD	Pending
09-005-107n	1/15/2009	1/15/2010	HI, IL, IN, IA, NE, PR	TBD	Pending
08-259-103n	10/15/2008	10/15/2009	HI	TBD	Pending
08-133-107n	6/1/2008	6/1/2009	IL (1), TX (1)	2	Pending
08-021-110n	4/1/2008	4/1/2009	IA	1	Pending
08-021-104n	3/20/2008	3/20/2009	IL (7), IN (11), IA (6), MN (4), MS (1), NE (4), WI (3)	36	Pending
07-242-103n	10/15/2007	10/15/2008	HI	5	Submitted
06-338-101n	1/29/2007	1/29/2008	HI	3	Submitted
05-308-03n	12/13/2005	12/13/2006	HI	1	Submitted

[1]Trials not yet planted as of April 1, 2009 are indicated as TBD (to be determined)
[2]Pending reports to be submitted within 6 months of the notification expiration date

Appendix 5. Literature Ranges for Compositional Analysis

Published values for compositional analytes of the corn forage and grain were compiled from literature sources to establish representative ranges for analytes typically found in corn (Watson, 1982; Watson, 1987; ILSI, 2006; OECD, 2002; and Codex, 2001). The ranges were then used in comparison with values determined in field trials of DAS-40278-9 corn and the non-transgenic control (Section VIII, Tables 17-23) and used to prepare plots of the compositional analysis results (Figures 26-32)

Literature ranges compiled for forage included proximates, fiber, and minerals (Table 5.1). The data compiled for grain included proximates and fiber (Table 5.2), minerals (Table 5.3), amino acids (Table 5.4), fatty acids (Table 5.5), vitamins (Table 5.6), and secondary metabolites and anti-nutrients (Table 5.7).

Table 5.1. Literature ranges for proximates, fiber, and minerals in forage

Analyte	Literature Reference (% Dry weight)		
	Watson (1982)	**ILSI (2006)**	**Combined Ranges**
Protein	3.5 - 15.9	3.14 - 11.6	3.14 - 15.9
Total Fat	0.7 - 6.7	0.296 - 4.57	0.296 - 6.7
Ash	1.3 - 10.5	1.53 - 9.64	1.3 - 10.5
Moisture	53.3 – 87.5	55.3 – 80.4	53.3 – 87.5
Carbohydrates[a]	66.9 - 94.5	76.4 – 92.1	66.9 - 94.5
Acid Detergent Fiber (ADF)	30 (average)	16.1 – 47.4	16.1 – 47.4
Neutral Detergent Fiber (NDF)	51 (average)	20.3 - 63.7	20.3 - 63.7
Total Dietary Fiber	19 - 42	35.9 – 62.8	19 – 62.8
Minerals (mg/100g dry wt.)			
Calcium	200 - 600	71.4 – 576.8	71.4 - 600
Phosphorus	150 - 550	93.6 – 370.4	93.6 - 550

[a] Carbohydrates are calculated as the percentage of dry weight = 100% total dry weight - % protein - % fat - % ash.

Table 5.2. Literature ranges for proximates and fiber in grain

Analyte	Literature Reference (% Dry weight)				
	Watson (1982)	Watson (1987)	OECD (2002)	ILSI (2006)	Combined Ranges
Protein	8 - 14	6 - 12	6 - 12.7	6.15 – 17.3	6 – 17.3
Total Fat	1.2 - 18.8	3.1 - 5.7	3.1 - 5.8	1.74 - 5.82	1.2 - 18.8
Ash	1.1 - 3.9	1.1 - 3.9	1.1 - 3.9	0.62 - 6.28	0.62 - 6.28
Moisture	7 - 23	7 - 23	7 - 23	6.1 – 40.5	6.1 – 40.5
Carbohydrate[a]	63.3 - 89.7	78.4 - 89.8	82.2 - 82.9	77.4 - 89.5	63.3 - 89.8
Acid Detergent Fiber (ADF)	3.0 - 4.3	3.3 - 4.3	3.0 - 4.3	1.82 - 11.3	1.82 - 11.3
Neutral Detergent Fiber (NDF)	8.3 - 11.9	8.3 - 11.9	8.3 - 11.9	5.59 - 22.6	5.59 - 22.6
Total Dietary Fiber	8.3- 11.9	NR[b]	NR	8.85 – 35.3	8.3 – 35.3

[a] Carbohydrates are calculated as the percentage of dry weight = 100% total dry weight - % protein - % fat - % ash.
[b] NR = not reported

Table 5.3. Literature ranges for minerals in grain

Analyte	Literature Reference (mg/100g)				
	Watson (1982)	Watson (1987)	OECD (2002)	ILSI (2006)	Combined Ranges
Calcium	10 – 100	10 – 100	3 - 100	1.27 – 20.8	1.27 - 100
Copper	0.09 – 1.0	0.09 – 1.0	0.09 – 1.0	0.073 – 1.85	0.073 – 1.85
Iodine	7.3 - 81	7.3 - 81	NR[a]	NR	7.3 - 81
Iron	0.1 - 10	0.1 - 10	0.1 - 10	1.04 – 4.91	0.1 – 10
Magnesium	90 - 1000	90 - 1000	82 - 1000	59.4 – 194	59.4 - 1000
Manganese	0.07 – 5.4	0.07 – 5.4	NR	0.169 – 1.43	0.07 – 5.4
Phosphorus	260 - 750	260 - 750	234 - 750	147 – 533.0	147 - 750
Potassium	320 - 720	320 - 720	320 - 720	181 - 603	181 - 720
Sodium	0 - 150	0 - 150	0 - 150	0.017 – 73.1	0 - 150
Zinc	1.2 – 3.0	1.2 – 3.0	1.2 – 3.0	0.65- 3.72	0.65 - 3.72
Chromium	0.006 – 0.016	0.006 – 0.016	NR	NR	0.006 – 0.016
Molybdenum	NR	NR	NR	NR	NR
Selenium	0.0045	0.001 – 0.1	0.001 – 0.1	0.005 – 0.075	0.001 – 0.1

[a] NR = not reported

Table 5.4. Literature ranges for amino acids in grain

Analyte	Literature Reference (% Dry weight)			
	Watson (1982)	OECD (2002)	ILSI (2006)	Combined Ranges
Aspartic Acid	0.58 - 0.72	0.48 - 0.85	0.34 – 1.21	0.34 –1.21
Threonine	0.29 - 0.39	0.27 - 0.58	0.22 - 0.67	0.22 - 0.67
Serine	0.42 - 0.55	0.35 - 0.91	0.24 - 0.77	0.24 - 0.91
Glutamic Acid	1.24 - 1.96	1.25 - 2.58	0.97 - 3.54	0.97 - 3.54
Proline	0.66 - 1.03	0.63 - 1.36	0.46 - 1.63	0.46 - 1.63
Glycine	0.26 - 0.47	0.26 - 0.49	0.18- 0.54	0.18- 0.54
Alanine	0.64 - 0.99	0.56 - 1.04	0.44 - 1.39	0.44 - 1.39
Cystine	0.12 - 0.16	0.08 - 0.32	0.13 - 0.51	0.08 - 0.51
Valine	0.21 - 0.52	0.21 -0.85	0.27 - 0.86	0.21 - 0.86
Methionine	0.10 - 0.21	0.10 - 0.46	0.12 - 0.47	0.10 - 0.47
Isoleucine	0.26 - 0.40	0.22 - 0.71	0.18 - 0.69	0.18 - 0.71
Leucine	0.78 - 1.52	0.79 - 2.41	0.64 - 2.49	0.64 - 2.49
Tyrosine	0.29 - 0.47	0.26 - 0.79	0.10 - 0.64	0.10 - 0.79
Phenylalanine	0.29 - 0.57	0.29 - 0.64	0.24 - 0.93	0.24 - 0.93
Histidine	0.2 - 0.28	0.15 - 0.38	0.14 - 0.43	0.14 - 0.43
Lysine	0.2 - 0.38	0.05 - 0.55	0.17 - 0.67	0.05 - 0.56
Arginine	0.29 - 0.59	0.22 - 0.64	0.12 - 0.64	0.12 - 0.64
Tryptophan	0.05 - 0.12	0.04 - 0.13	0.03 - 0.22	0.03 - 0.22

Table 5.5. Literature ranges for fatty acids in grain

Analyte	Literature Reference (% Total fatty acids)			
	Watson (1982)	Codex (2001)[a]	ILSI (2006)	Combined Ranges
8:0 Caprylic	NR[b]	ND	0.13 – 0.34	0.13 – 0.34
10:0 Capric	NR	ND	NR	ND
12:0 Lauric	NR	ND-0.3	0.687	ND – 0.687
14:0 Myristic	NR	ND-0.3	0.14-0.28	ND-0.3
14:1 Myristoleic	NR	NR	NR	NR
15:0 Pentadecanoic	NR	NR	NR	NR
15:1 Pentadecenoic	NR	NR	NR	NR
16:0 Palmitic	7 - 19	8.6 - 16.5	7.94 – 20.7	7 – 20.7
16:1 Palmitoleic	1.0	ND – 0.5	0.095 – 0.45	ND – 1.0
17:0 Heptadecanoic	NR	ND – 0.1	0.078 – 0.11	ND – 0.11
17:1 Heptadecenoic	NR	ND – 0.1	NR	ND – 0.1
18:0 Stearic	1 – 3	ND - 3.3	1.02 – 3.40	ND - 3.4
18:1 Oleic	20 - 46	20.0 - 42.2	17.4 - 40.2	17.4 - 46
18:2 Linoleic	35 - 70	34.0 - 65.6	36.2 – 66.5	34.0 - 70
18:3 Linolenic	0.8 - 2	ND - 2.0	0.57 – 2.25	ND - 2.25
20:0 Arachidic	0.1 - 2	0.3 – 1.0	0.28 – 0.97	0.1 - 2
20:1 Eicosenoic	NR[a]	0.2 – 0.6	0.17 – 1.92	0.17 – 1.92
20:2 Eicosadienoic	NR	ND – 0.1	0.12 – 0.53	ND – 0.53
20:3 Eicosatrienoic	NR	NR	0.275	0.275
20:4 Arachidonic	NR	NR	0.465	0.465
22:0 Behenic	NR[a]	ND - 0.5	0.11 – 0.35	ND – 0.5

[a] Data reported for maize oil.
[b] NR = not reported

Table 5.6. Literature ranges for vitamins in grain

Analyte	Literature Reference (ppm-Dry weight)				
	Watson (1982)	Watson (1987)	OECD (2002)	ILSI (2006)	Combined Ranges
Beta Carotene (Vitamin A)	2.5 (Average)	2.5 (Average)	0.49 – 2.18	0.19 – 46.8	0.19 – 46.8
Vitamin B1 (Thiamin)	3.0 - 8.6	3.0 - 8.6	2.3 - 8.6	1.3 - 40	1.3 - 40
Vitamin B2 (Riboflavin)	0.25 - 5.6	0.25 - 5.6	0.25 - 5.6	0.50 – 2.36	0.25 - 5.6
Vitamin B5 (Pantothenic acid)	NR[b]	NR	NR	NR	NR
Vitamin B6 (Pyridoxine)	9.6	5.3	4.6 - 9.6	3.68 – 11.3	3.68 – 11.3
Vitamin B12	NR	NR	NR	NR	NR
Vitamin C	NR	NR	NR	NR	NR
Vitamin D	NR	NR	NR	NR	NR
Vitamin E (alpha Tocopherol)	3.0 – 25	17 - 47 IU/kg[a]	NR	1.5 - 68.7	1.5 - 68.7
Niacin (Nicotinic acid,Vit. B3)	9.3 - 70	9.3 - 70	9.3 – 70	10.4 - 46.9	9.3 - 70
Folic Acid	100 - 683	0.3 (Average)	0.17 – 0.46	0.15 - 1.46	0.15 - 683

[a] IU = 1 mg of standard DL-α tocopherol.
[b] NR = not reported

Table 5.7. Literature ranges for secondary metabolites and anti-nutrients in grain

Analyte	Literature Reference (% Dry weight)		
	OECD (2002)	ILSI (2006)	Combined Ranges
Inositol	NR[b]	0.0089 - 0.377	0.0089 - 0.377
Furfural	NR	0.0003 - 0.0006	0.0003 - 0.0006
P-Coumaric Acid	0.003 - 0.03	0.0053 - 0.058	0.003 - 0.058
Ferulic Acid	0.02 - 0.3	0.029- 0.389	0.02 - 0.389
Phytic acid	0.45 - 1.0	0.11 - 1.57	0.11 - 1.57
Raffinose	0.21 - 0.31	0.02 - 0.32	0.02 - 0.32
Trypsin Inhibitor (TIU/mg) [a]	NR	1.09 - 7.18	1.09 - 7.18

[a] Abbreviation: TIU, trypsin inhibitor units
[b] NR = not reported

5.1. References

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Appendix 6. Glyphosate, 2,4-D, Quizalofop and Herbicide Resistant Weeds

6.1. Herbicide Tolerant Crops

Soon after the first weeds evolved resistance to herbicides, scientists began to consider altering crops to make them resistant to herbicides (Duke, 2005). Initially, non-transgenic methods were used until the early 1980s when the tools for producing transgenic crops were becoming available. The first transgenic herbicide-resistant crops included bromoxynil resistant cotton and canola. However, transgenic crops with resistance to broad-spectrum, non-selective herbicides were perceived as a better approach for weed management and for capturing market share. This was soon realized with development of glyphosate- and glufosinate-tolerant crops. Since these transgenic crops would tolerate the application of those broad spectrum herbicides, they could survive and prosper while reducing the amount and number of applications of herbicides by the growers (GEO-PIE, 2009).

Herbicide-tolerance (often called resistance) in plants employs one of two strategies (or a combination) to make the plant tolerant to the applied herbicide (GEO-PIE, 2009):

- the plant produces a new protein which detoxifies the herbicide, or
- the protein in the plant which is normally the target of the herbicide's action is replaced by a new protein which is unaffected by the herbicide.

Herbicide-tolerant crops which were available to farmers in 2005 are listed in Table 6.1 (Duke, 2005). Transgenes were only used to confer tolerance to bromoxynil, glufosinate, and glyphosate. The bromoxynil-tolerant crops are no longer sold. This leaves only glyphosate- and glufosinate-tolerant transgenic crops, and of those, glyphosate has had a strongest impact on weed management (Duke, 2005).

Growers choose glyphosate-tolerant crops because it makes weed control easier and more effective, increases profit, requires less tillage, and does not restrict crop rotations (Green, 2009). Thus, glyphosate-tolerant corn, soybeans, and cotton have experienced an unprecedented rapid adoption rate by U.S. farmers (Figure 6.1). The planting of glyphosate-tolerant crops has increased steadily since their introduction in 1996 (glyphosate-tolerant soybean plantings are >90% of all soybean planting in the US).

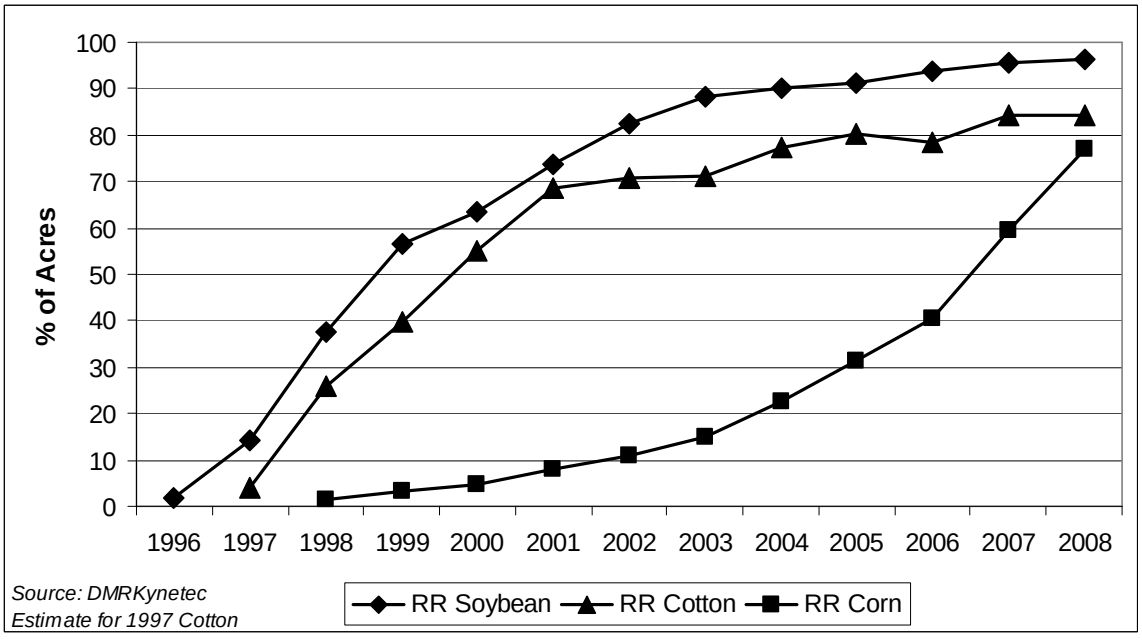
Many growers now rely only on glyphosate for their weed control in these crops (Foresman and Glasgow, 2008; Gustafson, 2008). This has significantly increased selection pressure for glyphosate-resistant weeds. Thus, introduction of combined event products ("stacks") which are tolerant to glyphosate plus at least one other class of herbicides is needed as a tool to delay selection for glyphosate-resistant weed species.

Table 6.1. Herbicide-tolerant crops available to farmers in North America in 2005

Herbicide	Crop	Year Available
Bromoxynil	Cotton[b]	1995
	Canola[b]	2000
Cyclohexanediones (sethoxydim)[ab]	Corn	1996
Glufosinate	Canola	1995
	Corn	1997
	Cotton	2004
Glyphosate	Soybean	1996
	Canola	1996
	Cotton	1997
	Corn	1998
Imidazolinones[a]	Corn	1993
	Canola	1997
	Wheat	2002
	Rice	2002
Sulfonylureas[a]	Soybean	1994
Triazines[a]	Canola	1984

[a]Not transgenic.
[b]No longer available by 2005

Figure 6.1. U.S. adoption rates of glyphosate-tolerant soybean, cotton and corn



6.2. Characteristics of Glyphosate, 2,4-D, and Quizalofop Herbicides

6.2.a. Glyphosate

Glyphosate is a nonselective, foliar applied herbicide which is registered for use on over one hundred food and feed crops, several non-food field crops (fallow, fencerows, woody ornamentals, golf courses, etc.), forestry uses (conifer release and reforestation), and non-crop areas where total vegetation control is desired (aquatics, rights of way, industrial sites, etc.) (US EPA, 1993). When applied at lower rates, glyphosate also is a plant growth regulator. Pre-plant or pre-emergence uses of glyphosate in food and feed crops include most of the major agricultural crops around the globe, including alfalfa, barley, buckwheat, corn, dry beans, grass forage/fodder/hay, lentils, millet, oats, pastures, rye, sorghum, soybeans, and wheat. In addition, it can be foliar applied over-the-top of recent glyphosate-tolerant transgenic crops, including corn, soybeans, cotton, canola, and sugarbeets.

Glyphosate is absorbed relatively rapidly through plant surfaces (Duke and Powles, 2008a). Leaf uptake rates vary considerably between species, accounting for at least some of the difference in glyphosate susceptibility between species. Once in the plant, glyphosate moves in the phloem with sugar to the growing points. Foliar post-emergence applications of glyphosate at 560 to 1120 g acid equivalent/hectare (ae/ha) control a broad spectrum of grass, broadleaf, and sedge weeds in agronomic crops.

The mode of action for glyphosate is unique in that it is the only herbicide that is highly effective at inhibiting an essential plant enzyme called EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), which produces EPSP from shikimate-3-phosphate and phosphoenolpyruvate in the shikimic acid pathway (WSSA, 2002). Many assume that this EPSPS inhibition leads to depletion of the aromatic amino acids tryptophan, tyrosine, and phenylalanine (Duke and Powles, 2008a). However, others support the view that increased carbon flow to the shikimate pathway by deregulation of the pathway by inhibiting EPSPS results in shortages of carbon for other essential pathways (Siehl, 1997). The EPSPS of all higher plants appears to be inhibited by glyphosate (Duke and Powles, 2008a), which makes it nonselective with activity on a wide range of plant species.

In general, glyphosate is an environmentally benign molecule (Franz, *et al*., 1997). It is degraded microbially in soil and water. Glyphosate binds tightly to most types of soil, so it is not available for uptake by roots of nearby plants (Monsanto, 2005). Therefore, even though glyphosate has a typical field half life of 47 days, crops can be planted immediately after application due to its strong adsorption to soil. Glyphosate is not appreciably metabolized in plants when applied at normal use rates for weed control. It is slowly metabolized to amino methylphosphonic acid (AMPA) (FAO, 1997; WSSA, 2002). Glyphosate is also one of the least toxic pesticides to animals (Duke and Powles, 2008a). The enzyme, EPSP synthase, is not present in humans or animals, which

contributes to the low risk to human health when glyphosate is used according to label directions (Monsanto, 2005).

6.2.b. 2,4-Dichlorophenoxyacetic Acid (2,4-D)

2,4-Dichlorophenoxyacetic acid (2,4-D) was introduced in 1946 as the first selective herbicide and rapidly became the most widely used herbicide in the world (Industry Task Force II, 2005). Today, it is still the third most widely used herbicide in the U.S. and Canada, as well as the most widely used herbicide globally. Approximately 46 million pounds of 2,4-D is used domestically in the U.S. annually, with 30 million pounds (66%) used by agriculture and 16 million pounds (34%) used in non-agriculture settings such as pasture/rangeland and lawn/garden (US EPA, 2006). 2,4-D is an ingredient in approximately 660 agricultural and home use products as a sole active ingredient and in conjunction with other active ingredients. Agriculturally, it is used on a variety of crops including corn, rice, sorghum, sugar cane, wheat, rangeland and pasture as well as being used on rights-of-way, roadsides, non-crop areas, forestry, lawn and turf care and on aquatic weeds (Industry Task Force II, 2005). A major use today of 2,4-D is in combination with other herbicides because it economically enhances the weed control spectrum of many other herbicides such as glyphosate, dicamba, mecoprop, ALS herbicides, etc (US EPA, 2006). 2,4-D controls many broadleaf weeds including carpetweed, dandelion, cocklebur, horseweed, morning glory, pigweed sp., lambsquarters, ragweed spp., shepherd's-purse and velvetleaf. It has little to no activity on grasses (Industry Task Force II, 2005).

In over 60 years since its discovery, probably few other compounds have been as thoroughly and extensively evaluated for health and safety as 2,4-D. There have been more than 40,000 research studies conducted and more than 140 peer-reviewed published epidemiologic studies specific to 2,4-D. In August, 2005, the U.S. Environmental Protection Agency (EPA) completed its reregistration assessment of 2,4-D. The EPA concluded that 2,4-D does not present risks of concern to human health when users follow its product instructions (US EPA, 2006). 2,4-D data has been reviewed by more than a dozen government and expert panels since 1986 and not one regulatory agency has ever identified 2,4-D as a human carcinogen.

The mode of action of 2,4-D is described as an "auxin mimic", meaning that it kills the target weed by mimicking auxin plant growth hormones like IAA. Auxins and synthetic auxinic herbicides all regulate virtually every aspect of plant growth and development (Mockaitis and Estelle, 2008). At low doses, auxinic herbicides possess similar hormonal properties to natural auxin (Kelley and Riechers, 2007). However, as rates increase, they can cause various growth abnormalities in sensitive dicots. Observable plant responses to 2,4-D can include epinasty, root growth inhibition, meristematic proliferation/callusing, leaf cupping/narrowing, stem cracking, adventitious root formation, senescence, and chlorosis. This uncontrolled and disorganized plant growth eventually leads to plant death when applied at effective doses (Tu *et. al*., 2001).

IAA and auxin herbicides work through stimulation of the ubiquination and degradation of the Aux/IAA family of transcriptional regulators. Degradation of these Aux/IAA proteins results in derepression of auxin-regulated genes that in turn leads to the physiological and morphological events associated with auxin action (Mockaitis and Estelle, 2008; Walsh, *et al*., 2006; Kelley and Riechers, 2007). Stated another way, high concentrations of IAA or auxin herbicides (like 2,4-D) promote ubiquitin mediated degradation of Aux/IAA protein repressors, which permits auxin response factor (ARF) dependent transcription of auxin-regulated genes. This results in "uncontrolled" growth which leads to plant death in susceptible species.

In the environment, 2,4-D is mainly degraded by soil microorganisms. Once it contacts soils, all 2,4-D forms are rapidly converted to the acid form and thus, the rate of soil dissipation is often the same as for the acid (Tu *et al*., 2001). 2,4-D has a relatively short soil half-life and no significant carryover effects to subsequent crops are encountered, adding to 2,4-D's herbicidal utility. 2,4-D has different levels of selectivity on certain plants, i.e., dicots are more sensitive than monocots. Differential metabolism of 2,4-D by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism of hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

6.2.c. Quizalofop

Aryloxyphenoxypropionate (AOPP) and cyclohexanedione (CHD) herbicides have been used widely to control grass weed species since their introduction in the 1970s and 1980s, respectively (Devine and Shimabukuro, 1994). These post graminicide herbicides are also frequently referred to as "fops" and "dims", respectively. While AOPP and CHD herbicides are two chemically dissimilar classes of herbicides, they both inhibit chloroplastic acetyl-CoA carboxylase (ACCase), which catalyzes the first committed step in fatty acid biosynthesis, causing plant death (Burton *et al.*, 1989).

Dicotyledonous plants contain a prokaryotic form of ACCase which is insensitive to AOPP herbicides and CHD herbicides (Devine and Shukla, 2000). In contrast, monocotyledonous plants contain a sensitive eukaryotic form of ACCase in the plastid. This is the primary reason that these herbicides are generally good graminicides, with little activity on dicot plants. In addition, some grass species, including some cereal crops, are tolerant of some of these herbicides due to their ability to metabolize the herbicides to inactive forms (Devine and Shimabukuro, 1994).

The herbicidal activity of quizalofop-ethyl ester was first reported in 1983 and quizalofop-ethyl was first approved for use in a registered herbicide product in the U.S. in 1988. However, all end use product registrations were cancelled prior to 1996 and it was replaced by the more active quizalofop-P-ethyl (pure R-enatiomer of quizalofop racemic mixture), which was first approved for use in a registered product in 1990. Quizalofop-P-

ethyl is a systemic herbicide which is absorbed from the leaf surface and translocated in the symplasm throughout the plant (WSSA, 2007). Although the rate of translocation is slow, it accumulates in the meristematic regions of the shoot and root. Quizalofop-P-ethyl ester diffuses readily across the plasmalemma. Once inside the cell, it is rapidly deesterified to quizalofop-P acid.

Quizalofop-P is used as a selective post-emergent herbicide for the control of annual and perennial grass weeds in potatoes, soybeans, sugar beet, peanuts, oilseed rape, sunflowers, vegetables, cotton and flax. Most non-graminaceous plants (dicots and sedges) are tolerant to quizalofop.

6.3. Evolution of Resistance to Glyphosate, 2,4-D, and Quizalofop

6.3.a. Glyphosate Resistance and Weed Shifts

Glyphosate Resistance

It was initially thought that evolution of glyphosate-resistant weeds would be very slow, and the levels of resistance would be very low (Bradshaw *et al*., 1997). This was based on the amount of glyphosate applied over many years, the repeated applications made to many perennial crops, the high level of herbicidal activity that it had demonstrated, and the uniqueness of its metabolic activity in the plant. More than twenty years after the launch of glyphosate, rigid ryegrass in Australia was reported as the first glyphosate-resistant weed in 1996 (Powles *et al*., 1998). About the same time, sales of glyphosate began to increase dramatically in the U.S. due to the launch of glyphosate-tolerant transgenic soybeans (1996), cotton (1997), and corn (1998). Rapid adoption of this new technology drove dramatic increases in the use of glyphosate, which resulted in increased selection pressure for glyphosate-resistant weeds.

Table 6.2 shows a summary of the fifteen glyphosate-resistant weed species that have been reported from 1996 to today. These data clearly show that glyphosate-resistance in weeds is expanding around the globe. Most notably, there have been reports of nine new weed species with some biotypes resistant to glyphosate in the U.S. since 2000 (Figure 6.2). Two of these glyphosate-resistant weed species have already become a significant problem for farmers across a large geographic area. *Conyza canadensis* infests at least two million hectares of glyphosate-tolerant crops in the U.S. (Main, *et al*., 2004) and glyphosate-resistance in Palmer amaranth has serious ramifications for future weed management in the Southeast U.S. due to its rapid growth rate, extremely competitive nature, and resistance to other herbicide modes of action (Culpepper, *et al*., 2008). Researchers have also reported that individual biotypes of seven of the 16 glyphosate-resistant species are also resistant to herbicides with other modes of action (Table 6.3). The Palmer amaranth, common waterhemp, and *Conyza canadensis* biotypes were reported in the corn and soybean growing states. Although herbicide options to control

these biotypes with multiple herbicide resistance will be more limited, 2,4-D is still a viable control option for the broadleaf weeds.

In addition, researchers in Virginia have been testing a biotype of common lambsquarters that survived 1120g/ha (1.0 lb ae/acre) glyphosate, and thus appears to have low level resistance to glyphosate (Hite, *et al*., 2007). Weed scientists in Ohio and Indiana have also identified a biotype of common lambsquarters in at least a dozen fields that appears to have low-level glyphosate-resistance (Curran, *et al*., 2007). The increased reports of glyphosate-resistant species, plus the geographic spread of their infestations, have caused some to raise concerns about the long term sustainability for glyphosate. Some researchers have stated that applying glyphosate alone over wide areas on highly variable and prolific weeds made the evolution of resistant weeds inevitable (Owen, 2001; Thill and Lemerle, 2001).

Table 6.2. Weed species with reported glyphosate-resistant biotypes
(Heap, 2009)

Common Name	Species Name	First Confirmed Report		Later Confirmed Reports in Other Countries
		Year	Country**	
Rigid ryegrass	Lolium rigidum	1996	Australia	USA, S. Africa, France, Spain
Goosegrass	Eleusine indica	1997	Malaysia	Colombia
Horseweed/Marestail*	Conyza canadensis	2000	USA	Brazil, China, Spain, Czech Rep
Italian regrass	Lolium multiflorum	2001	Chile	Brazil, USA, Spain, Argentina
Hairy fleabane	Conyza bonariensis	2003	S. Africa	Spain, Brazil, Colombia, USA
Buckhorn plantain	Plantago lanceolata	2003	S. Africa	
Common ragweed*	Ambrosia artemisiifolia	2004	USA	
Giant ragweed*	Ambrosia trifida	2004	USA	
Palmer amaranth*	Amaranthus palmeri	2005	USA	
Common waterhemp*	Amaranthus rudis	2005	USA	
Johnsongrasss*	Sorghum halepense	2005	Argentina	USA
Sourgrass	Digitaria insularis	2006	Paraguay	Brazil
Wild poinsettia*	Euphorbia heterophylla	2006	Brazil	
Junglerice	Echinochloa colona	2007	Australia	
Liverseedgrass	Urochloa panicoides	2008	Australia	

* Important weeds in U.S. corn, soybeans, and cotton.
** Nine new species confirmed resistant in USA since 2000.

Figure 6.2. Number of glyphosate-resistant weeds reported globally by year from 1996 to 2008 (Compiled from Heap, 2009)

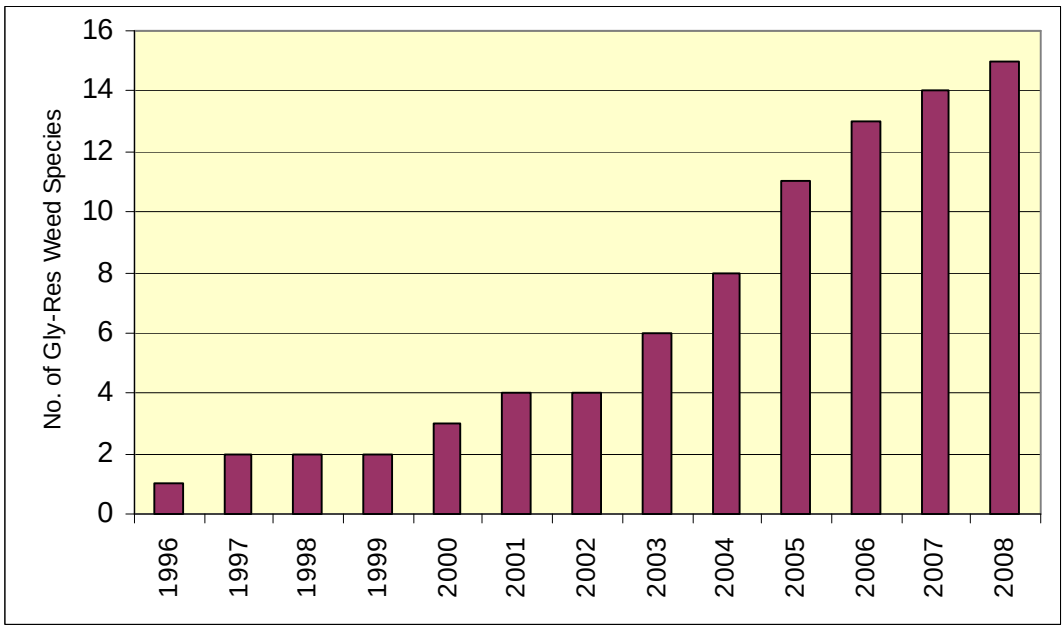


Table 6.3. Global reports of glyphosate-resistant weed biotypes with resistance to other herbicide modes of action
(Heap, 2009)

Common Name	Species Name	Year - Country (State)	Multiple Resistance to Other Herbicide MOAs
Palmer Amaranth	*Amaranthus palmeri*	2008 - USA (Mississippi)	ALS inhibitors
Common Waterhemp	*Amaranthus rudis*	2005 - USA (Missouri)	ALS, PPO
		2006 - USA (Illinois)	ALS inhibitors
Horseweed	*Conyza canadensis*	2003 - USA (Ohio)	ALS inhibitors
		2007 - USA (Mississippi)	Bipyridiliums
Goosegrass	*Eleusine indica*	1997 - Malaysia	ACCase inhibitors
Wild Poinsettia	*Euphorbia heterophylla*	2006 - Brazil	ALS inhibitors
Italian Ryegrass	*Lolium multiflorum*	2002 - Chile	ALS inhibitors
Rigid Ryegrass	*Lolium rigidum*	1999 - Australia (Victoria)	ACCase, ALS, Dinitroanilines
		2003 - South Africa	ACCase, Bipyridiliums

Weed Shifts

When glyphosate-tolerant crops are grown intensively with high reliance on glyphosate for weed control, species which can naturally resist or avoid glyphosate will become more prevalent. These "weed shifts" can occur more rapidly than selection for glyphosate-resistance (Shaner, 2000). Coble and Warren (1997) demonstrated that continuous use of glyphosate caused an increase in the infestation of morningglory (*Ipomoea*) species over a three year period compared with other herbicide programs. Some common hard to control weed species that could become "weed shifts" in U.S. corn and soybeans are listed below in Table 6.4.

Table 6.4. Potential weeds shifts with use of glyphosate in U.S. corn and soybeans (Duke and Powles, 2008b; Owen, 2008)

Common Name	Species Name
Asiatic dayflower	*Commelina communis*
Brazil callalily	*Richardia brasiliensis*
Broadleaf buttonweed	*Spermacoce latifolia*
Common waterhemp	*Amaranthus rudis*
Common lambsquarters	*Chenopodium album*
Eastern black nightshade	*Solanum ptycanthum*
Giant ragweed	*Ambrosia trifida*
Hemp sesbania	*Sesbania exaltata*
Kochia	*Kochia scoparia*
Marestail / Horseweed	*Conyza canadensis*
Morningglory spp.	*Ipomoea* spp.
Nutsedge spp.	*Cyperus* spp.
Prickly sida	*Sida spinosa*
Russian thistle	*Salsola iberica*
Tall waterhemp	*Amaranthus tuberculatus*
Tridax daisy	*Tridax procumbens*
Tropical spiderwort	*Commelina benghalensis*
Velvetleaf	*Abutilon theophrasti*

6.3.b. 2,4-D Resistance

The earliest documented reports of herbicide-resistant weeds were for resistance to 2,4-D in wild carrot (*Daucus carota*) (observed in 1952 but not reported until 1957) and spreading dayflower (*Commelina diffusa*) in 1957 (Heap, 2009). Today, a total of sixteen weed species have documented reports of 2,4-D resistant biotypes someplace around the globe (Table 6.5). Wild carrot in soybeans and roadsides, field bindweed in cropland, and prickly lettuce in cereals are the only ones reported on the U.S. mainland (Heap, 2009). Wild carrot, yellow bur-head, wild radish, musk thistle, and corn poppy are the only 2,4-D resistant weeds that have reported infestations in more than 1,000 acres. Some of these 2,4-D resistant biotypes have documented cross resistance to other auxin

herbicides or multiple resistance to some ALS-inhibiting herbicides. It is notable that most of these resistant species do not appear to be spreading, as indicated by few reports of additional sites after the initial report.

Table 6.5. Weed species with reported 2,4-D-resistant biotypes
(data from Heap, 2009).

Common Name	Species Name	Herbicides	Year	Country / State
Wild carrot	Daucus carota	2,4-D	1952	Ontario
		2,4-D	1993	Michigan
		2,4-D	1994	Ohio
Dayflower	Commelina diffusa	2,4-D	1957	Hawaii
Field bindweed	Convolvulus arvensis	2,4-D	1964	Kansas
Musk thistle	Carduus nutans	2,4-D, MCPA	1981	New Zealand
Scentless chamomile	Matricaria perforata	2,4-D	1975	France
		2,4-D	1975	United Kingdom
Gooseweed	Spenoclea zeylanica	2,4-D	1983	Phillipines
		2,4-D	1995	Malaysia
		2,4-D	2000	Thailand
Canada thistle	Cirsium arvense	2,4-D, MCPA	1985	Hungary
Globe fringerush	Fimbristylis miliacea	2,4-D	1989	Malaysia
Wild mustard	Sinapsi arvensis	2,4-D, most other auxins	1990	Manitoba
Corn poppy	Papaver rhoeas	2,4-D, tribenuron	1993	Spain
Yellow bur-head	Limnocharis flava	2,4-D	1995	Indonesia
		2,4-D, bensulfuron-methyl	1998	Malaysia
Italian thistle	Carduus pycnocephalus	2,4-D	1997	New Zealand
Wild radish	Raphanus raphanistrum	2,4-D	1999	Australia
Marshweed	Limnophila erecta	2,4-D, ALS herbicides	2002	Malaysia
Indian hedge mustard	Sisymbrium orientale	2,4-D, metsulfuron-methyl	2005	South Australia
Prickly lettuce	Lactuca serriola	2,4-D, dicamba, MCPA	2007	Washington

Few of these auxin resistant weeds have had a significant economic impact due to the wide array of alternatives that successfully control these resistant weeds (Heap, 1997). The overall incidence of auxinic herbicide-resistance after more than 60 years of use is low compared with other herbicide families such as the ALS inhibitors (imidazolinones, sulfonylureas, and sulfonamides), triazines, and ACCase herbicides in a much shorter period of use (Section IX.F.1, Figure 37). Furthermore, there is no widespread resistance to auxinic herbicides. It has been suggested by various researchers that the rarity of auxinic herbicide-resistant biotypes in the field is due to: a) a commonly held belief that these herbicides have multiple sites of action in the plant (Jasieniuk, *et al*., 1996), b) redundancy in auxin receptors (AFBs) and other components of the auxin signal response (Walsh, *et al*., 2006), c) moderate selection pressure and their use in mixtures with other herbicides (Kern *et al*., 2005), d) fitness penalties (Bourdot *et al*., 1996), and e) quantitative inheritance of the resistance trait (Cranston, *et al*., 2001).

The mechanism of resistance to auxinic herbicides has been investigated, in varying degrees for only a few of these resistant biotypes. Resistance mechanisms in these biotypes have proven to be difficult to elucidate. A lack of differences between biotypes in auxinic herbicide absorption, translocation, and metabolism has led to the hypothesis

that auxinic herbicide-resistance is most often likely due to differences at the target site or differences along the signal transduction pathway (Van Eerd, *et al*., 2005).

Patterns and mechanisms of cross resistance in auxin herbicide-resistant biotypes to other classes of auxin herbicides are not yet well understood. Further research is needed across a range of resistant species and biotypes to identify the potentially numerous gene mutations that cause resistance. It is also possible, but less likely, that a biotype might be resistant to all of these auxin herbicides. Due to the diversity of chemistry representing the synthetic auxin mode of action, it is unlikely plants will derive a single metabolic mechanism for tolerance to this class broadly: 2,4-D (phenoxy auxins), fluroxypyr (pyridyloxy auxin), dicamba (benzoate structure) or clopyralid (picolinate structure). Further research is needed to confirm or refute these cross resistance patterns.

To summarize, selection for auxin resistant weed bioytpes after more than 60 years of use has been slow, none show significant spread from initial sites, none are of significant economic importance, and none have been found in corn fields to date. Use of 2,4-D in DAS-40278-9 corn should not result in the chance for 2,4-D resistant weeds to become significant issue in corn. 2,4-D will likely be used in a mixture with one or more other herbicides. Other alternative herbicides which are effective on the same weeds can be used to control any 2,4-D resistant weeds that might occur.

6.3.c. Quizalofop Resistance

The aryloxyphenoxypropionate (AOPP - fops) and cyclohexanedione (CHD - dims) herbicides have been used widely to control grass weed species, and that heavy use of the ACCase-inhibitor herbicides has resulted in selection for resistant biotypes in at least 35 weed species around the globe (Heap, 2009). Resistance to ACCase-inhibitor herbicides was first reported in 1982 in two species; rigid ryegrass in Western Australia and blackgrass in the United Kingdom. Today there are fifteen weed species in the U.S. with documented resistance to one or more of the ACCase herbicides (Table 6.6), including some important grasses like wild oat, smooth crabgrass, large crabgrass, barnyardgrass, Italian ryegrass, perennial ryegrass, giant foxtail and Johnsongrass.

ACCase resistance is most often due to the presence of an insensitive form of ACCase (Devine, 1997). This has been identified as the cause for resistance in biotypes of blackgrass, wild oat, sterile oat, smooth crabgrass, goosegrass, Italian ryegrass, rigid ryegrass, littleseed canarygrass, giant foxtail, greenfoxtail, and Johsnongrass. Other less frequent resistance mechanisms of resistance include increased herbicide metabolism, repolarization of the plasma membrane electrogenic potential, and decreased absorption or translocation in the resistant biotype (Devine, 1997).

It has been difficult to predict cross-resistance patterns between the AOPP and CHD herbicides. A plant with resistance to either the AOPP or CHD herbicides may or may not be resistant to herbicides in the same family (UNL, 2007). Evolved resistance to ACCase herbicides can display quite different patterns of resistance within or across both

AOPP and CHD herbicides (Maneechote, *et al.*, 2005). Although one cannot assume that weed biotypes resistant to AOPP or CHD will always be cross-resistant to the other class of ACCase herbicides, testing has shown that cross-resistance does frequently occur. Cross-resistance between AOPP and CHD herbicides has been documented in giant foxtail, Johnsongrass, Chinese sprangletop, green foxtail, and large crabgrass (Ball, 2007). It is possible that other ACCase weed biotypes that have only been tested against one of these herbicide classes might also be cross-resistant to the other if tested.

Table 6.6. Weed species with reported ACCase-resistant biotypes
(Heap, 2009).

Common Name	Species Name	State	Year
Barnyardgrass	Echinochloa crus-galli	California	2000
Early Watergrass	Echinochloa oryzicola	California	2000
Giant Foxtail	Setaria faberi	Iowa	1994
		Wisconsin	1991
Italian Ryegrass	Lolium multiflorum	Georgia	1995
		Idaho	1991 1992 2005
		Maryland	1998
		North Carolina	1990
		Oregon	1987
		South Carolina	1990
		Tennessee	2006
		Virginia	1993
Itchgrass	Rottboellia exalta	Louisiana	1997
Johnsongrass	Sorghum halepense	Kentucky	1991
		Louisiana	1997
		Mississippi	1991
		Tennessee	1995
		Virginia	1995
Large Crabgrass	Digitaria sanguinalis	Wisconsin	1992
Late Watergrass	Echinochloa phyllopogon	California	1998
Little Seed Canary Grass	Phalaris minor	California	2001
Perennial Ryegrass	Lolium perenne	Arkansas	1995
Persian Darnell	Lolium persicum	Montana	1993
Purple Robust Foxtail	Setaria viridis var. robusta-purpurea	Minnesota	1999
Robust White Foxtail	Setaria viridis var. robusta-alba	Minnesota	1999
Smooth Crabgrass	Digitaria ischaemum	New Jersey	1996
Wild Oat	Avena fatua	Colorado	1997
		Idaho	1992
		Minnesota	1991
		Montana	1990 2002
		North Dakota	1991
		Oregon	1990
		Washington	1991

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Appendix 7. Stewardship of Herbicide Tolerant DAS-40278-9 Corn

Dow AgroSciences (DAS) takes product stewardship seriously and recognizes the importance of ongoing stewardship regarding both our agricultural chemical and biotechnology trait products. Dow AgroSciences will effectively steward the AAD-1 containing corn through both the agricultural chemical and seed business units using a variety of means with our sales force, commercial channels and grower customers.

7.1. Communication to Agricultural Chemical and Seed Customers

Technical Bulletins

Dow AgroSciences creates comprehensive technical bulletins on biotechnology and chemical products including elements on resistance management strategies, and ensures those are available to our channel and grower customers as well as available on our DAS customer websites. These bulletins help communicate key elements of the technology and how the products need be used effectively. The technical bulletins are also utilized with university cooperators, seed & chemical agronomists, crop consultants and other technical professionals.

Direct Mail

Dow AgroSciences uses direct mail newsletters sent on a monthly basis through both our chemical products business and seed brands. The Mycogen seed brand sends six "Let's Talk Agronomy" newsletters per year. These newsletters go directly to our farmer customers highlighting agronomics and production practices, as well as information regarding traits and other new technologies. The DAS chemical products newsletters contain product usage guidelines, positioning and best use practices. DAS has highlighted weed resistant management strategies and proper stewardship, and in the future will highlight proper stewardship of the AAD-1 herbicide program through both chemical products and seed brands newsletters.

Sales Literature

Dow AgroSciences also uses sales literature to promote our products and position proper usage. DAS uses this sales literature to properly position both trait and herbicide technologies properly as well as highlight weed resistance management strategies for effective use of the technology.

Information on Websites

The Dow AgroSciences seed brands websites focus on agronomic and proper hybrid placement. The websites also contain a specific section devoted to trait stewardship and following specific guidelines in planting transgenic traits (see the Mycogen Seed website, http://www.mycogen.com). In the future, websites can also feature AAD-1 information that will focus information towards trait and herbicide stewardship. The websites will also contain links to technical bulletin and herbicide resistance management strategies to provide a comprehensive resource.

Dow AgroSciences agricultural chemicals website (http://www.dowagro.com/usag/) features commercial and technical information and today contains information on pest resistance management such as rotating insecticides for effective stewardship. DAS can feature herbicide resistance management and effective stewardship of herbicide information labeled for AAD-1 corn upon commercial launch. Industry and university links will be included to expose the reader to a wider range of technical information.

Dow AgroSciences also has an internal Technology Transfer website where internal technical training and materials are posted. This information is made widely available to employees through links and kept up to date with regular information feeds.

7.2. Trait and Herbicide Field Testing

Dow AgroSciences uses a wide variety of field trial collaborators including universities, consultants, other biotech commercial partners and internal resources in reviewing hundreds of field trials every year, including AAD-1 trials. These field trials allow Dow AgroSciences to precisely characterize, position, and recommend the proper herbicide approach for best long term success and efficacy for the trait and herbicide usage. These also allow us to monitor a consistent set of field trial data over a long period of time to observe any efficacy trends and adjust our herbicide recommendations if necessary to mitigate resistance threats.

7.3. Ag Chemical Labeling

Key Dow AgroSciences herbicides carry a weed resistance management statement. Figure 7.1 is an example of the weed resistance management statement printed on DAS glyphosate brand labels. Dow AgroSciences can place a similar statement on all products labeled for use in conjunction with AAD-1 biotech corn to help communicate proper weed resistant management strategies.

7.4. Training and Education of Sales Representatives and Agronomists

Dow AgroSciences provides significant agronomic and herbicide usage training. This training is conducted for new sales representatives with updates provided to all sales representatives regularly by DAS Technical staff. Particular focus is on new products prior to launch and ensuring proper recommendations are communicated to our customer base for long-term product efficacy. DAS also employs extensive on-line training and includes agronomic training as needed. The Dow AgroSciences seed brand sales force and agronomists also engage in continual training including biotechnology trait technical training and positioning in addition to yearly product and agronomic training. The trait training includes specifics on stewardship and proper management of the traits. We produce product usage guides and technical use guides for current biotechnology traits and provide them to all growers planting biotechnology traits.

Figure 7.1. Example weed resistance management statement on DAS glyphosate brand labels

Weed Resistance Management

Glyphosate, the active ingredient in this product, is a group 9 herbicide (inhibitor of EPSP synthase). Some naturally occurring weed biotypes that are tolerant (resistant) to glyphosate may exist due to genetic variability in a weed population. Where resistant biotypes exist, the repeated use of herbicides with the same mode of action can lead to the selection for resistant weeds. Certain agronomic practices reduce the likelihood that resistant weed populations will develop, and can be utilized to manage weed resistance once it occurs.

To delay the selection for glyphosate resistant weeds, use the following practices:

Herbicide Selection:

- Rotate the use of glyphosate with non-glyphosate herbicides.
- Avoid using more than two applications of a glyphosate-based herbicide in a given field over a two-year period. Utilize tank mixes or sequential applications of herbicides with alternative modes of action if this is not possible.
- Use herbicides with alternative modes of action for burndown applications prior to planting Roundup Ready® crops that are likely to require more than one over-the-top application of glyphosate.
- Apply full rates of glyphosate at the specified time (correct weed size) to minimize escapes of tolerant weeds.

Crop Selection and Cultural Practices:

- Rotate Roundup Ready crops with conventional crops and use non-glyphosate herbicides to manage resistant volunteers.
- Use alternative weed control practices whenever possible, such as mechanical cultivation, delayed planting and weed-free crop seeds.
- Do not allow weed escapes to produce seeds, roots or tubers.
- Thoroughly clean plant residues from equipment before leaving fields suspected to contain resistant weeds.
- Scout fields after application to detect weed escapes or shifts in weed species.
- Report any incidence of repeated non-performance of this product against a particular weed species to the local retailer, county extension agent, or Dow AgroSciences representative.

Specific Directions:

- In burndown programs, always tank mix glyphosate with 2,4-D and/or other non-glyphosate herbicide. This product may be tank mixed with the products listed provided the product tank-mixed is registered for use on this site.
- Use soil-applied herbicides at full or reduced rates on some or all of your Roundup Ready crop fields to provide early season weed control, allow for optimal post-emergence applications of glyphosate, and to interrupt or delay selection for glyphosate resistant weeds.

7.5. Tracking Customer Satisfaction and Managing Issues

Dow AgroSciences is directly involved with farmer and distributor/retail customers on a daily basis throughout the U.S. Customer satisfaction is of ultimate importance to the continued success of DAS, so continual positioning and follow up on the joint usage of the AAD-1 traits and associated herbicides will be important in our launch and subsequent sales. As a result of our continual follow up directly with customers, we are able to implement and monitor the proper usage and stewardship of the AAD-1 technology.

DAS also electronically tracks any weed control non-performance issues and crop injury as serviced directly by our sales representatives. All new DAS sales representatives receive extensive in-field training on weed control issues and handling customer complaints. This complaint handling and data entry helps us track any emerging issues specific to products, pests (including resistant weeds) or crops and address them on a broad scale.

7.6. Involvement in Industry Groups

Dow AgroSciences is a participant in the Herbicide Resistance Action Committee (HRAC), an industry-based group supported by CropLife International. HRAC focuses on encouraging responsible attitudes towards herbicide usage, communicating herbicide resistance management strategies and supporting their implementation through practical guidelines.

HRAC engages in active collaboration with public and private researchers, especially in the areas of problem identification and devising and implementing herbicide management strategies.

Dow AgroSciences personnel also interact with academic weed scientists in tackling weed resistance management issues. DAS conducts joint trials at university sites as well as seeking input from universities regarding weed management. We also participate in a wide range of professional organizations including agronomy societies, seed trade groups, weed science societies, the National Corn Growers and their Biotech Working Group and many other industry organizations.