Application to Amend the Food Standards Code
- Food Produced Using Gene Technology

OECD Unique Identifier: DAS-40278-9

AAD-1 Maize

Submitting Company:

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SUMMARY

Dow AgroSciences Australia Ltd. (herein referred to as "DAS"), is submitting an application to vary the Code to approve the use of DAS-40278-9 Maize, a new food produced using gene technology.

DAS-40278-9 maize is a transgenic maize product that provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and aryloxyphenoxypropionate (AOPP) acetyl coenzyme A carboxylase (ACCase) inhibitors ("fop" herbicides). This herbicide-tolerance trait will provide overseas 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) as a selection agent in breeding nurseries.

DAS-40278-9 maize 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 maize 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 maize 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 maize. Segregation data for six generations confirmed the predicted inheritance of the *aad-1* gene.

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

Nutrient composition analyses of forage and grain was conducted to compare the composition of DAS-40278-9 maize 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 maize which was sprayed with either 2,4-D, quizalofop, both 2,4-D and quizalofop, or which was not sprayed with either herbicide. The compositional analyses indicate that DAS-40278-9 maize is substantially equivalent to conventional maize.

In summary, information collected during field trials and laboratory analyses presented herein demonstrate that DAS-40278-9 maize is as safe as conventional maize for food and feed uses.

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THE APPLICANT

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Dow AgroSciences is a top-tier agricultural company that combines the power of sciences and technology with the “Human Element” to constantly improve what is essential to human progress. Dow AgroSciences provides innovative technologies for crop protection, pest and vegetation management, seeds, traits and agricultural biotechnology to serve the world’s growing population.

ACRONYMS AND SCIENTIFIC TERMS

2,4-D	2,4-Dichlorophenoxyacetic acid
DAS-40278-9	Maize 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 fibre
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	Centre for Food Safety and Nutrition, US FDA
CHD	Cyclohexanedinone
DAS	Dow AgroSciences
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 maize 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 maize 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 fibre
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*

A. GENERAL INFORMATION ON THE APPLICATION

1. Purpose of the application

Dow AgroSciences (herein referred to as "DAS") has developed transgenic maize 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 maize 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).

This application to the Food Standards Australia New Zealand has been based on the submission generated for other overseas agencies. It is a component of the Dow AgroSciences global approval process, especially for export destinations of maize commodities and is consistent with Dow AgroSciences corporate policy of ensuring full regulatory compliance. As a result of this application, Dow AgroSciences Australia Ltd seek an amendment of Standard 1.5.2 by inserting: food derived from Herbicide Tolerant DAS-40278-9 maize line, into column 1 of the Table to clause 2, immediately after the last entry.

2. Justification for application

a. Advantage of the genetically modified food

DAS-40278-9 maize 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 maize will allow overseas 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 internationally 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, maize,

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). Glyphosate resistant weeds are also present in Australia including *Lolium rigidum* (Annual ryegrass), *Urochloa panicoides* (Liverseed grass) and *Echinochloa colona* (Barnyard grass). (Preston, C., 2005)

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. maize 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.

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 maize 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 maize plants also provides tolerance to AOPP ("fop") herbicides. This allows the use of these herbicides to control grasses in maize, 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 maize 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.

b. Safety of the genetically modified food

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).

Please refer to Part C, section 4 and 5 of this dossier for information relating to the potential allergenicity and toxicity of the novel protein.

c. Potential impact on trade

This application to the Food Standards Australia New Zealand has been based on the submission generated for other overseas agencies. It is a component of the Dow AgroSciences global approval process, especially for export destinations of maize commodities and is consistent with Dow AgroSciences corporate policy of ensuring full regulatory compliance. It is a necessary component of the global approval process since without such food import approvals, the cultivation and marketing of DAS-40278-9 in the USA will be significantly hampered. Dossiers are being submitted to the regulatory authorities of trade partners for import clearance may include Canada, Japan, Korea, Taiwan, European

Union, ANZ, South Africa, Brazil, Argentina, Mexico and Colombia. The benefit and market share implication are difficult to quantify, however, freedom to operate in the marketplace is a market requirement and will have an impact on these factors.

d. Costs and benefits for industry, consumers and government

The local cost implications are made up of DAS personnel time both locally and globally as well as the direct fees associated with the submission.

There are few price or employment implications which are directly related to the FSANZ assessment of DAS-40278-9. The trade implications however are clear since non-approval by FSANZ would impose a trade restriction on DAS-40278-9 and the products derived from these lines.

B. TECHNICAL INFORMATION ON THE GM FOOD

1. Nature and identity of the genetically modified food

a. Description of the GM organism

DAS-40278-9 maize is a transgenic maize 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 was developed using direct Whiskers-mediated transformation to stably incorporate the *aad-1* gene from *Sphingobium herbicidovorans* into maize. 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).
DAS-40278-9 maize will allow overseas growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance in weeds.

b. GM Organism Identification

This transformed maize is known as Event DAS-40278-9. No commercial name has yet been identified.

c. Food Identity

There is no intention to market food items containing maize derived from DAS-40278-9 with specific brands or names.

d. Products containing the food or food ingredients.

Refer to the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (Zea mays): Key Food and Feed Nutrients, Anti-nutrients and Secondary Plant Metabolites (2002), for the following aspects of the food uses of maize:

- Production of maize for food and feed
- Processing of maize
    - Wet Milling
    - Dry Milling
    - Masa Production

o Feed Processing

The majority of grain and forage derived from maize is used for animal feeds. Less than 10% if maize grain is processed for human food products. Maize grain is also processed into industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet-milling to produce starch and sweetener products. In addition to milling, maize germ can be processed to obtain maize oil.

Domestic production of maize in Australia (*ca.* 340,000t) and New Zealand is supplemented by import of a small amount of maize-based products, largely as high-fructose maize syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and maize chips. Other maize products such as maize starch are also imported. This is used by the food industry for the manufacture of dessert mixes and canned foods.

2. History and Use of the Host and Donor Organisms

a. Donor Organism

The donor organisms of each of the genetic elements inserted into DAS-40278-9 are listed in Table 1. Event DAS-40278-9 was generated using a linear *Fsp* I fragment from plasmid pDAS1740, containing the synthetic, plant-optimized *aad-1* gene from *Sphingobium hericidovorans*, and promoter and terminator sequences from *Zea mays*. A schematic map of the linearized fragment from pDAS1740 **(Figure 1)** illustrates the order and orientation of the sequences in the vector.

Table 1: Description, location and size of the genetic elements in 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 maize 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 places like plant root systems and clinical specimens. 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 (FSANZ Applications A446 and

A543) 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 neighbouring 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.

For information on the potential toxicity or allergenicity of the proteins see section C part 3 and 4 of this dossier.

b. Host Organism

Maize (*Zea mays* L.) is the only species usually included in the genus *Zea*, of the family Gramineae. It is a highly domesticated agricultural crop with well-characterised phenotypic and genetic traits. It reproduces sexually by wind-pollination and being a monoecious species has separate male staminate (tassels) and female pistillate (silk) flowers. This gives natural outcrossing between maize plants but it also enables the control of pollination in the production of hybrid seed. Typical of wind-pollinated plants, a large amount of redundant maize pollen is produced for each successful fertilisation of an ovule on the ear. Wind movements across the maize field cause pollen from the tassel to fall on the silks of the same or adjoining plants. Measuring about 0.1 mm in diameter, maize pollen is the largest of any pollen normally disseminated by wind from a comparably low level of elevation.

Repeated cycles of self-pollination leads to homogeneity of the genetic characteristics within a single maize plant (inbred). Controlled cross-pollination of inbred lines from chosen genetic pools combines desired genetic traits in a hybrid resulting in improved agronomic performance and yield increase. This inbred-hybrid concept and resulting yield response is the basis of the modern maize seed industry. Open pollination of hybrids in the field leads to the production of grain with properties from different lines and, if planted, would produce lower yields than those obtained with hybrids (Canadian Food Inspection Agency 1994).

Maize is extensively cultivated world-wide and has a long history of safe use. Production has benefited from many improvements, particularly since the 1920's when maize varieties were developed by conventional breeding between progeny of two inbreds to give hybrid varieties which are superior to open-pollinated varieties in their agronomic characteristics, such as increased grain yield. In present agricultural systems, hybrid maize varieties are used in most developed countries for consistent agronomic performance and production.

Maize, together with rice and wheat, is one of the most important cereal crops in the world with total production of 591 million tonnes in 2000 (FAOSTAT Database 2001). Of this world wide production, some 253 million tonnes (43%) is produced in the USA. The majority of grain and forage derived from maize is used as animal feed. Maize grain is also processed into industrial products, such as ethyl alcohol by fermentation and highly refined starch by wet-milling to produce starch and sweetener products. In addition to milling, the maize germ can be processed to obtain maize oil and for numerous other minor uses (White and Pollak 1995). No special processing is required to make maize safe to feed or eat.

For more information on the biology of *Zea mays* please refer to Regulatory Directive Dir94-11: The Biology of Zea mays L. (Corn/Maize) (CFIA, 1994).

Characterization of the recipient maize line

The publicly available maize line, Hi-II, was used as the recipient line for the generation of event DAS-40278-9 maize (Armstrong et al., 1991). Hi-II is a derivative of the A188 and B73 inbred maize 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 maize plants were subsequently crossed with elite proprietary inbred maize lines to derive maize hybrids containing DAS-40278-9.

3. Nature of the Genetic Modification

a. Transformation Method

The recipient maize line Hi-II was transformed using direct insertion of the DNA fragment from plasmid pDAS1740 via W*hiskers*-mediated transformation using silicon-carbide fibres (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, 2 fragments (1023 bp and 1235 bp respectively) each containing a portion of the ampicillin resistance gene sequence from the plasmid backbone, and 2 small fragments of 9 bp each containing sequences from the plasmid backbone as a result of 3 closely spaced *Fsp* I restriction

enzyme sites within the pDAS1740 plasmid **(Figure 2)**. The two smaller ampicillin resistance gene fragments and the two 9 bp 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 1).**

Figure 1: Schematic diagram of the linearized DNA fragment from pDAS1740 used in the Whiskers-mediated transformation

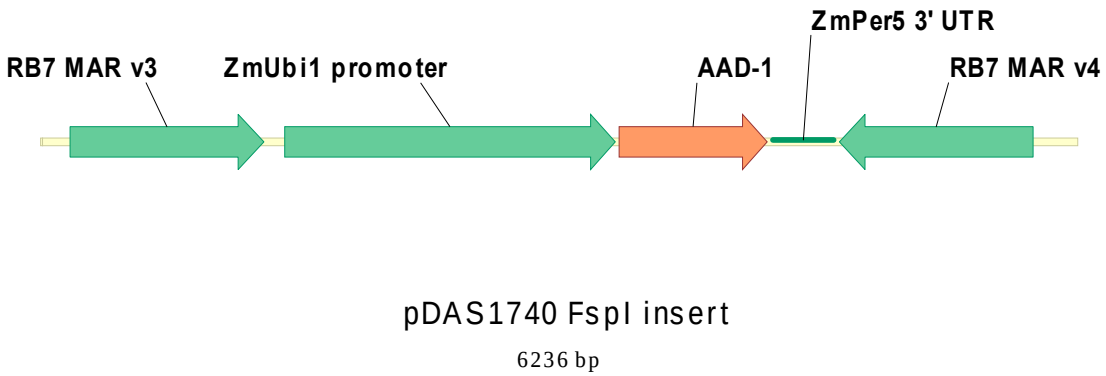
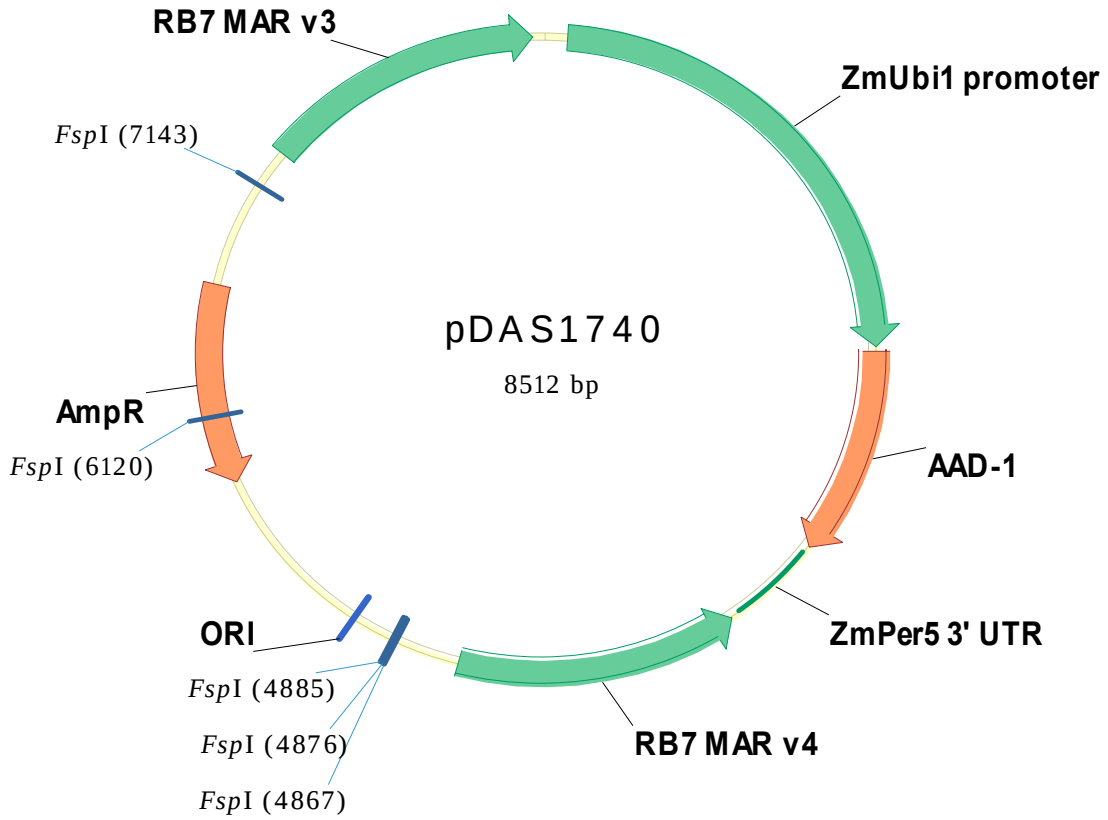


Figure 2: Schematic map of plasmid pDAS1740 including Fsp I restriction enzyme sites utilized to isolate the vector DNA fragment used in the Whiskers-transformation



Immature embryos of maize 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 fibres 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 aryloxyphenoxypropionate (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 maize lines to obtain T1 seed from the initially transformed T0 plants **(Figure 3).** A breeding diagram **(Figure 4)** demonstrates the pathway

from the original transformant to the creation of the hybrid. Furthermore, as different generations were used in the various studies performed throughout the data package, a table was created to clarify the generations, inbred lineage and controls were used in the specific studies and chapters **(Table 2).**

Figure 3: Schematic of the development of DAS-40278-9 maize outlining the pathway from the synthesis of the aad-1 gene to the selection of the DAS-40278-9 event as the leading commercial candidate

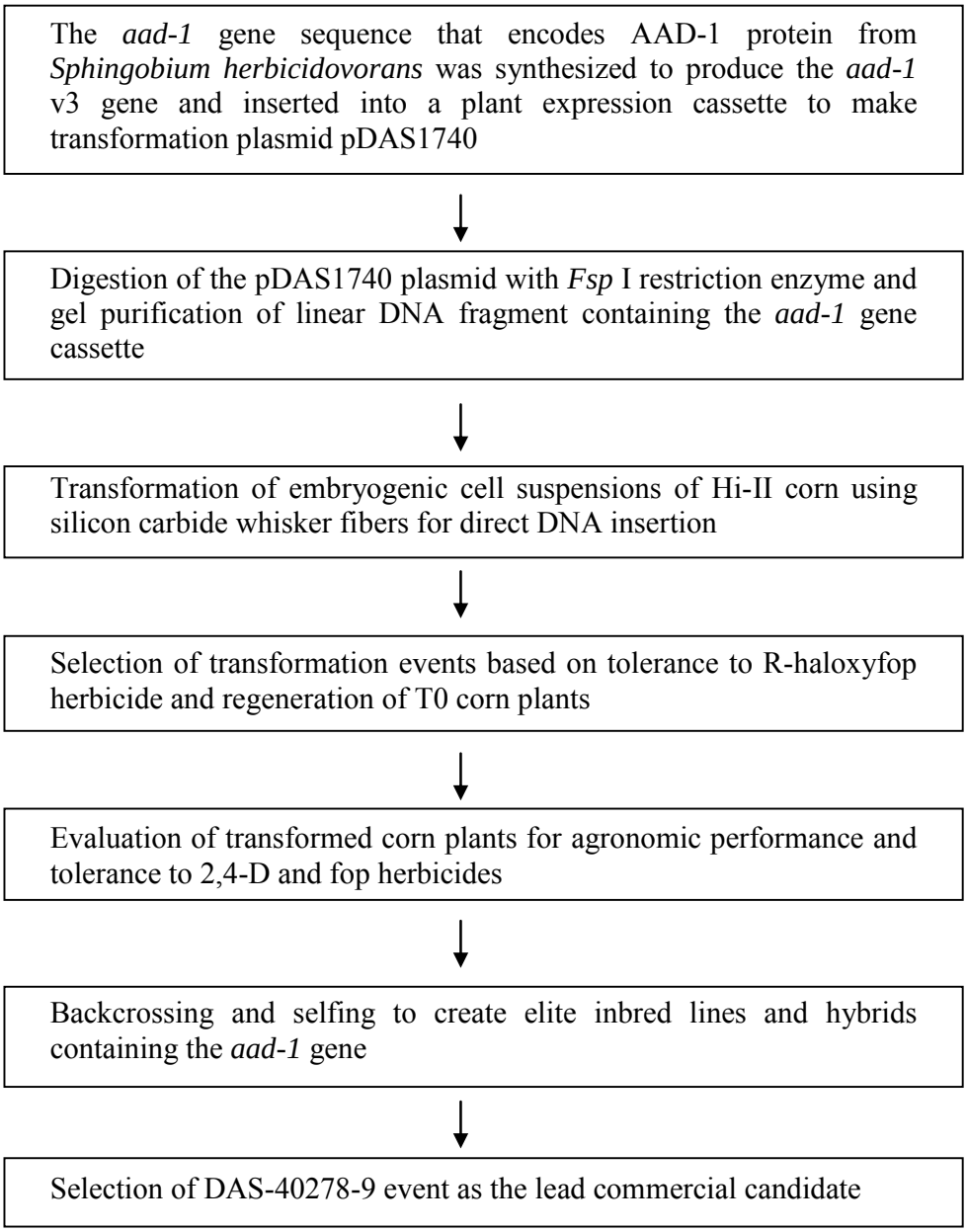
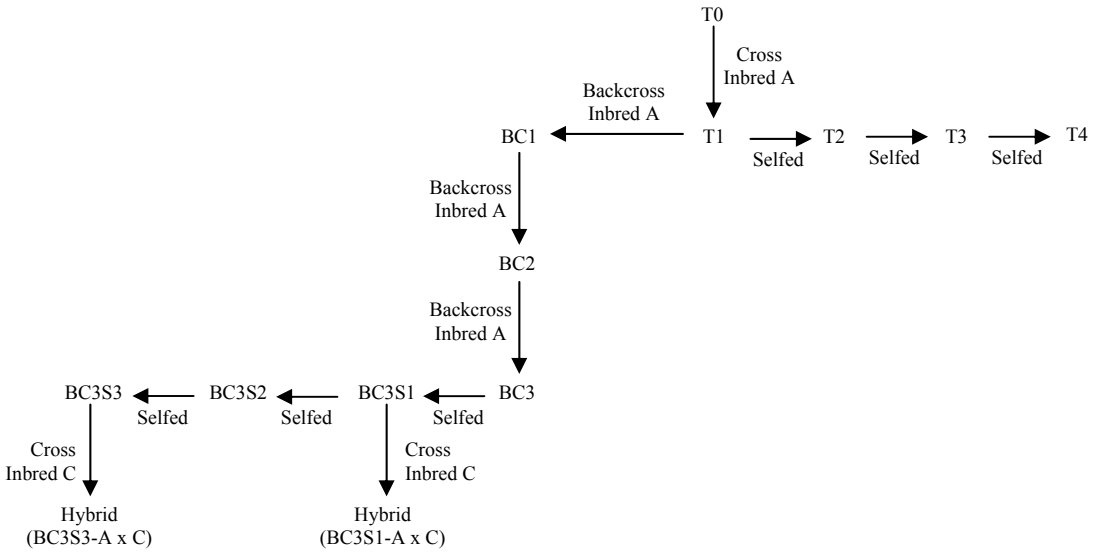


Figure 4: Breeding diagram for DAS-40278-9 maize demonstrating the pathway from the original transformant to the hybrid



Inbred A = DAS elite inbred XHH13 Inbred C = DAS elite inbred used to make hybrid T0 = original transformant T1 = first generation, derived from cross of T0 with elite inbred T2 = derived from self-pollination of T1 T3 = derived from self-pollination of T2 T4 = derived from self-pollination of T3	BC1 = first backcross with elite parental inbred BC2 = second backcross with elite parental inbred BC3 = third backcross with elite parental inbred 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

Table 2: Inbred lineage, DAS-40278-9 Maize Generations and controls used for analysis in specific chapters

Analysis	Data Package Section	Inbred Lineage	DAS-40278-9 Maize Generation	Control
Molecular Analysis	Section B, Part 3.d.	A	T3, T4, BC3S1, BC3S2, BC3S3	Inbred A
Segregation Analysis	Section B, Part 3.d.	A	T1, T2 BC1, BC2, BC3, BC3S1	--
Protein Characterization	Section C, Part 2.a-f.	A	BC3S1-A x C Hybrid	A x C Hybrid
Protein Expression	Section C, Part 2. a-f.	A	BC3S1-A x C Hybrid	A x C Hybrid
Composition	Section D, Part 1.	A	BC3S1-A x C Hybrid	A x C Hybrid

b. Bacteria used for manipulation

A standard lab strain of *E.coli* was used for all vector manipulations and for amplification of the plasmid DNA (pDAS1740) that was used for transformation.

c. Gene Construct and Vectors

Event DAS-40278-9 was generated using a linear *Fsp* I fragment from plasmid pDAS1740, containing the synthetic, plant-optimized *aad-1* gene from *Sphingobium hericidovorans*. A summary of the genetic elements is given in **Table 1**. A schematic map of the linearized fragment from pDAS1740 **(Figure 1)** illustrates the order and orientation of the sequences in the vector.

d. Molecular Characterisation

Molecular characterization of event DAS-40278-9 was conducted by Southern blot analyses in study 081052 performed by Zhuang et al (2009). The results demonstrate that the transgene insert in maize 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 maize.

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 3**

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 4** 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 maize 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.

Table 3: Location and length of probes used in Southern blot analysis
(Zhuang, 2009, p.28, Study 081052)

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 maize

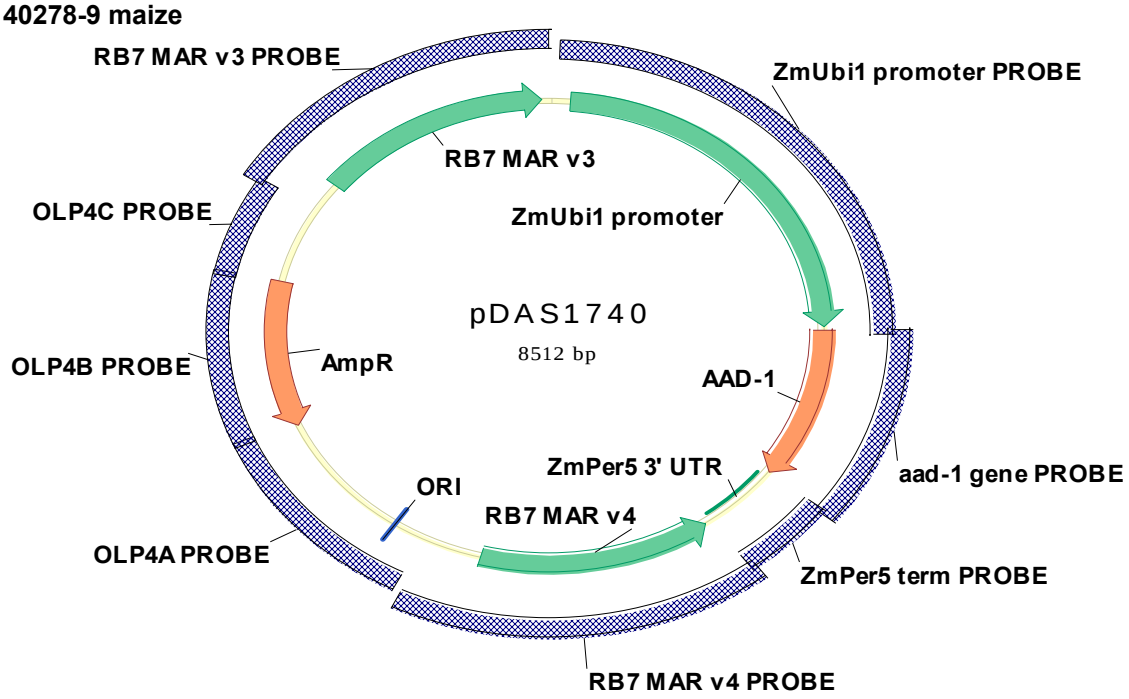


Table 4: Correlation of predicted and observed hybridizing fragments in Southern blot analysis
(Zhuang, 2009, pp.29-30, Study 081052)

DNA Probe	Restriction Enzymes		Figure	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
aad-1	*Eco*R I	pDAS1740	2.8	8512	8512
		XHH13	2.8	none	none
		DAS-40278-9	2.8	>3382 (border)	~12000
	Nco I	pDAS1740	2.9	8512	8512
		XHH13	2.9	none	none
		DAS-40278-9	2.9	>2764 (border)	~4000
	Sac I	pDAS1740	2.10	8512	8512
		XHH13	2.10	none	none
		DAS-40278-9	2.10	>4389 (border)	~16000
	Fse I / *Hind* III	pDAS1740	2.11	3361	3361
		XHH13	2.11	none	none
		DAS-40278-9	2.11	3361	3361
ZmUbi1 prom.	*Nco* I	pDAS1740	2.12	8512	8512, ~3600*
		XHH13	2.12	none	~3600*
		DAS-40278-9	2.12	>3472 (border)	~6300, ~3600*
	Sac I	pDAS1740	2.13	8512	8512, ~3800*
		XHH13	2.13	none	~3800*
		DAS-40278-9	2.13	>4389 (border)	~3800*, ~16000
	Fse I / *Hind* III	pDAS1740	2.14	3361	3361, ~6400*
		XHH13	2.14	none	~6400*

		DAS-40278-9	**2.14**	**3361**	**3361, ~6400*#**
ZmPer5 term.	***Nco* I**	**pDAS1740**	**2.15**	**8512**	**8512, ~3900***
		XHH13	**2.15**	**none**	**~3900***
		DAS-40278-9	**2.15**	**>2764 (border)**	**~4000, ~3900***
	***Sac* I**	**pDAS1740**	**2.16**	**8512**	**8512, ~9000***
		XHH13	**2.16**	**none**	**~9000***
		DAS-40278-9	**2.16**	**>1847 (border)**	**~1900, ~9000***
	***Fse* I / *Hin*d III**	**pDAS1740**	**2.17**	**3361**	**3361, ~2100***
		XHH13	**2.17**	**none**	**~2100***
		DAS-40278-9	**2.17**	**3361**	**3361, ~2100***
RB7 MAR4	***Nco* I**	**pDAS1740**	**2.18**	**8512**	**8512**
		XHH13	**2.18**	**none**	**none**
		DAS-40278-9	**2.18**	**>2764 (border) >3472 (border)**	**~4000 ~6300**
	***Sac* I**	**pDAS1740**	**2.19**	**8512**	**8512**
		XHH13	**2.19**	**none**	**none**
		DAS-40278-9	**2.19**	**>1847 (border) >4389 (border)**	**~1900 ~16000**
RB7 MAR3	***Nco* I**	**pDAS1740**	**2.20**	**8512**	**8512**
		XHH13	**2.20**	**none**	**none**
		DAS-40278-9	**2.20**	**>2764 (border) >3472 (border)**	**~4000 ~6300**
	***Sac* I**	**pDAS1740**	**2.21**	**8512**	**8512**
		XHH13	**2.21**	**none**	**none**
		DAS-40278-9	**2.21**	**>1847 (border) >4389 (border)**	**~1900 ~16000**
backbone	***Nco* I**	**pDAS1740**	**2.22**	**8512**	**8512**
		XHH13	**2.22**	**none**	**none**
		DAS-40278-9	**2.22**	**none**	**none**
	***Sac* I**	**pDAS1740**	**2.23**	**8512**	**8512**
		XHH13	**2.23**	**none**	**none**
		DAS-40278-9	**2.23**	**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-labelled 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

(Zhuang, 2009, p.31, Study 081052)

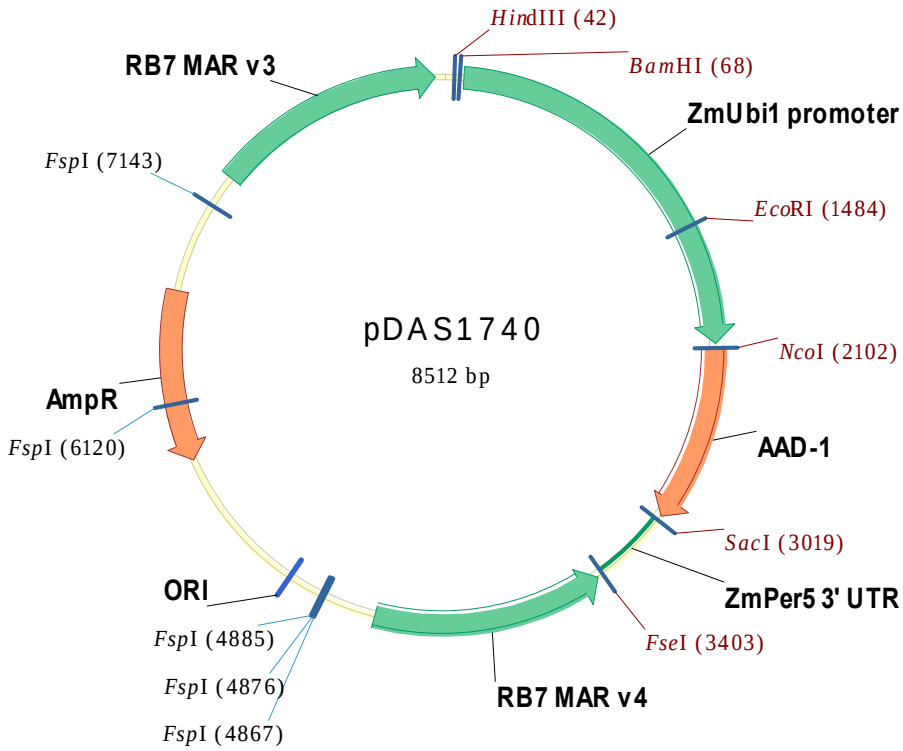
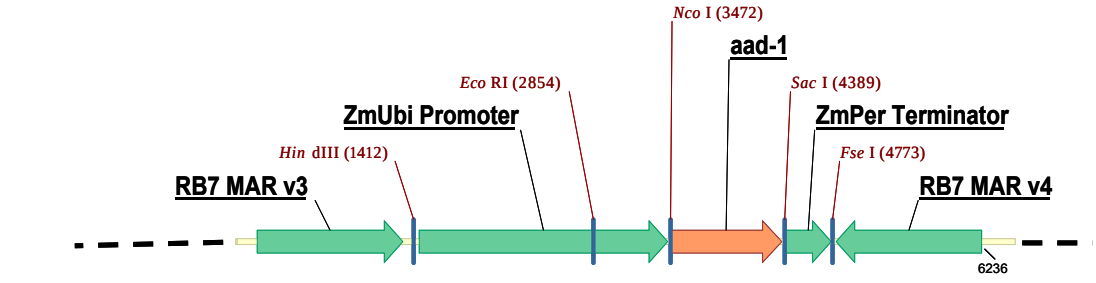


Figure 7: Restriction map of the DAS-40278-9 insertion site and predicted sizes of digested fragments
(Zhuang, 2009, p.33, Study 081052)



pDAS1740/FspI

6236 bp

EcoR I	>2854 bp	>3382 bp	
Nco I	>3472 bp	>2764 bp	
Sac I	>4389 bp	>1847 bp	
Hind III/Fse I	>1412 bp	3361 bp	>1463 bp

Genomic DNA for Southern blot analysis was prepared from leaf material of individual DAS-40278-9 maize plants from five distinct breeding generations in genetic background XHH13 (see breeding diagram **Figure 4**, Section B, Part 3a). Genomic DNA from leaves of conventional XHH13 maize plants was used as the control material. Plasmid DNA of pDAS1740 added to genomic DNA from the conventional XHH13 control maize served as the positive control for the transgene sequences.

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 (See Analysis of the Insert and Its Genetic Elements section below). 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 maize (T3, T4, BC3S1, BC3S2, BC3S3) were identical, indicating that the insertion is stably integrated in the maize genome (See Stability of the Insert Across Generations section below). No vector backbone sequences were detected in event DAS-40278-9. Additionally, the inheritance of DAS-40278-9 maize in segregating generations was investigated in a study by Zhuang et al (2009) Study 081120 using Southern blot analysis , protein detection, and herbicide screening methods, and all results confirmed the predicted inheritance of the transgene (See Segregation Analysis of DAS-40278-9 Maize below).

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 (Zhuang, 2009, p.21, Study 081052). 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 maize genome of event DAS-40278-9 **(Figures 8,9 and 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 maize genome of event DAS-40278-9 as shown in the restriction map in **Figure 7**.

Figure 8: Southern blot analysis of DAS-40278-9; aad-1 probe, EcoR I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize 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. Single hybridization bands of ~ 12 000 bp were observed within the lanes containing the DAS-40278-9 samples, and no bands were observed in the lanes containing the conventional maize XHH13 negative control. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.34-35, Study 081052)

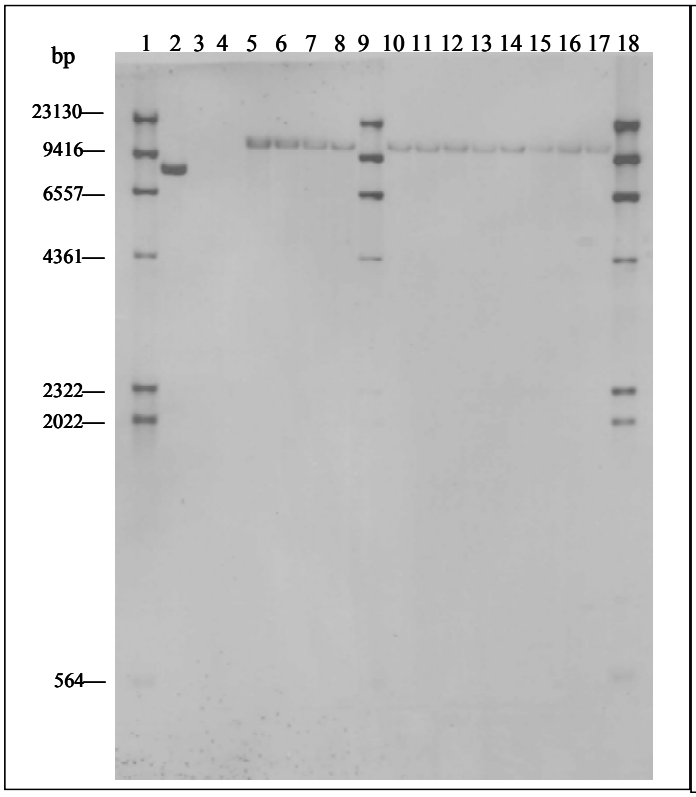
Panel A

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.

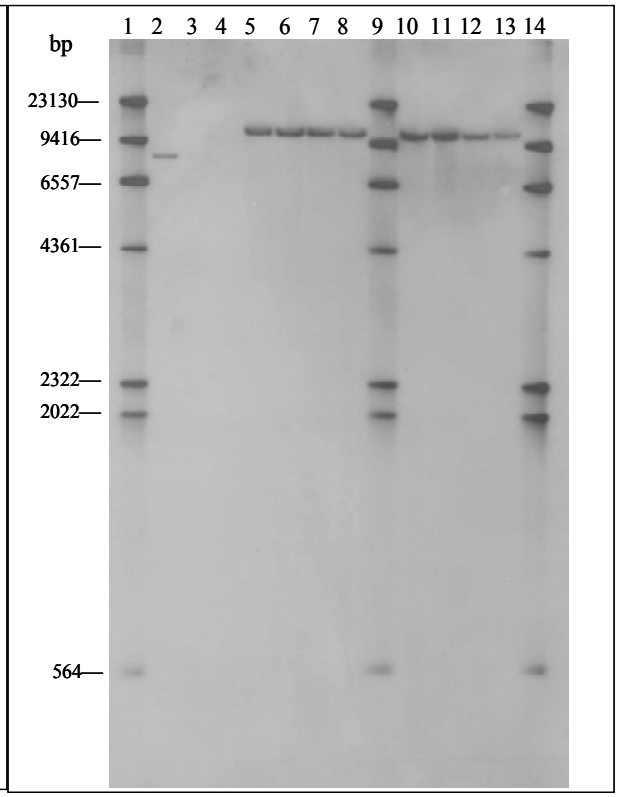


Figure 9: Southern blot analysis of DAS-40278-9; aad-1 probe, Nco I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Nco* I and probed with the *aad-1* gene probe. Nine (9) μg of DNA 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. Single hybridization bands of ~4000 bp were observed in the lanes containing the DAS-40278-9 samples, and no bands were observed in the lanes containing the conventional maize XHH13 negative control. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.36-37, Study 081052).

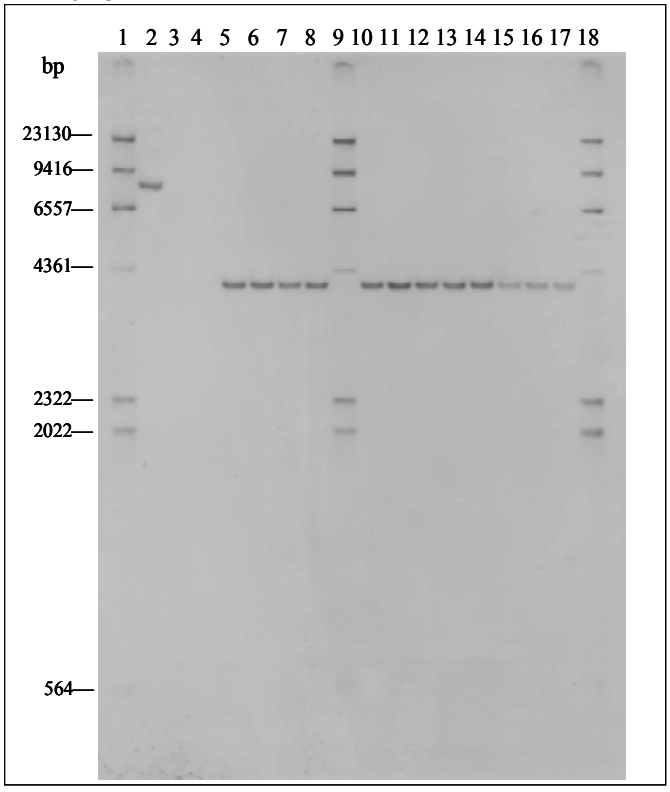
Panel A.

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.

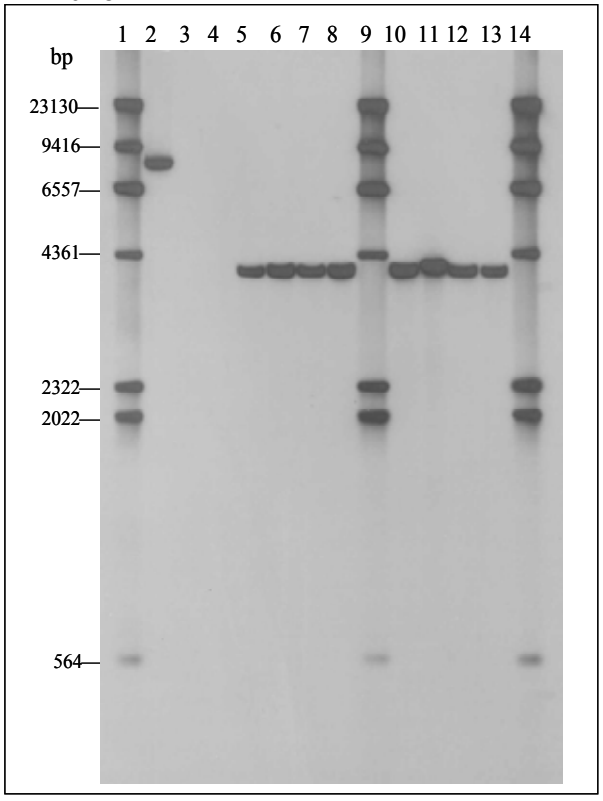


Figure 10: Southern blot analysis of DAS-40278-9; aad-1 probe, Sac I digest.
Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of
conventional maize XHH13 was digested with *Sac* I and probed with the *aad-1* gene probe. Nine (9) μg of DNA 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. Single hybridization bands of ~ 16 000 bp were observed in the lanes containing the DAS-40278-9 samples, and no bands were observed in the negative control lanes containing conventional maize XHH13. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.38-39, Study 081052).

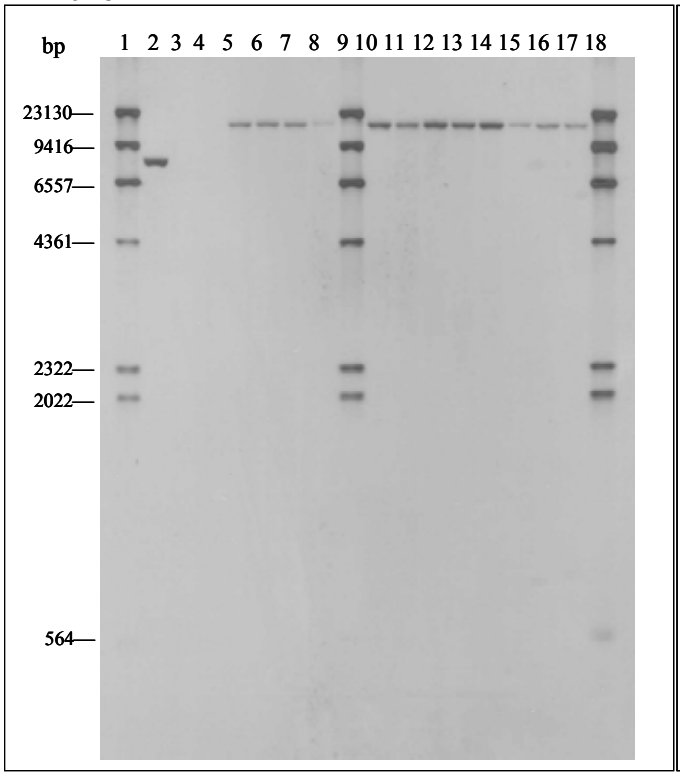
Panel A.

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.

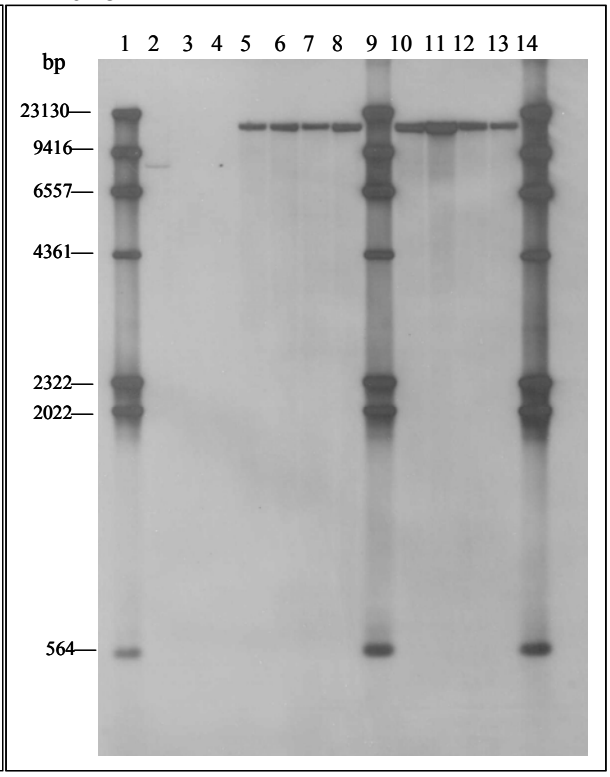


Figure 11: Southern blot analysis of DAS-40278-9; aad-1 probe, Fse I / Hind III digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Fse* I / *Hin*d III and probed with the *aad-1* gene 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. The double enzyme digest released a fragment of ~3361 bp in all lanes containing the DAS-40278-9 samples, as well as the positive control pDAS1740 sample. No bands were observed in the conventional maize XHH13 negative control lanes. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.40-41, Study 081052).

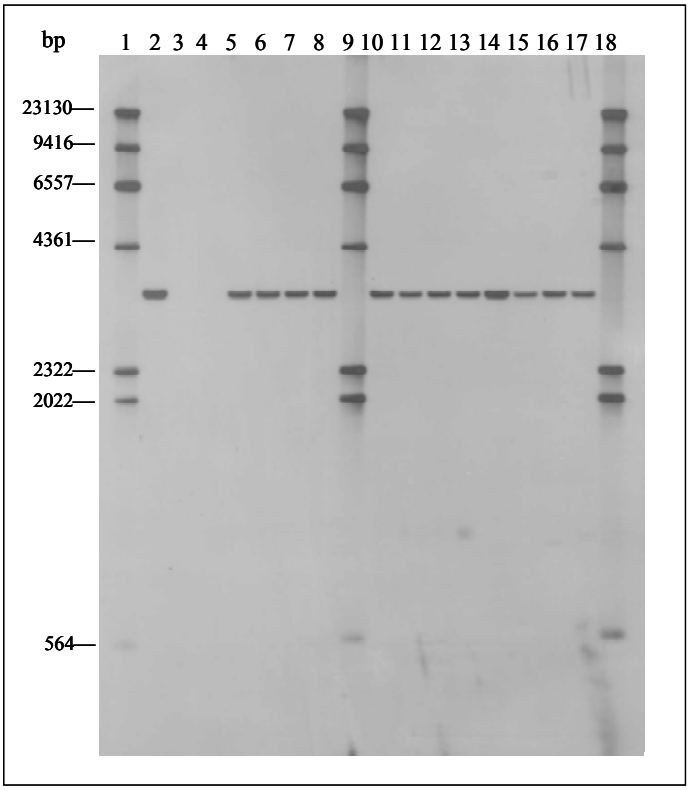
Panel A.

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

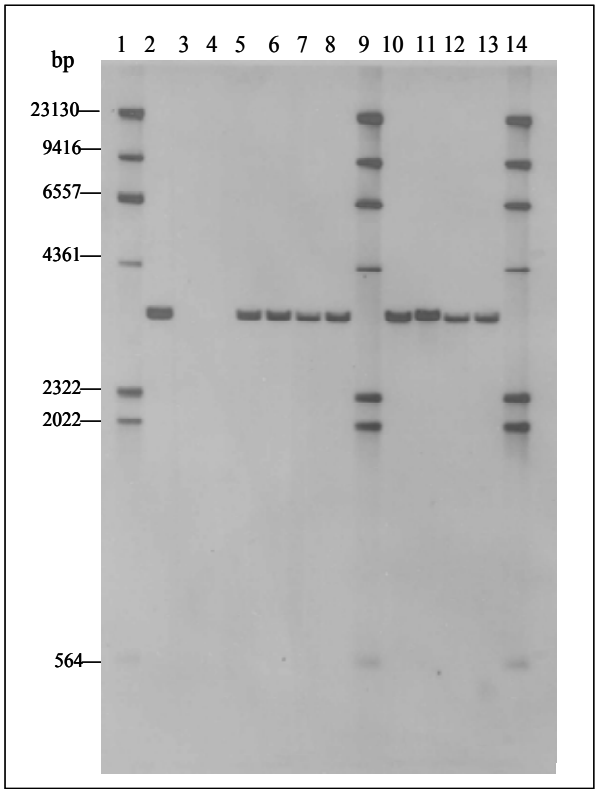
Panel B.

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.



Analysis of the ZmUbil 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 4).** 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 maize 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 maize 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 maize 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. The 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 maize genome of event DAS-40278-9 (Zhuang, 2009, p.22, Study 081052).

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 maize 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. A non-specific hybridization band of ~3600 bp was observed across all sample lanes, including the positive and negative control lanes containing XHH13. A specific hybridization band of ~6300 bp was observed only in the lanes containing the DAS-40278-9 samples, and not in the XHH13 negative controls. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.42-43, Study 081052).

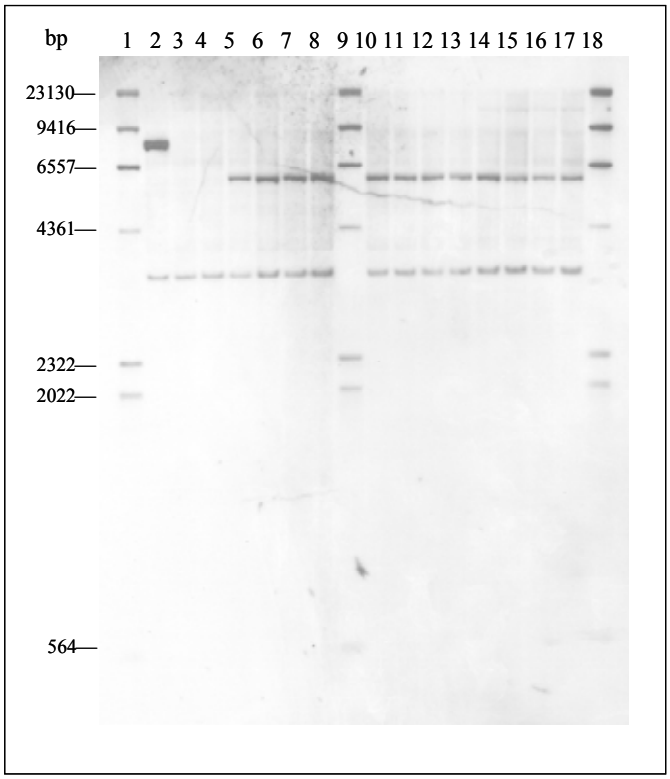
Panel A.

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.

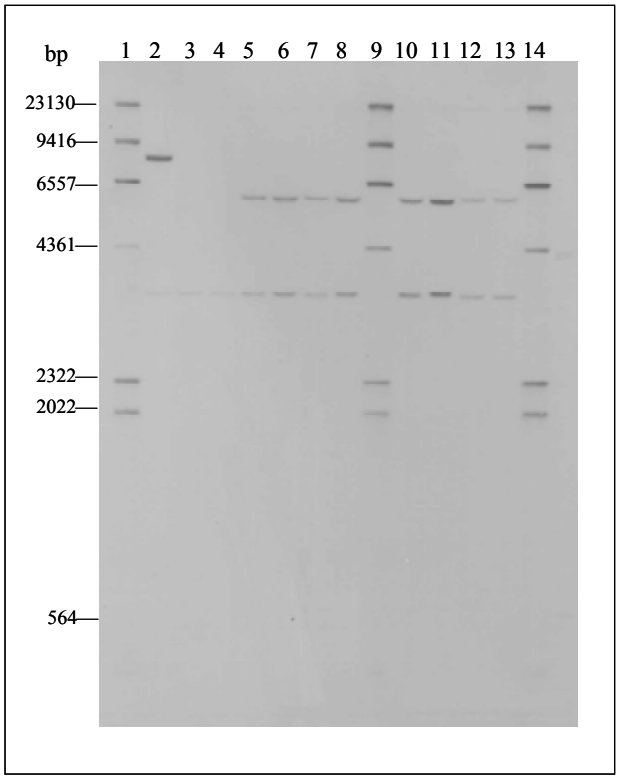


Figure 13: Southern blot analysis of DAS-40278-9; ZmUbi promoter probe, Sac I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Sac* I and probed with the ZmUbi promoter probe. Nine (9) 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. A non-specific hybridization band of ~3800 bp was observed across all sample lanes, including the positive and negative control lanes containing XHH13. A specific hybridization band of ~16 000 bp was observed only in the lanes containing the DAS-40278-9 samples, and not in the XHH13 negative controls. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.44-45, Study 081052).

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.**

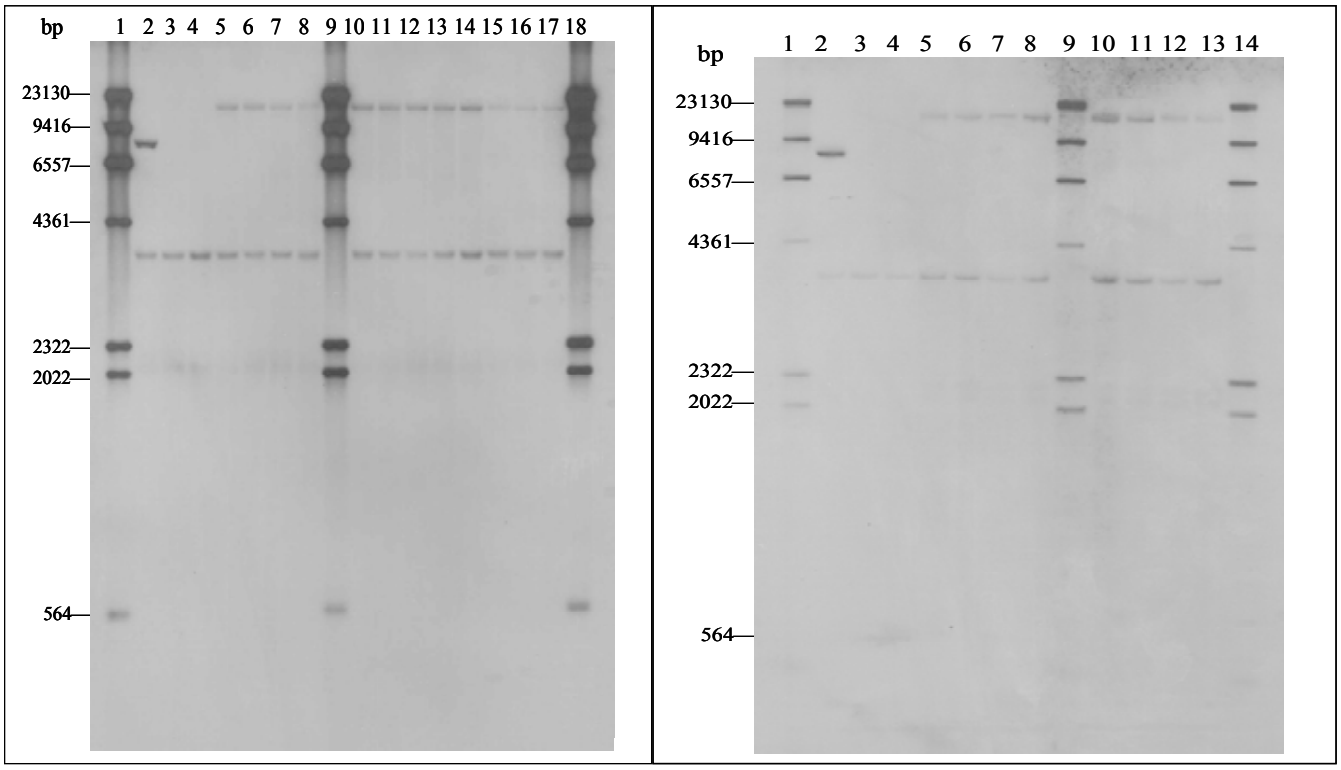


Figure 14: Southern blot analysis of DAS-40278-9; ZmUbi promoter probe, Fse I / Hind III digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Fse* I / *Hin*d III and probed with the ZmUbi 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. A hybridization band of ~ 3361 bp, which is the predicted size of the *aad*-1 PTU fragment, was observed in the lanes containing the DAS-40278-9 samples, and not in the lanes containing the negative control XHH13. A non-specific hybridization band of ~6400 bp was detected across all lanes, including the positive and negative control lanes containing XHH13. Sample name indicates test material, generation, and individual plant number used. **Note:** The ZmUbiI probe hybridized to the endogenous *ubi* gene in the maize 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 (Zhuang, 2009, pp.46-47, Study 081052).

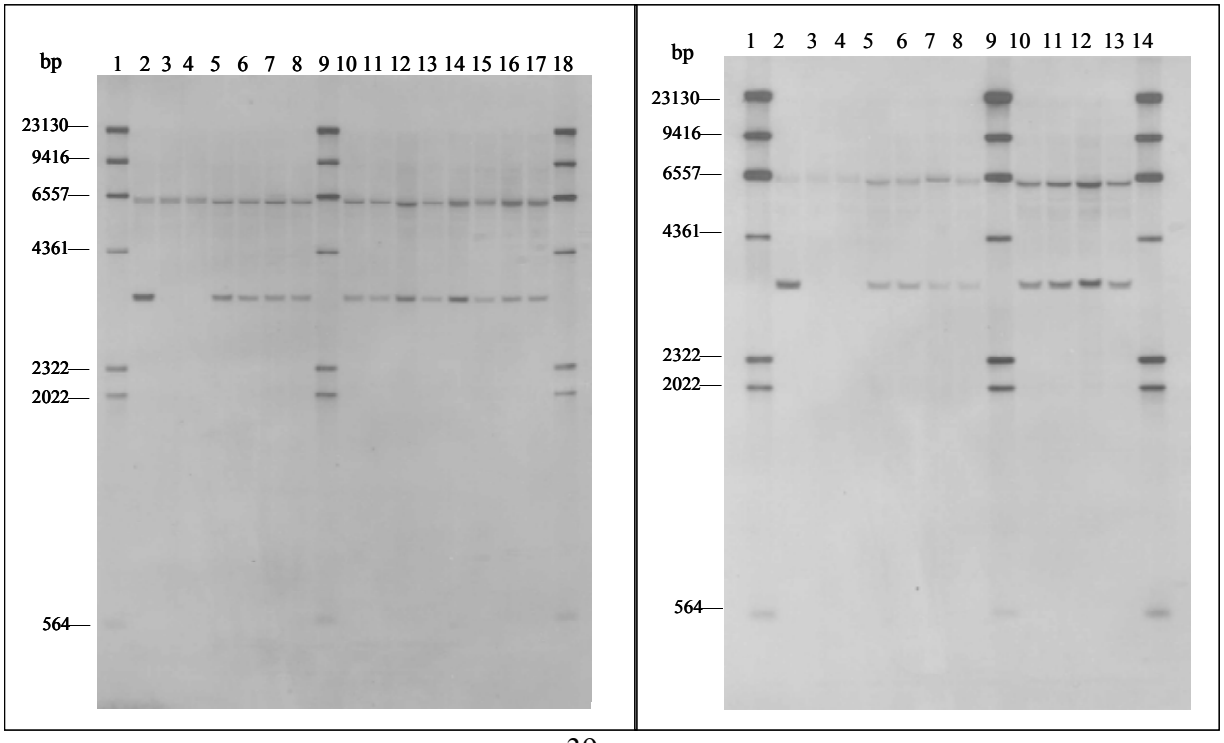
Panel A.

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.**



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 4)**. 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 maize 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 maize 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 **(Figure 17)**. The additional ~2100 bp band is the non-specific binding of the ZmPer5 terminator probe to the maize 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 maize genome of event DAS-40278-9 (Zhuang, 2009, pp.22-23, Study 081052).

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 maize 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. A non-specific hybridization band of ~3900 bp was detected across all lanes, including the positive and negative control lanes containing XHH13. A hybridization band of ~ 4000 bp was detected only in the lanes containing DAS-40278-9 samples, and not in the conventional control lanes. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.48-49, Study 081052).

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.**

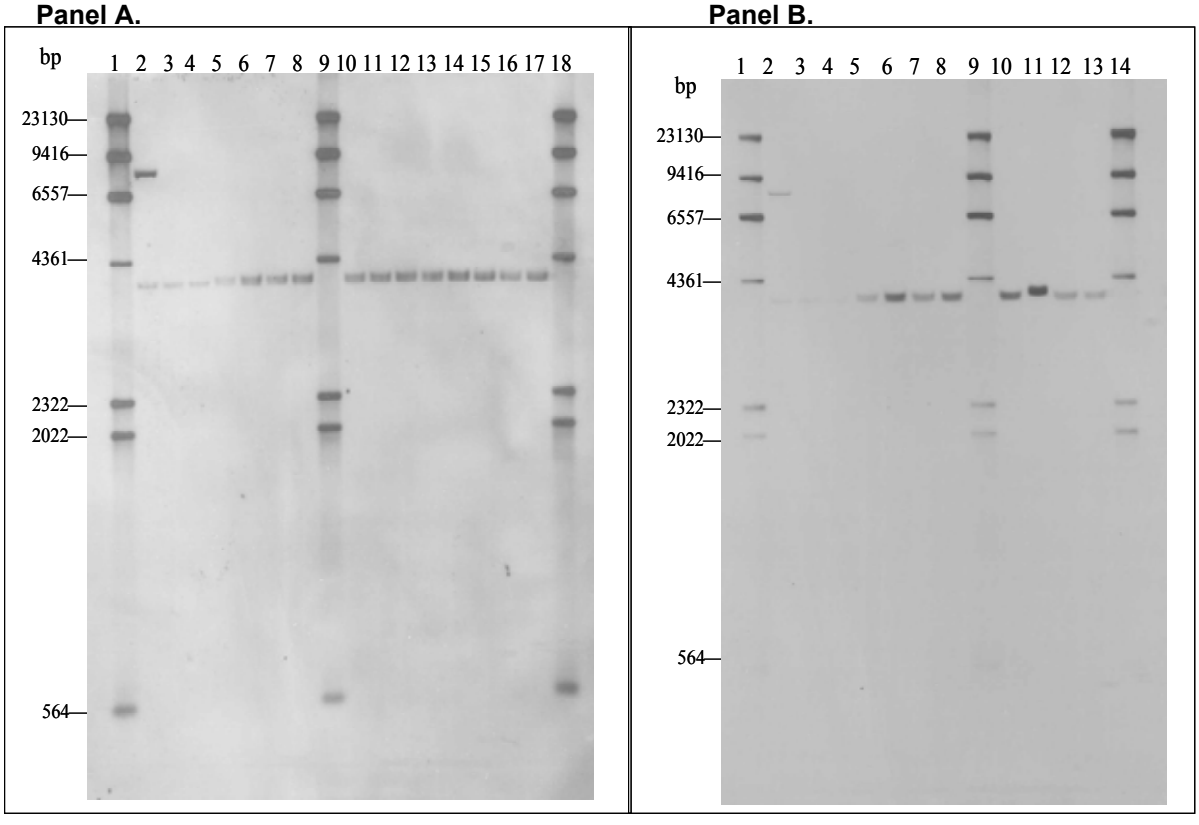


Figure 16: Southern blot analysis of DAS-40278-9; ZmPer terminator probe, Sac I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Sac* I and probed with the ZmPer terminator probe. Nine (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. A non-specific hybridization band of ~9000 bp was detected across all lanes, including positive and negative control lanes that contain XHH13. A hybridization band of ~1900 bp was observed only in lanes containing DAS-40278-9 samples, and not in the XHH13 negative control lanes. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.49-50, Study 081052).

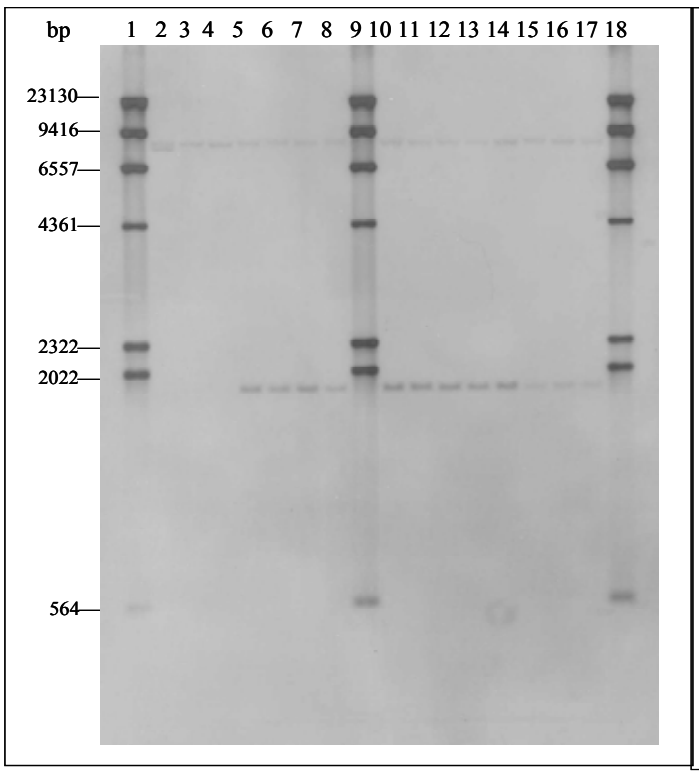
Panel A.

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.

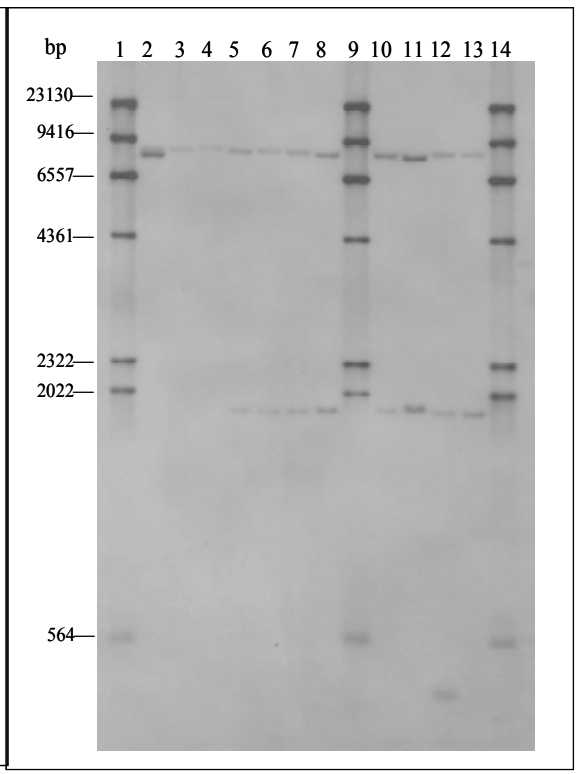


Figure 17: Southern blot analysis of DAS-40278-9; ZmPer terminator probe, Fse I / Hind III digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Fse* I / *Hin*d III and probed with the ZmPer 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. A non-specific hybridization band of ~ 2100 bp was detected across all lanes, including the positive and negative control lanes containing XHH13. A hybridization band of ~3361 bp, which is the predicted size of the *aad*-1 PTU, was detected in the lanes containing DAS-40278-9 samples, as well as the positive control lane with pDAS1740. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.52-53, Study 081052).

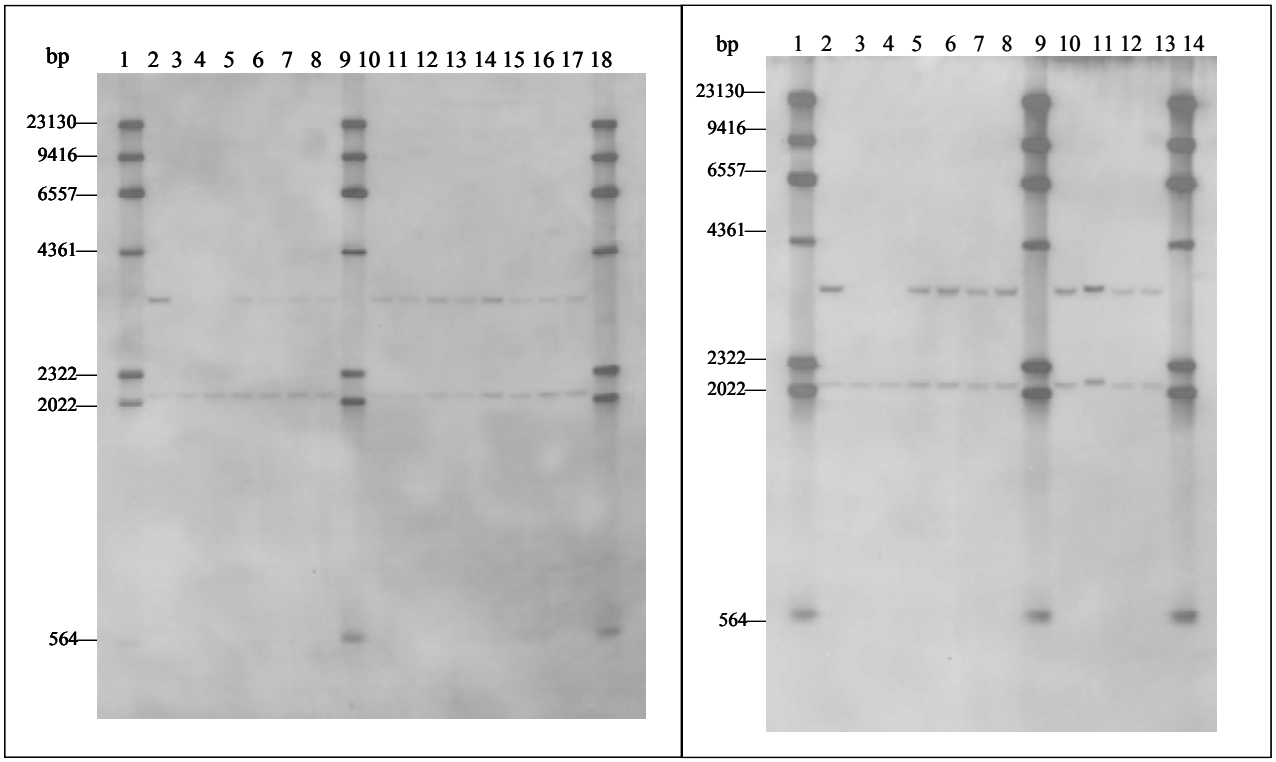
Panel A.

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.**



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 4)**. 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 4)**. 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 4)**. 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 maize 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 (Zhuang, 2009, pp. 23-24, Study 081052).

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 maize 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. Two hybridization bands of ~4000 bp and ~6300 were detected in the lanes containing DAS-40278-9 samples, and not in the XHH13 negative control lanes. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.54-55, Study 081052).

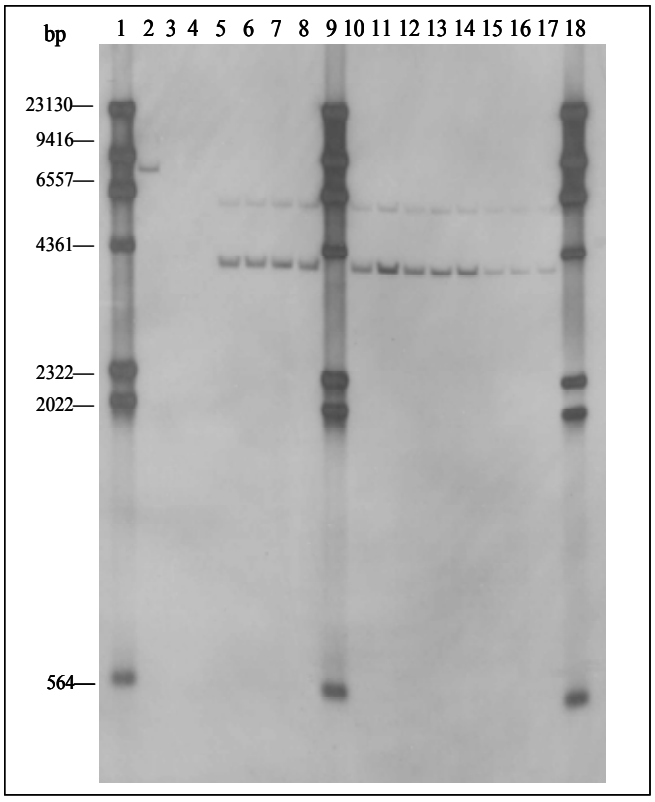
Panel A.

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.

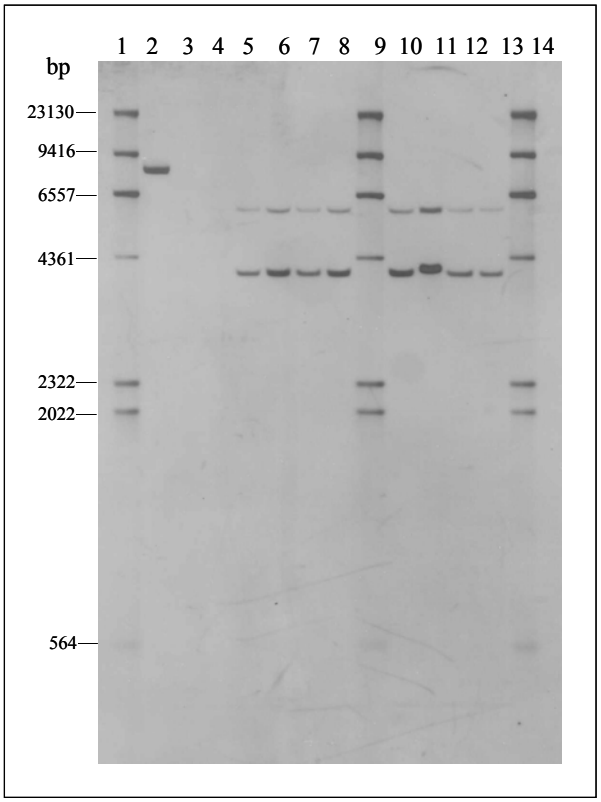


Figure 19: Southern blot analysis of DAS-40278-9; RB7 Mar v4 probe, Sac I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Sac* I and probed with the RB7 Mar v4 probe. Nine (9) μg of 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. Two hybridization bands of ~1900 bp and ~16 000 bp were detected in the lanes containing the DAS-40278-9 samples, and not in the lanes containing the XHH13 negative control. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.56-57, Study 081052).

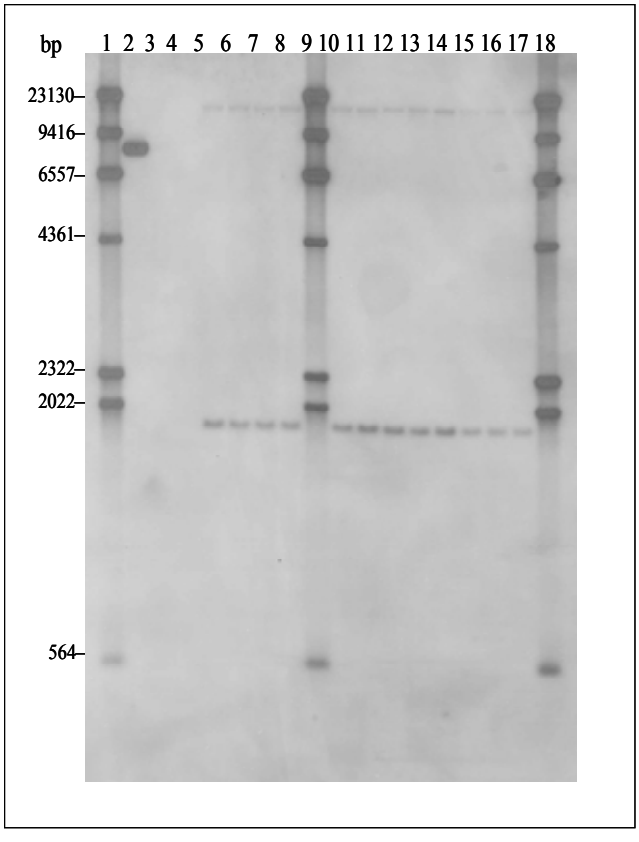
Panel A.

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.

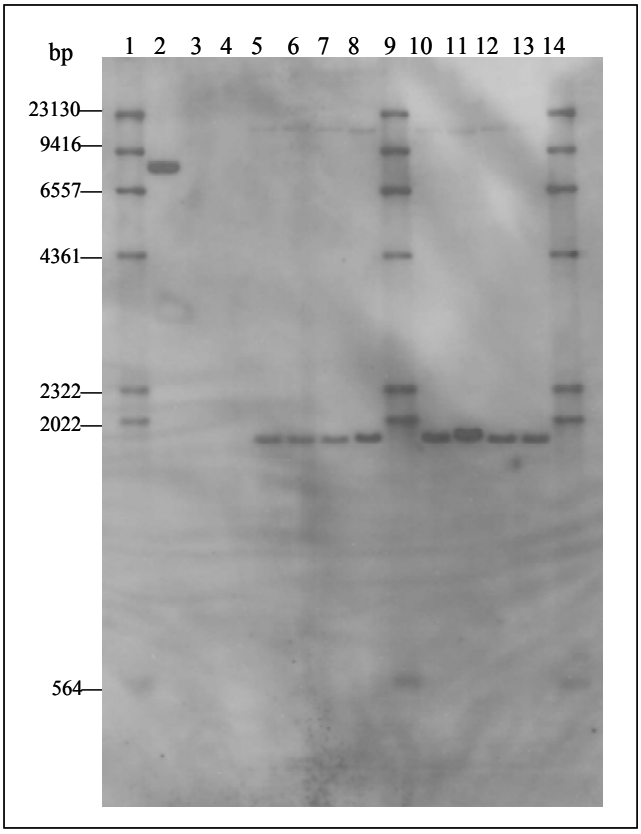


Figure 20: Southern blot analysis of DAS-40278-9; RB7 Mar v3 probe, Nco I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Nco* I and probed with the RB7 Mar v3 probe. Nine (9) μg of 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. Two hybridization bands of ~4000 bp and ~6300 were detected in the lanes containing DAS-40278-9 samples, and not in the XHH13 negative control lanes. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp. 58-59, Study 081052).

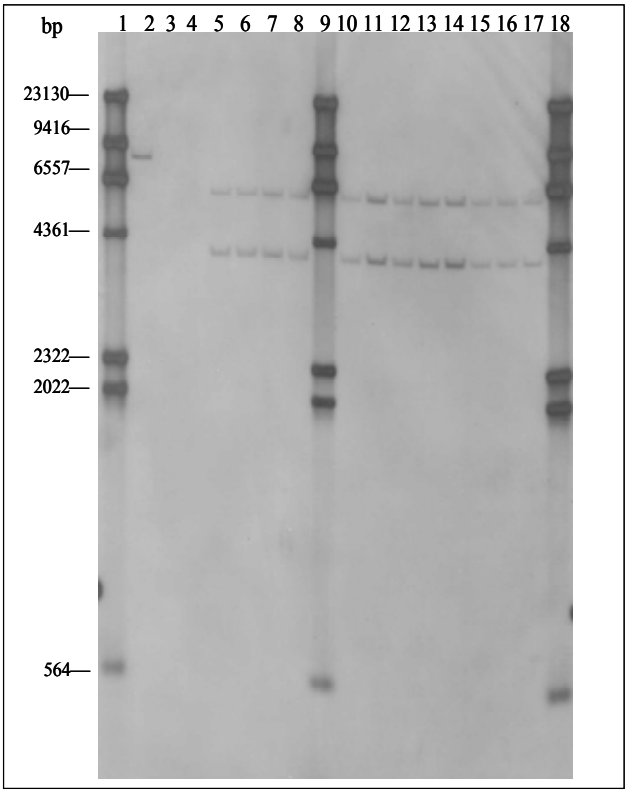
Panel A.

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.

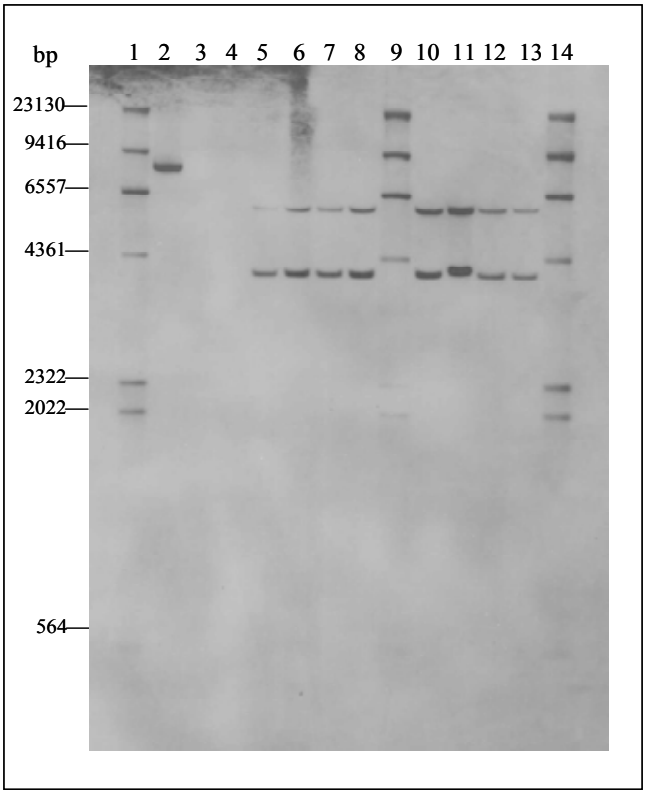


Figure 21: Southern blot analysis of DAS-40278-9; RB7 Mar v3 probe, Sac I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Sac* I and probed with the RB7 Mar v3 probe. Nine (9) μg of 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. Two hybridization bands of ~1900 bp and ~16 000 bp were detected in the lanes containing the DAS-40278-9 samples, and not in the lanes containing the XHH13 negative control. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.60-61, Study 081052).

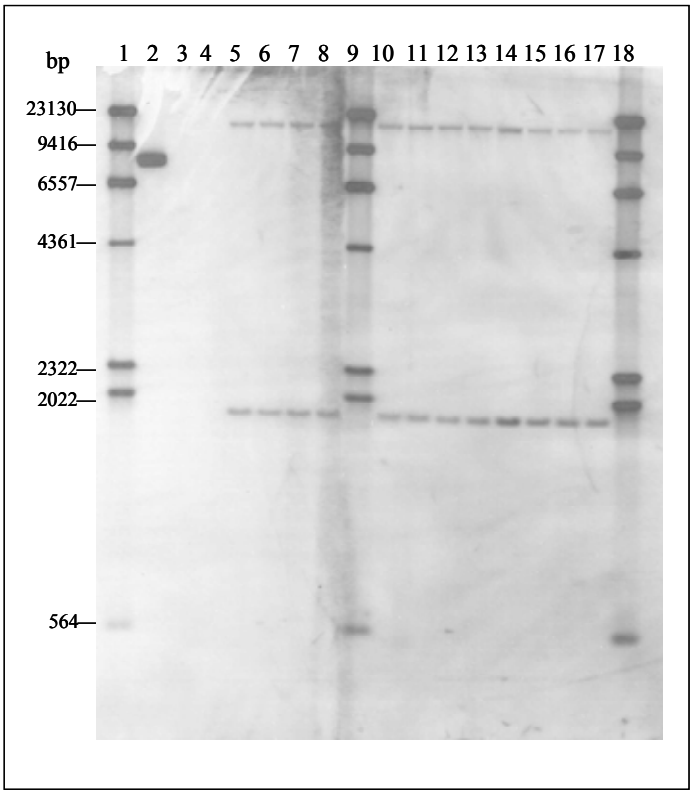
Panel A.

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

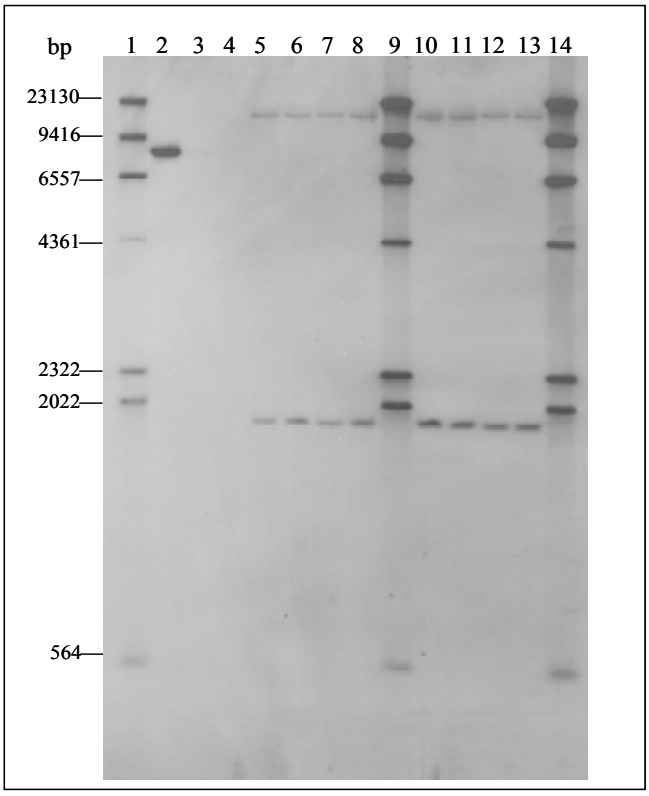
Panel B.

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.



Absence of Vector Backbone Sequences

To assess the absence of vector backbone sequences, equal molar ratio combinations of three DNA fragments **(Table 3)** covering nearly the entire *Fsp* I backbone region (4867-7143 bp in plasmid pDAS1740) 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 4)**. Following digestions with *Nco* I and *Sac* I, no specific hybridization signals were seen in the DAS-40278-9 samples **(Figures 22 and 23)**. The positive control lanes contained the expected hybridizing bands demonstrating that the probes were capable of hybridizing to any homologous DNA fragments if present in the samples. The results indicated that the PTU insertion in event DAS-40278-9 did not include any vector backbone sequences.

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 maize 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. No specific hybridization signals were detected in the lanes containing the DAS-40278-9 sample. A hybridized band was observed in the positive control lane, which contained the pDAS1740 plasmid + XHH13. Sample name indicates test material, generation, and individual plant number used (Zhuang, 2009, pp.62-63, Study 081052).

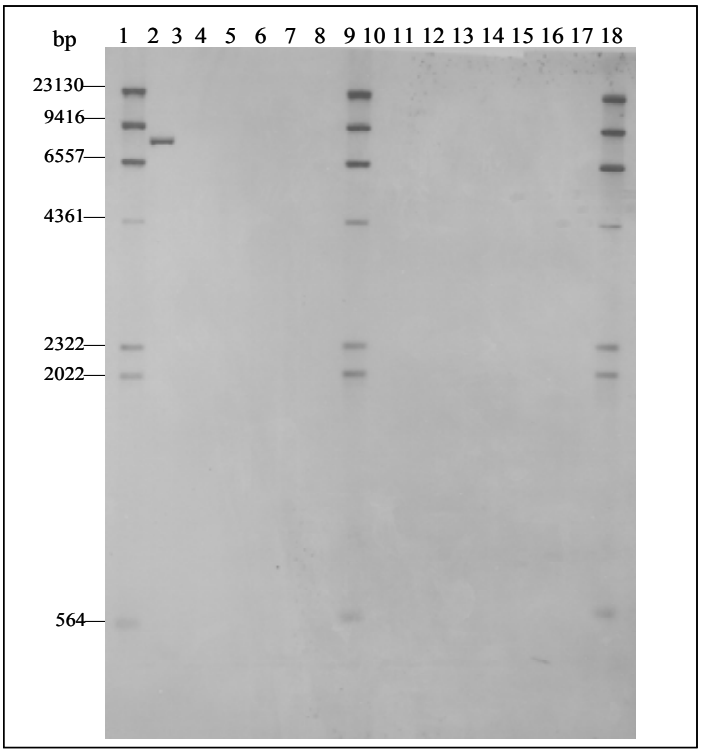
Panel A.

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.

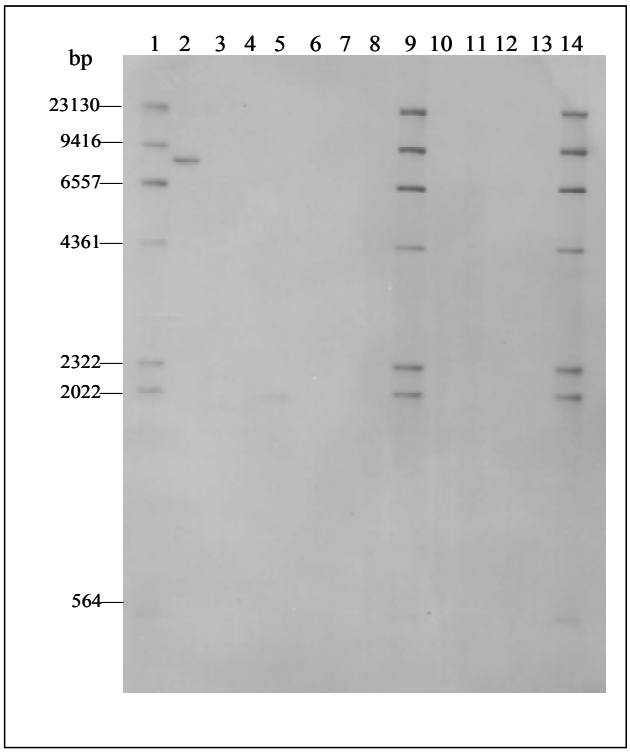


Figure 23: Southern blot analysis of DAS-40278-9; backbone probes, Sac I digest. Genomic DNA isolated from 4 individual plants each of 5 generations of event DAS-40278-9 and 2 individual plants of conventional maize XHH13 was digested with *Sac* I and probed with the backbone probes. Nine (9) μg of DNA 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. No specific hybridization signals were detected in the lanes containing the DAS-40278-9 sample. A hybridized band was observed in the positive control lane, which contained the pDAS1740 plasmid + 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* (Zhuang, 2009, pp.64-65, Study 081052).

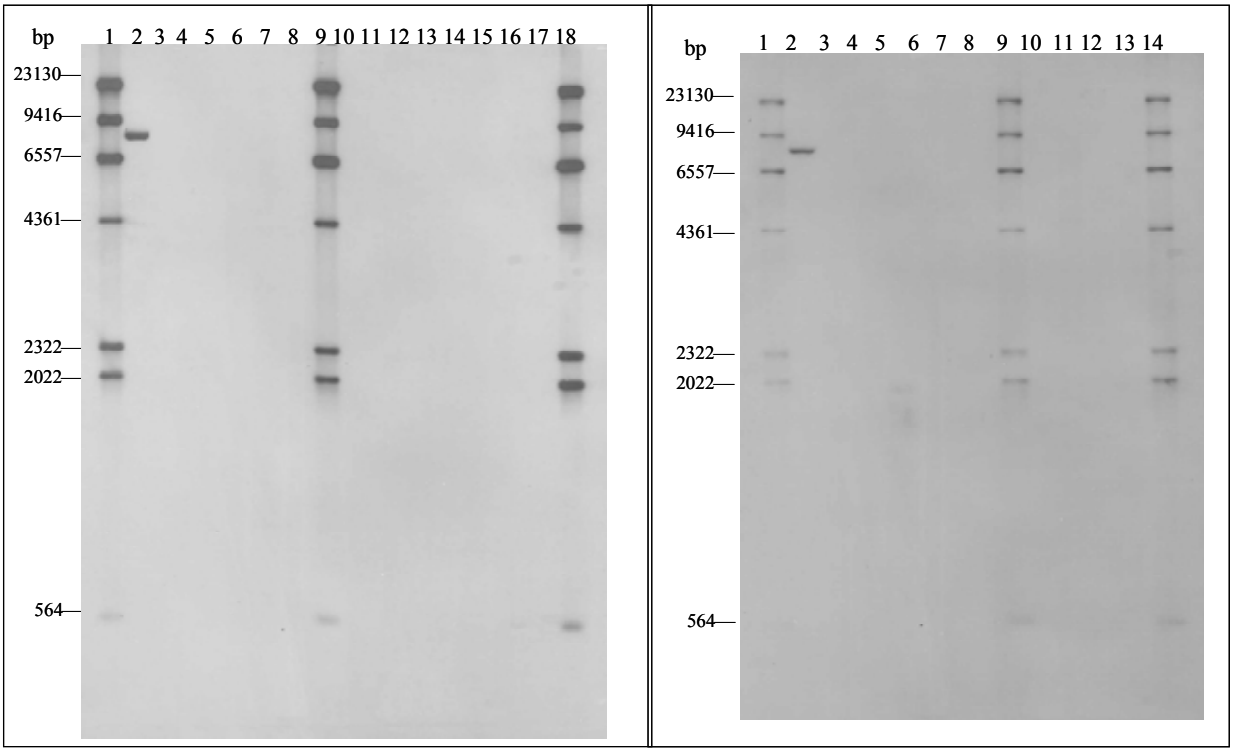
Panel A.

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.**



Summary of the Results for the Analysis of the Insert and Genetic Elements

The Southern blot data presented in the data package suggest that the insert in AAD-1 maize event DAS-40278-9 resulted from a single insertion of one intact copy of the *aad*-1 PTU from plasmid pDAS1740 at one locus in the maize genome. In addition, the results did not indicate ay rearrangements of the *aad*-1 PTU fragment, as all expected internal restriction enzyme sites appeared to be in intact and produced hybridizing fragments of expected size. Furthermore, the absence of vector backbone sequences was confirmed, indicating that only DNA contained within the pDAS1740/*Fsp* I fragment was integrated into maize even DAS-40278-9.

Segregation Analysis of DAS-40278-9 Maize

The stability of inheritance of the gene insert within a segregating generation was demonstrated in study 081120 by Zhuang et al (2009) with Southern analysis and protein detection of individual plants from a BC3S1 line **(Figure 4)** of DAS-40278-9 maize. 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 3)**. Hybridization of the *aad*-1 probe to *Nco* I digeted DNA was expected to yield a hybridization band of >2764 bp. A ~4000 bp hybridization band was observed in the 65 plant samples that had tested positive for AAD-1 protein, from which a representative Southern blot is presented **(Table 5, 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 maize 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 (*p*-value = 0.75) **(Table 6).**

The results from this characterization study indicate that the inheritance of the inserted DNA of event DAS-40278-9 is stable within a segregating generation (BC3S1). All 65 individual plants analysed indicated the insertion is equivalent in all individuals within the generation. The ration of 65 positive to 20 mull segregants in the BC3S1 generation fit the expected segregation ration of 31 based on a single locus. The Southern blot results indicated an intact copy of the *aad*-1 gene has been inserted into the maize genome and the result correlates with the Southern results from the across generation study (Zhuang, 2009, Study 081052).

Table 5: Correlation of the predicted and observed sizes of the hybridizing fragments in BC3S1 Southern blot analysis probed with aad-1
(Zhuang, 2009, p. 21, Study 081120).

DNA Probe	Restriction Enzymes		Figure	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
***aad*-1**	***Nco* I**	**pDAS1740**	**2.24**	**8512**	**8512**
		XHH13	**2.24**	**none**	**none**
		BC3S1*	**2.24**	**>2764 (border)***	**~4000**
		BC3S1**	**2.24**	**None****	**none**

1. Expected fragment sizes are based on the plasmid map of the pDAS1740 as shown in Figure 2.6.
2. Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labelled 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.

Figure 24: Southern blot analysis of DAS-40278-9; aad-1 probe, Nco I digest. Genomic DNA isolated from maize event DAS-40278-9 and conventional maize 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. A hybridization band of ~4000 bp was observed in the lanes of those DAS-40278-9 samples that tested positive for the AAD-1 protein. No bands were observed in the negative control lanes, or in the DAS-40278-9 samples that tested negative for the AAD-1 protein. Data from this southern blot is representative of the analysis performed on the 65 plant samples that tested positive for the AAD-1 protein (Zhuang, 2009, p. 27, Study 081120).

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				

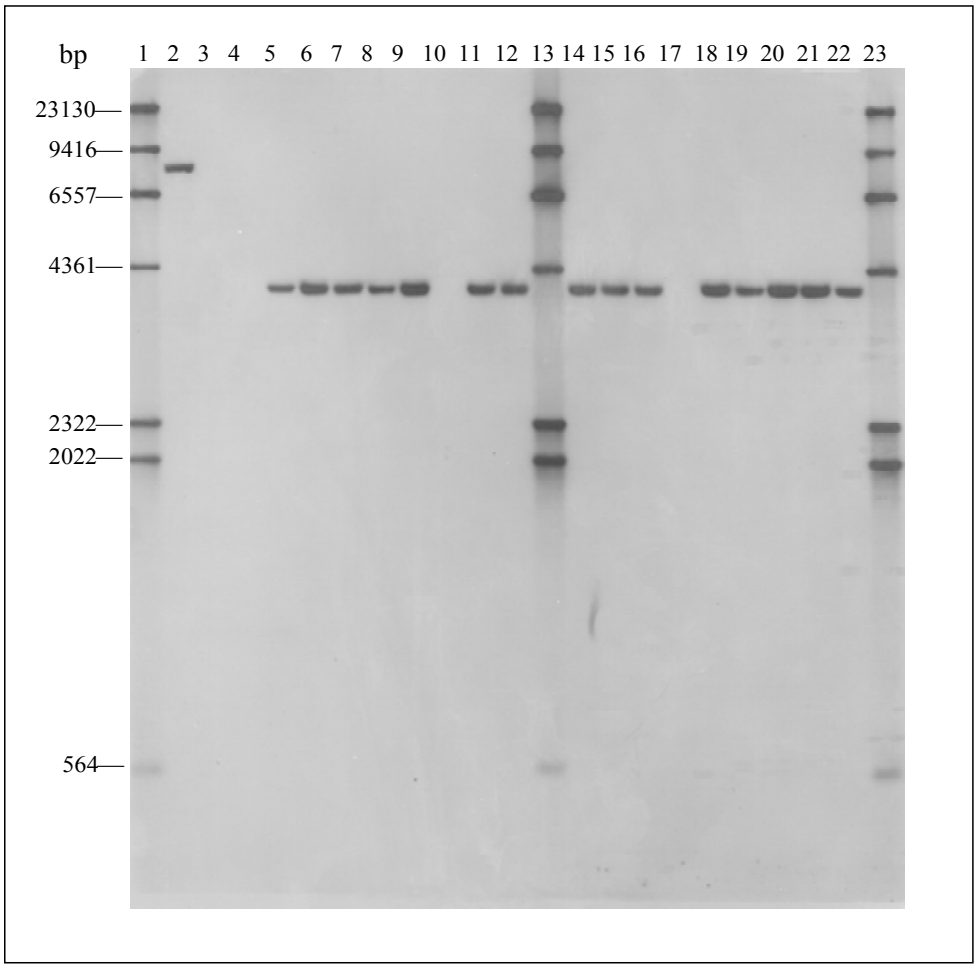


Table 6: Results of BC3S1 individual plant testing for segregation within a generation

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.

Segregation Analysis of Breeding Generations

The segregation ratios of six distinct breeding generations of DAS-40278-9 **(Figure 4)** were recorded and analysed using Chi-square analysis **(Table 7)**. 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 7: 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].

Conclusions

AAD-1 maize 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 maize 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.

Methods for Molecular Characterization of DAS-40278-9 Maize

DAS-40278-9 Maize Material

Transgenic maize seeds from five distinct generations of maize 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.

Control Maize 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.

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.

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.

Sample Collection and DNA Extraction

Leaf samples collected from greenhouse-grown maize 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 maize 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.

DNA Digestion and Electrophoretic Separation of the DNA Fragments

Genomic DNA extracted from the maize 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 maize 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.

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.

Probe Synthesis and Hybridization

The hybridization probes were generated using a PCR-based incorporation of a digoxigenin (DIG) labelled 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.

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

Detection

DIG-labelled 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 labelled 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.

e. Breeding Pedigree

The publicly available maize line, Hi-II, was used as the recipient line for the generation of event DAS-40278-9 maize (Armstrong et al., 1991). Hi-II is a derivative of the A188 and B73 inbred maize 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 maize plants were subsequently crossed with elite proprietary inbred maize lines to derive maize hybrids containing DAS-40278-9.

Figure 4 shows the breeding process and the generations used for molecular characterisation, within and between generation stability studies and commercial lead varieties.

f. Genetic Stability

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 maize. Identical fragment sizes were observed with all enzyme and probe combinations for four plants each from five distinct generations, indicating stability of inheritance and that the integration site is the same across all generations. As described above in sections titled Summary of Molecular Analysis and Analysis of the Insert and its Genetic Elements, results across all DAS-40278-9 samples were as expected, with intact internal restriction enzyme sites that produced hybridizing fragments that correlated with the predicted fragment size

(Table 4 and Figures 8-23), indicating stable inheritance of the intact, non-rearranged single copy insert across multiple generations of DAS-40278-9 maize.

4. Labelling of the GM Food

a. Novel Protein Presence in Final Food

Please refer to Section C, Part 2, C of this dossier for an analysis of the levels of AAD-1 protein measured in DAS-40278-9. Please specifically refer to the grain and forage results found in **Table 8** for data pertaining to maize food products.

b. Detection methodology

Please refer to Attachment 2 – Confidential Commercial Information.

C. SAFETY OF THE GENETICALLY MODIFIED FOOD

1. Antibiotic Resistance Marker Genes

a. Clinical Relevance

Information on the clinical and veterinary importance, if any, in Australia and New Zealand of the antibiotic to which any transferred antibiotic resistance gene confer resistance.

Not applicable

b. Therapeutic efficacy

Information on whether the presence in food of the enzyme or protein encoded by the antibiotic resistance marker gene would compromise the therapeutic efficacy of the orally administered antibiotic.

Not applicable.

c. Safety of the Gene Product

Please refer to Part A, Section 2 of this dossier.

d. End Use Viability (micro-organisms)

If the new GM organism is a micro-organism, information on whether it will remain viable in the final food.

Not applicable

2. Characterisation of the Novel Proteins

a. Biochemical function and phenotypic effect of novel proteins

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. AAD-1 is comprised of 296 amino acids and has a molecular weight of 33 kDa **(Figure 25).**

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

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

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 performed in study 080142 by Schafer (2009) to confirm the equivalency of the AAD-1 protein expressed *in planta* in maize line DAS-40278-9 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 maize 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 below and in the study by Schafer (2009) 080142 . 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 maize-derived AAD-1 protein. The 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).

Methods and Results for the Characterisation of the AAD1-Protein

DAS-40278-9 Maize 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.

Control Maize Material

Control maize line XHH13 had a genetic background representative of the DAS-40278-9 maize 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 maize.

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.

Protein Purification from DAS-40278-9 Maize 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.

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.

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.

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.

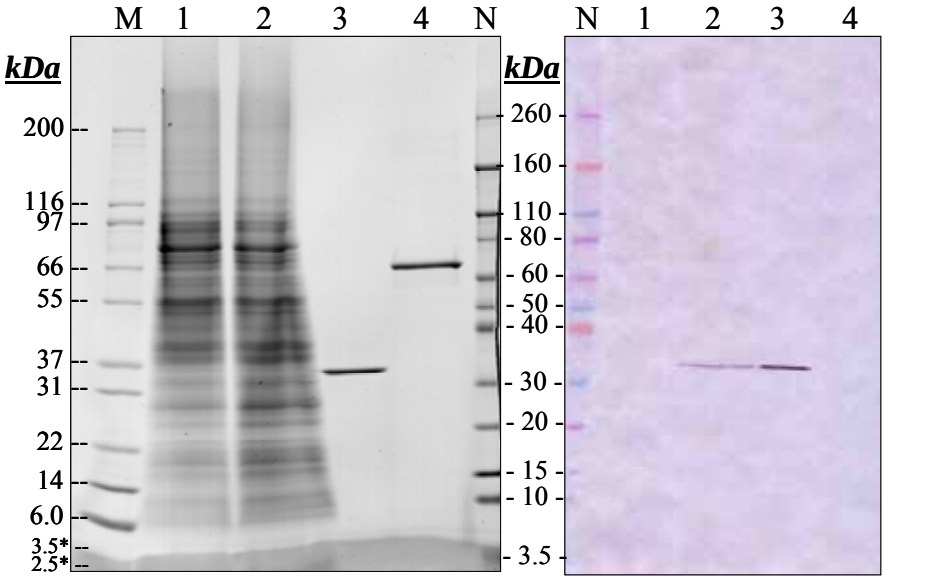
Results of the SDS-PAGE and Western Blot Analysis

In the microbe-derived AAD-1, the major protein band, as visualized on the Coomassie stained SDS-PAGE gel, was approximately 33 kDa (Figure B2.1). As expected, the corresponding plant-derived AAD-1 protein was identical in size to the microbe-derived protein. Predictably, the plant purified fractions contained a minor amount of non-immunoreactive impurities in addition to the AAD-1 protein. The co-purified proteins were likely retained on the column by weak interactions with the column matrix.

The microbe-derived AAD-1 and DAS-40278-9 plant tissue extract showed a positive signal of the expected size on the western blot using the anti-AAD-1 polyclonal antibody **(Figure 26)**. In the AAD-1 western blot analysis, no immunoreactive proteins were observed in the control XHH13 extract and no alternate size proteins (aggregates or degradation products) were seen in the samples from the transgenic plant.

Figure 26: 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.

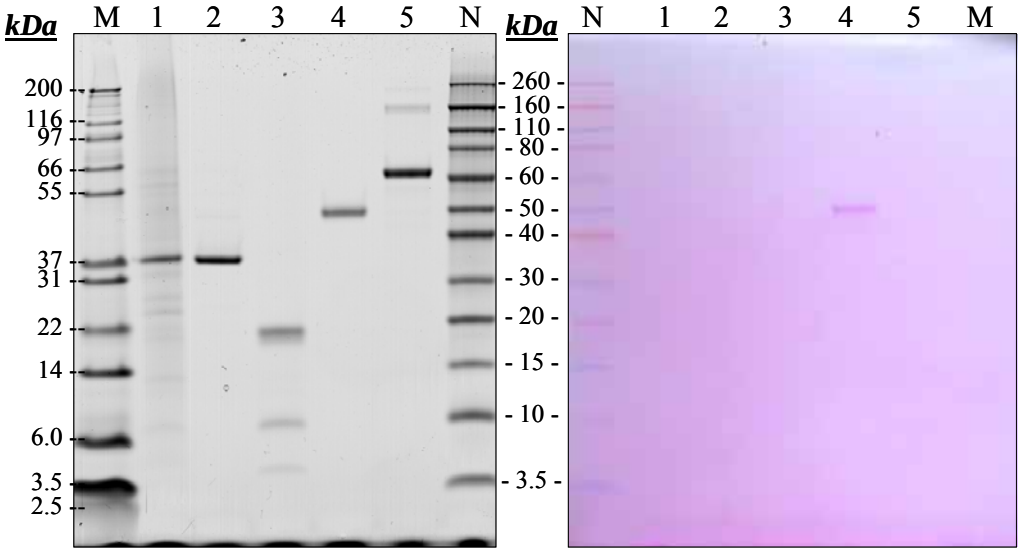


Panel A: SDS-PAGE gel Panel B: Western blot membrane

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

Results of Detection of Glycosylation

Figure 27: Glycosylation analysis of plant- and microbe-derived AAD-1 protein
No covalently-linked carbohydrates were detectable on the plant-derived or the microbe-derived AAD-1 proteins **(Figure 27)**. 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.
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

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 28** 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 28: 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
```

Results of Tryptic Peptide Fragment Sequencing

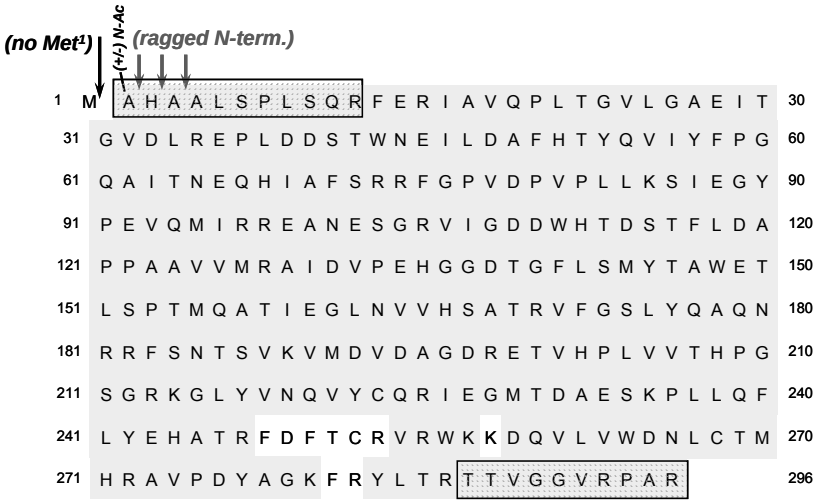
The sequences of the first 11 residues of the plant- and microbe-derived AAD-1 proteins were obtained by tandem mass spectrometry. The amino acid sequences for both proteins were A^{1} H A A L S P L S Q R^{11}, indicating the N-terminal methionine had been removed **(Table 9)**. These results suggest 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 9 and Figure 28)**. 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 10 and Figure 29)**.

Figure 29: 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

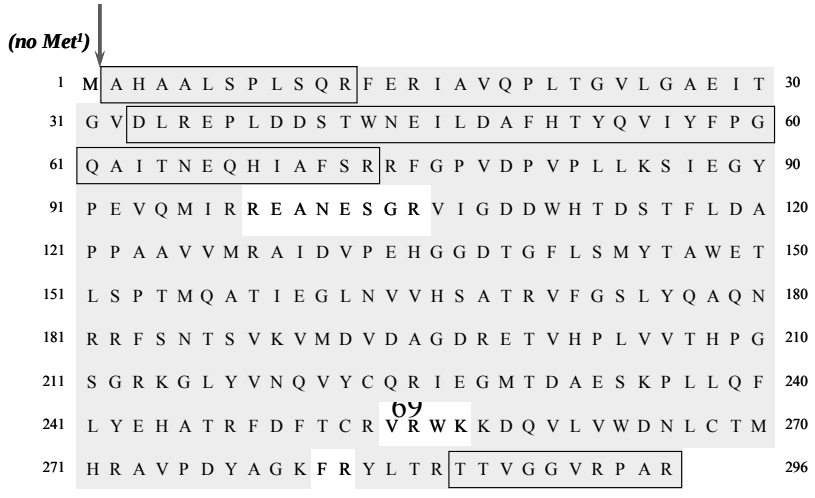


Table 9: Summary of N-terminal sequence data of plant- and microbe-derived AAD-1 proteins

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 10: 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

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) (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 catalyses the degradation reaction of the general class of herbicides known as aryloxyphenoxypropionates (AOPPs), such as quizalofop, to their corresponding inactive phenols (Wright et al., 2009).

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 maize are biochemically equivalent molecules.

b) Identification of Other Novel Substances

DCP

2,4-dichlorophenol (DCP) is a known primary degradate of 2,4-D in plants (Roberts, 1998). DCP has been observed as a degradate of 2,4-D in environmental matrices and is also observed in animal metabolism studies (Roberts, 1998; Barnekow *et al.,* 2001). The US tolerance expression for 2,4-D does not include DCP in the plant residue definition, however DCP was at one point included in the livestock meat and milk tolerance expression. In 2004 the US EPA's Health Effects Division (HED) Metabolism Assessment Review Committee (MARC) recommended that DCP be deleted from the livestock tolerance expression for 2,4-D. The MARC committee stated DCP is "not of concern for either the tolerance expression or for risk assessment at the levels expected in livestock tissues and considering the likely lower toxicity of 2,4-DCP compared to 2,4-D" (US EPA, 2003). This decision was included in the 2005 Registration Eligibility Decision (RED) document (US EPA, 2005) and posted in the 2007 Federal Register (US EPA, 2007). This action harmonizes US tolerances with CODEX, Japanese and European residue definitions which do not include DCP in any tolerance expression.

Quizalofop Phenol

Similar to the metabolism of 2,4-D to DCP, the formation of quizalofop phenol has been observed in soybean and cotton plants treated with quizalofop (Koeppe *et al*., 1990). Per the Federal Register (1997), Dupont provided the following information regarding the plant metabolism of quizalofop: “Quizalofop-p ethyl ester is metabolized by cleavage at three sites as follows: (i) Primary pathway is hydrolysis of the ethyl ester to form the quizalofop-p acid, then (ii) Cleavage of the enol ether linkage in the acid, between the phenyl and quinoxalinyl rings, to form phenols, and (iii) Cleavage of the ether linkage between the isopropanic group and the phenyl ring to form a phenol. The plant metabolism data show that quizalofop-p ethyl ester does not translocate, but is rapidly hydrolyzed to the corresponding acid; then the phenols conjugate with the plant sugars. Metabolism studies in soybeans using the racemic mixture quizalofop ethyl ester and the resolved D+ isomer show nearly identical pathways. The nature of the quizalofop-p ethyl ester residue in plants is adequately understood. The residues of concern are quizalofop-p ethyl ester and its acid metabolite, quizalofop-p, and the S enantiomers of both the ester and the acid, all expressed as quizalofop-p ethyl ester”. (For clarity, D+ and R isomers of quizalofop ethyl ester are the same, and similarly L- and S isomers of quizalofop ethyl ester are also the same.)

A review of the most recent EPA HED risk assessment document (US EPA, 2006) for quizalofop reiterated that: “HED has determined that the residues of concern in plant commodities are quizalofop-P ethyl, its acid metabolite quizalofop-P-, and the S-enantiomers of both compounds, each expressed as quizalofop-P ethyl”. Because EPA has not specifically included a phenol metabolite in the tolerance, it is evident that the EPA was not concerned about the potential exposure or toxicity of a phenol metabolite.

Similarly in the EU, the European Food Safety Authority (EFSA) (EFSA, 2008) recently published a section on quizalofop metabolism and resulting residue definition: “the metabolism proceeds primarily by the hydrolysis of the ester link to yield quizalofop (acid) followed by the loss of the propionyl moiety leading to the quizalofop-phenol metabolite. Further metabolism occurs by hydroxylation of the quinoxaline moiety giving the hydroxy-quizalofop, hydroxy-quizalofop-phenol and the dihydroxy-quizalofop-phenol. Pending further review, EFSA has defined the residue expression as: “Considering the metabolism studies performed with the three quizalofop ester variants, a common residue definition for monitoring and risk assessment was also proposed for propaquizafop, quizalofop-P-ethyl and quizalofop-P-tefuryl as: “Sum of quizalofop-esters, quizalofop and quizalofop conjugates expressed as quizalofop (sum of isomers)”.

c) Novel Protein Expression

Expression of AAD-1 Protein in Plant Tissues

A field expression study was conducted at locations in the U.S. and Canada in 2008 (Phillips, 2009, Study 090084). Six sites (Iowa, Illinois (2 sites), Indiana, Nebraska and Ontario, Canada) were planted with hybrid maize line DAS-40278-9 and a near-isoline control **(Figure 4).** The test sites represented regions of diverse agronomic practices and environmental conditions for maize, and the US locations are representative of the conditions present in Canada. Appropriate insect, weed, and disease control practices were applied to produce an agronomical acceptable crop. Four treatments of the DAS-40278-9 maize (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.* (1993) in **Table 11**). The soluble, extractable AAD-1 protein was measured using a quantitative enzyme-linked immunosorbent assay (ELISA) method.

A summary of the AAD-1 protein concentrations (averaged across sites) in the various maize 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. 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 which was likely due to a sampling error.

Table 11: Vegetative and reproductive stages of a maize plant.

From Ritchie *et al*.,1993

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 maize plant. However, all plants in a given field will not be in the same stage at the same time. When staging a field of maize, 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.

Table 8: Levels of AAD-1 protein measured in DAS-40278-9 Maize across locations

Maize 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

Methods for AAD-1 Expression Analysis

Experimental Design

The experimental design included six (6) field sites; Richland, IA; Carlyle, IL; Wyoming, IL; Rockville, IN; York, NE (Phillips, 2009, Study 080137); and Branchton, Ontario, Canada (Phillips, 2009, Study 080139) (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 maize plot was bordered by 2 rows of a non-transgenic maize hybrid of similar maturity. The entire trial site was surrounded by a minimum of 12 rows (or 30 ft) of a non-transgenic maize 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.

Sample Collection

Samples were collected of the leaves, pollen, root, forage, grain and the whole plant. Samples were shipped to Dow AgroSciences RSGA laboratories and maintained frozen until use. Samples of maize tissues were prepared for expression analysis by coarse grinding, lyophilizing and/or fine-grinding with a Geno/Grinder (Certiprep, Metuchen, New Jersey). No additional preparation was required for pollen.

Determination of AAD-1 Protein Concentration

Samples were analysed 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 maize 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 analysed 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 coloured product. Since the AAD-1 was bound in the antibody sandwich, the level of colour development was related to the concentration of AAD-1 in the sample (i.e., lower residue concentrations result in lower colour 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 analysed in duplicate wells with the average concentration of the duplicate wells being reported.

d) Post-Translational Modification in the New Host

The results of trypic peptide fragment sequencing (Part 2 a) 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 9 and Figure 28)**. 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 10 and Figure 29)**.

e) Novel Protein Silencing

None of the genes transferred to the maize lines have been silenced through mechanisms such as gene co-suppression.

f) Novel Protein History of Consumption

The bacterial strain that AAD-1 was derived from is *Sphingobium herbicidovorans* MH. This organism is a strictly aerobic, gram-negative α proteobacterium. The MH strain was initially isolated in 1990 as a *Flavobacterium* sp from 2,4-DP enriched soil (Horvath *et al*., 1990). The strain was reclassified as *Sphingomonas herbicidovorans* MH upon further characterization of its herbicide degrading capabilities (Zipper *et al*., 1996). The species *S. herbicidovorans* has, to date, only been isolated from soil.

S. herbicidovorans is a candidate for use in bioremediation of toxic pollutants based on the complement of xenobiotic degrading enzymes it possesses. Investigations of this organism have focused on the characterization of the herbicide metabolizing enzymes; AAD-1 facilitates the breakdown of phenoxy auxin and AOPP herbicides into carbon sources for the bacterium (Wright *et al.*, 2009).

There are no reports of *S. herbicidovorans* being implicated as a human pathogen or producing any allergens. Out of the ~20 recognized species of *Sphingobium*, only one, *S. yanoikuyae* has been isolated from a clinical environment. Other related genera however, are known to cause infrequent infections which are generally limited in virulence (Balkwill *et al*., 2006). Because of their ubiquity and adaptability, sphingomonads are often found in clinical settings, but usually not associated with infection. There are reports of sphingomonads producing antigenic glycolipids that may have use as therapeutics (Kinjo *et al*., 2008). Other related sphingomonads are known to produce sphingans (an extracellular gellan-like polysaccharide) which can be used in food as gelling agents, stabilizers, or suspending agents (van Kranenburg *et al*., 1999).

3. Potential Toxicity of the Novel Protein

a) Amino Acid Sequence Comparison to Known Toxins

The AAD-1 protein does not share meaningful amino acid sequence similarities with known toxins (Herman, 2007, Study 071022). 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.

b) Acute Oral Toxicity

An acute oral toxicity study with microbe-derived AAD-1 protein was conducted in mice at a level of 2000 mg AAD-1/kg after adjustment for purity (Wiescinski and Golden, 2007, Study 071128). All animals survived and no clinical signs were observed during the study. All animals gained weight by study termination on day 15. There were no treatment-related gross pathological observations. The report concludes that under the conditions of this study, the acute oral LD_{50} of AAD-1 in male and female mice was greater than 2000 mg/kg. The NOEL is >2000 mg/kg based on the fact that no mortality was observed and there were no observable effects (adverse or non-adverse effects) with the AAD-1 treated animals. In the US, based on this LD_{50} value, EPA would classify this substance as a category III for acute oral toxicity, indicating only slight toxicity has been observed. AAD-1 protein displays very low acute toxicity potential.

4. Potential Allergenicity of Novel Proteins

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. Studies were conducted to ascertain the potential allergenicity of the AAD-1 protein. These studies included: 1) bioinformatic search for amino-acid sequence homology with known allergens; 2) digestive fate in simulated gastric fluid (SGF), and 3) heat lability of the proteins.

The conclusion from that assessment is that the AAD-1 protein is unlikely to cause allergic or toxic reactions in humans or animals.

a) Source of Introduced Protein

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).

b) Amino Acid Sequence Comparison to Known Allergens

The AAD-1 protein had no meaningful homology to known allergens using a sequence evaluation program based on that formulated by the joint FAO/WHO Expert Consultation (2001) and by the Codex Alimentarius (Codex *Ad Hoc* Open-ended Working group on Allergenicity, 2001). This search looks for a match of at least eight contiguous amino acids or greater than 35% identity over 80-amino-acid stretches (sliding window) and no such matches were found (FARRP version 7.00) (Herman, 2007, Study 071029).

c) Structural Properties

Please refer to Section C, Part 3, B for information relating to the heat lability of the AAD-1 protein.

d) Serum Screening

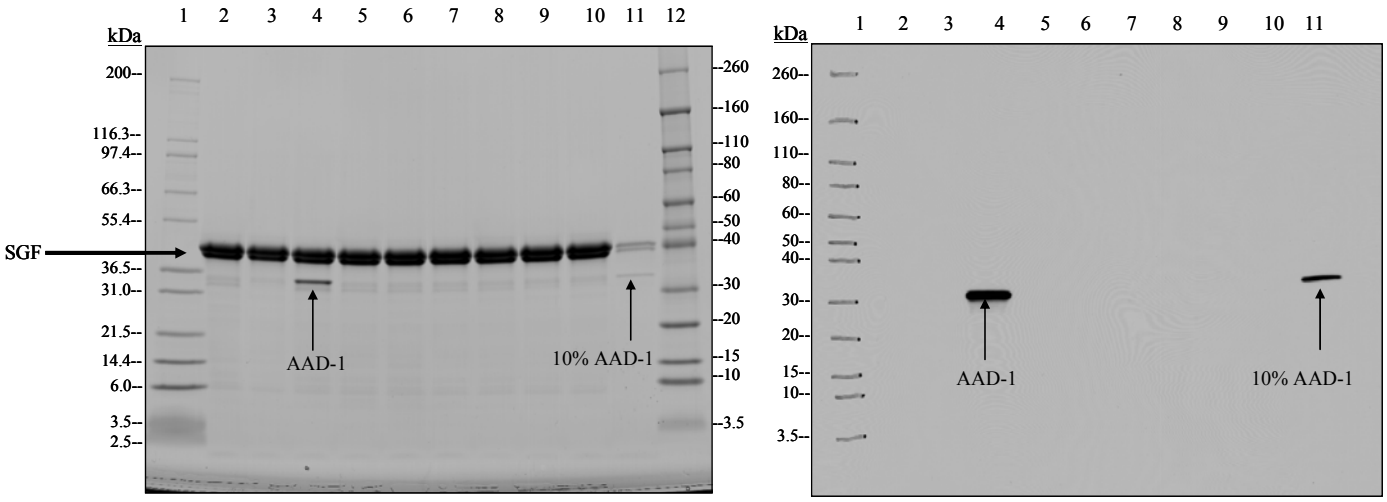
Not applicable.

e) Simulated Gastric Fluid and Heat Lability

Simulated Gastric Fluid

The digestibility of the AAD-1 protein was tested *in vitro* using a SGF (Embrey and Korjagin, 2008, Study 080062). For the SGF method, the microbially-produced (in *P. fluorescens*) AAD-1 protein was incubated in SGF (0.32% w/v pepsin at pH 1.2; U.S. Pharmacopeia) at a ratio of enzyme to protein equal to 1.5 mg pepsin to 1 nM substance solution (AAD-1: 1 nM equals 33 ug). At each scheduled time point (1, 2, 3, 5, 7, 10 and 15 minutes) 0.1 mL of the reaction mixture was removed and placed into a microcentrifuge tube containing 0.04 mL stop solution (200mM Na_2CO_3, pH 11.0). For the zero time point samples, 0.19 mL SGF solution was neutralized with 0.08 mL stop solution and then the protein sample was added for each protein (BSA = positive control, beta-Lactoglobulin = negative control and AAD-1). All samples were kept on ice after the stop solution was added. After all digestion time points were completed, the samples were mixed with Laemmli sample buffer and heated at 95^{o} C for 5 min. The samples were then analysed via SDS-PAGE and western blot analysis using an antibody specific to AAD-1. The results demonstrated that the AAD-1 protein was readily digested (not detectable at 1 minute) in SGF **(Figure 30)**.

Figure 30: SDS-PAGE (A) and Western blot (B) Analyses of the Simulated Gastric Fluid Digestion (SGF) of AAD-1
(Embrey and Korjagin, 2008, pp.20-21, Study 080062).
A: SDS-PAGE Gel B: Western blot



Lane	Sample	Amount Loaded for SDS-PAGE	Amount Loaded for Western Blot
1	Invitrogen Mark 12 MW markers	10µL	10µL
2	SGF Reagent Blank, 0 minute incubation	20 µL	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL	20 µL
4	Neutralized AAD-1 digestion	~0.879 µg	~0.088 µg
5	30-second AAD-1 digestion	~0.879 µg	~0.088 µg
6	1-minute AAD-1 digestion	~0.879 µg	~0.088 µg
7	2-minute AAD-1 digestion	~0.879 µg	~0.088 µg
8	4-minute AAD-1 digestion	~0.879 µg	~0.088 µg
9	8-minute AAD-1 digestion	~0.879 µg	~0.088 µg
10	16-minute AAD-1 digestion	~0.879 µg	~0.088 µg
11	10% Neutalized AAD-1 digestion	~0.088 µg	~0.0088 µg
12	Invitrogen Novex Sharp Prestained MW Markers	10 µL	10µL

Heat Lability

The thermal stability of the AAD-1 protein was evaluated by heating microbe-derived AAD-1 protein solutions for 30 min at 50, 70 and 95 °C and 20 min in an autoclave (120 °C @ ~117 kPa (~17 PSI)) in a phosphate based buffer (Schafer, 2008, Study 080059). The AAD-1 protein activity was measured by a modified enzyme assay based on the procedure described in Fukumori and Hausinger (1993). In the presence of Fe(II), the AAD-1 protein catalyses the conversion of dichlorphenoxyacetate to 2,4-dichlorophenol and glyoxylate concomitant with the decomposition of □-ketoglutarate to form succinate and carbon dioxide. The resulting phenol is measured with an AAPPC assay or the Emerson reaction (Emerson, 1943). Phenols react with 4-aminoantipyrine in the presence of alkaline oxidizing agents (potassium ferricyanide) at a pH of 10.0 to form a stable reddish-brown antipyrine dye (AAPPC). The amount of colour produced is a function of the concentration of phenols and was measured with a microplate reader (Molecular Devices, Model #: SPECTRAmax 190 ROM v3.13) at 510 nm. All heating conditions virtually eliminated the enzymatic activity of the AAD-1 protein.

Summary of Allergenicity Potential of AAD-1 Protein

Based on the lack of significant amino-acid-sequence homology to known allergens, and the lack of enzymatic and heat stability, the AAD-1 protein is considered to have a low risk of allergenic potential.

Conclusions

The arylalkanoate 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 analysing 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 (Codex, 2003). Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the AAD-1 amino acid sequence. The AAD-1 protein hydrolyses 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 maize 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.

5. Compositional Analysis

a) Grain and Forage Composition

Compositional analysis was performed on maize forage and grain to investigate the equivalency between DAS-40278-9 maize (unsprayed, sprayed with 2,4-D, sprayed with quizalofop, and sprayed with 2,4-D and quizalofop) and the near-isoline control maize. Trials were conducted at six test sites located within the major maize-producing regions of the U.S and Canada using hybrid maize 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.

The experimental design for composition analysis was the same as that used for protein expression analysis, with six (6) field sites including 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 maize plot was bordered by 2 rows of a non-transgenic maize hybrid of similar maturity. The entire trial site was surrounded by a minimum of 12 rows (or 30 ft) of a non-transgenic maize 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.

Forage and grain samples were collected for compositional analysis from test and control plots. One forage sample consisting of the aerial portion (no roots) of 3 whole plants was collected from each test and control entry in blocks 2, 3, and 4. Plants used for sampling contained self-pollinated ears. Grain samples were collected at typical harvest maturity from plants that were previously self-pollinated. One individual sample was collected from each test and control entry in each of blocks 2, 3 and 4. Each sample contained five (5) ears. All samples were maintained frozen at approximately -20 °C until analysis.

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

The results of the nutritional analysis for maize forage and grain were compared with values reported in literature. A summarization of the compositional data used for comparison can be found in **Tables 12-19**. 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).

Compositional Analyses of Maize Forage

An analysis of the protein, fat, ash, moisture, carbohydrate, ADF, NDF, calcium and phosphorus in maize 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 20.** For the across-site and individual-site analysis, all proximate, fibre and mineral mean values were within literature ranges **(Figure 31)**. 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

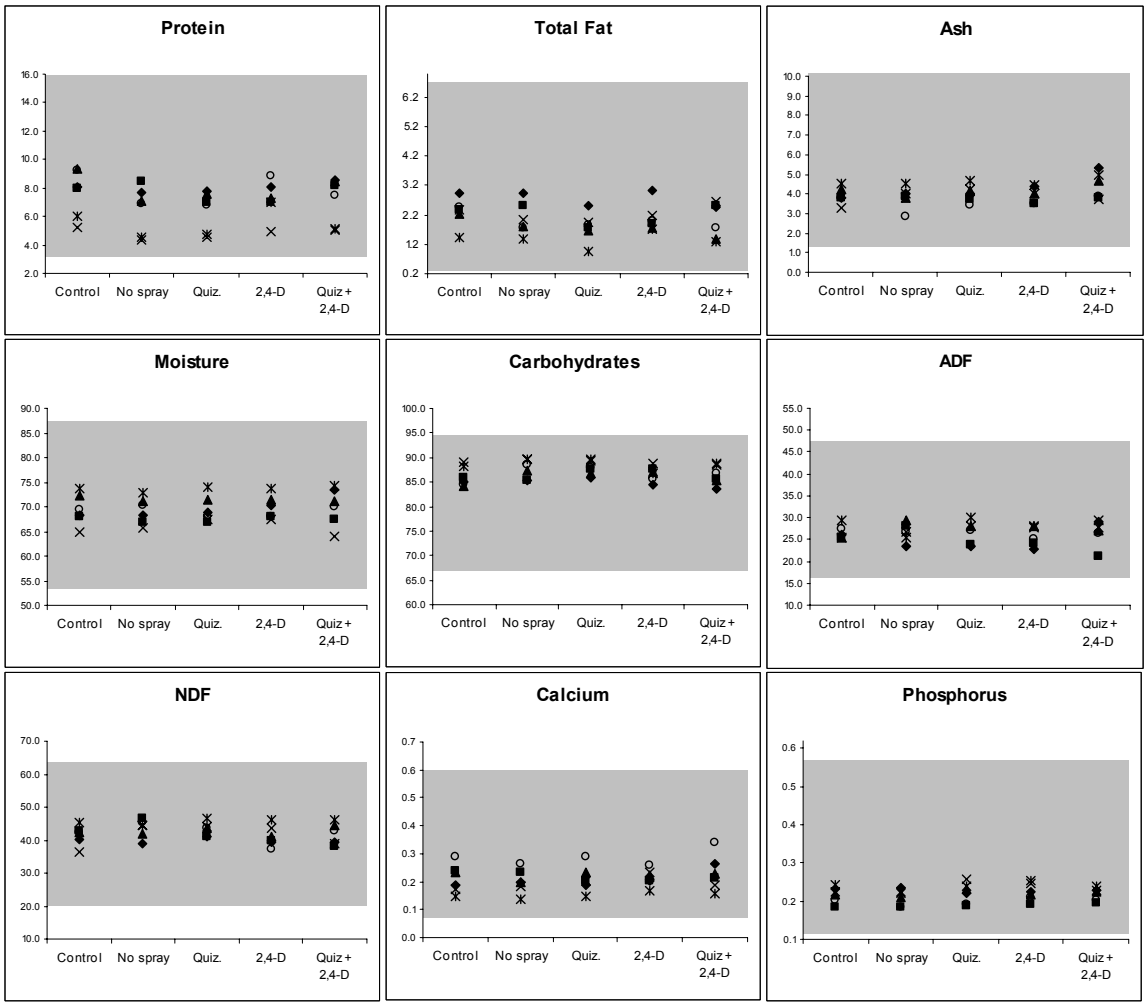
unsprayed AAD-1 entry was observed, but without a significant FDR adjusted p-value. These differences are not biologically meaningful since all across-site results for these analytes were within the reported literature ranges for maize, and differences from the control were <23 %.

Table 20: Summary of the proximate, fiber and mineral analysis of maize forage
(Phillips, 2009, p.30, Study 090084)

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)
Fibre (% dry weight)							
Acid Detergent Fibre (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 Fibre (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 D.
[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 31: Proximate, fiber, and mineral analysis of maize forage
(Phillips, 2009, p.44, Study 090084**)**



Compositional Analyses of Maize Grain

Proximate and Fibre Analysis of Grain

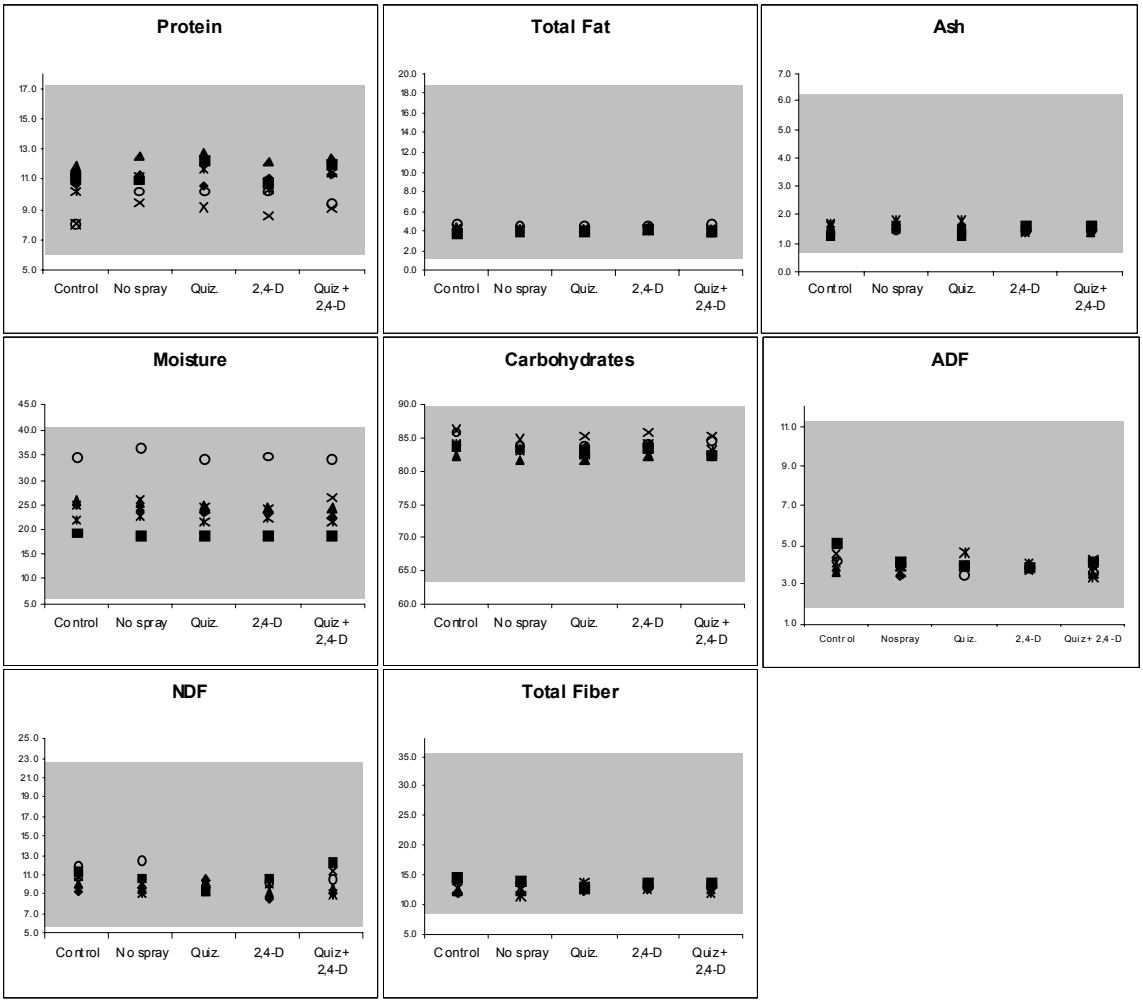
A summary of the results for proximates (protein, fat, ash, moisture, and carbohydrates) and fibre (ADF, NDF and total dietary fibre) in maize grain across all locations is shown in **Table 21**. All results for proximates and fibre were within literature ranges **(Figure 32)**, and no significant differences in the across-site analysis were observed between the control and DAS-40278-9 maize entries for fat, ash, NDF and total dietary fibre. 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 < 12% and all values were within literature ranges, the differences are not biologically meaningful.

Table 21: Summary of the proximate and fiber analysis of maize grain
(Phillips, 2009, p.35, Study 090084)

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])
Fibre (% dry weight)							
Acid Detergent Fibre (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 Fibre (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 Fibre	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 D.
[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 32: Proximate and fibre analysis of maize grain
(Phillips, 2009, p.45, Study 090084)
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix D, Table D.2) are shaded.



Mineral Analysis of Grain

An analysis of maize 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 22**. All results were within the reported literature ranges **(Figure 33).** 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

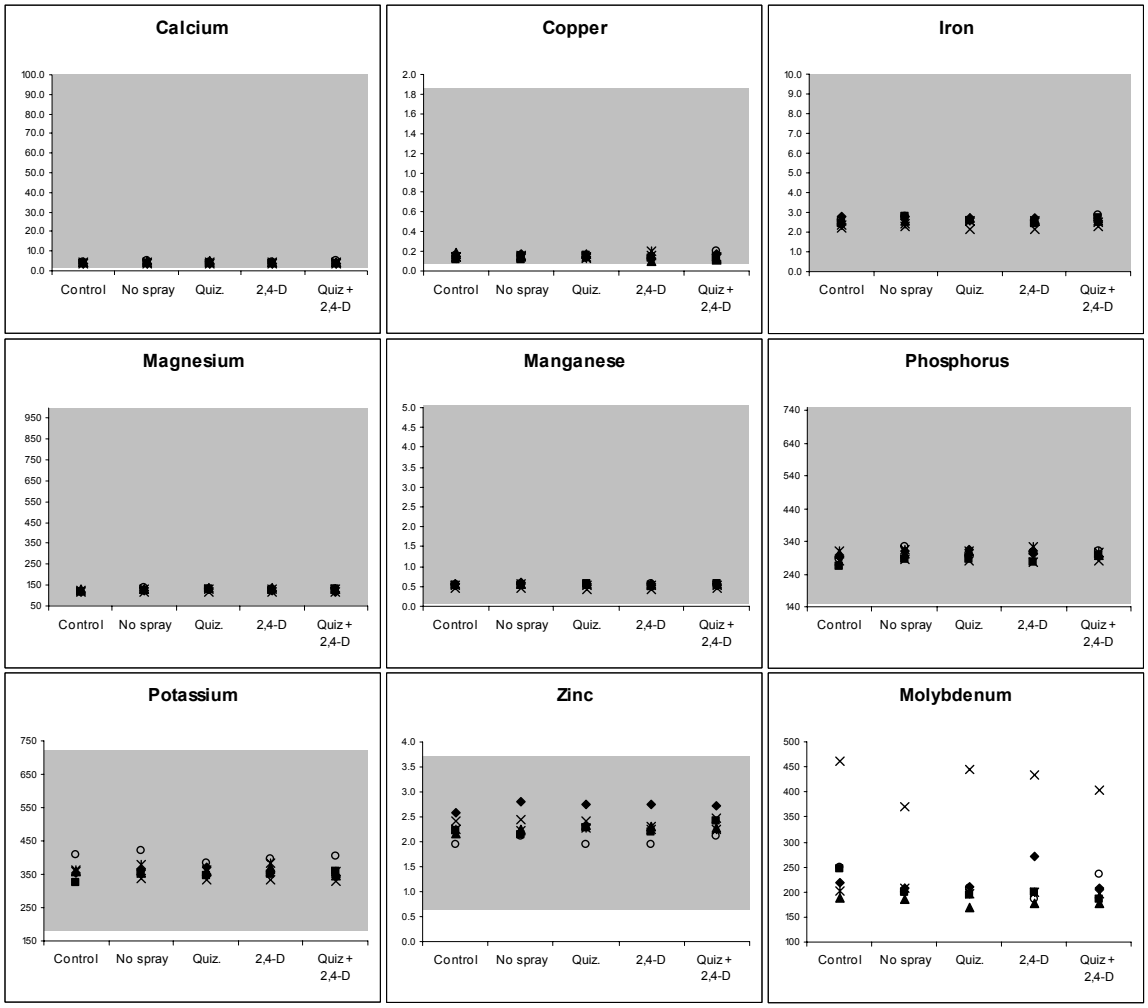
was not present. Additionally, these differences from the control were < 13%, and all values were within literature ranges, when available.

Table 22: Summary of the mineral analysis of maize grain
(Phillips, 2009, p.36, Study 090084)

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 D.
[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 33: Mineral analysis of maize grain
(Phillips, 2009, p.46, Study 090084).
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix D, Table D.3) are shaded. Grain was also analysed for chromium, iodine, selenium and sodium, but results were less than the limit of quantitation.



Amino Acid Analysis of Grain

Maize samples were analysed for amino acid content in the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both maize, and a summary of the results over all locations are shown in **Table 23**. Levels of all amino acids were within the reported literature ranges **(Figure 34)**, 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 < 15%, not observed across all sites, and all mean values were within reported literature ranges.

Table 23: Summary of the amino acid analysis of maize grain
(Phillips, 2009, p.37, Study 090084)

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])
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 D.
[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 34: Amino acid analysis of maize grain
(Phillips, 2009, p.47 Study 090084)

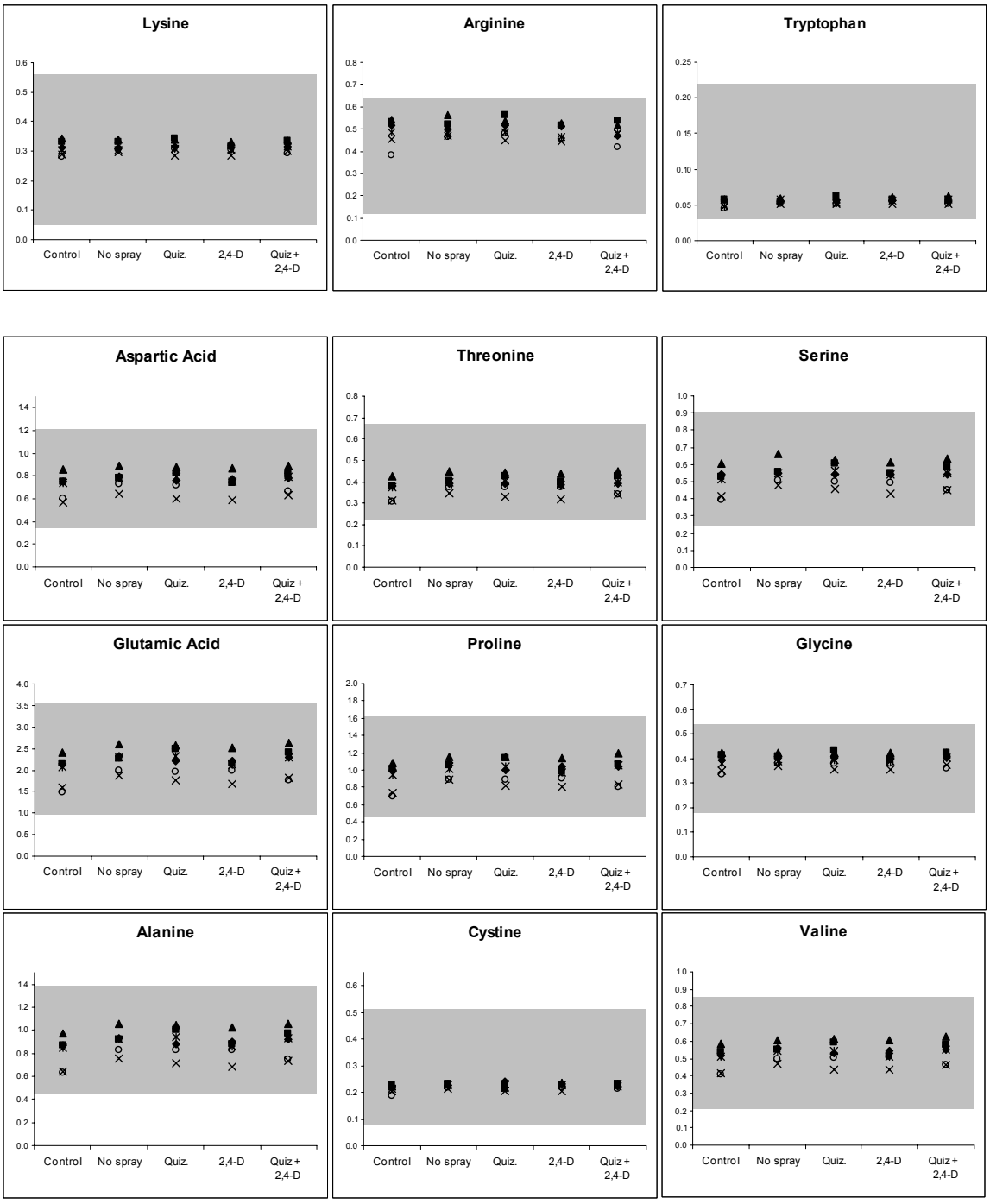
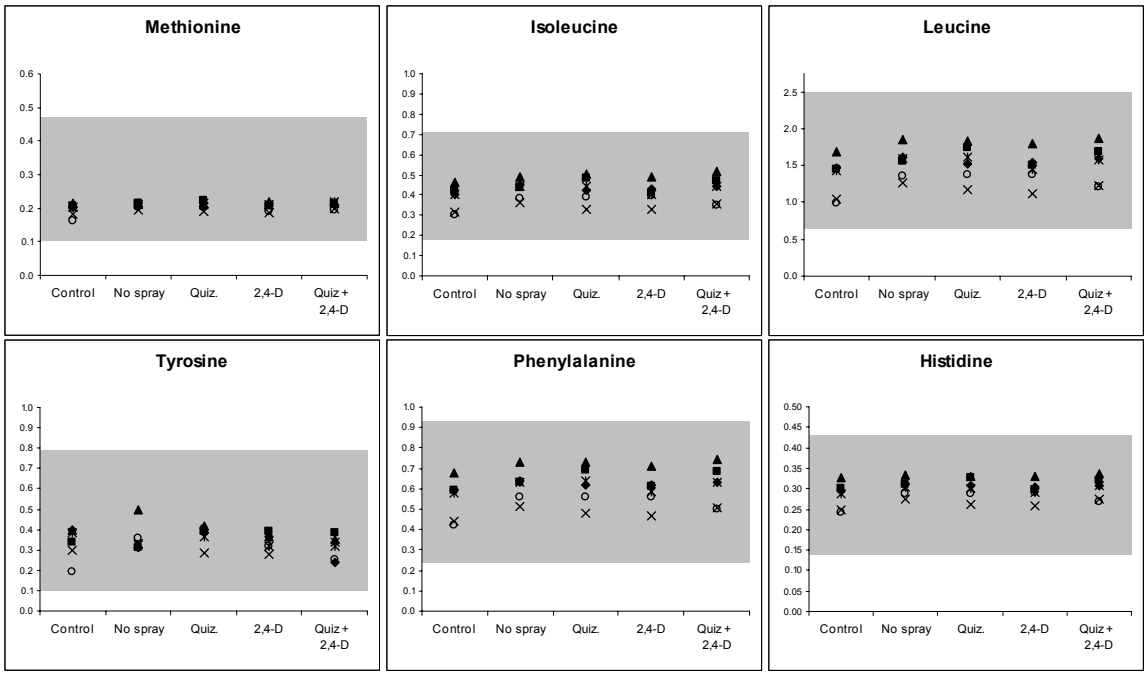


Figure 34 (cont.). Amino acid analysis of maize grain
(Phillips, 2009, p.47, Study 90084)



Fatty Acid Analysis of Grain

An analysis of maize grain samples for fatty acids was performed. A summary of the results across all locations is shown in **Table 24**. All results for the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both maize grain samples analysed for these fatty acids were within the published literature ranges **(Figure 35)**. 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.

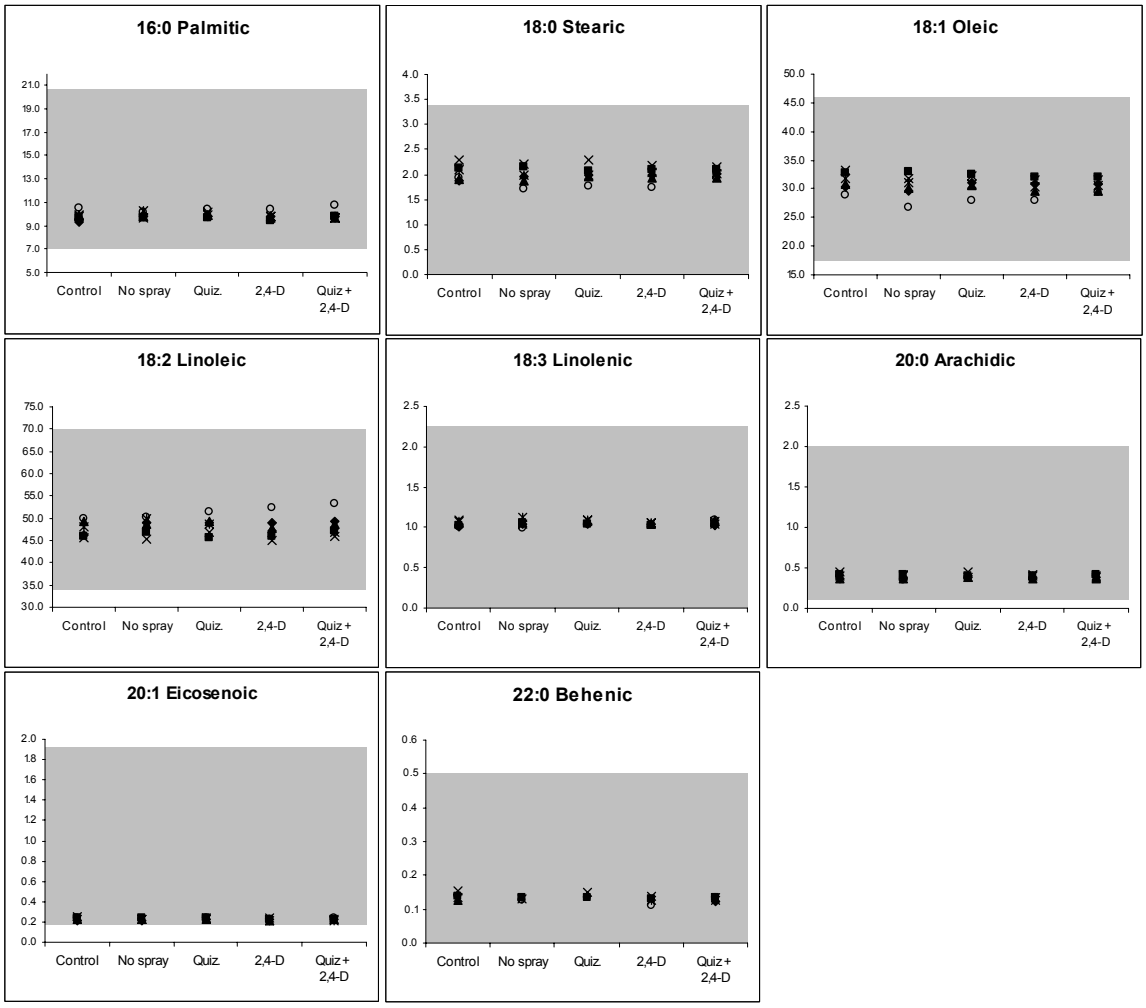
Table 24: Summary of the fatty acid analysis of maize grain
(Phillips, 2009, p.39, Study 090084).

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)
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 D.
[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 35: Fatty acid analysis of maize grain
(Phillips, 2009, p.49, Study 090084)
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix D, Table D.5) are shaded. Grain was also analysed 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.



Vitamin Analysis of Grain

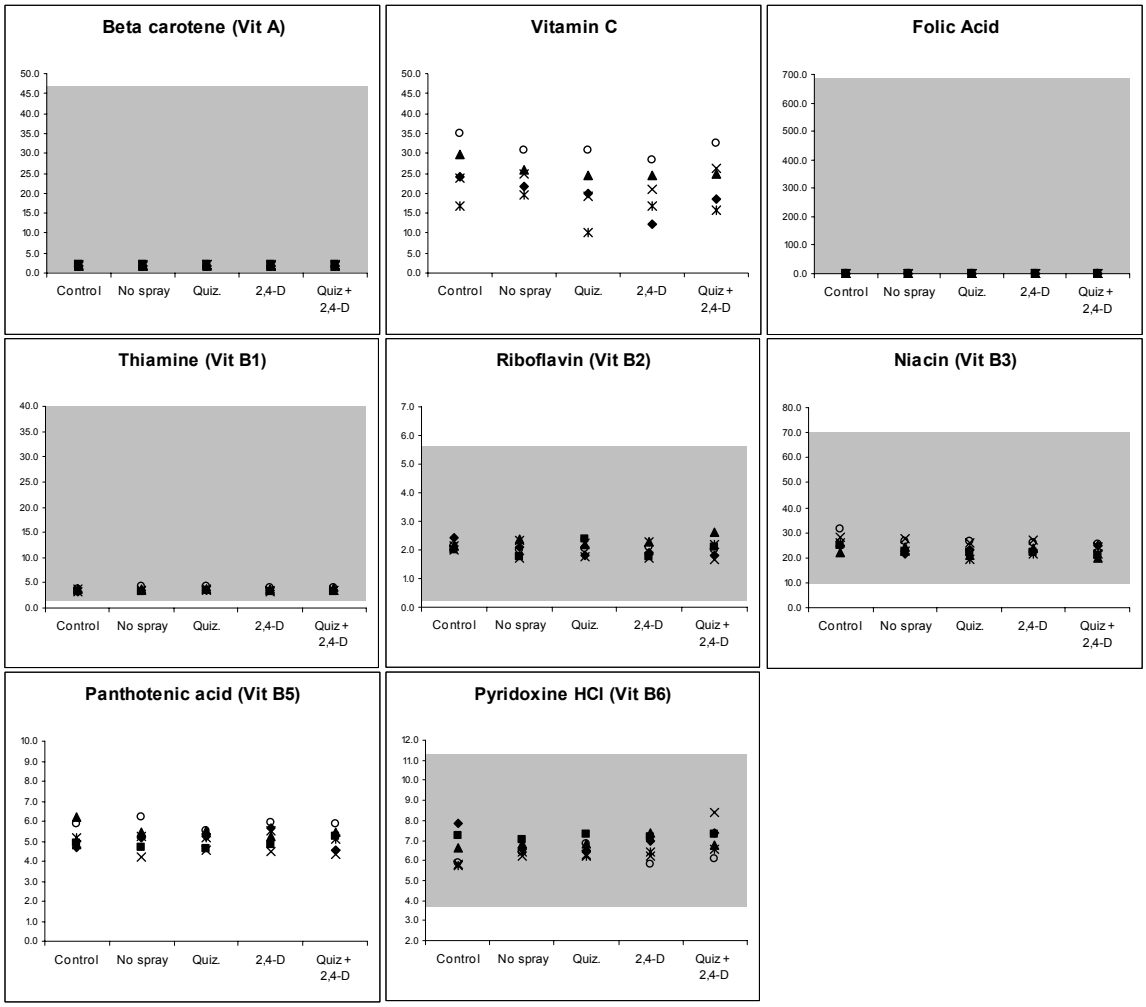
The levels of vitamin A, B1, B2, B5, B6, B12, C, D, E, niacin, and folic acid in maize grain samples from the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both maize entries were determined. A summary of the results across all locations is shown in **Table 25.** Mean results 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 36)**. 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 25: Summary of the vitamin analysis of maize grain
(Phillips, 2009, p.41, Study 090084)

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 D.
[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 36: Vitamin analysis of maize grain
(Phillips, 2009, p.50, Study 090084)
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix D, Table D.6) are shaded. Grain was also analysed for Vitamin E, Vitamin B12 and Vitamin D, but results were less than the limit of quantitation.



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 maize. A limited number of significant differences between unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D or AAD-1 + both maize 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 maize. 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 maize and the control maize line **(Figures 31-37)**. 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 maize to conventional maize.

Literature Ranges for Compositional Analysis

Published values for compositional analytes of the maize forage and grain were compiled from literature sources to establish representative ranges for analytes typically found in maize (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 maize and the non-transgenic control **(Tables 20-26)** and used to prepare plots of the compositional analysis results **(Figures 31-37).**

Literature ranges compiled for forage included proximates, fibre, and minerals **(Table 13)**. The data compiled for grain included proximates and fibre **(Table 14),** minerals **(Table 15)**, amino acids **(Table 16)**, fatty acids **(Table 17)**, vitamins **(Table 18)**, and secondary metabolites and anti-nutrients **(Table 19)**.

Table 12: Literature ranges for proximates 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 13: Literature ranges for fiber, and minerals in forage

Analyte	Literature Reference (% Dry weight)		
	Watson (1982)	**ILSI (2006)**	**Combined Ranges**
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

Table 14: 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 15: 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 16: 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 17: 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 18: 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 19: 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

b) 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 maize grain samples from the control, unsprayed AAD-1, AAD-1 + quizalofop, AAD-1 + 2,4-D and AAD-1 + both maize entries were determined. A summary of the results across all locations is shown in **Table 26**. For the across-site analysis, all values were within literature ranges **(Figure 37)**. 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.

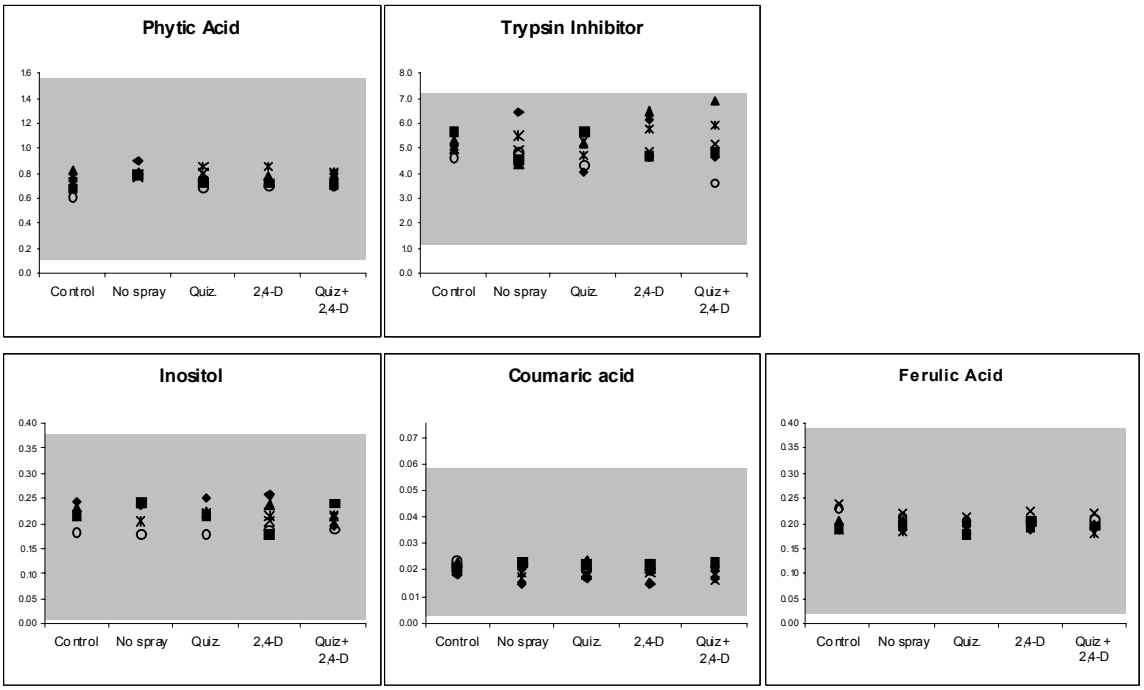
Table 26: Summary of the secondary metabolite and anti-nutrient analysis of maize grain
(Phillips, 2009, p.42, Study 090084)

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 D.
[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 37: Secondary metabolite and anti-nutrient analysis of maize grain

(Phillips, 2009, p. 51, Study 090084)
Values at each location shown: diamond = IA, square = IL1, triangle = IL2, X = IN, star = NE, and circle = ON. Combined literature ranges (Appendix D, Table D.7) are shaded. Grain was also analysed for furfural and raffinose, but results were less than the limit of quantitation.



c) Allergenic Proteins

Maize is a food source which is generally recognised as safe. The comparative analysis indicate that Maize line DAS-40278-9 is as safe as the non-GM counterpart.

D. NUTRITIONAL IMPACT

1. Human Nutritional Impact

Estimate of Dietary Exposure of AAD-1

Protein expression levels of AAD-1 in DAS-40278-9 were used with conservative (i.e. protective) human dietary consumption data for maize to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the AAD-1 protein in DAS-40278-9 maize, indicating no concern for adverse effects from acute dietary exposure through maize.

Potential Human Exposure to AAD-1 Protein via Maize

The field expression of AAD-1 protein in Event DAS-40278-9 maize has been measured using an AAD-1 specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of maize. Protein expression was analyzed in leaf, root, pollen, whole plant and grain tissues collected throughout the growing season from DAS 40278 maize plants treated with 2,4-D, quizalofop, both 2,4-D and quizalofop, or not treated with either herbicide. In general, the results showed low level expression of the AAD-1 protein with or without herbicide treatments and across environments, indicating a low exposure risk to humans and animals. In maize grain collected at growth stage R6 to maturity, the average value of AAD-1 protein across treatments was 4.81 ng/mg tissue on a dry weight basis (Table 3.2). The full range of values was 1.07 to 9.10 ng/mg tissue, but the use of an average expression value is most appropriate, because maize grain is a highly blended commodity (making consumption of single-servings of grain at the maximum expression-level highly unlikely). Use of this value is a conservative and protective estimate for exposure to the AAD-1 protein from maize; actual dietary exposure to the protein will be lower because: 1) there will be protein degradation during transport and storage, 2) grain containing AAD-1 will be mixed with non-AAD-1 grain, 3) for humans, consumption of maize products is often in food forms which are cooked and heat is known to denature this protein and 4) a portion of the consumer dietary exposure to maize is in forms where the protein concentrations will be reduced by processing, such as in maize syrup. It is also known that oils contain very little protein.

A conservative acute consumption (i.e. exposure) estimate is made based on global data published by the World Health Organization (WHO). WHO has established a maximum consumption of each food commodity for acute exposures for the entire world, based on maximum inputs from multiple countries (FAO WHO, 2008). **Table 27** includes 97.5th

percentile values for all possible commodities associated with maize and maize. For AAD-1 maize, the appropriate maximum consumption value is associated with the "GC 645 Maize" group with an upper limit for maize reported by France. Other information for sweet so and popcorn are presented here for completeness as well, however there are no plans for introduction of the AAD-1 trait into these related commodities. Information for maize oil is presented here for completeness, but it is known that the oils and other highly refined fractions do not contain significant amounts of protein. Moreover, total acute consumption across all these entities cannot be calculated, because it is not appropriate to add 97.5th percentile values for individual commodities for survey results from different countries.

Table 27: Estimates of Acute Maize Consumption from the GEMS/Food Highest 97.5th Percentile "Eater-Only" Worldwide

		Consumption[a] (g/kg/day)	
Commodity	**Country with Reported Maximum**	**General Population**	**Children ≤6 years**
GC 645 maize	**France**	**4.06**	**6.17**
VO 447 sweet corn[b] (corn on the cob)	Thailand	7.16	11.52
GC 656 popcorn	Japan	3.33	3.33
OR 645 maize oil, refined	Netherlands	0.89	0.68

[a]Total acute consumption across these entities cannot be calculated because, it is not appropriate to add 97.5th percentile values for individual commodities survey results from different countries; FAO WHO, 2009

When the WHO "GC 645 maize" acute consumption information is coupled to the AAD-1 field expression level of 4.81 ng/mg tissue, an upper limit for acute exposure to AAD-1 protein via maize is estimated as:

- **0.0195 mg protein/kg bw/day, for general population (i.e. adults)**
- **0.0297 mg protein/kg bw/day, for children of 6 years or younger**

A dietary exposure estimate for AAD-1 protein in livestock diets was developed by coupling field expression information for AAD-1 protein from DAS-40278-9 maize plants with livestock dietary consumption assumptions for maize and maize forage. In addition, the relevance of the exposure estimate is placed into context, based on the mammalian toxicity information.

Maize grain and forage are used for animal feeds. An assessment for livestock exposure is presented here based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures of US EPA (US EPA, 2008). The MRBD guidance has been used to construct a maximum maize grain contribution for swine, poultry and cattle based on the average value for AAD-1 of 4.81 ng/mg (or ppm) in DAS-40278-9 maize grain (BNF 120, page 59, Table 11). For cattle, the field expression levels of AAD-1 in forage (collected at R4) are also applicable.

The average value of AAD-1 protein in maize forage plants (across treatments) was 7.05 ng/mg tissue (dry weight basis) and the maximum value observed was 11.6 ng/mg tissue. The presence of AAD-1 protein in maize is not anticipated to have impact for feed ration formulation, because nutrient composition analyses have shown that DAS-40278-9 maize is substantially equivalent to conventional maize.

These livestock diets have been built based on the traditional use of the unmodified counterpart per US EPA procedures; and estimates of dietary exposure are conservative (and protective) in that they have assumed 100% replacement of the unmodified counterpart. US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (US EPA, 2008). The above request was for the broiler chicken but the US EPA has stated "The laying hen is the reference animal since the required poultry feeding study uses the hen as the study animal." Thus, information on both the laying and the broiler chicken are provided:

Beef: Finishing or feedlot beef (body weight at slaughter, 1200 lb or **544 kg,** daily feed intake of 20 lb or **9 kg** dry matter feed). Feedlot rations in the finishing stage consist of high amounts of grain or grain supplements (80% Carbohydrate content (CC)), forages (15% Roughage (R)), and protein sources (5% Protein content (PC)) in last 120 to 180 days (4 to 6 months) before slaughter at **16 to18 months of age.**

Dairy: Mature lactating cow (body weight, 1350 lb or **612 kg**, daily feed intake of 53 lb or **24 kg dry matter feed,** and producing average of 90 lb of milk a day). Feed rations include forages (45% R), grain or grain supplements (45% CC), and protein source (10% PC). Dairy cows generally calve at **24 to 28 months of age**. The usual length of lactation is 250 to 450 days, with a 305 day lactation being the standard. Dairy cows are usually slaughtered after 2 or 3 calves. The average productive life span of the mature lactating dairy cow is 3 to 4 years.

Poultry: Chicken Broiler: (body weights for frying and rotisserie chickens range from 3 to 4 lb (an average of **1.59 kg**). Rotisserie chickens have an average life span of **38 to 42 days**. The broiler diet contains 85% CC and 15% PC. EPA did not name an average daily intake for broiler so instead the feed value for an average US broiler has been scaled to **1 kg of feed** based on the commonly used OECD reference animal of 1.2 kg feed for a 1.9 kg chicken.

Poultry: Chicken: Laying hen (body weight, 4.2 lb or **1.9 kg**, average daily intake of 52 grams or **0.052 kg of feed**). Laying hens are usually slaughtered **after 18 months**. A daily ration includes grain or grain supplement (75% CC) and protein source (25% PC).

Swine: Finishing or Market hog (body weight, up to 250 lb or **113 kg**, average daily intake of 6.8 lb or **3.1 kg of feed**). Hogs are slaughtered in **5 to 8 months**. In general, daily ration consists of high grain or grain supplement (85% CC) and oilseed meal (15% PC).

The above assumptions apply for finishing animals in US feedlots. For cattle, a younger animal would receive a higher percentage of forage than grain, but analysis of younger animals would not result in substantially different overall conclusion given the low toxicity of the AAD-1 protein. In addition, the worse case value of 11.6 ppm for forage has been assumed here at 100% AAD-1 event DAS-40278-9 maize. In reality, exposure via forage will be lower, given the average of 7.05 ppm in forage and market adoption of DAS-40278-9 maize will not be 100 percent. The resulting intake dietary burden for animal feeds is totaled in **Table 28**.

Table 28: Intake Animal Dietary Burdens for Livestock

			Dietary Contribution (%)						Animal Dietary Burden (ppm)				
Feed stuff	T	DM (%)	Beef	Dairy	Poultry (broiler)	Poultry (layer)	Pig	AAD-1 (ppm)	Beef	Dairy	Poultry (broiler)	Poultry (layer)	Pig
Maize, grain	CC	88	80	45	85	75	85	**4.81**	4.37	2.46	4.09	3.61	4.09
Maize, forage/ silage	R	40	15	45	Nu	Nu	Nu	**11.6**	4.35	13.05			
								Total	**8.72**	**15.53**	**4.09**	**3.61**	**4.09**

T= Type; DM=dry matter; Nu = Not used

Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal in **Table 29**.

Table 29: Livestock Daily Dose Estimates of AAD-1 Protein from Maize Feeds

	Chicken (Broiler)	Chicken (Layer)	Dairy	Beef	Pig
Body weight (kg)	1.59	1.9	612	544	113
Daily Maximum Feed (kg)	1	0.052	24	9	3.1
Maximum AAD-1 intake (mg/kg feed)	4.09	3.61	15.51	8.72	4.09
Maximum intake (mg/kg bw)	2.57	0.10	0.61	0.15	0.11

The highest exposed animal is the broiler chicken with 2.57 mg AAD-1/kg bw. When this value is compared to the acute no observable effect level (NOEL) of >2000 mg/kg bw, there is an adequate margin of safety for livestock; there is an even larger Margin of Exposure (MOE) for livestock mammals. Variations in livestock feed diets elsewhere in the world could results in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for AAD-1 protein in DAS-40278-9 maize.

2. Animal Feeding Studies

No animal feeding study with the GM food has yet been conducted.

REFERENCES

Dow AgroSciences Internal Studies

Cleveland, C. B., Herman, R. A., Tagliani, L. A. 2009. Human and Livestock Exposure Assessment for AAD-1 Protein in DAS-40278-9 Maize. Study ID 091115. July 15, 2009.

Embrey, S.K., Korjagin, V.A. 2008. In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenas-1 (abbreviation AAD-1). Study ID 080062

Herman, R.A., Larrinua, I.M. 2007. AAD1 Amino-Acid Homology Search for Similarity to Toxins. Study ID 071022

Herman, R.A. 2007. AAD1 Amino-Acid Homology Search for Similarity to Allergins. Study ID 071029

Phillips, A.M., Herman, R.A., Thomas, A.D., M. Sosa. 2009. Field Expression, Nutrient Composition Analysis and Agronomic Characteristics of a Hybrid Maize Line Containing Aryloxyalkanoate Dioxygenase-1 (AAD-1) – Event DAS-40278-9 (Protocols 080137 and 080139). Study ID 090084

Schafer, B.W. 2008. Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1. Study ID 080059

Schafer, BW., Embrey, S.K. 2009. Characterization of the Aryloxyalkanoate Dioxygenase-1 (AAD-1) Protein Derived from Transgenic Maize Event DAS-40278-9. Study ID 080142

Please refer to Attachment 2 – Confidential Commercial Information.

Wiescinski, M.S., Golden, R.M. 2007. AAD-1: Acute Oral Toxicity Study in CRL:CD1(ICR) Mice. Study ID 071128

Zhuang, M., Mo, J., Poorbaugh, J.D., Richey, K.A., Cruse, J., Thomas, A. 2009. Molecular Characterization of AAD-1 Corn Event DAS-40278-9. Study ID 081052

Zhuang, M., Poorbaugh, J.D., Mo. J., Richey, K.A., Cruse, J. 2009. Molecular Characterization of AAD-1 of AAD-1 Corn Event DAS-40278-9 within a Single Generation. Study ID 081120

Published References

Abranches, R., Shultz, R. W., Thompson, W. F., Allen, G. C. 2005. Matrix attachment regions and regulated transcription increase and stabilize transgene expression. Plant Biotechnology Journal 3: 535-543.

Ainley, M., Armstrong, K., Belmar, S., Folkerts, O., Hopkins, N., Menke, M.A., Pareddy, D., Petolino, J. F., Smith, K., Woosley, A. 2002. Regulatory Sequences from Transgenic Plants. U.S. Patent US6384207.

Allen, G.C., Spiker, S., Thompson, W.F. 2000. Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Molecular Biology 43: 361-376.

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.

Balkwill, D.L, Fredreickson, J.K., Romine, M.F. 2006. *Sphingomonas* and Related Genera. Prokaryotes 7:605-629

Barnekow, D.E., Hamburg, A.W., Puvanesarajah, V., Guo, M. 2001. Metabolism of 2,4-Dichlorophenoxyacetic Acid in Laying Hens and Lactating Goats. Journal of Agricultural and Food Chemistry 49:156-163.

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.

Bower, S., Burke, E., Harding, N.E., Patel, Y.N., Schneider, J.C., Meissner, D., Morrison, N.A. and Bezanson, R. 2006. Mutant bacterial strains of the genus Sphingomonas deficient in production of polyhydroxybutyrate and a process of clarification of sphingans and compositions thereof. U.S. Patent #20060121578.

Canadian Food Inspection Agency (CFIA). 1994. Biology Document BIO1994-11: The Biology of *Zea mays* (L.) (Maize).

Christensen, A.H., Quail, P.H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Research 5: 213-218.

Christensen, A.H., Sharrock, R.A. and Quail, P.H. 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Molecular Biology 18(4): 675-689.

Codex Alimentarius Commission. 2001. Information paper on familiarity. Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology. CX/FBT/01/7, February, 2001. Joint FAO/WHO Food Standard Programme, Rome.

Codex Alimentarius Commission. 2003. Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, Annex 1: Assessment of Possible Allergenicity, CAC/GL 45-2003, pg 9-11.
www.codexalimentarius.net/download/standards/10021/CXG_045e.pdf

Emerson, E. 1943. The condensation of aminoantipirene. A new colour test for phenolic compounds. *J. Organomet. Chem.* 8: 417–428

European Food Safety Authority (EFSA) 2008. Summary of the *EFSA Scientific Report* (2008) 205, 1-216 Conclusion on the pesticide peer review of quizalofop-P. Issued on 26 November 2008.

Federal Register, 1997. FRL-5758-3, Vol. 62, No. 242, December 17, 1997, page 66080**,**
http://frwebgate.access.gpo.gov/cgi-bin/getpage.cgi?position=all&page=66080&dbname=1997_register , accessed December 10, 2009.

Fukumori, F. and Hausinger, R. P. 1993. Purification and Characterization of 2, 4-Dichlorophenoxyacetate/□-Ketoglutarate Dioxygenase. J. Biol. Chem. 268, 15: 24311-24317.

FAO WHO. 2008. Highest Reported 97.5th Percentile Consumption Figures (Eaters Only) for Various Commodities by the General Population and Children Ages 6 and Under. http://www.who.int/foodsafety/chem/en/acute_hazard_db1.pdf

FAOSTAT Database (2001) 'Provisional 2001 production and production indices data.' (FAO, Rome)

Hall, G., Allen, G.C., Loer, D.S., Thompson, W.F., Spiker, S. 1991. Nuclear scaffolds and scaffold-attachment regions in higher plants. Proceedings of the National Academy of Sciences of the United States of America 88(20), 9320-9324.

Han, K.-H., Caiping, M., Strauss, S.H. 1997. Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar. Transgenic Research 6: 415-420.

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

Horvath, M., Ditzelmuller, G., Loidl, M., Streichsbier, F. 1990. Isolation and characterization of a 2-(2,4-dichlorophenoxy) proprionic acid-degrading soil bacterium. Appl. Microbiol. Biotech. 33:213-216

ILSI. 2006. International Life Sciences Institute Crop Composition Database. Version 3.0. http://www.cropcomposition.org/

Kinjo, Y., Pei, B., Bufali, S., Raju, R., Richardson, S.K., Imamura, M. Fujio, M., Wu, D., Khurana, A., Kawahara, K., Wong, C.-H., Howell, A.R., Seeberger, P.H., Kronenberg, M. 2008. Natural Sphingomonas glycolipids vary greatly in their ability to activate natural killer T cells. Chem Biol. 15(7):654-664

Koeppe, M. K., J.J. Anderson, and L.M. Shalaby. 1990. Metabolism of [^{14}C]Quizalofop-ethyl in Soybean and Cotton Plants. J. Agric. Food Chem. 38:1085-1091

Lal, R., Dogra, C., Malhotra, S., Sharma, P. and Pal, R. 2006. Diversity, distribution and divergence of *lin* genes in hexachlorocylohexane-degrading sphingomonads. Trends in Biotechnology 24 (3): 121-130.

Johnsen, A.R., Wick, L.Y. and Harms, H. 2005. Principles of microbial PAH-degradation in soil. Environmental Pollution 133(1): 71-84.

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

OECD Consensus Document on Compositional Considerations for New Varieties of Maize (Zea mays): Key Food and Feed Nutrients, Anti-nutrients and Secondary Plant Metabolites (2002)

http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/env-jm-mono(2002)25

Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" 2004.
http://www.oecd.org/document/57/0,3343,en_2649_34385_31562937_1_1_1_1,00.html

OECD. 2002. Organisation for Economic Co-operation and Development, Consensus document on compositional considerations for new varieties of maize (Zea mays): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. ENV/JM/MONO (2002)25. 42p.
http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/env-jm-mono(2002)25

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.

Petolino, J.F., Arnold, N.L. 2009. Whiskers-mediated maize transformation. In Methods in Molecular Biology: Transgenic Maize, Vol. 526, M. Paul Scott (ed.), Humana Press, pp 59-67.

Polevoda, B. and Sherman, F. 2003. N-terminal Acetyltransferases and Sequence Requirements for N-terminal Acetylation of Eukaryotic Proteins. Journal of Molecular Biology 325, 595-622.

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

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

Pollock, T. and Armentrout, R. 1999. Planktonic/sessile dimorphism of polysaccharide-encapsulated sphingomonads. Journal of Industrial Microbiology and Biotechnology 23 (4-5): 436-441.

Preston, C, 2005. Australian Glyphosate Resistance Register. National Glyphosate Sustainability Working Group. www.weeds.crc.org.au/glyphosate

Proteometrics LLC
http://www.proteometrics.com/

Ritchie, S.W., Hanway, J.J., Benson, G.O. 1993. How a Corn Plant Develops. Cooperative Extension Service, Special Report 48, Iowa State University http://www.extension.iastate.edu/hancock/info/Corn+Develop+Stages.htm

Roberts, T. (ed.) 1998. Metabolic Pathways of AgroChemicals, Part One: Herbicides and Plant Growth Regulators, The Royal Society of Chemistry, Cambridge.

US EPA 2003. 2,4-D HED MARC Decision Document, DP Barcodes D293119 and D293128, Chemical ID No 030001, Case no, 0073, Meeting Date 9/3/03, Memorandum TXR No. 0052264, December 3, 2003.

US EPA 2005. Reregistration Eligibility Decision for 2,4-D, EPA738-R-05-002, June 2005.

US EPA 2006. D310869, Quizalofop-P ethyl: Human Health Risk Assessment for New Uses on Barley, Flax, Sunflower and Wheat, August 2006, http://www.regulations.gov/search/Regs/home.html#docketDetail?R=EPA-HQ-OPP-2007-1089 , accessed December 10, 2009.

US EPA 2007. Federal Register, Vol 72, No. 108, 31221, June 6, 2007.

US EPA 2008. HED Memo; Revisions of Feedstuffs in Table 1 of OPPTS Test Guideline 860.1000 and Guidance on Constructing Maximum Reasonably Balanced Diets (MRBD), Docket number EPA-HQ-OPP-2007-0639-0025.

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/

van Kranenburg, R., Boels, I.C., Kleerebezem, M., M de Vos, W. 1999. Genetics and engineering of microbial exopolysaccharides for food: approaches for the production of existing and novel polysaccharides. Curr. Op. Biotech. 10:498-504

Verma, D., Verma, M., Dey, M., Jain, R.K., Wu, R. 2005. Molecular dissection of the tobacco Rb7 matrix attachment region (MAR): Effect of 5' half on gene expression in rice. Plant Science 169: 704-711.

Watson, S. A. 1982. Corn: amazing maize, CRC Handbook of Processing and Utilization in Agriculture, vol. II, Part 1. Plant Products I. A. Wolf (ed.) CRC Press Inc., pp. 3-29, Florida.

Watson, S. A. 1987. Structure and composition, Corn: Chemistry and Technology, S. A. Watson and P. E. Ransted (eds.), American Association of Cereal Chemists, Inc., pp. 53-82, Minnesota.

White PJ, Pollak LM. 1995. Corn as a food source in the United States: Part II. Processes, products, composition, and nutritive values. *Cereal Foods World* 40, 756-762.

Wright, T.R., Lira, J.M., Merlo, D.J. and Hopkins, N. 2005. Novel Herbicide Resistance Genes. U.S. Patent # 2009/0093366.

Yanisch-Perron C., Vieira J., Messing J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-119

Zipper, C., Nickel, K., Angst, W., Kohler, H.-P. E. 1996. Complete Microbial degradation of both enantiomers of the chiral herbicide mecoprop [(RS)-2-(4-Chloro-2-Methylphenoxy)propionic Acid] in an enantioselective manner by *Sphingomonas herbicidovorans* sp. nov. Applied and Environmental Microbiology 62(12):4318-4322

ATTACHMENT 1