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

OECD Unique Identifier: DAS-68416-4

AAD-12 Soybean

Volume 1 of 2

Submitting Company:

Dow AgroSciences Australia Pty Ltd.
Submitted by:

Sarah Russell French
Regulatory Specialist
Regulatory Sciences & Government Affairs
Dow AgroSciences Australia Pty Ltd.
Level 5, 20 Rodborourgh Rd
Frenchs Forest
NSW 2086
Telephone: +61297763415
FAX: +61297763199
Email: skrussellfrench@dow.com

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SUMMARY

Dow AgroSciences Australia Ltd (herein referred to as "DAS"), is submitting an application to amend the Code to approve the use of DAS-68416-4 Soybean, a new food produced using gene technology.

DAS-68416-4 soybean is a transgenic soybean product that provides tolerance to the herbicides 2,4-Dichlorophenoxyacetic acid (2,4-D) and glufosinate. This herbicide-tolerant soybean will provide growers with greater flexibility in selection of herbicides for the improved control of economically important weeds; allow an increased application window for effective weed control; and provide an effective weed resistance management solution to the increased incidence of glyphosate resistant weeds.

DAS-68416-4 soybean plants have been genetically modified to express the aryloxyalkanoate dioxygenase-12 (AAD-12) and phosphinothricin acetyltransferase (PAT) proteins. The AAD-12 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-12* gene, which expresses the AAD-12 protein, was derived from *Delftia acidovorans,* a gram-negative soil bacterium. The PAT enzyme acetylates the primary amino group of phosphinothricin rendering it inactive. The *pat* gene expressing the PAT protein was derived from *Streptomyces viridochromogenes*.

The *aad-12* and *pat* genes were introduced into DAS-68416-4 soybean using *Agrobacterium*-mediated transformation. Molecular characterization by Southern analyses of the DAS-68416-4 event confirmed that a single, intact insert of the *aad-12* and *pat* genes were stably integrated into the soybean genome. A single copy of each of the genetic elements of the *aad-12* expression cassette is present and the integrity of the inserted DNA fragment was demonstrated in three 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-68416-4 soybean. Segregation data for breeding generations confirmed the predicted inheritance of the *aad-12* and *pat* genes.

The AAD-12 and PAT proteins in DAS-68416-4 soybean were characterized biochemically and measured using AAD-12 and PAT specific enzyme linked immunosorbent assays (ELISA). Protein expression was analysed in leaf, root, whole plant and grain tissues collected throughout the growing season from DAS-68416-4 plants treated with 2,4-D, glufosinate, both 2,4-D and glufosinate, or not treated with either herbicide. The results showed a low level of expression of the AAD-12 and PAT proteins across herbicide treatments and environments, indicating a low exposure to humans and animals.

The AAD-12 protein was assessed for any potential adverse effects to humans or animals resulting from the environmental release of crops containing the AAD-12 protein. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 protein. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-12 amino acid sequence. The AAD-12 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-12 protein. Glycosylation analysis of the plant- and microbe-derived AAD-12 proteins revealed no detectable covalently linked carbohydrates. Results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals. The safety of the PAT protein has been assessed previously and it has been approved for use in canola, corn, cotton, soybeans, and sugar beets.

Nutrient composition analyses of forage and grain was conducted to compare the composition of DAS-68416-4 soybean with the composition of a non-transgenic control. Compositional analyses were used to evaluate any changes in the levels of key nutrients and anti-nutrients in DAS-68416-4 soybean which was sprayed with either 2,4-D, glufosinate, both 2,4-D and glufosinate, or which was not sprayed with either herbicide. Along with the agronomic data, the compositional analyses indicate that DAS-68416-4 soybean is substantially equivalent to conventional soybean and will not exhibit unexpected or unintended effects with respect to plant pest risk.

In summary, information collected during field trials and laboratory analyses presented herein demonstrate that DAS-68416-4 soybean is safe as conventional soybean for food and feed uses.

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THE APPLICANT

This application is submitted by:

Dow AgroSciences Australia Pty. Ltd.
20 Rodbourgh Rd
Frenchs Forest
NSW 2086

Locked Bag 502
Frenchs Forest
NSW 2086

The primary contact is:

Sarah Russell French
Regulatory Specialist
Dow AgroSciences Australia Pty. Ltd.
Ph: +61 2 9776 3415
Fax: +61 2 9776 3199
Email: skrussellfrench@dow.com

The Managing Director of Dow AgroSciences Australia Pty. Ltd is:

Mr Peter Dryden
Ph: +64 6 751 2400
Email: dryden@dow.com

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
A	Acre
aad-12	Gene from *Delftia acidovorans* which encodes the AAD-12 protein
AAD-12	Aryloxyalkanoate Dioxygenase-12 protein
ADF	Acid detergent fiber
ae	Acid equivalent
ae/A	Acid equivalent per acre
ae/ha	Acid equivalent per hectare
ai	Active ingredient
ai/A	Active ingredient per acre
ai/ha	Active ingredient per hectare
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service, USDA
AtUbi10	Ubiquitin promoter from *Arabidopsis thaliana*
AtuORF1	3' untranslated region from *Agrobacterium tumefaciens*
AtuORF23	3' untranslated region from *Agrobacterium tumefaciens*
bp	Base pair
CFIA	Canadian Food Inspection Agency
CFSAN	Center for Food Safety and Nutrition, US FDA
CsVMV	Promoter from cassava vein mosaic virus
DAS	Dow AgroSciences LLC
DAS-68416-4	Soybean line containing event DAS-68416-4
DCP	2,4-Dichlorophenol
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (US)
ESI-LC/MS	Electrospray ionization-liquid chromatography mass spectrometry
Event DAS-68416-4	OECD identifier for the soybean event expressing the AAD-12 protein
FDA	Food and Drug Administration (US)
FDR	False Discovery Rate
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
ha	Hectare
ILSI	International Life Sciences Institute
Kb	Kilobase pair
kDa	Kilodalton
L	Liter
LOD	Limit of Detection
LOQ	Limit of Quantitation

MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
Maverick	Publicly available soybean line used in transformation to produce event DAS-68416-4
MCPA	4-chloro-2-methylphenoxyacetic acid
MOA	Mode of action
NDF	Neutral detergent fiber
OECD	Organisation for Economic Co-operation and Development
pat	Gene from *Streptomyces viridochromogenes* which encodes the PAT protein
PAT	Phosphinothricin N-acetyl transferase protein
PBN	US FDA Pre-market Biotechnology Notice
pDAB4468	DNA vector carrying the transgenes (*aad-12* and *pat*) for insertion into the plant genome
Pf	*Pseudomonas fluorescens*
PTU	Plant transcription unit consisting of promoter, gene, and terminator sequences
RB7 MAR	Matrix attachment region (MAR) from *Nicotiana tabacum*
RCB	Randomized complete block
SCN	Soybean cyst nematode
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
spp	species
subsp	subspecies
T-DNA	Transfer DNA
USDA	United States Department of Agriculture

A. GENERAL INFORMATION ON THE APPLICATION

1. Purpose of the application

Dow AgroSciences LLC (herein referred to as "DAS") has developed transgenic soybean plants that are tolerant to the herbicides 2,4-Dichlorophenoxyacetic acid (2,4-D) and glufosinate. DAS-68416-4 is the unique identifier of these plants, 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, 2002).

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 soybean commodities and is consistent with Dow AgroSciences corporate policy of ensuring full regulatory compliance. As a result of this application, Dow AgroSciences Australia Ltd seeks an amendment of Standard 1.5.2 by inserting: food derived from Herbicide Tolerant -68416-4 soybean 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-68416-4 soybean 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-68416-4 soybean will allow overseas growers to effectively 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., 2007).

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 soybeans 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.

DAS-68416-4 soybeans also provide tolerance to glufosinate herbicides. Glufosinate is a non-selective, contact herbicide that controls a broad spectrum of annual and perennial grasses and broadleaf weeds. GLufosinate – tolerant soybeans have been established in the global market place for several years. The tolerance to glufosinate allows use of an additional mode of action as part of effective herbicide resistance management strategies. Glufosinate herbicides can also be used as selection agents in breeding nurseries to select herbicide-tolerant plants to maintain seed trait purity.

b. Safety of the genetically modified food

The donor organism, *Delftia acidovorans* (formerly designated as *Pseudomonas acidovorans* and *Comamonas acidovorans)* is a non glucose-fermenting, gram-negative, non spore-forming rod present in soil, fresh water, activated sludge, and clinical specimens (von Gravenitz 1985, Tamaoka *et al.* 1987, Wen *et al.*, 1999).

Delftia acidovorans can be used to transform ferulic acid into vanillin and related flavour metabolites (Toms and Wood, 1970; Ramachandra Rao and Ravishankar, 2000; Shetty *et al.*, 2006). This utility has led to a history of safe use for *Delftia acidovorans* in the food processing industry. For example, see US Patent 5,128,253 "Bioconversion process for the production of vanillin" issued on July 7, 1992 to Kraft General Foods (Labuda *et al.*, 1992).

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 soybean 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-68416-4 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, South Africa, Brazil, Argentina, Mexico and China. 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-68416-4. The trade implications however are clear since non-approval by FSANZ would impose a trade restriction on DAS-68416-4 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-68416-4 soybean is a transgenic soybean product that provides tolerance to the herbicides 2,4-Dichlorophenoxyacetic acid (2,4-D) and glufosinate.

DAS-68416-4 soybean was developed using *Agrobacterium*-mediated transformation to introduce the *aad-12* and *pat* genes into soybean.

Two gene expression cassettes were present in the pDAB4468 vector for insertion into soybeans. The *aad-12* expression cassette contained in the T-DNA insert of pDAB4468 is designed to express the plant-optimized aryloxyalkanoate dioxygenase (*aad-12*) gene that encodes the AAD-12 protein. The *aad-12* gene was isolated from *Delftia acidovorans* 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 native and plant-optimized DNA sequences of *aad-12* are 79.7% identical. The *aad-12* gene encodes a protein of 293 amino acids that has a molecular weight of approximately 32 kDa. The insertion of *aad-12* into soybean plants confers tolerance to herbicides such as 2,4-D by production of the aryloxyalkanoate dioxygenase-12 enzyme (AAD-12).

The *pat* gene expressing the PAT protein was derived from *Streptomyces viridochromogenes* and has been used in numerous other transgenic crops produced in the U.S. (FDA 1998, FDA 2001, FDA 2003, FDA 2004a, FDA 2004b, FDA 2004c, USDA 1996, USDA 2001, USDA 2004, USDA 2005)

DAS-68416-4 soybean 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 soybean is known as Event DAS-68416-4. No commercial name has yet been identified.

c. Food Identity

There is no intention to market food items containing soybean derived from DAS-68416-4 with specific brands or names.

d. Products containing the food or food ingredients.

Refer to the OECD Consensus Document on the Biology of *Glycine max* (L.) Merr. (Soybean), 2000, for information related to the following aspects of soybean biology:

- general description, including taxonomy, morphology, and the uses of soybean as a crop plant
- agronomic practices
- centres of origin
- reproductive biology
- cultivated *Glycine max* as a volunteer weed
- ability to cross inter-species/genus, introgressions into relatives, and interactions with other organisms
- summary of the ecology of *Glycine max*

Soybeans are crushed to form two derivatives, meal and oil. The main product derived from soybean that is used in Australia is meal for animal feed. Soybean meal is particularly high in protein and is the preferred meal for pig and poultry productions systems. It is also used in the production of pet food. Domestic production of soybean in Australia (~30,000t) and New Zealand is supplemented by import of soybean-based products, predominantly meal, to meet the requirements by the animal industry. Soybean oil is also imported for table oil use or processed into margarines or mayonnaise and used by the food industry or the consumer.

2. History and Use of the Host and Donor Organisms

Part B Section 2 DAS Reports

Song, P., Cruse, J., Poorbaugh, J., Thomas, A. (2009) Molecular Characterisation of AAD-12 Soybean Event DAS-68416-4 within a Single Segregating Generation. Dow AgroSciences LLC Study ID 091071.

Song, P., Cruse, J., Thomas, A., (2009). Molecular Characterisation of AAD-12 Soybean Event DAS-68416-4. Dow AgroSciences LLC Study ID: 081087.

a. Donor Organism

The donor organisms of each of the genetic elements inserted into DAS-68416-4 are listed in **Table 1.** Soybean event DAS-68416-4 was generated by *Agrobacterium*-mediated transformation using the plasmid pDAB4468 (Figure 1). The T-DNA insert in the plasmid contains a synthetic, plant-optimized sequence of the *aad-12* gene from *Delftia acidovorans* and the *pat* gene from *Streptomyces viridochromogenes*

Figure).

Table 1. Genetic elements of the T-DNA insert from plasmid pDAB4468. (Song et al, 2009, Study ID 081087).

Location on T-DNA insert of pDAB4468[1]	Genetic Element	Size (base pairs)	Description
1−24	T-DNA Border B	24	Transferring DNA sequences
25−160	Intervening sequence	136	Sequence from Ti plasmid pTi15955 (Barker *et al.*, 1983)
161−1326	RB7-MAR	1166	Matrix attachment region (MAR) from *Nicotiana tabacum* (Hall *et al.*, 1991)
1327−1421	Intervening sequence	95	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
1422−2743	AtUbi10	1322	*Arabidopsis thaliana* polyubiquitin UBQ10 comprising the promoter, 5' untranslated region and intron (Norris *et al.,* 1993)

2744−2751	Intervening sequence	8	Sequence used for DNA cloning
2752−3633	*aad-12*	882	Synthetic, plant-optimized version of an aryloxyalkanoate dioxygenase gene from *Delftia acidovorans* (Wright *et al*., 2007)
3634−3735	Intervening sequence	102	Sequence used for DNA cloning
3736−4192	AtuORF23	457	3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 23 (ORF23) of *Agrobacterium tumefaciens* pTi15955 (Barker *et al*., 1983)
4193−4306	Intervening sequence	114	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
4307−4819	CsVMV	513	Promoter and 5' untranslated region derived from the cassava vein mosaic virus (Verdaguer *et al.*, 1996)
4820−5371	*pat*	552	Synthetic, plant-optimized version of phosphinothricin N-acetyl transferase (PAT) gene, isolated from *Streptomyces viridochromogenes* (Wohlleben *et al.,* 1988)
5372−5484	Intervening sequence	113	Sequence from plasmid pCRI2.1 (Invitrogen Cat. No. K205001) and multiple cloning sites
5485−6188	AtuORF1	704	3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of *Agrobacterium tumefaciens* pTi15955 (Barker *et al.*, 1983)
6189−6416	Intervening sequence	228	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
6417−6440	T-DNA border A	24	Transferring DNA sequence
6441−6459	Intervening sequence	19	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
6460−6483	T-DNA border A	24	Transferring DNA sequence
6484−6770	Intervening sequence	287	Sequence from Ti plasmid pTi15955 (Barker *et al.*, 1983)
6771−6794	T-DNA border A	24	Transferring DNA sequence

[1] Base pair position.

Figure 1. Plasmid map of pDAB4468. (Song et al, 2009, Study ID 081087).

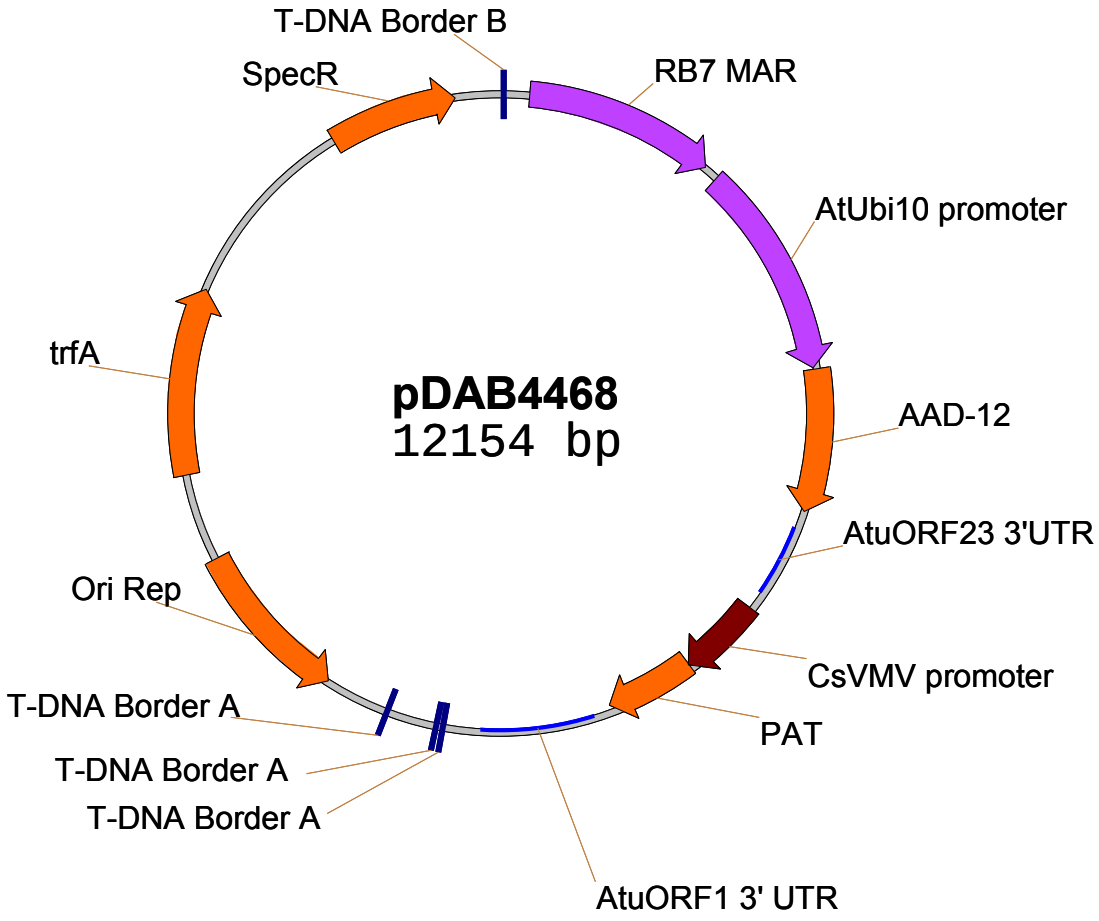
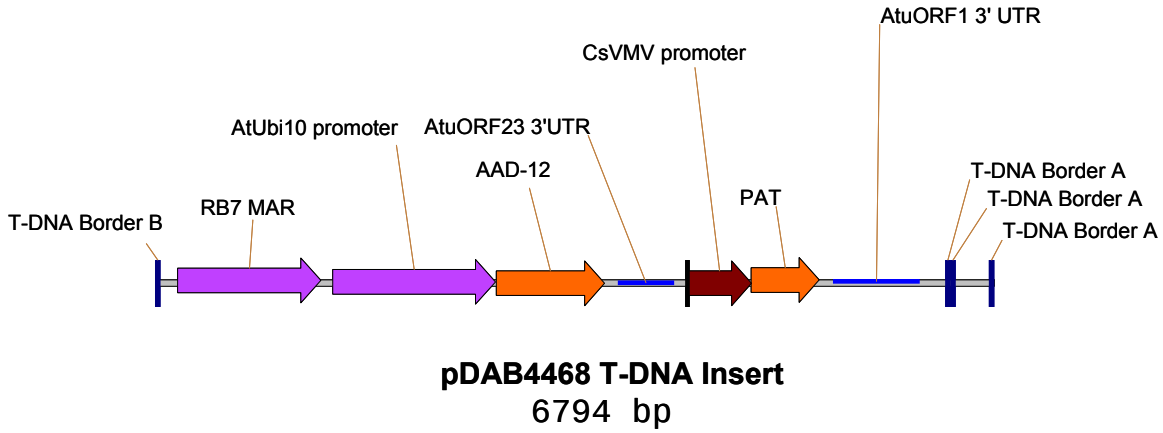


Figure 2. Diagram of T-DNA insert in plasmid pDAB4468. (Song et al, 2009, Study ID 081087).



Two gene expression cassettes were present in the pDAB4468 vector for insertion into soybeans. The *aad-12* expression cassette contained in the T-DNA insert of pDAB4468 is designed to express the plant-optimized aryloxyalkanoate dioxygenase (*aad-12*) gene that encodes the AAD-12 protein. The *aad-12* gene was isolated from *Delftia acidovorans* 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 native and plant-optimized DNA sequences of *aad-12* are 79.7% identical. The *aad-12* gene encodes a protein of 293 amino acids that has a molecular weight of approximately 32 kDa. The insertion of *aad-12* into soybean plants confers tolerance to herbicides such as 2,4-D by production of the aryloxyalkanoate dioxygenase-12 enzyme (AAD-12).

Expression of *aad-12* in the T-DNA insert of pDAB4468 is controlled by the AtUbi10 promoter from *Arabidopsis thaliana* and AtuORF23 3' UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The AtUbi10 promoter is known to drive constitutive expression of the genes it controls (Norris *et al.,* 1993).

A matrix attachment region (MAR) of RB7 from *Nicotiana tabacum* was included at the 5' end of the *aad-12* PTU (plant transcriptional unit, includes promoter, gene, and terminator sequences) to potentially facilitate expression of the *aad-12* 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). A MAR was included at the 5' end of *aad-12* PTU to potentially increase the consistency of *aad-12* expression in transgenic plants.

The *pat* expression cassette contained in the T-DNA insert of pDAB4468 is designed to express the plant-optimized phosphinothricin *N*-acetyl transferase (*pat*) gene that encodes the PAT protein. The *pat* gene was isolated from *Streptomyces viridochromogenes* 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 *pat* gene into soybean genome confers tolerance to glufosinate and was used as a selectable marker during the soybean transformation. The *pat* gene encodes a protein of 183 amino acids that has a molecular weight of approximately 21 kDa. The *pat* gene has been widely used both as a selectable marker and herbicide tolerance trait in products previously reviewed by FDA and

deregulated by USDA (e.g., FDA 1998, FDA 2001, FDA 2003, FDA 2004a, FDA 2004b, FDA 2004c, USDA 1996, USDA 2001, USDA 2004, USDA 2005)

Expression of the *pat* gene in the T-DNA insert of pDAB4468 is controlled by the CsVMV promoter from cassava vein mosaic virus and AtuORF1 3' UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The cassava vein mosaic virus is a double stranded DNA virus which infects cassava plants (*Manihot esculenta* Crantz) and has been characterized as a plant pararetrovirus belonging to the caulimovirus subgroup. The CsVMV promoter is known to drive constitutive expression of the genes it controls (Verdaguer *et al.*, 1996).

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

b. Host Organism

Soybean (*Glycine max)* is a diploidized tetraploid (2n=40), in the family *Leguminosae,* subfamily *Papilionoideae,* tribe *Phaseoleae,* genus *Glycine* Willd, subgenus *Soja* (Moench). It is an erect, bushy herbaceous annual that can reach a height of 1.5m. Three types of growth habit can be found amongst soybean cultivars: determinate, semi-determinate and indeterminate. Determinate growth is characterized by the cessation of vegetative activity of the terminal bud when it becomes an inflorescence at both auxiliary and terminal racemes. Indeterminate genotypes continue vegetative activity throughout the flowering period. Semi-determinate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period.

The primary leaves are unifoliate, opposite and ovate, the secondary leaves are trifoliolate and alternate, and compound leaves with four or more leaflets are occasionally present. The nodulated root system consists of a taproot from which emerges a lateral root system. The plans of most cultivars are covered with fine trichomes, but some glabrous types exist. The papilionaceous flowers consist of a tubular alyx of five sepals, a corolla of 5 petals, one pistil and nine fused stamens with a single separate posterior stamen. The pod is straight or slightly curved, varies in length from 2-7cm and consists of two halves of a single carpel which are joined by a dorsal and ventral suture. The shape of the seed, usually oval, can vary amongst cultivars from spherical to elongate and flattened. The stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation and cross pollination is usually less than one percent. A soybean plant can produce as many as 400 pods, with 2 to 20 pods at a single node. Each pod contains 1-5 seeds. Neither the seed pod, nor the seed, has morphological characteristics that encourage animal transportation.

Soybean is primarily grown for the production of seed, has a multitude of uses in the food and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use.

The United States, Brazil, Argentina and China produced 200 million metric tonnes of soybeans in 2005 which was 90% of the total global production.

Characterization of the recipient soybean line

The publicly available cultivar ‘Maverick’ was used as the recipient line for the generation of DAS-68416-4 soybean.

Maverick was originally developed by the Missouri and Illinois Agricultural Experiment Stations at the Universities of Missouri and Illinois, respectively, and released in 1996 (Sleper *et al.*, 1998). Maverick was developed because of its resistance to the soybean cyst nematode (SCN) and higher yield compared with SCN-resistant cultivars of similar maturity. Maverick is classified as a late Group III maturity (relative maturity 3.8). Maverick has purple flowers, grey pubescence, brown pods at maturity, and dull yellow seed with buff hila. Maverick is resistant to phytophthora rot but is susceptible to brown stem rot and sudden death syndrome.

3. Nature of the Genetic Modification

Part B Section 3 DAS Reports

Song, P., Cruse, J., Poorbaugh, J., Thomas, A. (2009) Molecular Characterisation of AAD-12 Soybean Event DAS-68416-4 within a Single Segregating Generation. Dow AgroSciences LLC Study ID 091071.

Song, P., Cruse, J., Thomas, A., (2009). Molecular Characterisation of AAD-12 Soybean Event DAS-68416-4. Dow AgroSciences LLC Study ID: 081087.

a. Transformation Method

Transgenic soybean (*Glycine max*) DAS-68416-4 was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. The disarmed *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 2006), carrying the binary vector pDAB4468 was used to initiate transformation.

Agrobacterium-mediated transformation was carried out using a modified procedure of Zeng *et al.* (2004). Briefly, soybean seeds (cv Maverick) were germinated on basal media and cotyledonary nodes were isolated and infected with *Agrobacterium.* Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium*. Glufosinate selection was employed to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

Terminal leaflets of selected plantlets were leaf painted with glufosinate to screen for putative transformants. The screened plantlets were transferred to the greenhouse, allowed to acclimate and then leaf-painted with glufosinate to reconfirm tolerance. Surviving plantlets were deemed to be putative transformants. The screened plants were sampled and molecular analyses for the confirmation of the selectable marker gene and/or the gene of interest were carried out. T_0 plants were allowed to self fertilize in the greenhouse to give rise to T_1 seed.

Figure 7 shows the steps used to develop DAS-68416-4 soybean.

Figure 8 is a breeding diagram for DAS-68416-4 soybean including identification of the generations used in the various safety assessment studies.

Figure 3. Development of DAS-68416-4 soybean.

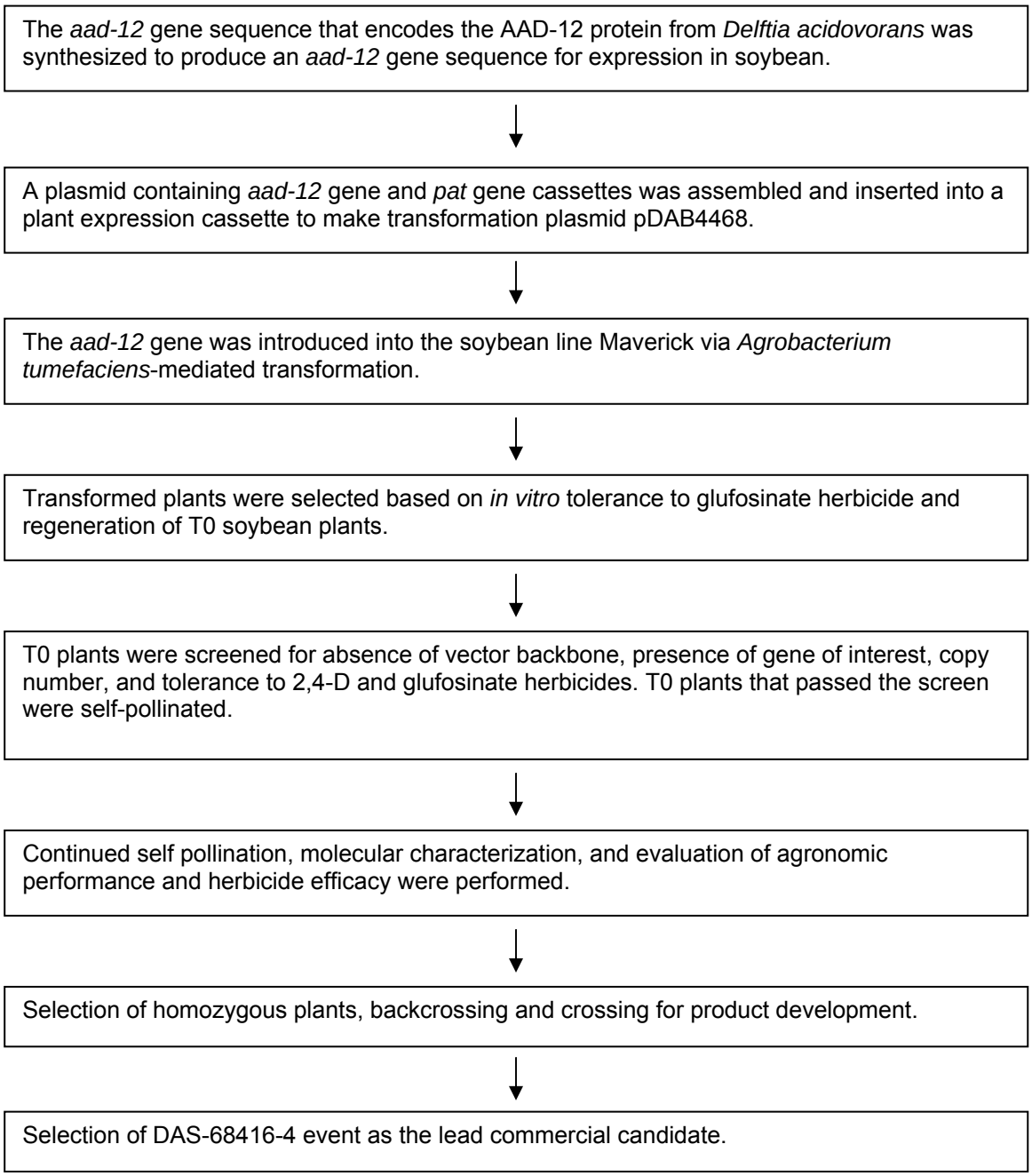
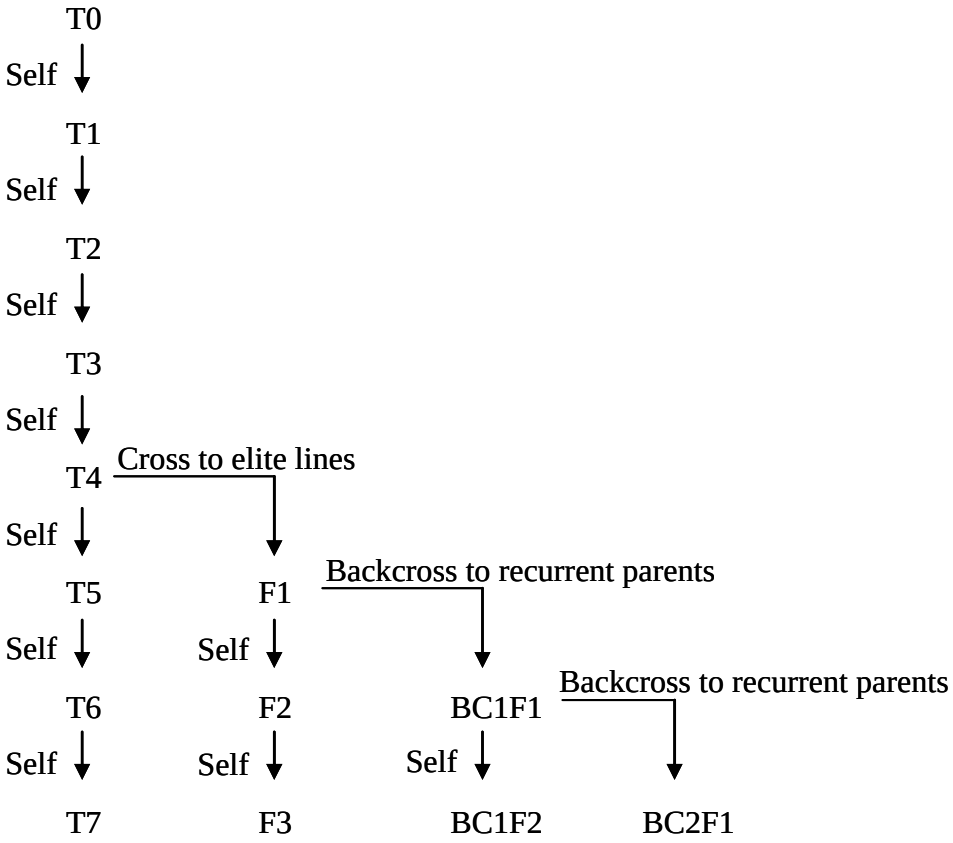


Figure 4. Breeding diagram of DAS-68416-4 soybean.



Analysis	PBN Section(s)	DAS-68416-4 Soybean Generation Used	Control
Molecular Analysis	**4.3**	**T3, T4, T5**	**Maverick**
Segregation Analysis	**4.4**	**F2**	**Maverick**
Protein Characterization	**6**	**T5**	**Maverick**
Protein Expression	**6**	**T4**	**Maverick**
Composition	**7.3**	**T4**	**Maverick**

b. Bacteria used for manipulation

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

c. Gene Construct and Vectors

Soybean event DAS-68416-4 was generated by *Agrobacterium*-mediated transformation using the plasmid pDAB4468 (**Figure 1**). The T-DNA insert in the plasmid contains a synthetic, plant-optimized sequence of the *aad-12* gene from *Delftia acidovorans* and the *pat* gene from *Streptomyces viridochromogenes* (**Figure 2**).

d. Molecular Characterisation

Molecular characterization of event DAS-68416-4 was conducted by Southern blot analysis in study IDs 081087 and 091071 performed by Song, P., Cruse, J and Thomas, A. and Song, P., Cruse, J., Poorbaugh A., and Thomas A, respectively (2009). The results demonstrate that the transgene insert in soybean event DAS-68416-4 occurred as a simple integration of the T-DNA insert from plasmid pDAB4468, including a single, intact copy of the *aad-12* and *pat* expression cassettes. The event is stably integrated and inherited across and within breeding generations, and no plasmid backbone sequences are present in DAS-68416-4 soybean.

Detailed Southern blot analysis was conducted using probes specific to the gene coding sequences, promoters, terminators, and other regulatory elements contained in the pDAB4468 transformation plasmid. The locations of each probe on the pDAB4468 plasmid are described in **Table 1** and shown in **Figure 1**. The expected and observed fragment sizes with specific digest and probe combinations, based on the known restriction enzyme sites of the pDAB4468 plasmid are shown in **Table 3**, **Figure 6** and **Figure 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 T-DNA insert of pDAB4468 and b) border fragments in which a known enzyme site is located within the T-DNA insert and a second site is located in the soybean genome flanking the insert. 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 determine the number of DNA insertions and to specifically identify the event. Please refer to Attachment 2 – Confidential Commercial Information for data relating to the sequencing of the insert and border regions.

Genomic DNA for Southern blot analysis was prepared from leaf material of individual DAS-68416-4 soybean plants from four distinct breeding generations. Genomic DNA from leaves of non-transgenic variety Maverick was used as the control material. Plasmid DNA of pDAB4468 added to genomic DNA from the conventional control served as the positive control for the Southern blot analysis. Materials and methods used for the Southern analyses are described further on in this section. .

The expected restriction fragments of the inserted DNA are shown in **Figure 7**. Southern blot analysis showed that event DAS-68416-4 contains a single intact copy of the *aad-12* and *pat* expression cassettes integrated at a single locus). No vector backbone sequences were detected in event DAS-68416-4. The hybridization patterns across four sample sets representing three generations of DAS-68416-4 soybean (T3, T4, and T5) were identical, indicating that the insertion is stably integrated in the soybean genome. The inheritance of DAS-68416-4 soybean in segregating generations was investigated using Southern blot analysis, detection of the AAD-12 protein, and detection of the *aad-12* gene; all results confirmed the predicted inheritance of the transgene in a single locus.

Table 2. List of probes and their positions in plasmid pDAB4468. (Song et al, 2009, Study ID 081087).

Probe Name	Size (bp)	Location in pDAB4468
Flanking B	303	11894 – 42
RB7	1010	306 – 1315
AtUbi10	771	1411 – 2181
aad-12	882	2752 – 3633
AtuORF23	413	3762 – 4174
CsVMV	478	4332 – 4809
pat	552	4820 – 5371
AtuORF1	684	5474 – 6157
Flanking A	339	6793 – 7131
Ori Rep	1068	7111 – 8178
Backbone 2	1728	8157 – 9884
Backbone 1	1310	9854 – 11163
Spec R	789	11092 – 11880

Figure 5. **Location of probes on pDAB4468 used in Southern blot analysis of DAS-68416-4 soybean.** (Song et al, 2009, Study ID 081087).

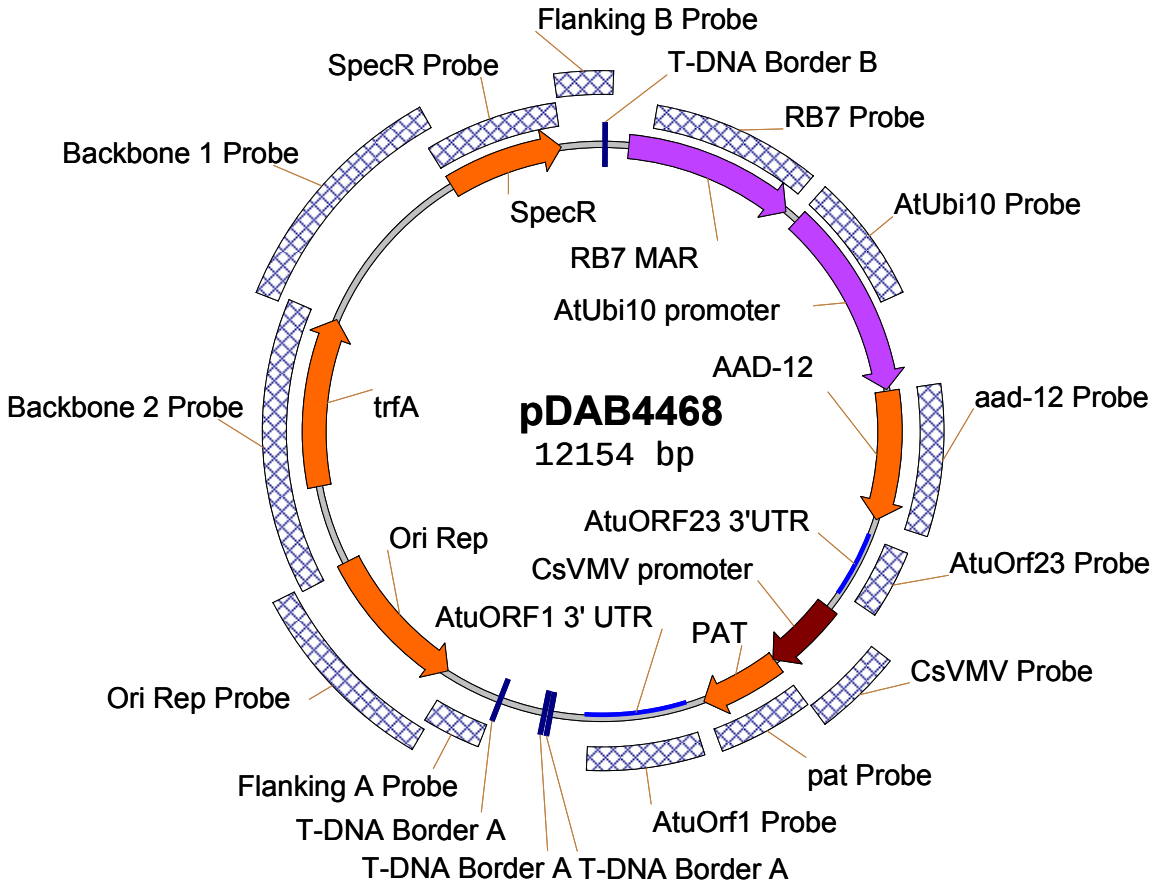


Table 3. Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-68416-4 soybean. (Song et al, 2009, Study ID 081087).

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
aad-12	*Nco* I	Plasmid pDAB4468	Figure 8, Figure 11	7957	7957
		DAS-68416-4	Figure 8, Figure 11	> 4043*	~5500*
		Control (Maverick)	Figure 8, Figure 11	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure , Figure 11	12146	12146
		DAS-68416-4	Figure , Figure 11	> 6229*	~8500*
		Control (Maverick)	Figure , Figure 11	none	none
	Nhe I/*Xho* I	Plasmid pDAB4468	Figure 10, Figure 11	12148	12148
		DAS-68416-4	Figure 10, Figure 11	> 6229*	~7200*
		Control (Maverick)	Figure 10, Figure 11	none	none
pat	*Nco* I	Plasmid pDAB4468	Figure , Figure	7957	7957
		DAS-68416-4	Figure , Figure	> 4043*	~5500*
		Control (Maverick)	Figure , Figure	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure , Figure	12146	12146
		DAS-68416-4	Figure , Figure	> 6229*	~8500*
		Control (Maverick)	Figure , Figure	none	none
	Nhe I/*Xho* I	Plasmid pDAB4468	Figure , Figure 19	12148	12148
		DAS-68416-4	Figure , Figure 19	> 6229*	~7200*
		Control (Maverick)	Figure , Figure 19	none	none
aad-12	*Pst* I (Release PTU)	Plasmid pDAB4468	Figure 12, Figure 13	2868	2868
		DAS-68416-4	Figure 12, Figure 13	2868	2868
		Control (Maverick)	Figure 12, Figure 13	none	none
AtUbi10		Plasmid pDAB4468	Figure 14, Figure	2868	2868
		DAS-68416-4	Figure 14, Figure 13	2868	2868
		Control (Maverick)	Figure 14, Figure 13	none	none
AtuORF23		Plasmid pDAB4468	Figure 15, Figure 13	2868	2868
		DAS-68416-4	Figure 15, Figure 13	2868	2868
		Control (Maverick)	Figure 15, Figure 13	none	none
pat	*Pst* I/*Xho* I (Release PTU)	Plasmid pDAB4468	Figure 20, Figure 21	1928	1928
		DAS-68416-4	Figure 20, Figure 21	1928	1928
		Control (Maverick)	Figure 20, Figure 21	none	none
CsVMV		Plasmid pDAB4468	Figure 22, Figure 21	1928	1928
		DAS-68416-4	Figure 22, Figure 21	1928	1928
		Control (Maverick)	Figure 22, Figure 21	none	none
AtuORF1		Plasmid pDAB4468	Figure 23, Figure 21	1928	1928
		DAS-68416-4	Figure 23, Figure 21	1928	1928
		Control (Maverick)	Figure 23, Figure 21	none	none
RB7	*Bam*H I/*Nco* I	Plasmid pDAB4468	Figure 24, Figure 26	2617	2617
		DAS-68416-4	Figure 24, Figure 26	2617	2617

		Control (Maverick)	Figure 24, Figure 26	none	none
AtUbi10		Plasmid pDAB4468	Figure 25, Figure 26	2617	2617
		DAS-68416-4	Figure 25, Figure 26	2617	2617
		Control (Maverick)	Figure 25, Figure 26	none	none

Table 3. Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-68416-4 soybean cont. (Song et al, 2009, Study ID 081087).

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
Flanking A	*Nco* I	Plasmid pDAB4468	Figure 27, Figure 31	7957	7957
		DAS-68416-4	Figure 27, Figure 31	none	none
		Control (Maverick)	Figure 27, Figure 31	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 29, Figure 31	12146	12146
		DAS-68416-4	Figure 29, Figure 31	none	none
		Control (Maverick)	Figure 29, Figure 31	none	none
Backbone1	*Nco* I	Plasmid pDAB4468	Figure 27, Figure 31	4197, 7957	4197, 7957
		DAS-68416-4	Figure 27, Figure 31	none	none
		Control (Maverick)	Figure 27, Figure 31	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 29, Figure 31	12146	12146
		DAS-68416-4	Figure 29, Figure 31	none	none
		Control (Maverick)	Figure 29, Figure 31	none	none
SpecR	*Nco* I	Plasmid pDAB4468	Figure 27, Figure 31	4197	4197
		DAS-68416-4	Figure 27, Figure 31	none	none
		Control (Maverick)	Figure 27, Figure 31	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 29, Figure 31	12146	12146
		DAS-68416-4	Figure 29, Figure 31	none	none
		Control (Maverick)	Figure 29, Figure 31	none	none
Flanking B	*Nco* I	Plasmid pDAB4468	Figure , Figure 32	4197	4197
		DAS-68416-4	Figure , Figure 32	none	none
		Control (Maverick)	Figure , Figure 32	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 30, Figure 32	12146	12146
		DAS-68416-4	Figure 30, Figure 32	none	none
		Control (Maverick)	Figure 30, Figure 32	none	none
Backbone2	*Nco* I	Plasmid pDAB4468	Figure , Figure 32	7957	7957
		DAS-68416-4	Figure , Figure 32	none	none
		Control (Maverick)	Figure , Figure 32	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 30, Figure 32	12146	12146
		DAS-68416-4	Figure 30, Figure 32	none	none
		Control (Maverick)	Figure 30, Figure 32	none	none
Ori-Rep	*Nco* I	Plasmid pDAB4468	Figure , Figure 32	7957	7957
		DAS-68416-4	Figure , Figure 32	none	none
		Control (Maverick)	Figure , Figure 32	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 30, Figure 32	12146	12146
		DAS-68416-4	Figure 30, Figure 32	none	none
		Control (Maverick)	Figure 30, Figure 32	none	none

Note: * These bands include border region of soybean genome;

1. Expected fragment sizes are based on the plasmid map of the pDAB4468 and its T-DNA insert as shown in Figure 6 and Figure 7,
2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker II fragments. Due to the incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

Figure 6. Plasmid map of pDAB4468 with restriction enzyme sites used for Southern blot analysis. (Song et al, 2009, Study ID 081087).

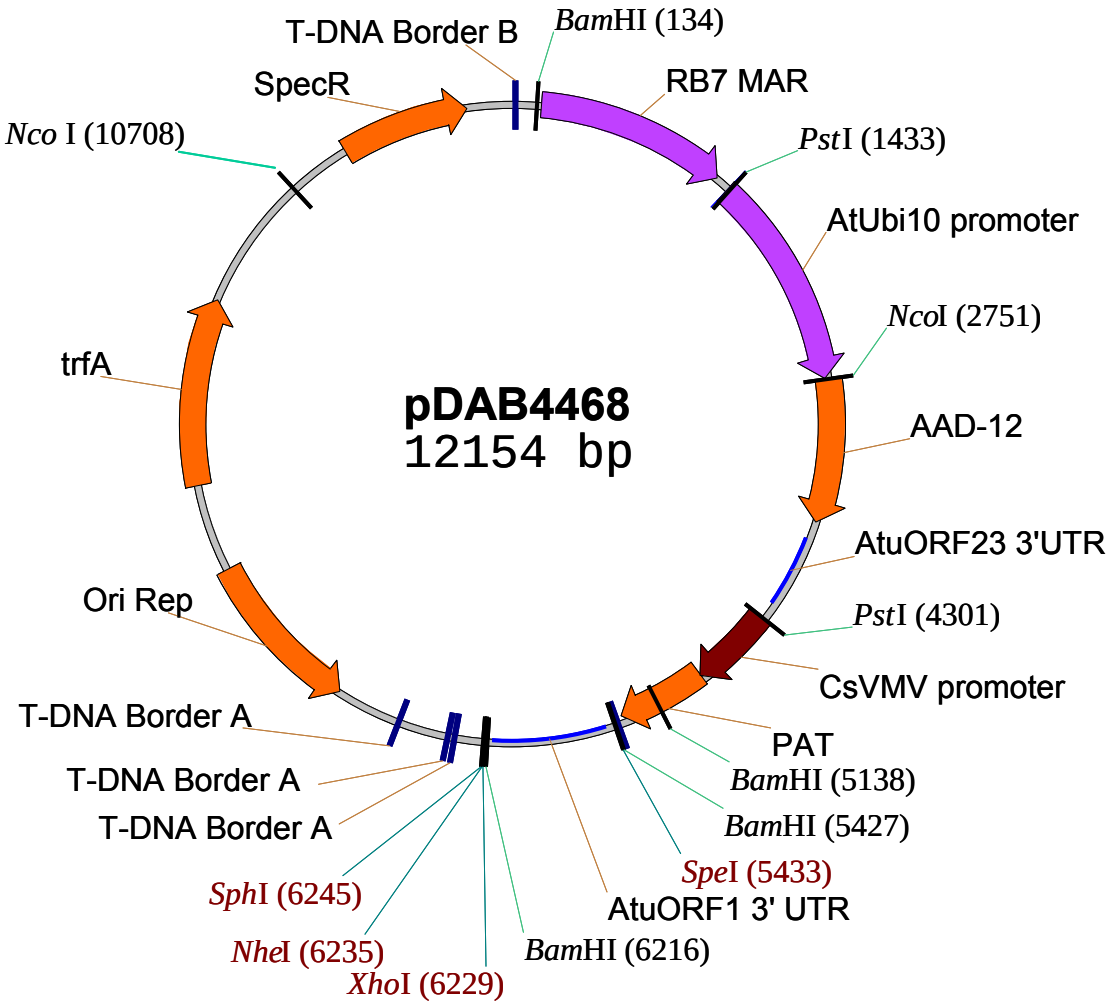
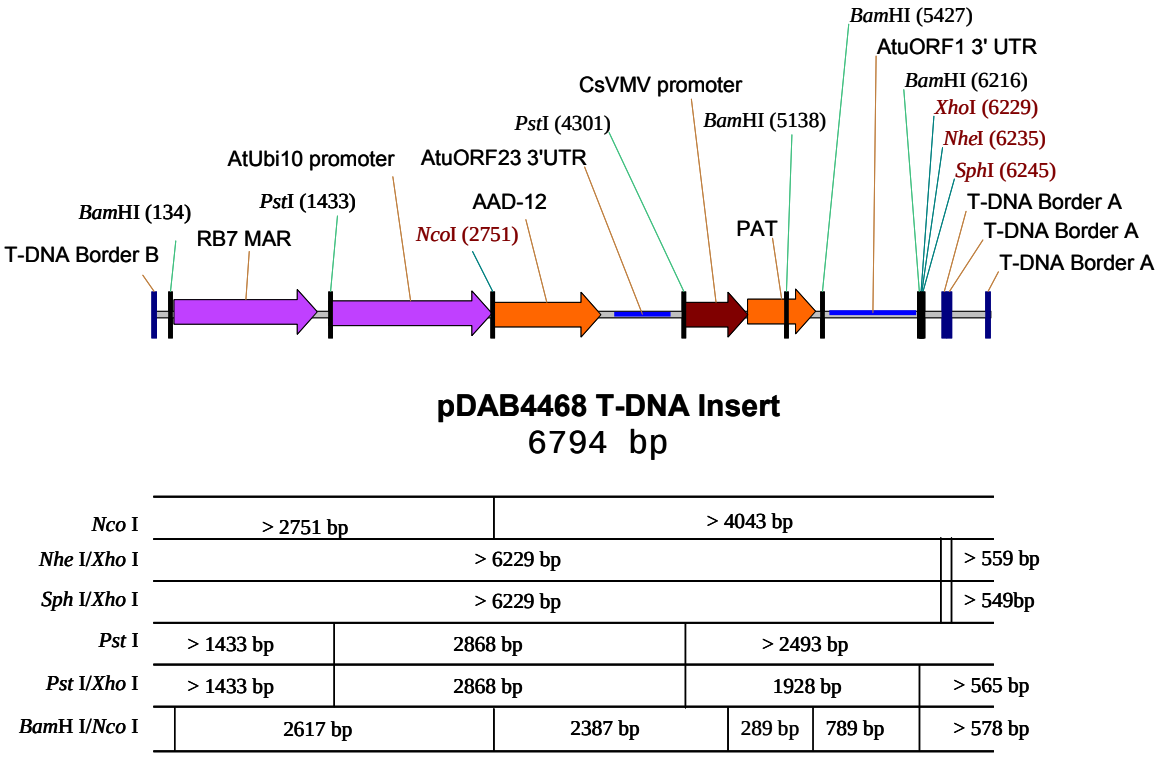


Figure 7. pDAB4468 T-DNA insert, restriction enzymes used in DNA digestion and expected hybridization bands. (Song et al, 2009, Study ID 081087).



Analysis of the aad-12 Gene

To characterize the *aad-12* gene insert in event DAS-68416-4, restriction enzymes *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I were used. These enzymes possess unique restriction sites in the pDAB4468 T-DNA insert. Border fragments of >4043 bp, >6229 bp, >6229 bp were predicted to hybridize with the *aad-12* gene probe following digestion with *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes respectively (**Table 3**). The results showed single hybridization bands of ~5500 bp, ~8500 bp and ~7200 bp respectively when *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes were used, indicating a single insertion site of *aad-12* in the soybean genome of event DAS-68416-4 (**Figure 8, Figure 9, Figure 10, Figure 11)**. An enzyme digestion with *Pst* I was conducted to release a PTU (plant transcription unit) fragment of 2868 bp which contains the AtUbi10 promoter, *aad-12* gene, and AtuORF23 terminator sequences. The predicted 2868 bp fragment was observed following the *Pst* I digestion and hybridization with *aad-12* probe (**Figure 12, Figure 13A**). Results obtained from the individual and double enzyme digestions indicated that a single copy of an intact *aad-12* expression cassette from pDAB4468 was inserted into the soybean genome of event DAS-68416-4 as shown in the restriction map in **Figure 7**.

Analysis of the AtUbi10 Promoter

Restriction enzyme *Pst* I was used to characterize the AtUbi10 promoter region for *aad-12* in event DAS-68416-4. *Pst* I digestion was expected to release a PTU (plant transcription unit) fragment of 2868 bp which contains the AtUbi10 promoter, *aad-12* gene, and AtuORF23 terminator sequences. The predicted 2868 bp fragment was observed following the *Pst* I digestion and hybridization with AtUbi10 promoter probe (**Figure 13B, Figure 14**). The AtUbi10 promoter was further characterized with a double digestion of *Bam*H I and *Nco* I which releases a fragment of 2617bp containing AtUbi10 promoter and RB7 MAR element. The predicted 2617 bp fragment was detected following the

enzyme digestion and hybridization with AtUbi10 promoter probe (**Figure 25, Figure 26B**). Results obtained with *Pst* I or *Bam*H I/*Nco* I digestion of the DAS-68416-4 sample followed by AtUbi10 promoter probe hybridization further confirmed that a single copy of an intact *aad-12* PTU from plasmid pDAB4468, along with a RB7 MAR element at its 5' end, was inserted into the soybean genome of event DAS-68416-4.

Analysis of the AtuORF23 3'UTR

The terminator sequence, AtuORF23, for *aad-12* in event DAS-68416-4 was characterized using *Pst* I digestion, followed by hybridization of AtuORF23 probe. *Pst* I was expected to release a PTU (plant transcription unit) fragment of 2868 bp which contains the AtUbi10 promoter, *aad-12* gene, and AtuORF23 terminator sequences. The predicted 2868 bp fragment was observed following the enzyme digestion and hybridization with AtuORF23 probe (**Figure 13C, Figure 15**). Results obtained with *Pst* I digestion of the DAS-68416-4 sample followed by AtuORF23 probe hybridization further confirmed that a single copy of an intact *aad-12* PTU from plasmid pDAB4468 was inserted into the soybean genome of event DAS-68416-4.

Analysis of the pat Gene

To characterize the *pat* gene insert in event DAS-68416-4, restriction enzymes *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I were used. These enzymes possessed unique restriction sites in the pDAB4468 T-DNA insert. Border fragments of >4043 bp, >6229 bp, >6229 bp were predicted to hybridize with the *pat* gene probe following digestion with *Nco* I, *Sph* I/*Xho* I and *Nhe* I/*Xho* I enzymes respectively (**Table 3**). The results showed single hybridization bands of ~5500 bp, ~8500 bp and ~7200 bp respectively when *Nco* I, *Sph* I/*Xho* and *Nhe* I/*Xho* I enzymes were used, indicating a single site of *pat* gene insertion in the soybean genome of event DAS-68416-4 (**Figure 16, Figure 17, Figure 18, Figure 19**). An enzyme digestion with *Pst* I/*Xho* I was conducted to release a PTU (plant transcription unit) fragment of 1928 bp which contained the CsVMV promoter, *pat* gene, and AtuORF1 terminator sequences. The predicted 1928 bp fragment was observed following the enzyme digestion and hybridization with *pat* probe (**Figure 20, Figure 21A**). Results obtained from the individual and double enzyme digestions indicated that a single copy of an intact *pat* expression cassette from pDAB4468 was inserted into the soybean genome of event DAS-68416-4 as shown in the restriction map in **Figure 7**.

Analysis of the CsVMV Promoter

Restriction enzyme combination of *Pst* I/*Xho* I was used to characterize the CsVMV promoter region for *pat* in event DAS-68416-4. *Pst* I/*Xho* I digestion was expected to release a PTU (plant transcription unit) fragment of 1928 bp which contains the CsVMV promoter, *pat* gene, and AtuORF1 terminator sequences. The predicted 1928 bp fragment was observed following the enzyme digestion and hybridization with CsVMV promoter probe (**Figure 21B, Figure 22**). Results obtained with *Pst* I/*Xho* I digestion of the DAS-68416-4 sample followed by CsVMV promoter probe hybridization further confirmed that a single copy of an intact *pat* PTU from plasmid pDAB4468 was inserted into the soybean genome of event DAS-68416-4.

Analysis of the AtuORF1 3'UTR

The terminator sequence, AtuORF1, for *pat* in event DAS-68416-4 was characterized using *Pst* I/*Xho* I double digestion, followed by hybridization of AtuORF1 probe. The double digestion of *Pst* I/*Xho* I was expected to release a PTU (plant transcription unit) fragment of 1928 bp which contained the CsVMV promoter, *pat* gene, and AtuORF1 terminator sequences. The predicted 1928 bp fragment was observed following the enzyme digestion and hybridization with AtuORF1 probe (**Figure 21C, Figure 23**). Results obtained with *Pst* I/*Xho* I double digestion of the DAS-68416-4 sample followed by AtuORF1 probe hybridization further confirmed that a single copy of an intact *pat* PTU from plasmid pDAB4468 was inserted into the soybean genome of event DAS-68416-4.

Analysis of the RB7 MAR

Restriction enzyme combination of *BamH* I and *Nco* I was selected to characterize the RB7 MAR elements from the T-DNA insert in pDAB4468 (**Table 3**). A double digestion with *BamH* I and *Nco* I was expected to release a fragment of 2617 bp containing the RB7 MAR and AtUbi10 promoter. The predicted 2617 bp fragment was observed following the double enzyme digestion and hybridization with RB7 MAR and Atubi10 probe, respectively (**Figure 24, Figure 26A, and Figure 26B**). Results obtained with *BamH* I/*Nco* I double digestion of the DAS-68416-4 sample followed by hybridization with RB7 MAR (**Figure 25, Figure 26B**) further confirmed that a single copy of an intact RB7 MAR, along with an intact *aad-12* PTU from plasmid pDAB4468, was inserted into the soybean genome of event DAS-68416-4.

Absence of Vector Backbone DNA

To verify that no plasmid vector backbone sequences exist in event DAS-68416-4, six probes covering the whole backbone region of pDAB4468 were used to hybridize the blots from digestions with *Nco* I and *Sph* I/*Xho* I (**Table 2, Figure 5**). For the T5 generation, a blot from digestion with *Nhe* I/*Xho* I was also hybridized with backbone probes. The probes were grouped into 2 sets by mixing them with equal ratio for hybridization purposes. Probe Set 1 included backbone1, flanking A, and *Spec*R, and Probe Set 2 included backbone 2, flanking B, and *Ori-Rep* (**Figure 5, Table 2**). The blots were hybridized with Probe Set 1, and then followed by Probe Set 2 after complete removal of previously deployed probes. No hybridization signals were detected in any sample across the T2 to T5 generations except for the positive controls, indicating no backbone sequences from pDAB4468 were incorporated into event DAS-68416-4.

Figure 8. Southern blot analysis of Nco I digest with aad-12 probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Nco *I and hybridized with* aad-12 *probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 15 was due to a larger amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).*

Lane	*Description*	*Lane*	*Description*
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

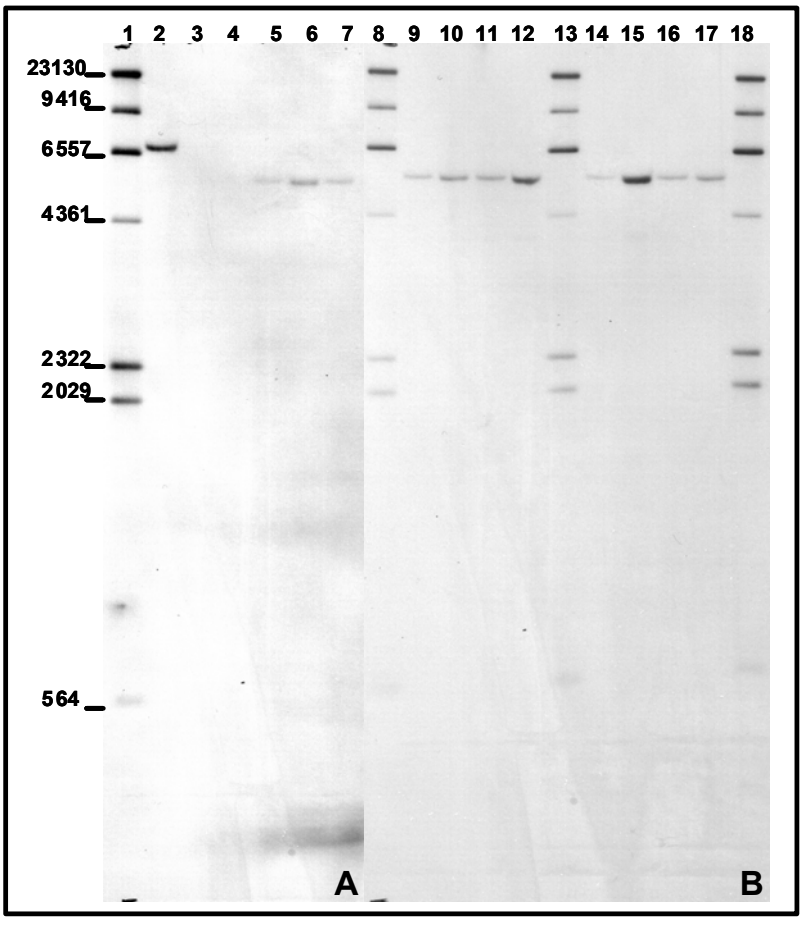


Figure 9. Southern blot analysis of *Sph* I/*Xho* I digest with *aad-12* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Sph *I/*Xho *I and hybridized with* aad-12 *probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 10 and 15 were due to a larger amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).*

Lane	*Description*	*Lane*	*Description*
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

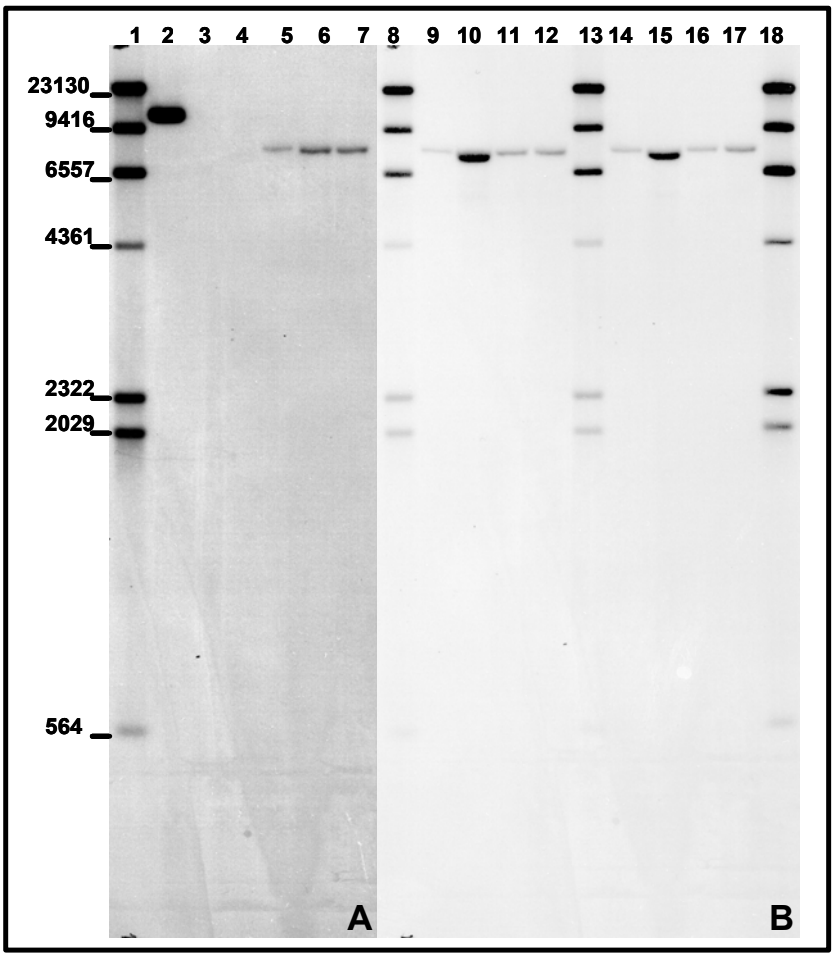


Figure 10. Southern blot analysis of *Nhe* I/*Xho* I digest with *aad-12* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Nhe *I/*Xho *I and hybridized with* aad-12 *probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 9 was due to a larger amount of DNA recovered after digestion).*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

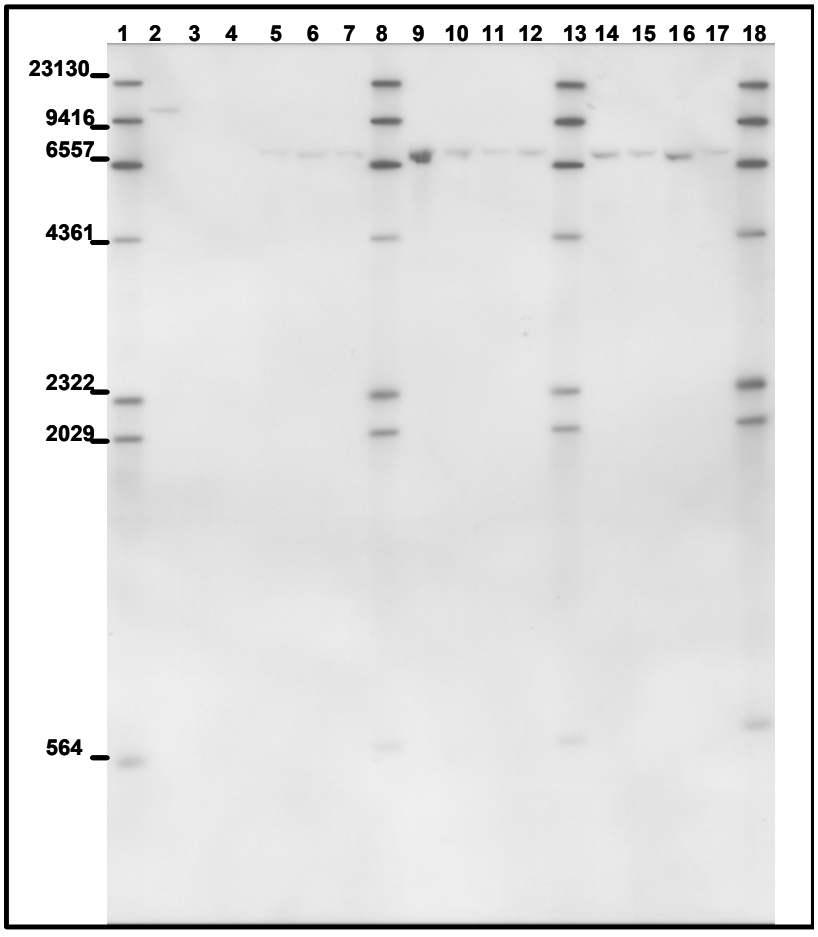


Figure 11. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with *aad-12* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 and the non-transgenic control was digested with Nco *I,* Sph *I/*Xho *I, and* Nhe *I/*Xho *I and hybridized with* aad-12 *probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 19 was due to a larger amount of DNA recovered after digestion).*

Lane	***Description***	***Enzyme***	***Lane***	***Description***	***Enzyme***
1	*DNA molecular marker (bp)*		*12*	*T5 #1*	Sph *I*/ Xho *I*
2	*pDAB4468 + control (Maverick) #2*	Nco *I*	*13*	*T5 #4*	
3	*control (Maverick) #2*		*14*	*T5 #6*	
4	*control (Maverick) #3*		*15*	*T5 #8*	
5	*T5 #1*		*16*	*pDAB4468 + control (Maverick) #2*	Nhe *I*/ Xho *I*
6	*T5 #4*		*17*	*control (Maverick) #2*	
7	*T5 #6*		*18*	*control (Maverick) #3*	
8	*T5 #8*		*19*	*T5 #1*	
9	*pDAB4468 + control (Maverick) #4*	Sph *I*/ Xho *I*	*20*	*T5 #4*	
10	*control (Maverick) #4*		*21*	*T5 #6*	
11	*control (Maverick) #5*		*22*	*T5 #8*	

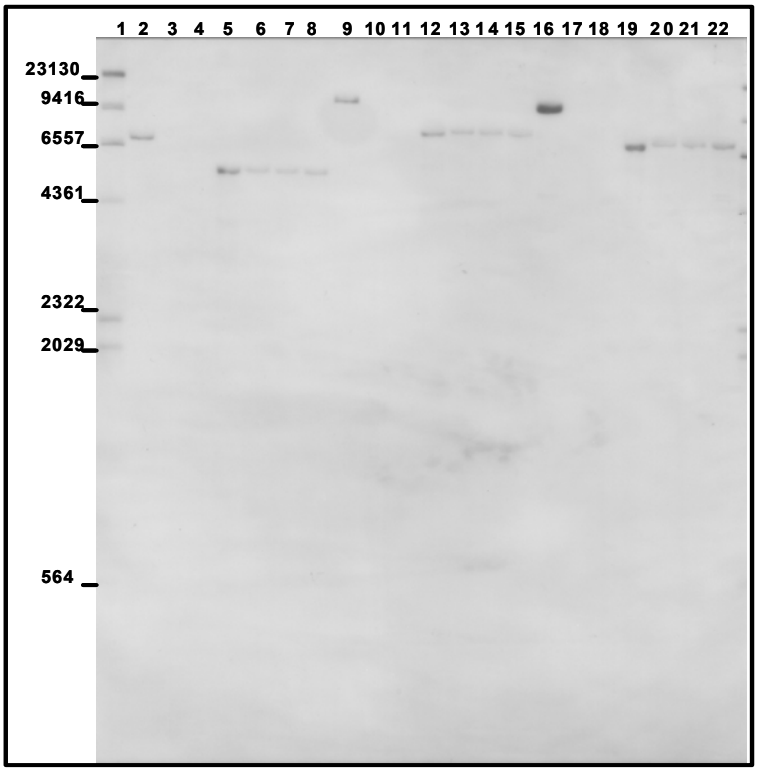


Figure 12. Southern blot analysis of *Pst* I digest with *aad-12* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Pst *I and hybridized with* aad-12 *probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).*

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T2 #2	14	T5 #2
6	T2 #7	15	T5 #4
7	T2 #8	16	T5 #5
8	DNA molecular marker (bp)	17	T5 #7
9	T3 #3	18	DNA molecular marker (bp)

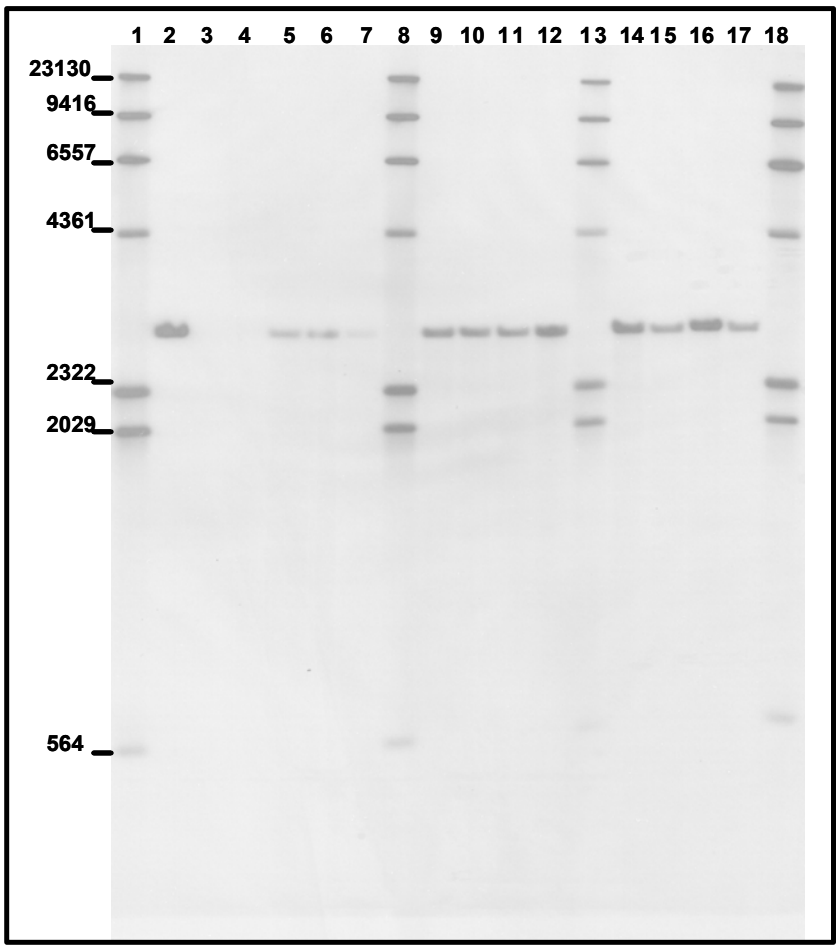


Figure 13. Southern blot analysis of *Pst* I digest of T5 generation with *aad-12*, AtUbi10, and AtuORF23 probes. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 generation and the non-transgenic control were digested with Pst *I and hybridized with* aad-12 *(Panel A), AtUbi10 (Panel B), and ORF23 probes (Panel C). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.*

Lane	***Description***
1	*DNA molecular marker (bp)*
2	*pDAB4468 + control (Maverick) #2*
3	*control (Maverick) #2*
4	*control (Maverick) #3*
5	*T5 #1*
6	*T5 #4*
7	*T5 #6*
8	*T5 #8*

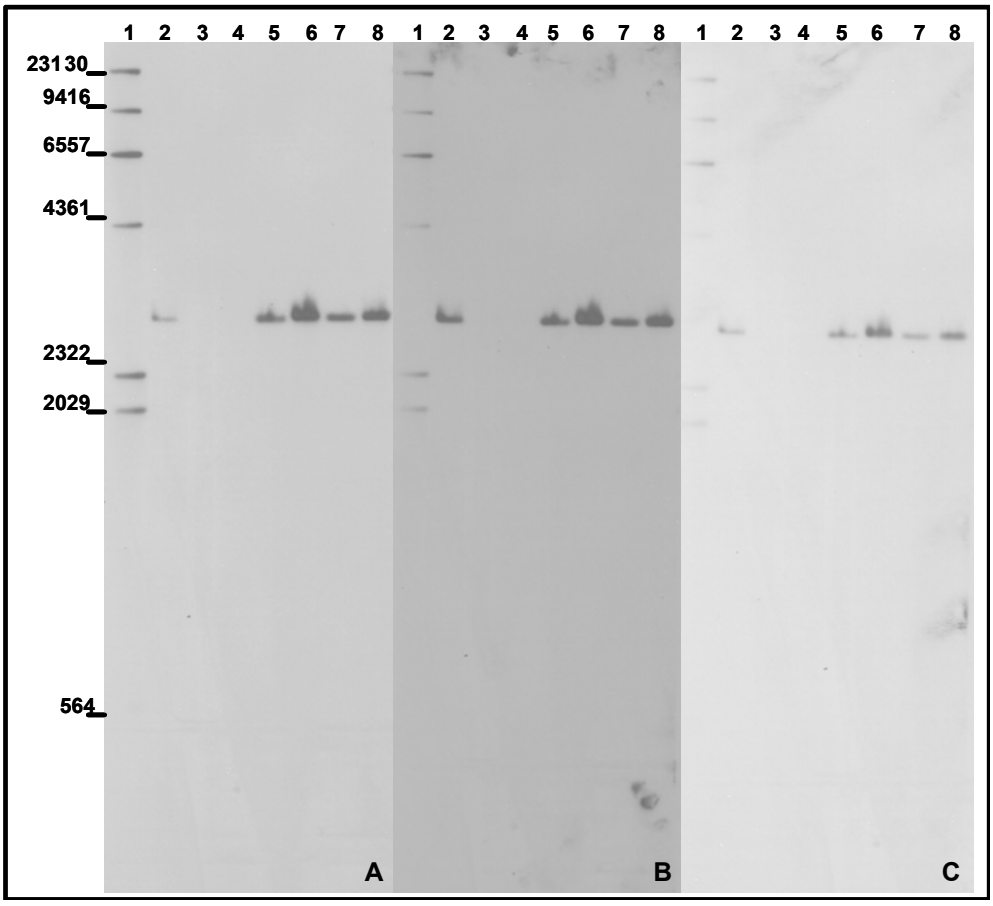


Figure 14. Southern blot analysis of *Pst* I digest with AtUbi10 probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Pst *I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

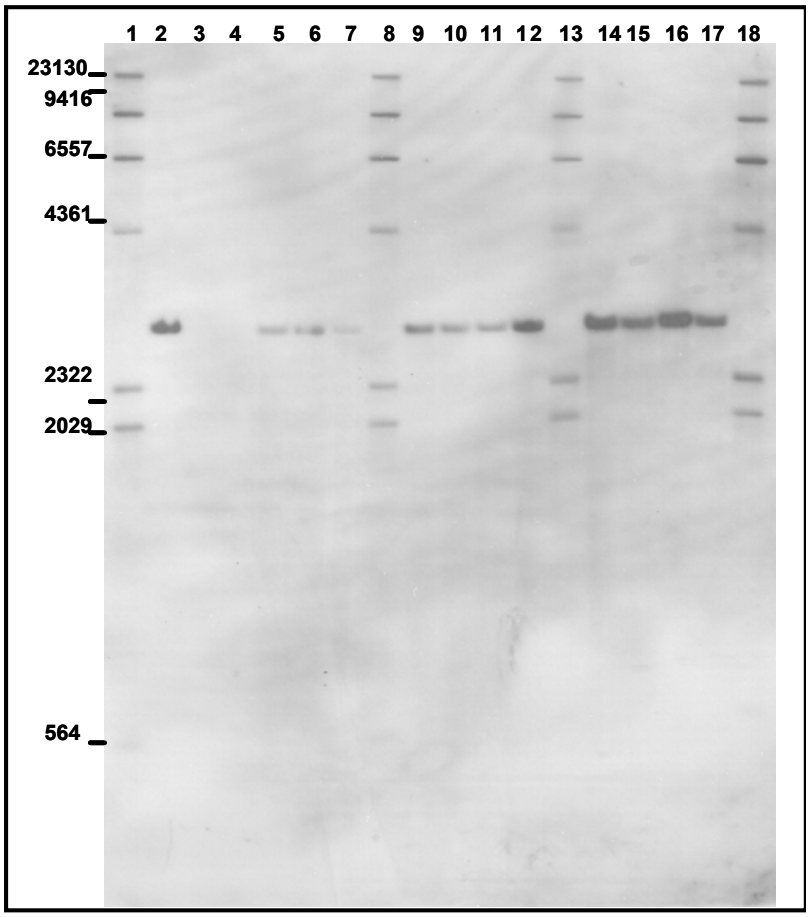


Figure 15. Southern blot analysis of *Pst* I digest with AtuORF23 probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Pst *I and hybridized with AtuORF23 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

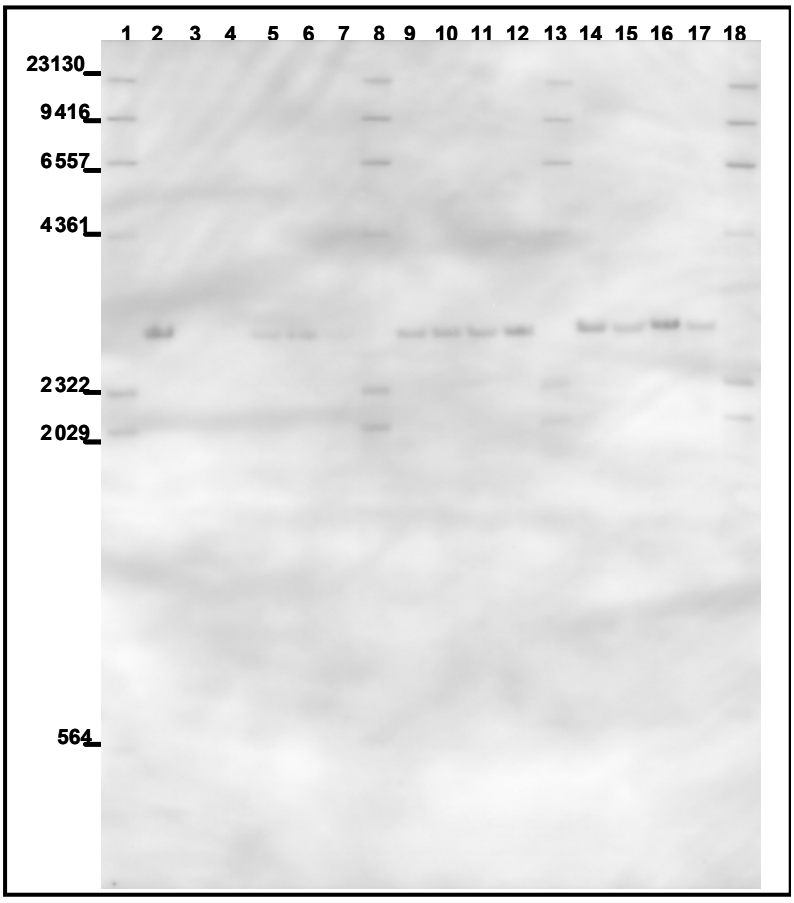


Figure 16. Southern blot analysis of *Nco* I digest with *pat* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Nco I and hybridized with pat probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 12 and 15 were due to the greater amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T2 #2	14	T5 #2
6	T2 #7	15	T5 #4
7	T2 #8	16	T5 #5
8	DNA molecular marker (bp)	17	T5 #7
9	T3 #3	18	DNA molecular marker (bp)

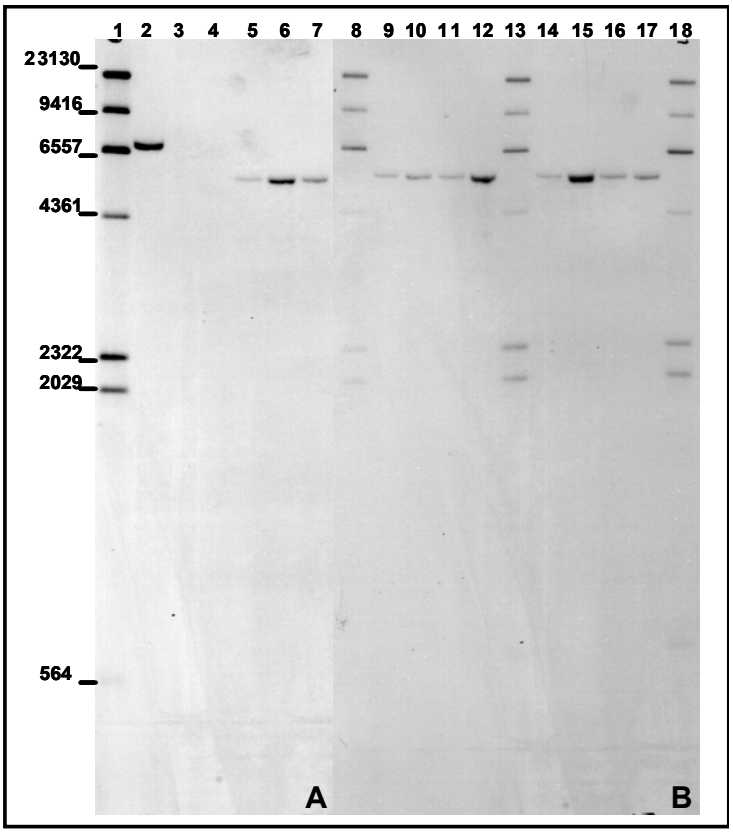


Figure 17. Southern blot analysis of *Sph* I/*Xho* I digest with *pat* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Sph *I/*Xho *I and hybridized with pat probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 10 and 15 were due to the greater amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container.)*

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T2 #2	14	T5 #2
6	T2 #7	15	T5 #4
7	T2 #8	16	T5 #5
8	DNA molecular marker (bp)	17	T5 #7
9	T3 #3	18	DNA molecular marker (bp)

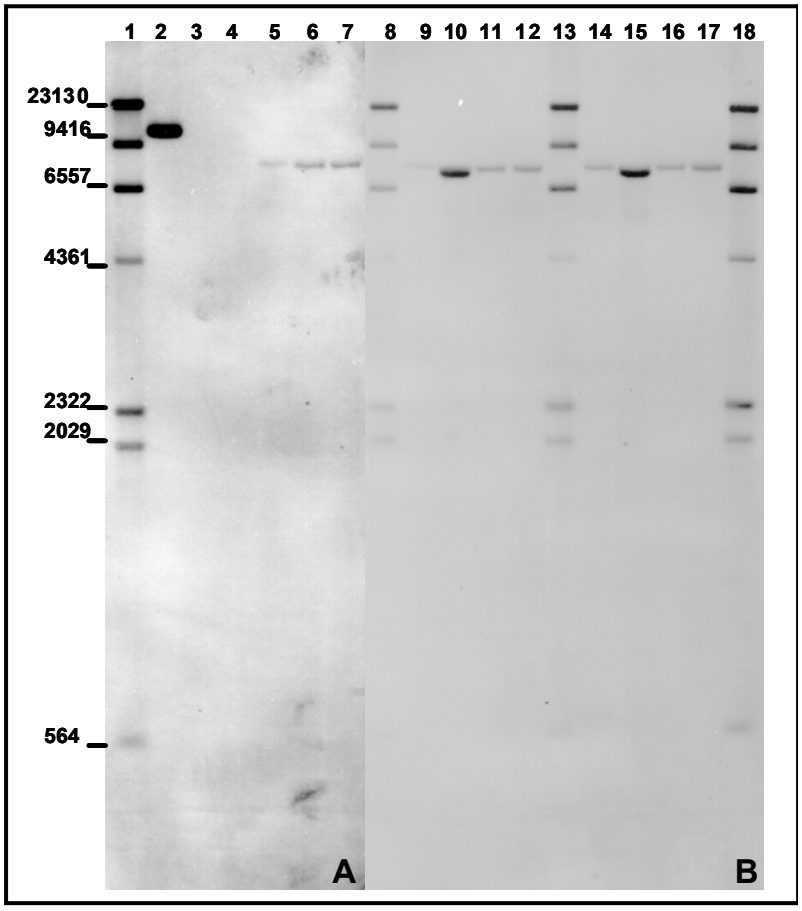


Figure 18. Southern blot analysis of *Nhe* I/*Xho* I digest with *pat* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Nhe *I/*Xho *I and hybridized with pat probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 9 was due to the greater amount of DNA recovered after digestion).*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

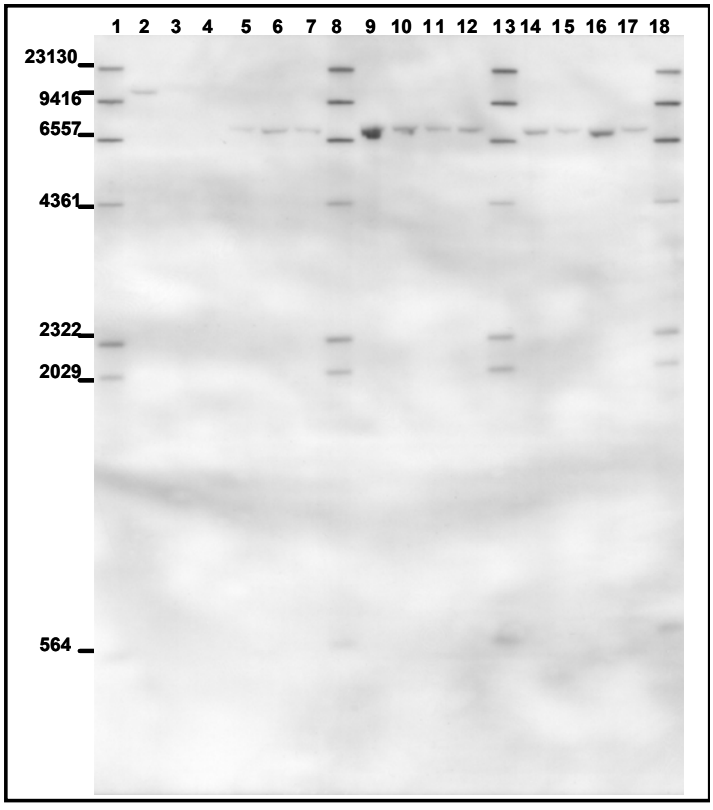


Figure 19. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with *pat* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 and the non-transgenic control was digested with Nco *I,* Sph *I/*Xho *I and* Nhe *I/*Xho *I and hybridized with pat probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 19 was due to the greater amount of DNA recovered after digestion. The faint band in Lane 16 is probably degraded plasmid DNA).*

Lane	***Description***	***Enzyme***	***Lane***	***Description***	***Enzyme***
1, 23	*DNA molecular marker (bp)*		*12*	*T5 #1*	Sph *I*/ Xho *I*
2	*pDAB4468 + control (Maverick)l #2*	Nco *I*	*13*	*T5 #4*	
3	*control (Maverick) #2*		*14*	*T5 #6*	
4	*control (Maverick) #3*		*15*	*T5 #8*	
5	*T5 #1*		*16*	*pDAB4468 + control (Maverick) #2*	Nhe *I*/ Xho *I*
6	*T5 #4*		*17*	*control (Maverick) #2*	
7	*T5 #6*		*18*	*control (Maverick) #3*	
8	*T5 #8*		*19*	*T5 #1*	
9	*pDAB4468 + control (Maverick) #4*	Sph *I*/ Xho *I*	*20*	*T5 #4*	
10	*control (Maverick) #4*		*21*	*T5 #6*	
11	*control (Maverick) #5*		*22*	*T5 #8*	

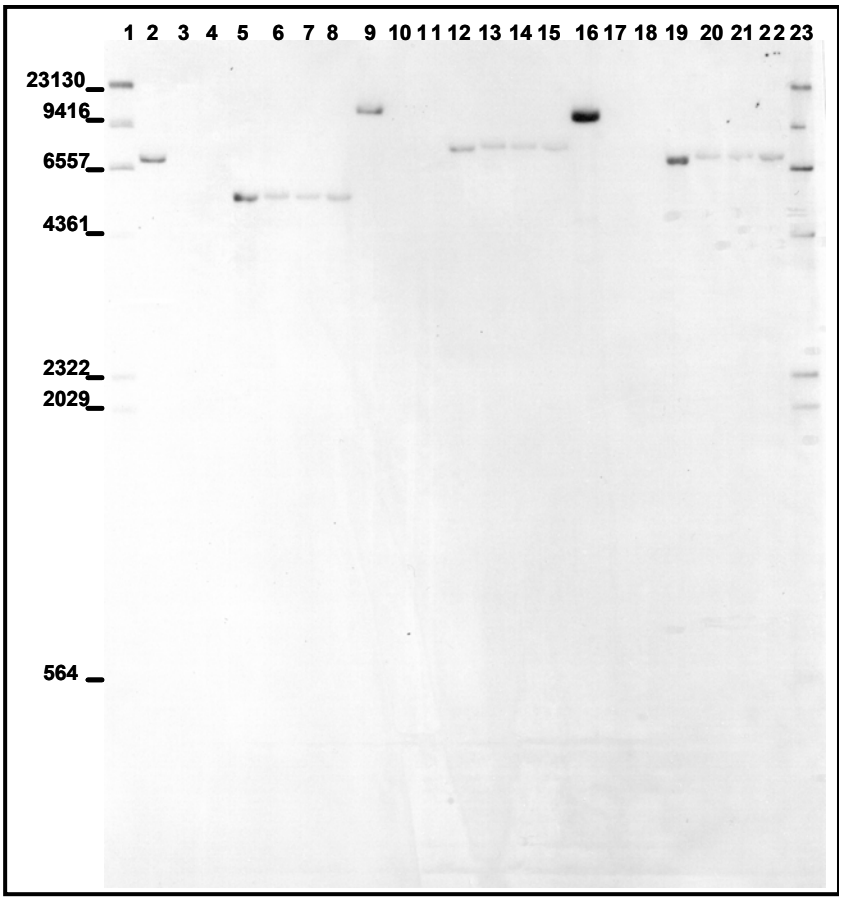


Figure 20. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Pst *I*/Xho *I and hybridized with pat probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #3*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

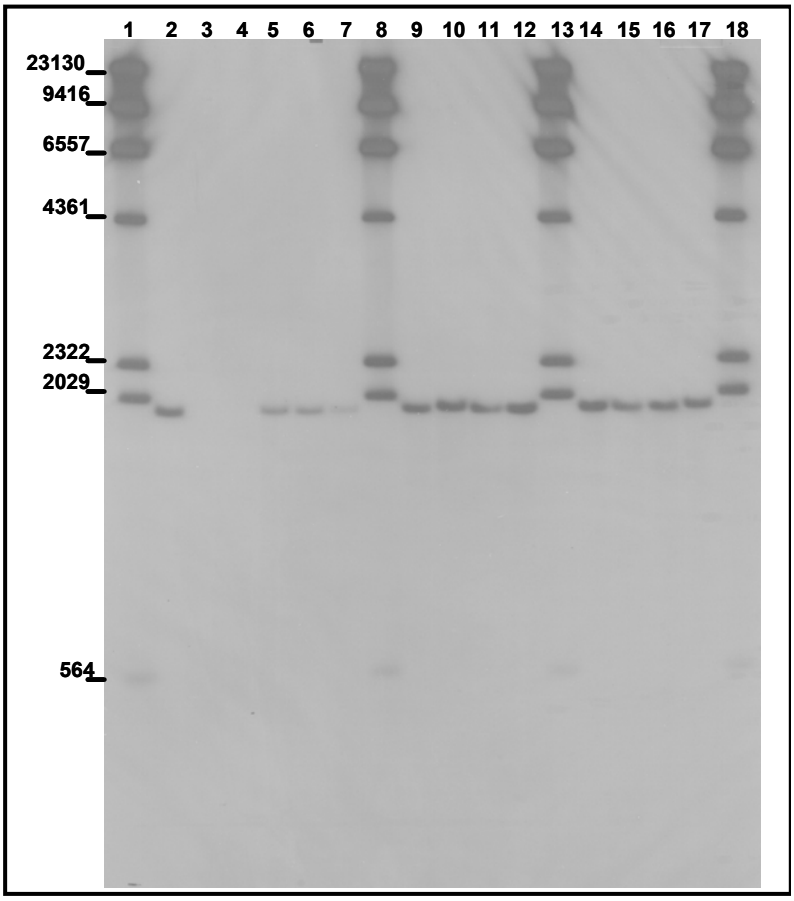


Figure 21. Southern blot analysis of *Pst* I/*Xho* I digest of T5 generation with *pat,* CsVMV, and AtuORF1 probes. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 generation and the non-transgenic control were digested with Pst *I/*Xho *I and hybridized with pat (Panel A), CsVMV (Panel B), and AtuORF1 probes (Panel C). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.*

Lane	***Description***
1	*DNA molecular marker (bp)*
2	*pDAB4468 + control (Maverick) #2*
3	*control (Maverick) #2*
4	*control (Maverick) #3*
5	*T5 #1*
6	*T5 #4*
7	*T5 #6*
8	*T5 #8*

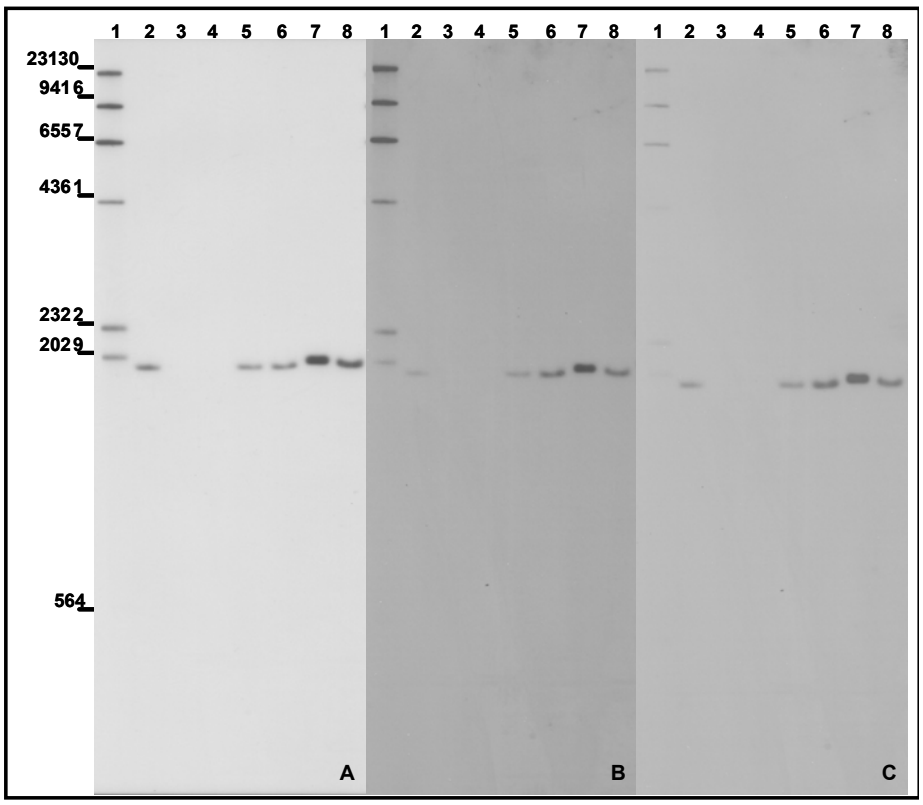


Figure 22. Southern blot analysis of *Pst* I/*Xho* I digest with CsVMV probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Pst *I/*Xho *I and hybridized with CsVMV probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.*

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #3	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T2 #2	14	T5 #2
6	T2 #7	15	T5 #4
7	T2 #8	16	T5 #5
8	DNA molecular marker (bp)	17	T5 #7
9	T3 #3	18	DNA molecular marker (bp)

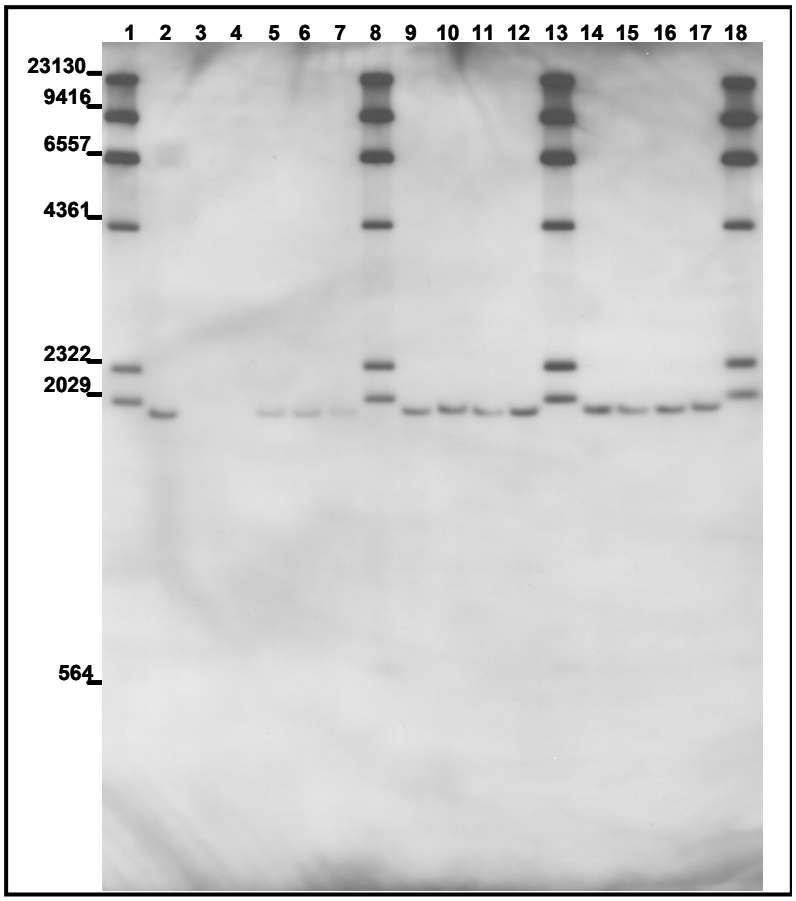


Figure 23. Southern blot analysis of *Pst* I/*Xho* I digest with AtuORF1 probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Pst *I/*Xho *I and hybridized with AtuORF1 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #3*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

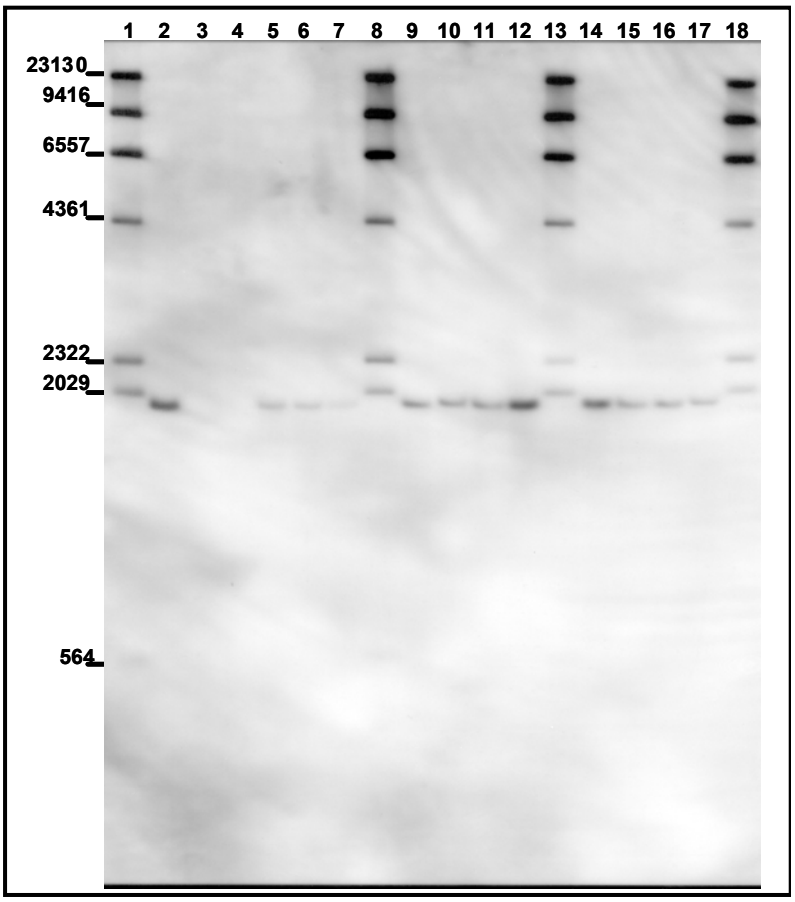


Figure 24. Southern blot analysis of *Bam*H I/*Nco* I digest with RB7 probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Bam*H I/*Nco *I and hybridized with RB7 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signals in Lane 6 and 7 were due to the less amount of DNA recovered after digestion).*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

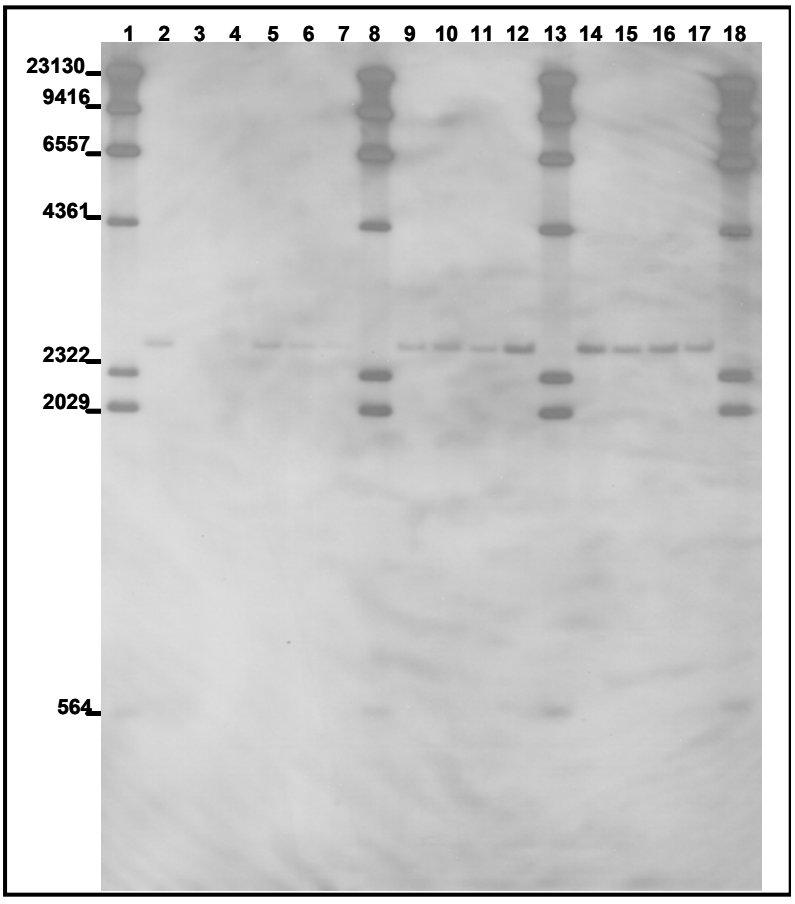


Figure 25. Southern blot analysis of *Bam*H I/*Nco* I digest with AtUbi10 probe. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3, T4, T5 and the non-transgenic control were digested with Bam*H I/*Nco *I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

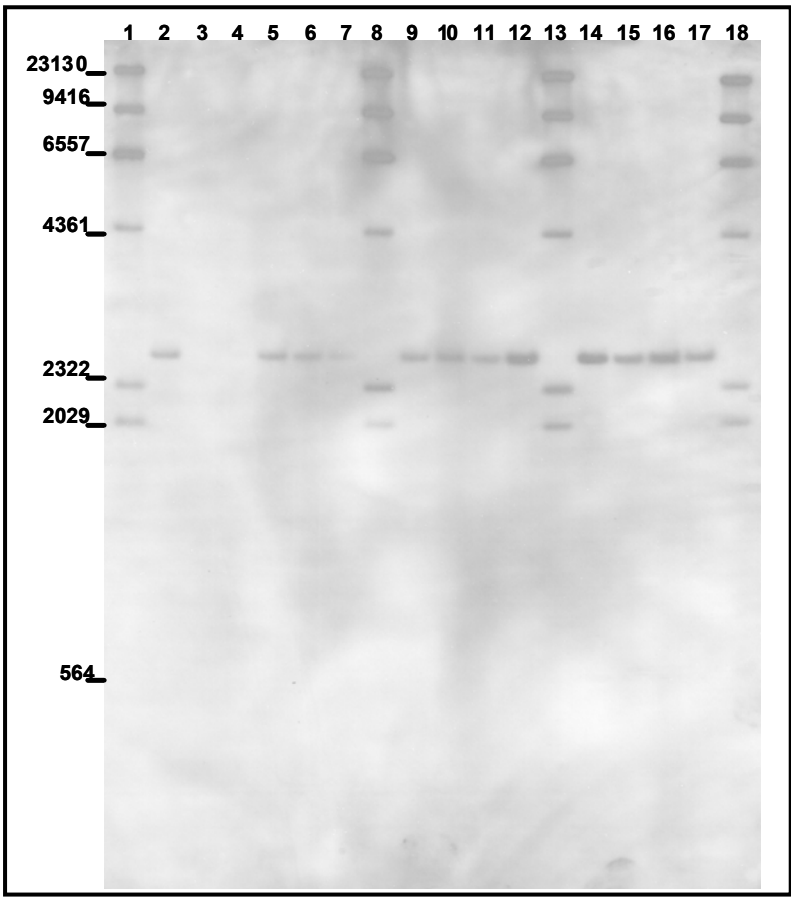


Figure 26. Southern blot analysis of *Bam*H I/*Nco* I digest of T5 generation with RB7 and AtUbi10 probes. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 generation and the non-transgenic control were digested with BamH I/Nco I and hybridized with RB7 (Panel A), AtUbi10 (Panel B). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

Lane	*Description*
1	*DNA molecular marker (bp)*
2	*pDAB4468 + control (Maverick) #2*
3	*control (Maverick) #2*
4	*control (Maverick) #3*
5	*T5 #1*
6	*T5 #4*
7	*T5 #6*
8	*T5 #8*

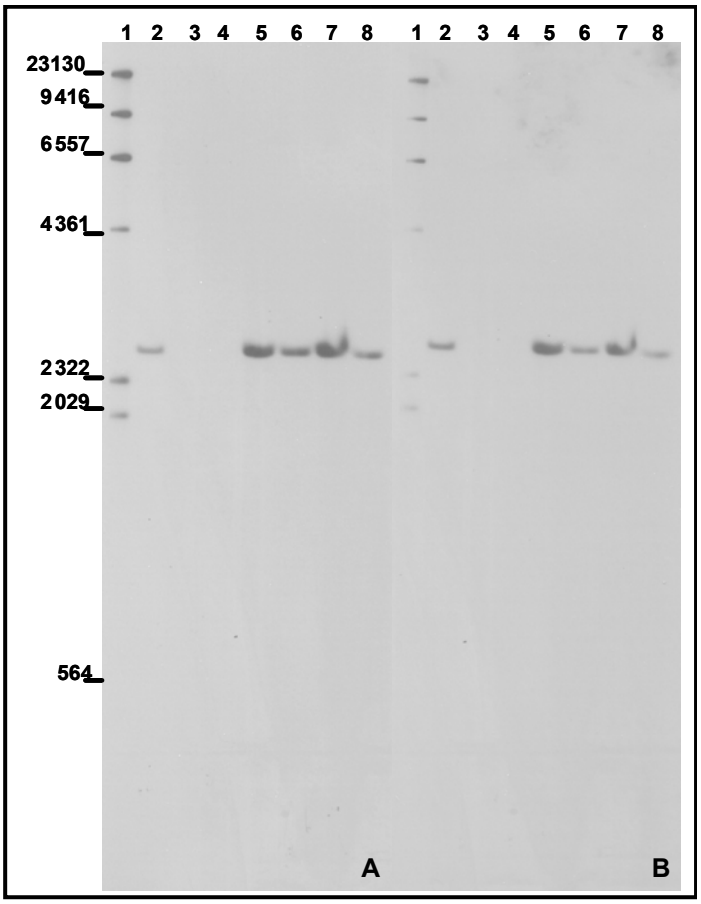


Figure 27. Southern blot analysis of *Nco* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T4 (Panel B) and the non-transgenic control were digested with Nco *I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and SpecR). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).*

Lane	***Description***	***Lane***	***Description***
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

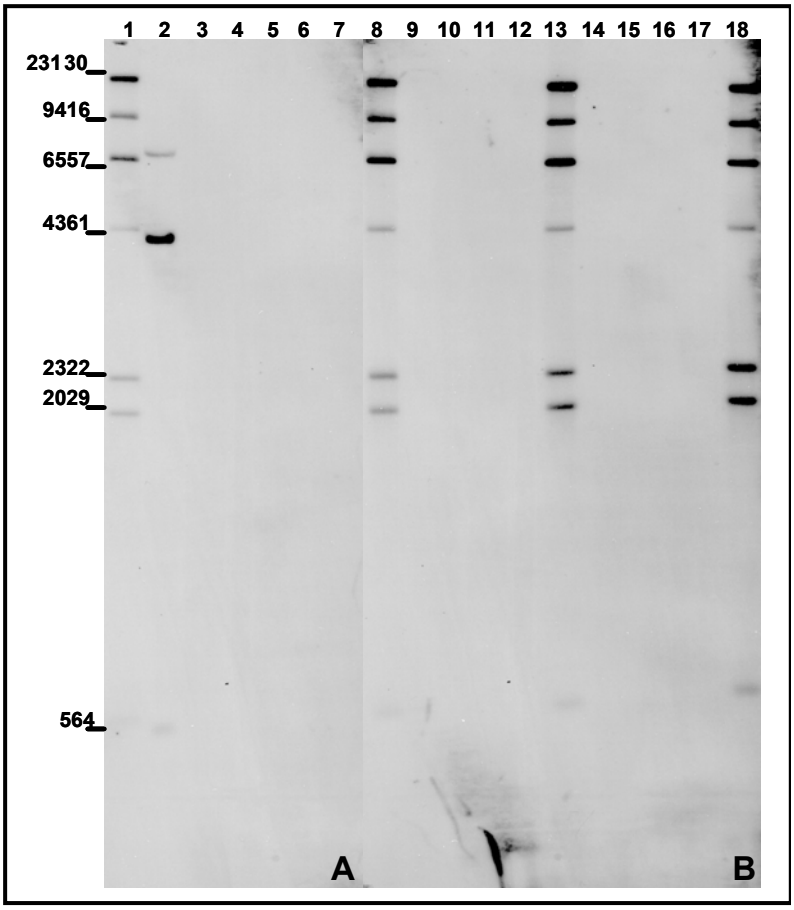


Figure 28. Southern blot analysis of *Nco* I digest with backbone probe set 2 from plasmid pDAB4468 vector backbone. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Nco *I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and Ori-Rep) probes. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).*

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T2 #2	14	T5 #2
6	T2 #7	15	T5 #4
7	T2 #8	16	T5 #5
8	DNA molecular marker (bp)	17	T5 #7
9	T3 #3	18	DNA molecular marker (bp)

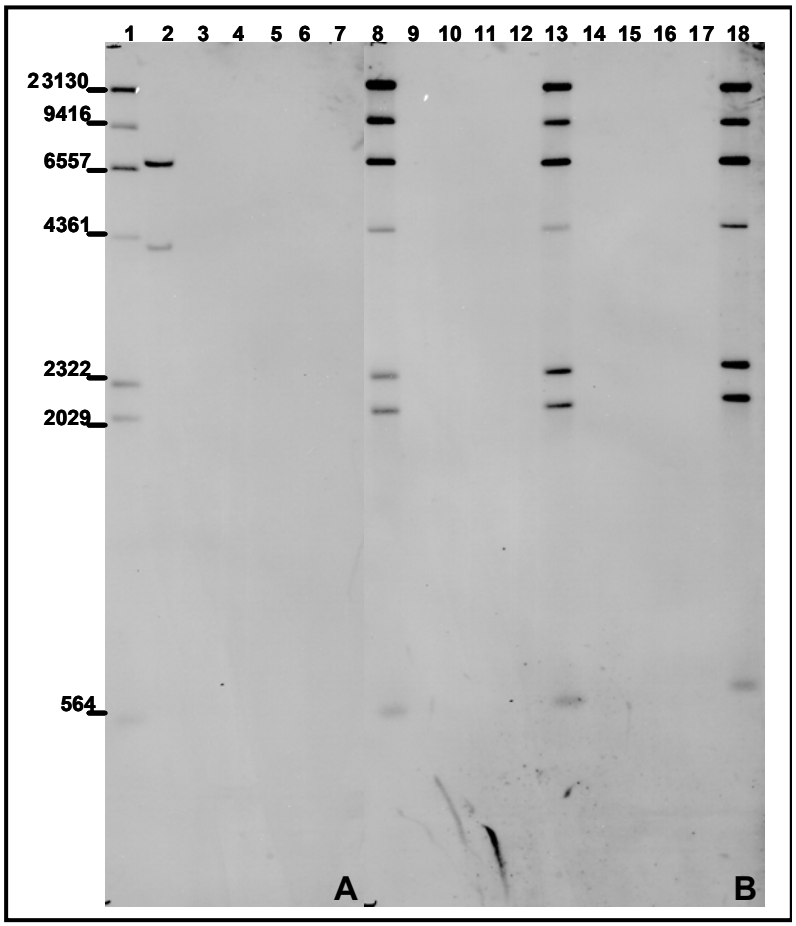


Figure 29. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Sph *I/*Xho *I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and* SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).*

Lane	Description	Lane	Description
1	DNA molecular marker (bp)	10	T4 #6
2	pDAB4468 + control (Maverick) #2	11	T4 #9
3	control (Maverick) #3	12	T4 #10
4	control (Maverick) #2	13	DNA molecular marker (bp)
5	T2 #2	14	T5 #2
6	T2 #7	15	T5 #4
7	T2 #8	16	T5 #5
8	DNA molecular marker (bp)	17	T5 #7
9	T3 #3	18	DNA molecular marker (bp)

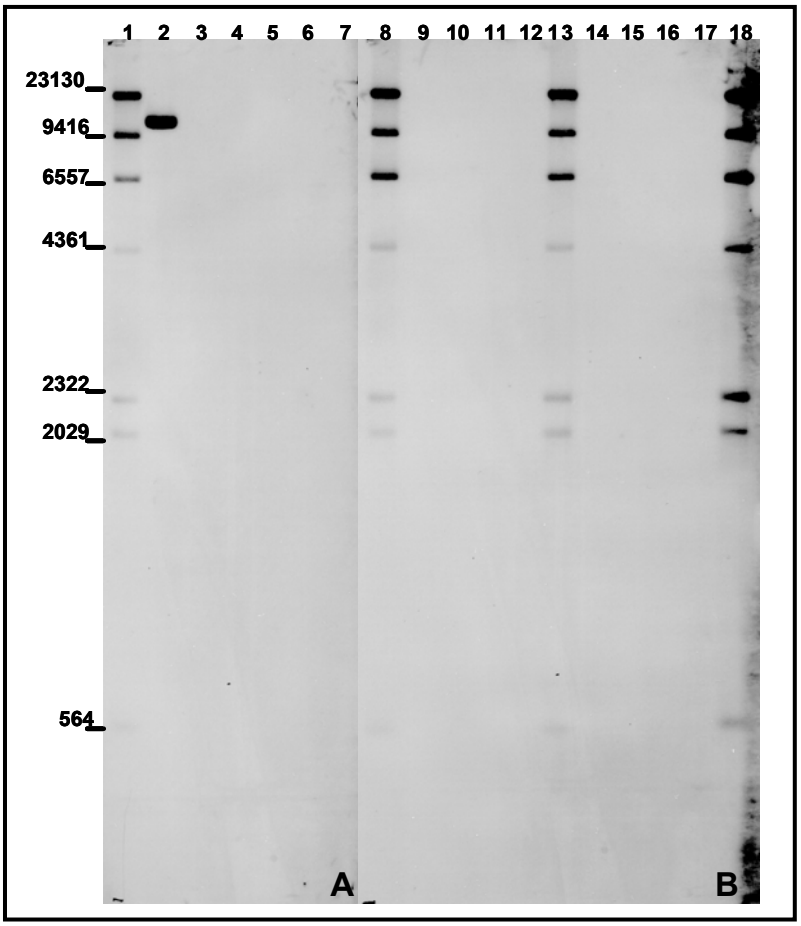


Figure 30. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 2 plasmid pDAB4468 vector backbone. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generations T3 (Panel A), T4, T5 (Panel B) and the non-transgenic control were digested with Sph *I/*Xho *I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and* Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).*

Lane	*Description*	*Lane*	*Description*
1	*DNA molecular marker (bp)*	*10*	*T4 #6*
2	*pDAB4468 + control (Maverick) #2*	*11*	*T4 #9*
3	*control (Maverick) #3*	*12*	*T4 #10*
4	*control (Maverick) #2*	*13*	*DNA molecular marker (bp)*
5	*T2 #2*	*14*	*T5 #2*
6	*T2 #7*	*15*	*T5 #4*
7	*T2 #8*	*16*	*T5 #5*
8	*DNA molecular marker (bp)*	*17*	*T5 #7*
9	*T3 #3*	*18*	*DNA molecular marker (bp)*

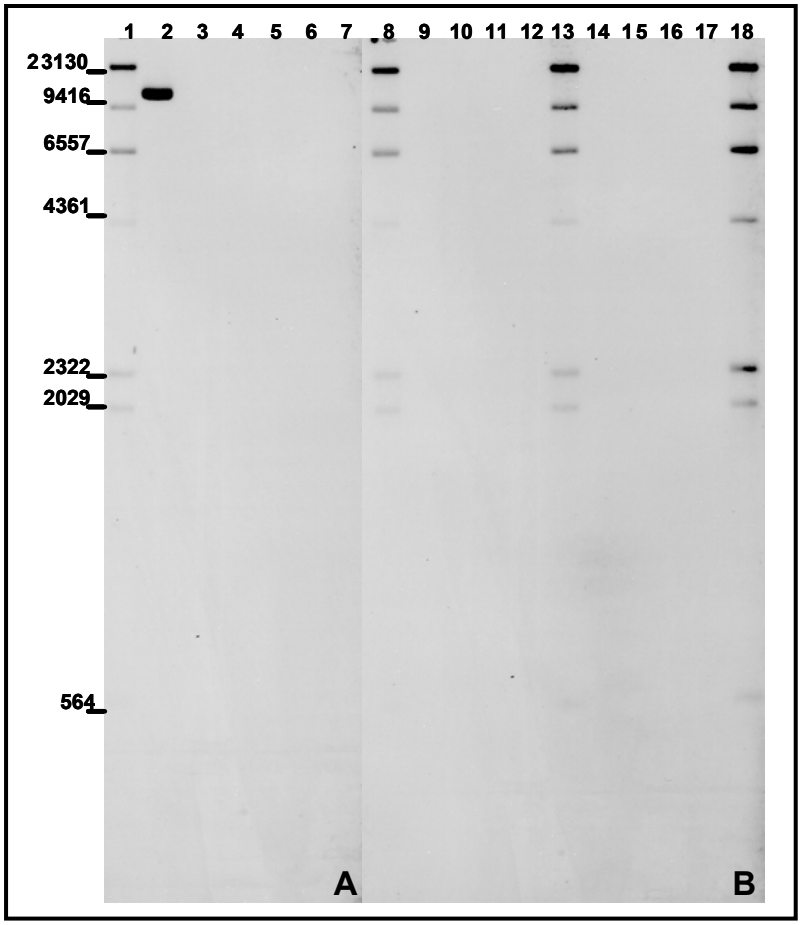


Figure 31. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generations with the backbone probe set 1 from pDAB4468 vector backbone. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 and the non-transgenic control was digested with Nco *I,* Sph *I/*Xho *I, and* Nhe *I/*Xho *I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and* SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: the faint bands in lane 16 may be a result of hybridization to the degraded plasmid DNA).*

Lane	***Description***	***Enzyme***	***Lane***	***Description***	***Enzyme***
1, 23	*DNA molecular marker (bp)*		*12*	*T5 #1*	Sph *I*/Xho *I*
2	*pDAB4468 + control (Maverick) #2*	Nco *I*	*13*	*T5 #4*	
3	*control (Maverick) #2*		*14*	*T5 #6*	
4	*control (Maverick) #3*		*15*	*T5 #8*	
5	*T5 #1*		*16*	*pDAB4468 + control (Maverick) #2*	Nhe *I*/Xho *I*
6	*T5 #4*		*17*	*control (Maverick) #2*	
7	*T5 #6*		*18*	*control (Maverick) #3*	
8	*T5 #8*		*19*	*T5 #1*	
9	*pDAB4468 + control (Maverick) #4*	Sph *I*/Xho *I*	*20*	*T5 #4*	
10	*control (Maverick) #4*		*21*	*T5 #6*	
11	*control (Maverick) #5*		*22*	*T5 #8*	

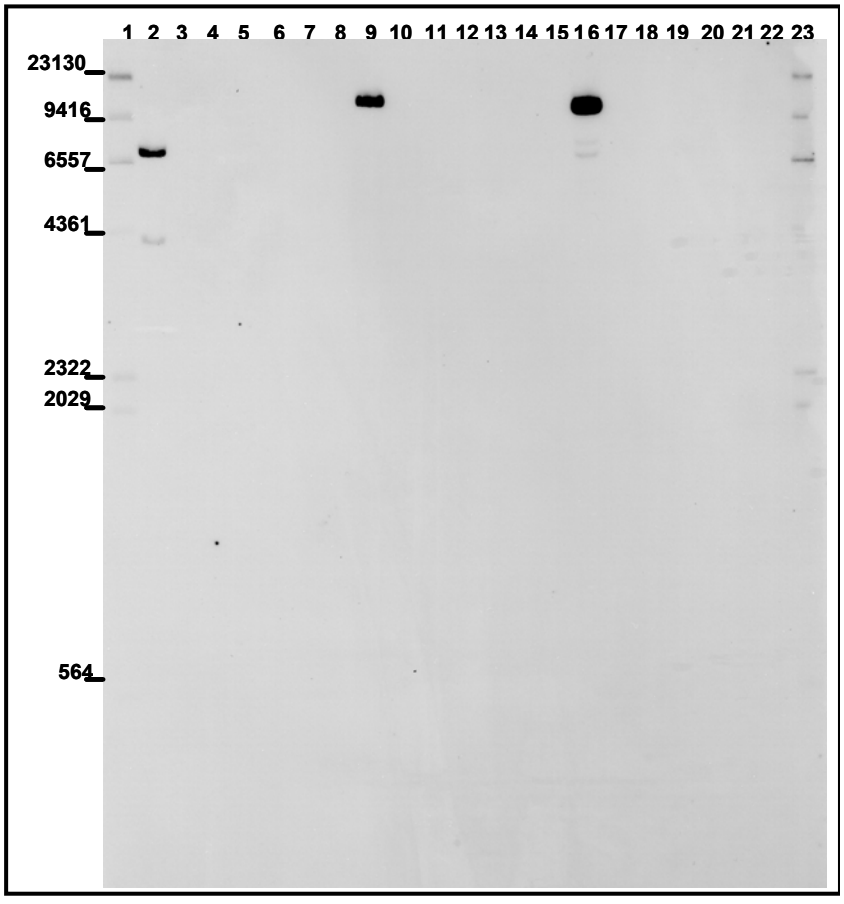
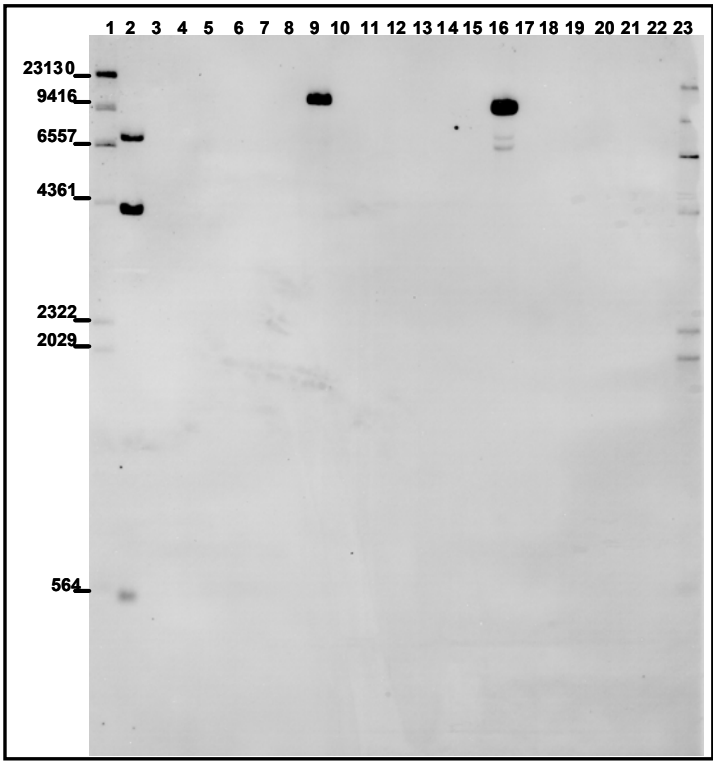


Figure 32. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with the backbone probe set 2 from pDAB4468 vector backbone. (Song et al, 2009, Study ID 081087).

DNA isolated from individual plants of soybean event DAS-68416-4 generation T5 and the non-transgenic control was digested with Nco *I,* Sph *I/*Xho *I and* Nhe *I/*Xho *I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and* Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The faint bands in lane 16 may be a result of hybridization to the degraded plasmid DNA).*

Lane	***Description***	***Enzyme***	***Lane***	***Description***	***Enzyme***
1, 23	*DNA molecular marker (bp)*		*12*	*T5 #1*	Sph *I*/Xho *I*
2	*pDAB4468 + control (Maverick) #2*	Nco *I*	*13*	*T5 #4*	
3	*control (Maverick) #2*		*14*	*T5 #6*	
4	*control (Maverick) #3*		*15*	*T5 #8*	
5	*T5 #1*		*16*	*pDAB4468 + control (Maverick) #2*	Nhe *I*/Xho *I*
6	*T5 #4*		*17*	*control (Maverick) #2*	
7	*T5 #6*		*18*	*control (Maverick) #3*	
8	*T5 #8*		*19*	*T5 #1*	
9	*pDAB4468 + control (Maverick) #4*	Sph *I*/Xho *I*	*20*	*T5 #4*	
10	*control (Maverick) #4*		*21*	*T5 #6*	
11	*control (Maverick) #5*		*22*	*T5 #8*	



Genetic and Molecular Analysis of a Segregating Generation

The inheritance pattern of the transgene insert within a segregating generation was demonstrated with protein expression detection and Southern analysis of individual plants from a F2 population of DAS-68416-4 soybean. The F2 generation was generated by crossing T4 plants of DAS-68416-4 soybean with a conventional soybean line. The F1 plants were self pollinated to produce the F2 seeds.

A total of 147 F2 seedlings were leaf tested for the presence or absence of the AAD-12 protein using an AAD-12 specific lateral flow strip test kit. Of the 147 plants tested, 102 plants were positive for AAD-12 protein expression, and 45 plants were negative (segregated null) (**Table 4**). Statistical analysis using a χ^2 goodness of fit test indicated the phenotypic segregation ratio of the plants with positive AAD-12 protein expression versus negative is consistent with the 3:1 segregation ratio characteristic of the Mendelian inheritance pattern of a single dominant trait.

Similarly, Southern blot analysis was used to determine the genetic equivalence of the inserted DNA among the same F2 individual plants. Among 147 emerged plants, four plants (2 positive and 2 negative for AAD-12 protein expression) died prior to proceeding with DNA extraction. To further confirm if the phenotypic segregation matched the genotypic makeup of the tested F2 population, genomic DNA samples from each of the remaining 143 plants, along with DNA samples from the non-transgenic control, were analysed by Southern blot using *Nco* I restriction enzyme digestion followed by hybridization with *aad-12* and *pat* probes. All the DNA samples from AAD-12 expression positive plants displayed a ~5500 bp expected single band of the 3' border of the transgene insert when digested by *Nco* I and hybridized with either the *aad-12* or *pat* probes (**Table 5**). Two representative Southern blots are presented in **Figure 33** and **Figure 34**. The hybridization patterns across all the individual plants that tested positive for AAD-12 protein expression were identical, which indicated that all individual plants contained the same insert and were equivalent to one another. None of the DNA samples from AAD-12 protein expression negative plants and non-transgenic control showed any hybridization bands. The Southern blot analysis data matches what was observed in the AAD-12 protein expression testing, *i.e*., individual plants which tested positive for AAD-12 expression displayed the expected hybridization bands, while plants negative for AAD-12 protein expression (segregated nulls) did not have any hybridization signals. As observed in the protein expression testing, the ratio of *aad-12* or *pat* hybridization positive versus negative plants in the F2 population also fit the expected 3:1 segregation ratio characteristic of the Mendelian inheritance pattern of a single gene (**Table 6**).

Table 4. Results of F2 individual plants tested for of AAD-12 expression within a single segregating generation. (Song et al, 2009, Study ID 091071).

Generation	Total plants tested	AAD-12 protein positive	AAD-12 protein negative	Expected ratio	P-value[a]
F2	147	102	45	3:1	0.116

[a] Based on a chi-squared goodness of fit test

Table 5. Predicted and observed hybridizing fragments in Southern blot analysis of F2 population.
(Song et al, 2009, Study ID 091071).

Restriction Enzymes	DNA Probe	Sample Source	Southern Blot Figure	Expected Fragment Sizes (bp) [a]	Observed Fragment Size (bp)[b]
Nco I	*aad-12*	pDAB4468	Figure 33	7429	7429
		Control (Maverick)	Figure 33	none	none
		AAD-12 positive plants in F2	Figure 33	>4043(border)	~5500
		AAD-12 negative plants in F2 (null segregants)	Figure 33	none	none
	pat	pDAB4468	**Error! Not a valid result for table.** 34	7429	7429
		Control (Maverick)	**Error! Not a valid result for table.** 34	none	none
		AAD-12 positive plants in F2	**Error! Not a valid result for table.** 34	>4043(border)	~5500
		AAD-12 negative plants in F2 (null segregants)	**Error! Not a valid result for table.** 34	none	none

a. Expected fragment sizes are based on the plasmid map of the pDAB4468 as shown in Figure.

b. 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.

Table 6. Results of F2 individual plants analysed by Southern blot with *aad-12* and *pat* probes within a single segregating generation. (Song et al, 2009, Study ID 091071).

Generation	Total plants analysed	Southern hybridization positive	Southern hybridization negative	Expected ratio	P-value[a]
F2	143	100	43	3:1	0.162

[a] Based on a chi-squared goodness of fit test

Figure 33. Southern blot analysis of *Nco* I digest with *aad-12* probe. (Song et al, 2009, Study ID 091071).

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was digested with *Nco* I and hybridized with *aad-12* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgenic copy per soybean genome.

Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-72	1, 12, 22	Molecular Marker	11	416-90
2	pDAB4468+ Maverick C6	13	416-73	2	pDAB4468+ Maverick C2	13	416-91
3	Maverick C6	14	416-74	3	Maverick C2	14	416-92
4	Maverick C10	15	416-75	4	Maverick C3	15	416-93
5	416-66	16	416-76	5	416-83	16	416-94
6	416-67	17	416-77	6	416-85	17	416-95
7	416-68	18	416-78	7	416-86	18	416-96
8	416-69	19	416-79	8	416-87	19	416-98
9	416-70	20	416-80	9	416-88	20	416-99
10	416-71	21	416-82	10	416-89	21	416-100

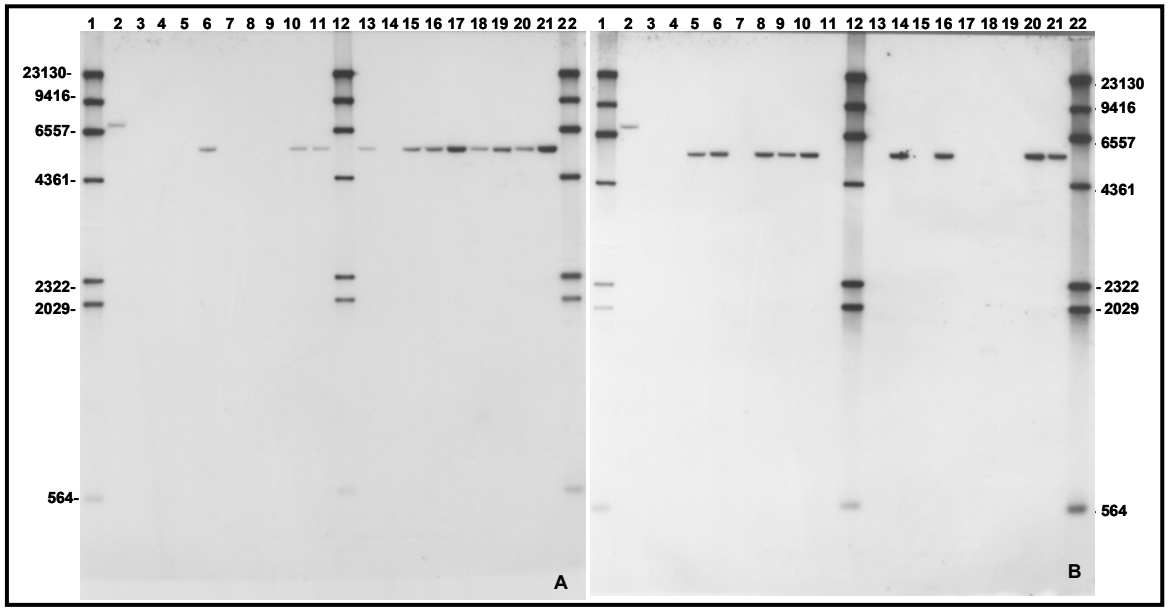
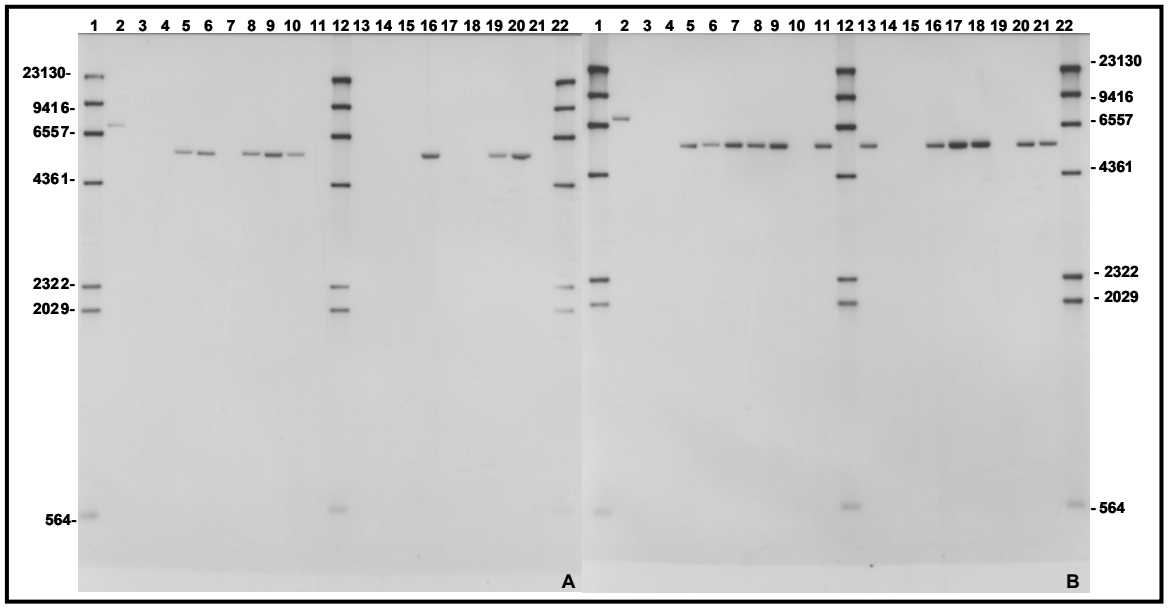


Figure 34. Southern blot analysis of *Nco* I digest with *pat* probe. (Song et al, 2009, Study ID 091071).

DNA isolated from individual plants of soybean event DAS-68416-4 F2 population and non-transgenic Maverick was digested with *Nco* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid controls contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgenic copy per soybean genome.

Panel A				Panel B			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1, 12, 22	Molecular Marker	11	416-40	1, 12, 22	Molecular Marker	11	416-56
2	pDAB4468+ Maverick C2	13	416-41	2	pDAB4468+ Maverick C2	13	416-57
3	Maverick C2	14	416-42	3	Maverick C2	14	416-58
4	Maverick C3	15	416-43	4	Maverick C3	15	416-59
5	416-34	16	416-44	5	416-50	16	416-60
6	416-35	17	416-45	6	416-51	17	416-61
7	416-36	18	416-46	7	416-52	18	416-62
8	416-37	19	416-47	8	416-53	19	416-63
9	416-38	20	416-48	9	416-54	20	416-64
10	416-39	21	416-49	10	416-55	21	416-65



Segregation Analysis of Breeding Generations

Chi-square analysis of trait inheritance data from a F2 breeding generation was conducted to determine the Mendelian inheritance of *aad-12* in DAS-68416-4 soybeans. The presence or absence of *aad-12* was determined using a gene-specific fluorescence-based detection method specific for *aad-12*. The expected segregation ratio of 3:1 for plants containing *aad-12* (homozygous + hemizygous) versus plants not containing *aad-12* was observed (**Table 3**).

Table 7. Results of F2 individual plants tested for *aad-12* within a single segregating generation. (Song et al, 2009, Study ID 091071).

Generation	Total plants tested	*aad-12* gene positive	*aad-12* gene negative	Expected ratio	P-value[a]
F2	6774	5056	1718	3:1	0.492

[a] Based on a chi-squared goodness of fit test

Summary of the Genetic Characterization

AAD-12 soybean event DAS-68416-4 was produced using *Agrobacterium*-mediated transformation with the plasmid pDAB4468. The T-DNA insert of pDAB4468 consists of the *aad-12* gene, controlled by the AtUbi10 promoter and AtuORF23 3' UTR regulatory sequences, the *pat* gene, controlled by the CsVMV promoter and AtuORF1 3' UTR regulatory sequences, and a RB7 MAR element at the 5' of the AtUbi10 promoter. Various breeding generations were developed and used to examine the integrity, stability, and inheritance of the *aad-12* and *pat* transgenic insert in soybean event DAS-68416-4.

Molecular characterization of soybean event DAS-68416-4 by Southern blot analysis confirmed the insertion of a single intact copy of the *aad-12* and *pat* expression cassettes from the T-DNA insert of pDAB4468. No additional DNA fragments from the *aad-12* and *pat* expression cassettes were identified in DAS-68416-4 and no plasmid backbone sequences were present. DAS-68416-4 was also shown to be stably integrated across three distinct breeding generations (T3, T4, and T5) and displayed the expected inheritance pattern of a single insert/locus in a generation (F2) that was segregating for the DAS-68416-4 event.

Methods for Molecular Characterization of DAS-68416-4 Soybean

DAS-68416-4 Soybean Material

Transgenic soybean seeds from three distinct generations of soybean containing event DAS-68416-4 were planted in the greenhouse. After at least two weeks of growth, leaf punches were taken from each plant and were tested for AAD-12 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-12 protein. Refer to Attachment 2 – Confidential Commercial Information for the validated method.

Control Soybean Material

Seeds from the unmodified Maverick were planted in the greenhouse. The Maverick seeds had a genetic background representative of the transgenic seeds but did not contain the *aad-12* gene.

Reference Materials

DNA of the plasmid pDAB4468 was added to samples of the Maverick control genomic DNA at a ratio approximately equivalent to 1 copy of the transgene per soybean genome 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 in the T-DNA insert of pDAB4468 and the vector backbone were produced via polymerase chain reaction (PCR) amplification using pDAB4468 plasmid DNA as a template, followed by purification.

Sample Collection and DNA Extraction

Labeled leaf samples were collected from green house for DNA extraction or being stored in -80°C freezer for future use. Genomic DNA was extracted with 2 methods. Method 1 is based on the method of Guillemant, 1992. Briefly, leaf samples were ground individually in liquid nitrogen, and then extraction buffer was added to samples at a ratio of about 3:1 plus 10 µL of RNase-A (Qiagen, Valencia, catalog # 1007885). After precipitation using isopropyl alcohol, crude DNA samples were purified using PCI (phenol:chloroform:isoamyl alcohol = 25:24:1, Sigma, St. Louis, MO, catalog #: P2069) and CI (chloroform:isoamyl alcohol = 24:1, Sigma, St. Louis, MO, catalog # C0549) extraction. DNA was precipitated again by addition of 1/10 volume of 3 M NaOAc and equal volume of isopropyl alcohol. The precipitated DNA was rinsed with 70% ethanol, then dissolved in appropriate volume of 0.1X TE buffer. Method 2 is based on the modified CTAB method. Briefly, leaf samples were individually ground in liquid nitrogen followed by the addition of extraction buffer (~5:1 ratio milliliter CTAB extraction buffer: gram leaf tissue) and RNase-A (>10 µL) (Qiagen, Valencia, catalog # 1007885). After approximately 2 hours of incubation at ~65 °C with gentle shaking, samples were spun down and the supernatants were extracted with equal volume of chloroform:octanol = 24:1 (chloroform, Sigma, Catalog # 366922-4L;

octanol, Sigma, catalog # O4504-100mL). DNA was precipitated by mixing the supernatants with equal volume of precipitation buffer (1% CTAB, Sigma, Catalog # H6269-2506; 50 mM Tris-HCl, Invitrogen, Catalog # 15568-025; 10 mM EDTA, AcruGene, Catalog # 51234). The precipitated DNA was dissolved in high salt TE buffer (1X TE pH8.0, thermo, Catalog # 17890; 1.0M NaCl, AccuGene, Catalog # 51202) followed by precipitation with isopropyl alcohol (Mallinckrot, Catalog # 3031-08). The precipitated DNA was rinsed with 70% ethanol, air-dried, then dissolved in appropriate volume of 1 X TE buffer (pH8.0).
To check the quality of the resultant genomic DNA, an aliquot of the DNA samples was electrophoretically separated on a 1% agarose gel containing ethidium bromide (~1 µg/mL) with 1X TBE buffer (89 mM Tris-Borate, 20 mM EDTA, pH 8.3).
The gel was visualized under ultraviolet (UV) light to confirm that the DNA was not degraded and that the RNA had been removed by the RNase-A. The concentration of DNA in solution was determined by a picogreen kit (Invitrogen, Carlsbad, CA, catalog # P7589) in a fluorometer (Bio-TEK, FLX800).

DNA Digestion and Electrophoretic Separation of the DNA Fragments

Genomic DNA extracted from the soybean 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°C overnight. The positive control sample was prepared by combining pDAB4468 plasmid DNA with genomic DNA from the Maverick control (at a ratio approximately equivalent to 1 copy of the transgene per soybean genome) and was digested using the same procedures and restriction enzymes as the transgenic DNA samples. DNA from the Maverick 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) and re-suspended 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 crosslinking through UV treatment.

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 pDAB4468. 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 cultivar 'Maverick' was used as the recipient line for the generation of DAS-68416-4 soybean.

Maverick was originally developed by the Missouri and Illinois Agricultural Experiment Stations at the Universities of Missouri and Illinois, respectively, and released in 1996 (Sleper *et al.*, 1998). Maverick was developed because of its resistance to the soybean cyst nematode (SCN) and higher yield compared with SCN-resistant cultivars of similar maturity. Maverick is classified as a late Group III maturity (relative maturity 3.8). Maverick has purple flowers, grey pubescence, brown pods at maturity, and dull yellow seed with buff hila. Maverick is resistant to phytophthora rot but is susceptible to brown stem rot and sudden death syndrome.

Transformed Maverick soybean plants were subsequently crossed with elite proprietary inbred soybean lines to derive soybean hybrids containing DAS-68416-4.

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 four sample sets across three distinct generations (T3, T4, and T5) of event DAS-468416-4. Prior to initiation of Southern blot analysis, all plants were tested for AAD-12 protein expression using a lateral flow strip test kit to allow confirmation of AAD-12 expression positive plants. All of the genetic element probes: *aad-12* gene, AtUbi10 promoter,

AtuORF23 terminator, CsVMV promoter, *pat* gene, AtuORF1 terminator, and RB7 MAR, and the backbone of plasmid pDAB4468, were hybridized with the DAS-68416-4 soybean samples. Results across all DAS-68416-4 samples in three generations were as expected (**Table 3, Figure 8-Figure 23**), indicating stable integration and inheritance of the intact, single copy insert across multiple generations of DAS-68416-4 soybean.

4. Labelling of the GM Food

Part B Section 4 DAS Reports

Smith-Drake, J.K., Dunville, C.M., Phillips, A.M., Herman, R.A. (2009) Field Expression, Nutrient Composition Analysis and Agronomic Characteristics of Transformed Soybean Cultivar (DAS-68416-4) Containing Aryloxyalkanoate Dioygenase (AAD-12) and Phosphinoticin Acetyltransferase (PAT). Dow AgroSciences LLC Study ID 080003.

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-12 protein measured in DAS-68416-4. Please specifically refer to the results found in **Table 10** for data pertaining to soybean 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

As described in Section A, Part 3, D of this dossier, Southern blot analysis confirms that the PTU insertion into DAS-68416-4 soybean does not contain any vector backbone from the plasmid pDAB4468. Therefore the spectinomycin resistance gene (*SpecR*, **Figure 1**) in plasmid pDAB4468 was not inserted into the soybean genome in event DAS-68416-4.

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

Part C Section 2 DAS Reports

Lira, J.M., (2010), Strain Review for *Delftia acidovorans,* the Source Organism for *aad-12.* Dow AgroSciences LLC Study ID: JML100001

Schafer, B.W., Embrey, S. K. (2009). Characterization of Aryloxyalknaoate Dioxygenase-12 (AAD-12) Protein Derived from Transgenic Soybean Event DAS-68416-4. Dow AgroSciences LLC Study ID 081113.

Schafer, B.W., Embrey, S. K. (2009). Characterization of Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-68416-4. Dow AgroSciences LLC Study ID 081132.

Smith-Drake, J.K., Thomas, A.S., Sosa, M.J. (2009). Field Expression of a Transformed Soybean Cultivar (DAS-68416-4) Containing Aryloxyalkanoate Dioxygenase-12 (AAD-12) and Phosphinotricin Acetyltransferase (PAT). Study ID: 080003.

a. Biochemical function and phenotypic effect of novel proteins

Identity of the AAD-12 Protein

The aryloxyalkanoate dioxygenase (AAD-12) protein was derived from *Delftia acidovorans*, a gram-negative soil bacterium. The amino acid sequence is identical to the native enzyme sequence except for the addition of an alanine at position number 2. The additional alanine codon encodes part of an *Nco* I restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. This additional codon serves the dual purpose of facilitating subsequent cloning operations and improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized coding regions are 99.3% identical, differing only at amino acid number 2. The AAD-12 protein is comprised of 293 amino acids and has a molecular weight of ~32 kDa (**Figure 35**).

Figure 35. Amino acid sequence of the AAD-12 protein.

001 MAQTTLQITPTGATLGATVTGVHLATLDDAGFAALHAAWLQHALLIFPGQ
051 HLSNDQQITFAKRFGAIERIGGGDIVAISNVKADGTVRQHSPAEWDDMMK
101 VIVGNMAWHADSTYMPVMAQGAVFSAEVVPAVGGRTCFADMRAAYDALDE
151 ATRALVHQRSARHSLVYSQSKLGHVQQAGSAYIGYGMDTTATPLRPLVKV
201 HPETGRPSLLIGRHAHAIPGMDAAESERFLEGLVDWACQAPRVHAHQWAA
251 GDVVVWDNRCLLHRAEPWDFKLPRVMWHSRLAGRPETEGAALV

Identity of the PAT Protein

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium. The *pat* transgene in DAS-68416-4 encodes a protein sequence that is identical to the native PAT protein (Accession number: Q57146). PAT is comprised of 183 amino acids and has a molecular weight of ~21 kDa.

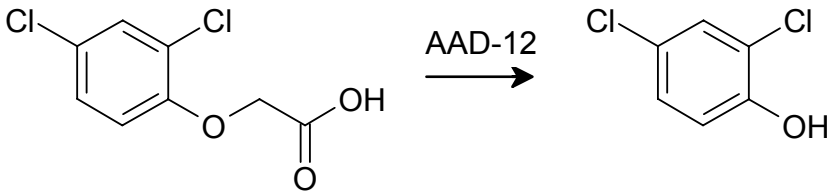
Figure 36. Amino acid sequence of the PAT protein.

001 MSPERRPVEIRPATAADMAAVCDIVNHYIETSTVNFRTEPQTPQEWIDDL
051 ERLQDRYPWLVAEVEGVVAGIAYAGPWKARNAYDWTVESTVYVSHRHQRL
101 GLGSTLYTHLLKSMEAQGFKSVVAVIGLPNDPSVRLHEALGYTARGTLRA
151 AGYKHGGWHDVGFWQRDFELPAPPRPVRPVTQI

Mode of Action of the AAD-12 Protein

Expression of the AAD-12 protein in transgenic crops provides tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) by catalyzing the conversion of 2,4-D to 2,4-dichlorophenol (DCP) (Müller *et al.*, 1999; Westendorf *et al.*, 2002 and 2003; Wright *et al.,* 2007), a herbicidally inactive compound. See **Figure 37** for a representative reaction pathway.

Figure 37. Degradation reaction of 2,4-D catalyzed by AAD-12.



2,4-D DCP

AAD-12 is also able to degrade related achiral phenoxyacetate herbicides such as MCPA ((4-chloro-2-methylphenoxy)acetic acid) and pyridyloxyacetate herbicides such as triclopyr and fluroxypyr to their corresponding inactive phenols and pyridinols, respectively. DAS currently has no plans to commercialize the use any herbicides other than 2,4-D and glufosinate on DAS-68416-4 soybeans.

Enzyme Specificity

AAD-12 has enantiomeric selectivity for the (S)-enantiomers of the chiral phenoxy acid herbicides (*e.g.*, dichlorprop and mecoprop), but does not catalyze degradation of the (R)-enantiomers. It is the R-enantiomers in this class of chemistry that are herbicidally active, therefore AAD-12 does not provide tolerance to commercially-available chiral phenoxy acid herbicides.

Biochemical Characterization of the AAD-12 Protein

Large quantities of purified AAD-12 protein are required to perform safety assessment studies. Because it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans, 2004), the AAD-12 protein was microbially-produced using *Pseudomonas fluorescens (Pf)*. After fermentation, cells were lysed and cell debris and unbroken cells were removed by centrifugation and the supernatant was filtered through a 0.2 µm filter. The AAD-12 protein was purified

to homogeneity in two column steps using anion exchange and hydrophobic interaction chromatography.

Characterization studies were performed to confirm the equivalency of the AAD-12 protein expressed *in planta* in soybean line DAS-68416-4 with the *Pf* microbe-derived AAD-12 protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycoprotein detection, and protein sequence analysis by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization-liquid chromatography mass spectrometry (ESI-LC/MS) were used to characterize the biochemical properties of the protein. The materials and methods used for the biochemical characterization of the DAS-68416-4 soybean- and microbe-derived AAD-12 proteins are described in detail below.

Using these methods, the AAD-12 protein from *Pf* and the transgenic soybean event DAS-68416-4 were shown to be biochemically equivalent, thereby supporting the use of the microbially-produced protein in safety assessment studies.

Biochemical Characterization of the PAT Protein

Characterization of the biochemical properties of the plant-derived PAT protein was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), lateral flow strips and western blot analysis. Using these methods the PAT protein produced in DAS-68416-4 soybean was shown to be equivalent to that produced in other transgenic crops (USDA 1996, USDA 2001, USDA 2004, USDA 2005).

Methods and Results for the Characterisation of the AAD-12 Protein

DAS-68416-4 Transgenic Soybean Material

Greenhouse-grown DAS-68416-4 soybean plants (T5 generation) were used as the plant source of the AAD-12 protein. Prior to use, individual plants were leaf tested to confirm expression of the AAD-12 protein using a rapid lateral flow test strip according to the manufacturer's instructions. Leaves (and some stems) from AAD-12 expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

Control Soybean Material

Control soybean line Maverick had a genetic background representative of the DAS-68416-4 soybean plants, but did not contain the *aad-12* gene. Absence of AAD-12 expression in the control plants was confirmed by immunoassay using an AAD-12 specific rapid lateral flow test strip. Leaves (and some stems) of control plants were harvested, lyophilized, ground and stored under the same conditions as the DAS-68416-4 soybean.

Reference Material

Recombinant AAD-12 microbial protein was produced in *Pseudomonas fluorescens* (*Pf*) and purified to a lyophilized powder. The microbe-derived AAD-12 protein preparation was stored dry and resuspended in a buffer to maintain activity prior to use.

Protein Purification of AAD-12 from DAS-68416-4 Soybean Plant Tissue

The AAD-12 protein was extracted from lyophilized leaf tissue in a PBST (Phosphate Buffered Saline with 0.05% Tween 20, pH 7.4) based buffer with added stabilizers, and the soluble proteins were collected by centrifugation. The supernatant was filtered and the soluble proteins were allowed to bind to Phenyl Sepharose (PS) beads (GE Healthcare). After an hour of incubation, the PS beads were washed with PBST and the bound proteins were eluted with Milli-Q water. Sodium chloride was added to increase the conductivity and the PS purified proteins were loaded onto an anti-AAD-12 immunoaffinity column which had been conjugated with an AAD-12 specific polyclonal antibody. The non-bound proteins were collected from the column and the column was washed extensively with pre-chilled PBS (phosphate buffered saline, pH 7.4). 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 leaf tissue from event DAS-68416-4 and Maverick was mixed with PBST buffer containing ~2.0% 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 briefly centrifuged. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived AAD-12, 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-12 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-12 protein was analysed for evidence of glycosylation by electrophoresis with microbe-derived AAD-12 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-12 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 Sequence Analysis of Plant- and Microbe-Derived AAD-12 Protein

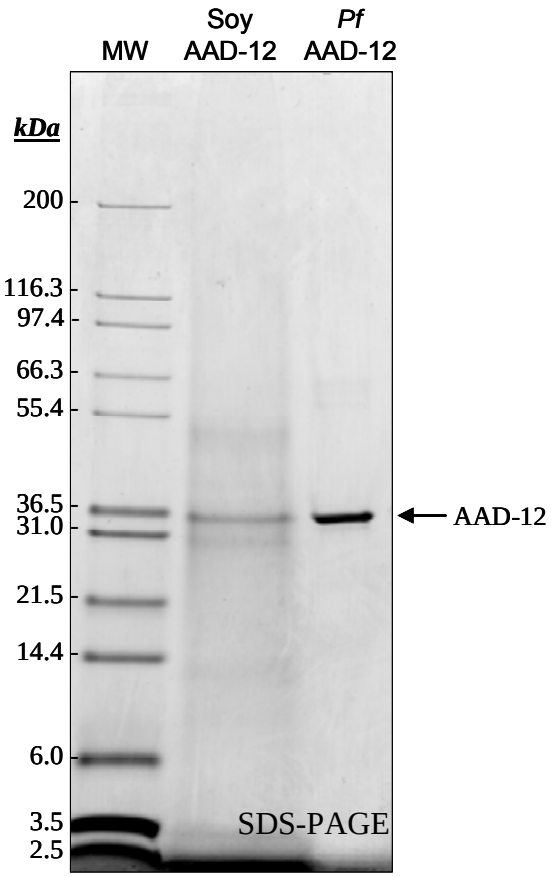
The immunoaffinity purified AAD-12 plant-derived protein was subjected to in-solution digestion by trypsin and Asp-N followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray-ionization liquid chromatography/mass spectrometry (ESI-LC/MS). The peptide fragments of the plant-derived AAD-12 protein (including the N- and C-termini) were analysed and compared with the sequence of the microbe-derived protein.

Results of the SDS-PAGE and Western Blot Analysis of Crude Extracts – AAD-12

In the microbe-derived AAD-12, the major protein band, as visualized on the Coomassie stained SDS-PAGE gel, was approximately 32 kDa (**Figure 38**). As expected, the corresponding plant-derived AAD-12 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-12 protein. The co-purified proteins were likely retained on the column by weak interactions with the column matrix (Holroyde *et al*., 1976, Kennedy and Barnes, 1983 and Williams *et. al.*, 2006).

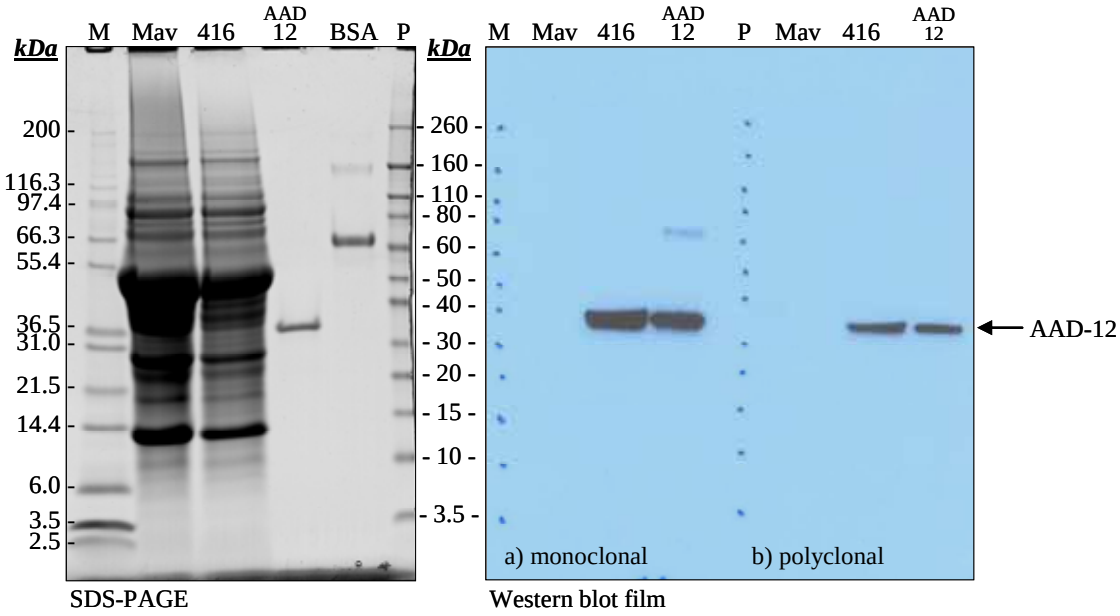
The microbe-derived AAD-12 and DAS-68416-4 plant tissue extract showed a positive signal of the expected size on the western blot using the anti-AAD-12 polyclonal antibody (**Figure 39**). In the AAD-12 western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the samples from the transgenic plant. The monoclonal antibody did detect a small amount of the AAD-12 dimer in the microbe-derived protein. These results add to the evidence that the protein expressed in soybean is not glycosylated which would add to the overall protein molecular weight.

Figure 38. SDS-PAGE of soybean- and microbe-derived AAD-12. (Schafer et al, 2009, Study ID 081113).



Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
Soy	Soybean-Derived AAD-12 (DAS-68416-4)	500 µL
Pf	Microbe-Derived AAD-12	1000 ng

Figure 39. SDS-PAGE and western blot of soybean- and microbe-derived AAD-12 protein extracts. (Schafer et al, 2009, Study ID 081113).



Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
Mav	Nontransgenic Soybean Extract	32 µL
416	Event DAS-68416-4 extract	32 µL
AAD-12	Microbe-Derived AAD-12	~785 ng
BSA	Bovine Serum Albumin (BSA)	~785 ng
P	Novex Prestained MW Markers	10 µL

Methods and Results for Characterization of PAT Protein

DAS-68416-4 Transgenic Soybean Material

Greenhouse-grown DAS-68416-4 T5 plants were used as the plant source of the PAT protein. Prior to use, individual plants were leaf tested to confirm expression of the PAT protein using a rapid lateral flow test strip according to the manufacturer's instructions. Leaves (and some stems) from PAT expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

Control Soybean Material

Control soybean line Maverick had a genetic background representative of the DAS-68416-4 soybean plants, but did not contain the pat gene. Absence of PAT expression in the control plants was confirmed by immunoassay using a PAT specific rapid lateral flow test strip. Leaves (and some stems) of control plants were harvested, lyophilized, ground and stored under the same conditions as the DAS-68416-4 soybean.

Reference Material

Recombinant PAT microbial protein was produced in Pseudomonas fluorescens (Pf) and purified to homogeneity. The microbe-derived PAT protein preparation was aliquoted and stored at -80 ºC to maintain activity.

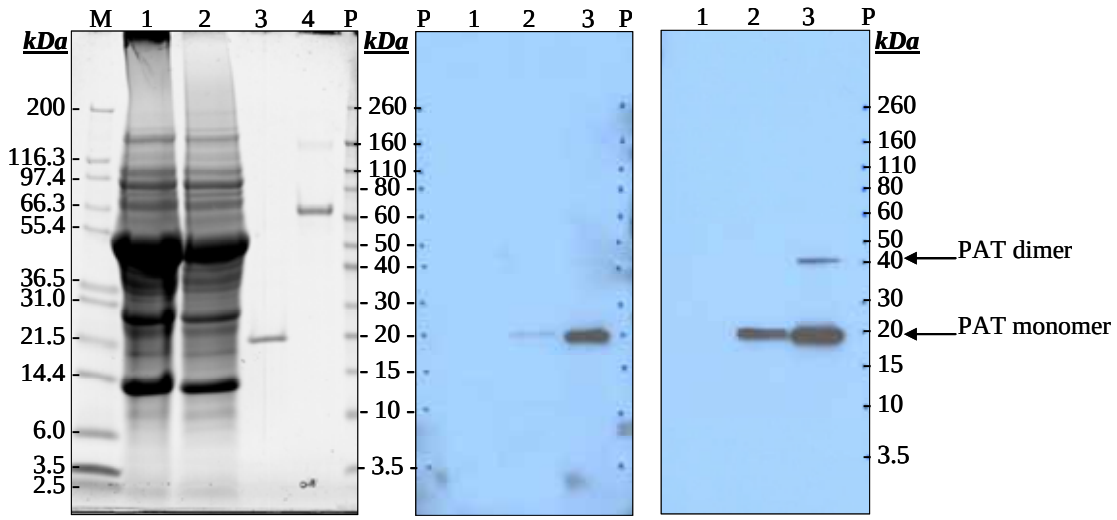
SDS-PAGE and Western Blot Analysis of Crude Extracts

Lyophilized leaf tissue from event DAS-68416-4 and Maverick was mixed with PBST buffer containing ~2.0% 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 briefly centrifuged. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived PAT, 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 cut in half with one probed with a PAT specific polyclonal rabbit antibody and the remaining half probed with a PAT specific monoclonal antibody. A chemiluminescent substrate was used to visualize the immunoreactive bands.

Results of the SDS-PAGE and Western Blot Analysis of Crude Extracts - PAT

The soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal antibodies and showed the expected band at approximately 21 kDa (**Figure 40**, Panel B and C). In the PAT western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the transgenic soybean extract. This result adds to the evidence that the protein expressed in soybean is not post-translationally modified which would have added to the overall protein molecular weight.

Figure 40. SDS-PAGE and western blots of DAS-68416-4 and non-transgenic Maverick soybean. (Schafer et al, 2009, Study ID 081132).



Panel A: SDS-PAGE

Panel B: Polyclonal antibody western blot

Panel C: Monoclonal antibody western blot

Lane	Sample	Amount
M	Invitrogen Mark12 molecular weight markers	10 □L
1	Non-transgenic (Maverick) soybean extract	40 □L
2	Transgenic (Event DAS-68416-4) soybean extract	40 □L
3	Microbe-derived PAT protein (TSN105742)750 ng gel, 35 ng blot	
4	Bovine serum albumin (BSA)780 ng gel	
P	Novex Sharp prestained molecular weight markers	10 □L

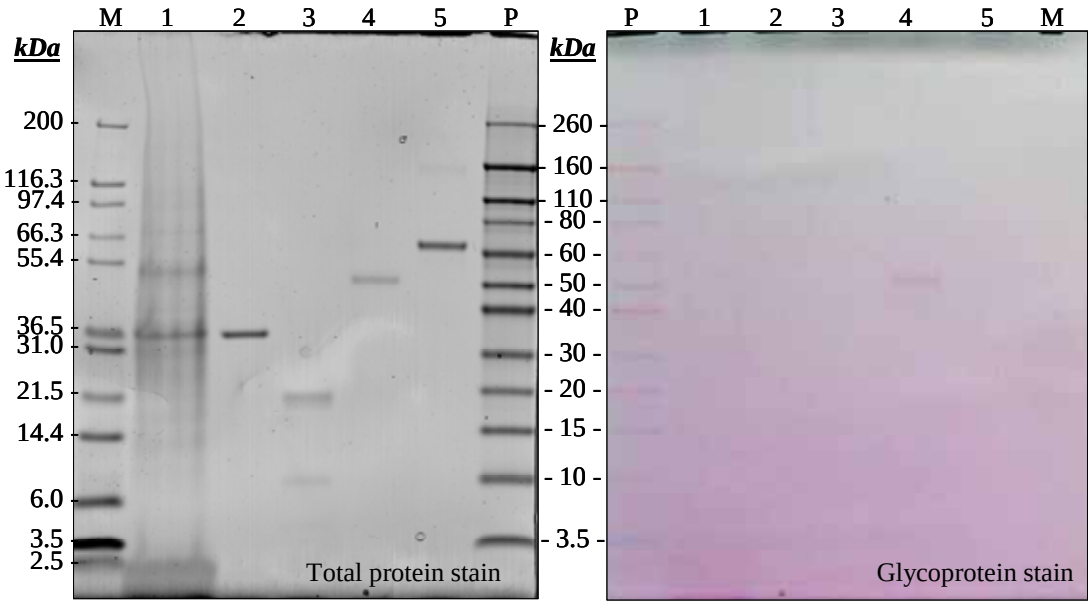
Conclusions

The PAT protein produced in DAS-68416-4 soybean was shown to be equivalent to that produced in other transgenic crops (USDA 1996, USDA 2001, USDA 2004, USDA 2005).

Post-Translation Glycosylation

No covalently-linked carbohydrates were detectable on the plant- or microbe-derived AAD-12 proteins (**Figure 41**). Horseradish peroxidase, a glycoprotein, was used as a positive indicator for glycosylation. Soybean trypsin inhibitor and bovine serum albumin, both non-glycoproteins, served as negative controls.

Figure 41. Glycosylation analysis of soybean- and microbe-derived AAD-12 proteins. (Schafer et al, 2009, Study ID 081113).
Note: The immunoaffinity-purified, soybean-derived AAD-12 protein, microbe-derived AAD-12, 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.



Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
1	Soybean-Derived AAD-12 (Frac 3)	500 µL
2	Microbe-Derived AAD-12	500 ng
3	Soybean Trypsin Inhibitor (STI)	500 ng
4	Horseradish Peroxidase (HRP)	500 ng
5	Bovine Serum Albumin (BSA)	500 ng
P	Novex Prestained MW markers	10 µL

MALDI-TOF and ESI/LC- MS Tryptic and Asp-N Peptide Mass Fingerprints of AAD-12 Proteins

Following digestion of the plant-derived AAD-12 protein by trypsin and Asp-N, the masses of the detected peptides were compared with those deduced based on potential cleavage sites in the sequence of the AAD-12 protein. **Figure 42** illustrates the theoretical peptide cleavage which was generated *in silico* using PAWs software (Proteometrics LLC).

The trypsin and Asp-N digestion of soybean-derived AAD-12 protein yielded high detection of the expected peptides, resulting in 73.4% coverage of the AAD-12 protein sequence. The analysis confirmed the plant-derived protein amino acid sequence matched that of the microbe-derived AAD-12 protein and that of the predicted amino acid sequence. Results of these analyses indicated that the amino acid sequence of the soybean-derived AAD-12 protein was equivalent to the *P. fluorescens*-expressed protein.

Tryptic and Asp-N Peptide N- and C-terminal Sequence Analysis of AAD-12

The N-terminal sequence of the first 27 residues of the plant-derived and all 292 residues of the microbe-derived AAD-12 protein was obtained by mass spectrometry. The amino acid sequences for N-terminus of both proteins was A^{2} H A A L S P L S Q I T P T G A T L G A T V T G V H L A T L^{27}, indicating the N-terminal methionine had been removed (**Table 8** and **Figure 43**). 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, the N-terminal peptide of the AAD-12 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, 2000; Polevoda and Sherman, 2002).

The C-terminal sequences of the plant- and microbe-derived AAD-12 proteins were determined to be identical to the expected sequences (**Table 9** and **Figure 44**).

Figure 42. Theoretical trypsin (top panel) and Asp-N (bottom panel) cleavage of the AAD-12 protein. (Schafer et al, 2009, Study ID 081113).

Note: Alternating blocks of upper (black) and lower (red) 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.

Digestion at K (lysine) and R (arginine)

1	M A Q T T L Q I T P T G A T L G A T V T G V H L A T L D D A	30
31	G F A A L H A A W L Q H A L L I F P G Q H L S N D Q Q I T F	60
61	A K r F G A I E R i g g g d i v a i s n v k A D G T V R q h	90
91	s p a e w d d m m k V I V G N M A W H A D S T Y M P V M A Q	120
121	G A V F S A E V V P A V G G R t c f a d m r A A Y D A L D E	150
151	A T R a l v h q r S A R h s l v y s q s k L G H V Q Q A G S	180
181	A Y I G Y G M D T T A T P L R P L V K v h p e t g r p s l l	210
211	i g r H A H A I P G M D A A E S E R f l e g l v d w a c q a	240
241	p r V H A H Q W A A G D V V V W D N R c l l h r A E P W D F	270
271	K l p r V M W H S R l a g r p e t e g a a l v	293

Digestion at D (aspartate)

1	M A Q T T L Q I T P T G A T L G A T V T G V H L A T L d D A	30
31	G F A A L H A A W L Q H A L L I F P G Q H L S N d q q i t f	60
61	a k r f g a i e r i g g g D I V A I S N V K A d g t v r q h	90
91	s p a e w D d m m k v i v g n m a w h a D S T Y M P V M A Q	120
121	G A V F S A E V V P A V G G R T C F A d m r a a y D A L d e	150
151	a t r a l v h q r s a r h s l v y s q s k l g h v q q a g s	180
181	a y i g y g m D T T A T P L R P L V K V H P E T G R P S L L	210
211	I G R H A H A I P G M d a a e s e r f l e g l v D W A C Q A	240
241	P R V H A H Q W A A G d v v v w D N R C L L H R A E P W d f	270
271	k l p r v m w h s r l a g r p e t e g a a l v	293

Figure 43. Sequence coverage in the tryptic and Asp-N peptide mapping analysis of plant-derived AAD-12 protein with MALDI-TOF and ESI/LC MS. (Schafer et al, 2009, Study ID 081113).

Note: The numbers on the left and right sides of the protein sequence indicate the amino acid residue numbers. Letters highlighted in gray represent tryptic peptide sequence detected by MALDI-TOF MS and ESI-LC/MS. Underlined letters represent Asp-N peptide sequence detected. The overall sequence coverage was 73.4%. The down arrow indicates the N-terminal methionine was removed by an aminopeptidase and the N-terminal alanine was N-acetylated.

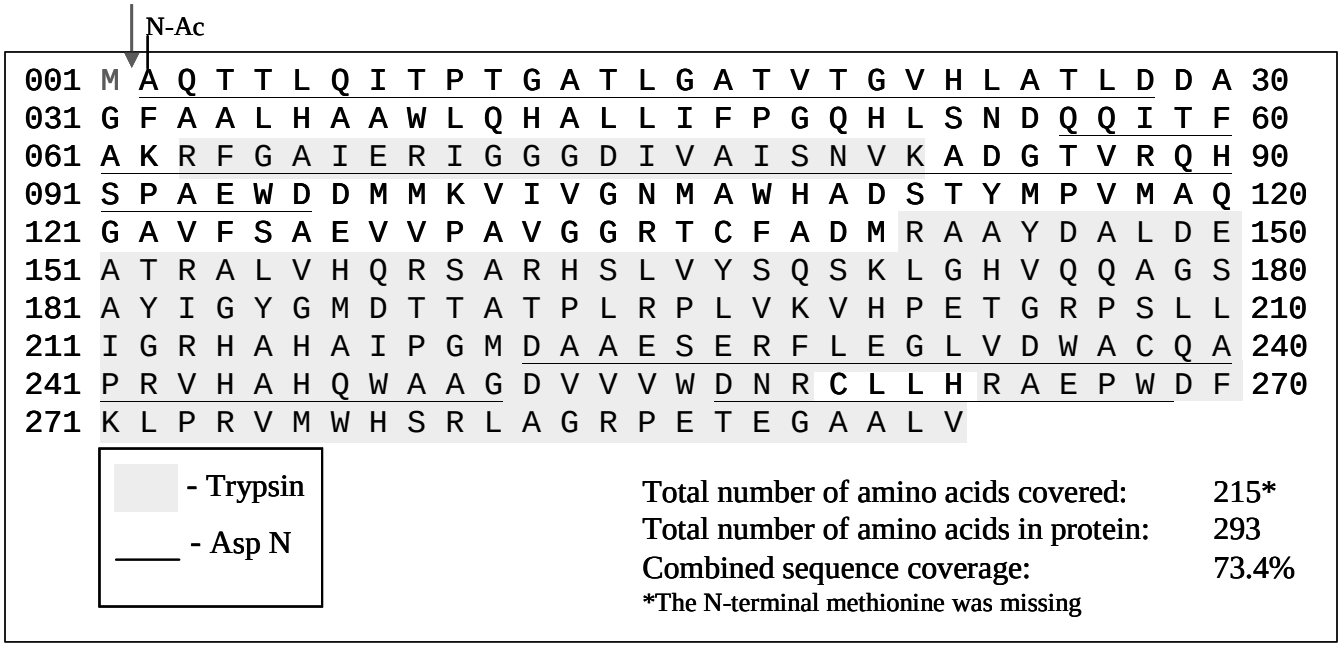
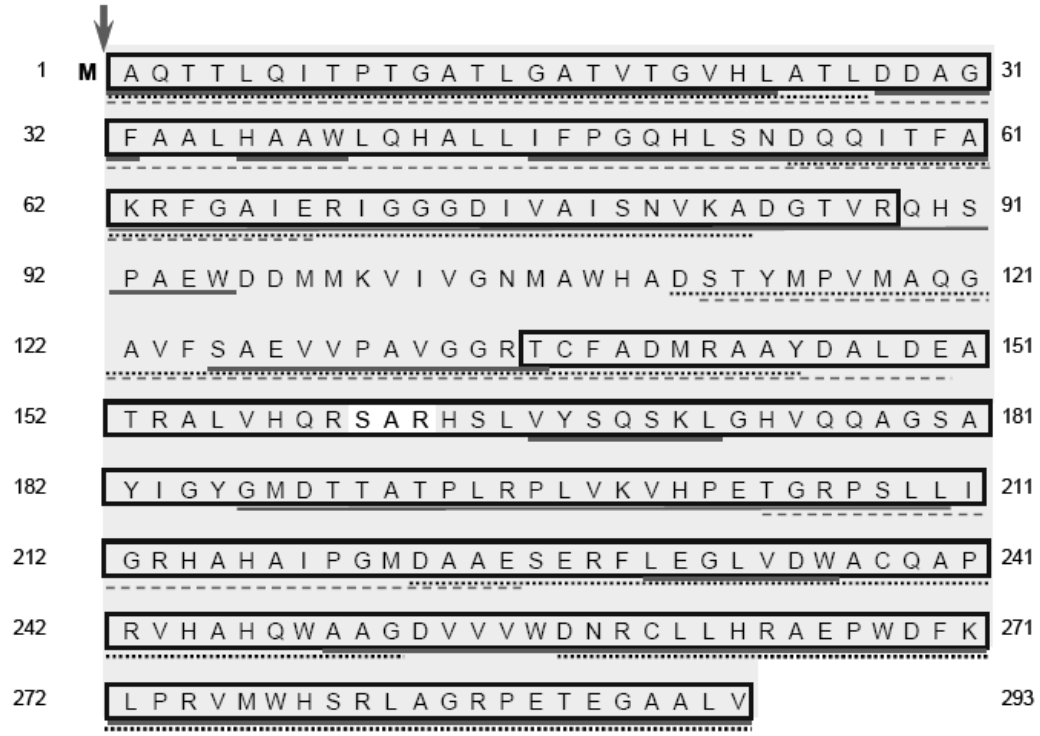
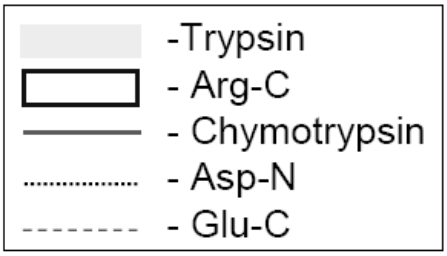


Figure 44. Sequence coverage in the peptide mapping analysis of microbe-derived AAD-12 protein with MALDI-TOF and ESI/LC MS. (Schafer et al, 2009, Study ID 081113).

Note: The numbers on the left and right sides of the protein sequence indicate the amino acid residue numbers. Letters highlighted in gray represent tryptic peptide sequence detected by MALDI-TOF MS and ESI-LC/MS. Letters in boxes indicates sequence coverage detected with Arg-C digestion. Underlined letters indicates sequence coverage detected with Asp-N, chymotrypsin and Glu-C digestions. The overall sequence coverage was 99.7%. The down arrow indicates the N-terminal methionine was removed by an aminopeptidase





Total number of amino acids covered:	292*
Total number of amino acids in protein:	293
Combined sequence coverage:	99.7%

*The N-terminal methionine was missing

Table 8. Summary of N-terminal sequence data of AAD-12 soybean- and microbe derived proteins. (Schafer et al, 2009, Study ID 081113).

Source	Expected N-terminal Sequence[1]
P. fluorescens	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Soybean Event DAS-68416-4	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Source	**Detected N-terminal Sequence[2]**
P. fluorescens	A^{2} Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Soybean Event DAS-68416-4[3]	$^{N\text{-}Ac}A^{2}$ Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}

[1]Expected N-terminal sequence of the first 27 amino acid residues of *P. fluorescens*- and soybean-derived AAD-12.

[2]Detected N-terminal sequences of *P. fluorescens*- and soybean-derived AAD-12.

[3]The MALDI-TOF MS data for the N-terminal peptide revealed that the soybean-derived AAD-12 protein was acetylated (*N-Acetyl*-A Q T T L Q I T P T G A T L G A T V T G V H L A T L D).

Notes:

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

A:	alanine	D:	Aspartate	G:	glycine
H:	histidine	I:	isoleucine	L:	leucine
M:	methionine	P:	proline	Q:	glutamine
T:	threonine	V:	valine		

Table 9. Summary of C-terminal sequence data of AAD-12 soybean- and microbe derived proteins. (Schafer et al, 2009, Study ID 081113).

Source	Expected C-terminal Sequence[1]
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-68416-4	^{281}L A G R P E T E G A A L V^{293}
Source	**Detected C-terminal Sequence[2]**
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-68416-4	^{281}L A G R P E T E G A A L V^{293}

[1]Expected C-terminal sequence of the last 13 amino acid residues of *P. fluorescens*- and soybean-derived AAD-12.

[2]Detected C-terminal sequences of *P. fluorescens*- and soybean-derived AAD-12.

Notes:

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

Amino acid residue abbreviations:

A:	alanine	E:	glutamate	G:	glycine
L:	leucine	P:	proline	R:	arginine
T:	threonine	V:	valine		

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 Australian, CODEX, Japanese and European residue definitions which do not include DCP in any tolerance expression.

c. Novel Protein Expression

To determine if any novel open reading frames (>30 amino acids) were created or endogenous coding sequences were interrupted by the insertion of the DAS-68416-4 insert into the soybean genome, DNA sequences of the insert and its flanking border regions were determined. In total, 10212 bp of event DAS-68416-4 genomic sequence were confirmed, comprising 2730 bp of the 5' flanking border sequence, 1082 bp of the 3' flanking border sequence, and 6400 bp of the DNA insert. In addition, 3867 bp of DNA sequences from the original locus, including the 5' and 3' borders, were confirmed. Analysis of the sequence spanning the junctions between the insert and its borders indicated that no novel open reading frames (>30 amino acids) resulted from the DNA insertion in event DAS-68416-4 and also that no endogenous open reading frames (>30 amino acids) were disrupted by the DAS-68416-4 integration in the native soybean genome.

Expression of AAD-12 Protein in Plant Tissues

A field expression study was conducted at six locations in U.S. and Canada during 2008. Six sites (Iowa, Illinois, Indiana, Nebraska and Ontario, Canada (2 sites)) were planted with DAS-68416-4 soybean and the conventional control (Maverick). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Four treatments of the DAS-68416-4 soybean (unsprayed, sprayed with 2,4-D, sprayed with glufosinate, or sprayed with both 2,4-D and glufosinate) were tested. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at V5 and V10 stage, and root and forage were collected at the R3 stage of

development. The grain was collected at the R8 stage of development (Gaska, 2006). The soluble, extractable AAD-1 protein was measured using a validated enzyme-linked immunosorbent assay (ELISA) method. AAD-12 protein levels for all tissue types were calculated on ng/mg dry weight basis. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed below. A summary of the AAD-12 protein concentrations (averaged across sites) in the various soybean matrices is shown in **Table 10**. Average expression values ranged from 15.48 ng/mg dry weight in R3 stage root to 66.08 ng/mg dry weight in V5 stage leaf tissue. Expression values were similar for the all sprayed treatments as well as for the plots sprayed and unsprayed with 2,4-D and glufosinate herbicides. No AAD-12 protein was detected in the control tissues across the six locations.

Table 10. Summary of AAD-12 protein levels in tissues collected from DAS-68416-4 produced in the U.S. and Canada during 2008. (Smith-Drake et al, 2009, Study ID 080003).

Tissue	Treatment	AAD-12 ng/mg Tissue Dry Weight		
		Mean	Std. Dev.	Range
V5 Leaf	DAS-68416-4 Unsprayed	51.42	25.22	26.37 - 97.66
	DAS-68416-4 + Glufosinate	50.63	23.69	28.03 - 94.00
	DAS-68416-4 + 2,4-D	51.68	25.41	27.16 - 100.79
	DAS-68416-4 + Glufosinate and 2,4-D	66.08	37.82	25.14 - 164.58
V10 Leaf	DAS-68416-4 Unsprayed	53.95	20.85	29.83 - 90.89
	DAS-68416-4 + Glufosinate	56.06	21.95	25.06 - 91.95
	DAS-68416-4 + 2,4-D	55.24	20.62	30.84 - 91.80
	DAS-68416-4 + Glufosinate and 2,4-D	57.07	22.97	32.02 - 95.16
Root	DAS-68416-4 Unsprayed	17.10	5.68	8.80 - 27.62
	DAS-68416-4 + Glufosinate	15.48	4.58	6.30 - 23.08
	DAS-68416-4 + 2,4-D	16.01	6.64	3.16 - 27.91
	DAS-68416-4 + Glufosinate and 2,4-D	16.66	6.81	1.84 - 26.50
Forage	DAS-68416-4 Unsprayed	41.11	25.72	5.70 - 91.17
	DAS-68416-4 + Glufosinate	39.35	24.47	5.49 - 87.96
	DAS-68416-4 + 2,4-D	40.56	25.58	5.02 - 88.02
	DAS-68416-4 + Glufosinate and 2,4-D	39.65	22.41	4.96 - 69.62
Grain	DAS-68416-4 Unsprayed	16.47	3.55	9.40 - 21.86
	DAS-68416-4 + Glufosinate	16.94	3.15	11.9 - 22.74
	DAS-68416-4 + 2,4-D	16.47	3.78	9.71 - 21.95
	DAS-68416-4 + Glufosinate and 2,4-D	16.21	3.62	9.91 - 23.40

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the AAD-12 ELISA in the tissue matrices were as follows:

Tissue	LOD (ng/mg DW)	LOQ (ng/mg DW)
Leaf (V5)	0.50	1.00
Leaf (V10)	0.50	1.00
Root	0.50	1.00
Forage	0.50	1.00
Grain	0.50	1.00

Expression of the PAT Protein in Plant Tissues

A field expression study was conducted at six locations in U.S. and Canada during 2008. Six sites (Iowa, Illinois, Indiana, Nebraska and Ontario, Canada (2 sites)) were planted with DAS-68416-4 soybean and the conventional control (Maverick). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Four treatments of the DAS-68416-4 soybean (unsprayed, sprayed with 2,4-D, sprayed with glufosinate, or sprayed with both 2,4-D and glufosinate) were tested. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at V5 and V10 stage, and root and forage were collected at the R3 stage of development. The grain was collected at the R8 stage of development (Gaska, 2006). The soluble, extractable PAT protein was measured using a validated enzyme-linked immunosorbent assay (ELISA) method. PAT protein levels for all tissue types were calculated on ng/mg dry weight basis. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed below.

A summary of the PAT protein concentrations (averaged across sites) in the various soybean matrices is shown in **Table 11**. Average expression values ranged from 1.73 ng/mg dry weight in R3 stage root to 11.76 ng/mg dry weight in V10 stage leaf tissue. Expression values were similar for the all sprayed treatments as well as for the plots sprayed and unsprayed with 2,4-D and glufosinate herbicides. No PAT protein was detected in the control tissues across the six locations.

Table 11. Summary of PAT protein levels in tissues collected from DAS-68416-4 produced in the U.S. and Canada during 2008. (Smith-Drake et al, 2009, Study ID 080003).

Tissue	Treatment	PAT ng/mg Tissue Dry Weight		
		Mean	Std. Dev.	Range
V5 Leaf	DAS-68416-4 Unsprayed	9.17	2.99	4.33 - 13.75
	DAS-68416-4 + Glufosinate	9.83	2.66	3.67 - 13.78
	DAS-68416-4 + 2,4-D	9.01	3.03	4.87 - 13.92
	DAS-68416-4 + Glufosinate and 2,4-D	10.05	3.76	3.00 - 15.03
V10 Leaf	DAS-68416-4 Unsprayed	10.94	1.31	8.43 - 13.35
	DAS-68416-4 + Glufosinate	11.51	1.69	9.08 - 14.44
	DAS-68416-4 + 2,4-D	11.76	2.02	7.49 - 14.81
	DAS-68416-4 + Glufosinate and 2,4-D	11.58	1.45	9.26 - 14.15
Root	DAS-68416-4 Unsprayed	1.73	0.51	0.47 - 2.84
	DAS-68416-4 + Glufosinate	1.92	0.45	1.01 - 2.67
	DAS-68416-4 + 2,4-D	1.73	0.68	0.42 - 2.83
	DAS-68416-4 + Glufosinate and 2,4-D	1.93	0.55	0.36 - 2.68
Forage	DAS-68416-4 Unsprayed	3.63	2.88	0.06 - 12.54
	DAS-68416-4 + Glufosinate	4.81	3.75	0.40 - 12.10
	DAS-68416-4 + 2,4-D	5.28	4.20	0.12 - 12.13
	DAS-68416-4 + Glufosinate and 2,4-D	4.73	3.63	0.45 - 12.35

Grain	DAS-68416-4 Unsprayed	2.73	0.34	1.96 - 3.37
	DAS-68416-4 + Glufosinate	2.74	0.28	2.29 - 3.39
	DAS-68416-4 + 2,4-D	2.79	0.26	2.21 - 3.13
	DAS-68416-4 + Glufosinate and 2,4-D	2.82	0.23	2.43 - 3.25

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the PAT ELISA in the tissue matrices were as follows:

Tissue	**LOD** (ng/mg DW)	**LOQ** (ng/mg DW)
Leaf (V5)	0.06	0.12
Leaf (V10)	0.06	0.12
Root	0.06	0.12
Forage	0.06	0.12
Grain	0.06	0.12

Methods for AAD-12 and PAT Protein Expression Analysis

Experimental Design

The experimental design included six (6) field sites; Iowa, Illinois, Indiana, Nebraska and Ontario, Canada (2 sites) (referred to as IA, IL, IN, NE, ON1 and ON2). Each site consisted of one plot of each treatment per block, with 3 blocks per location. Plot size was 2 rows by 25 feet. Plots were arranged in a randomized complete block (RCB) design, with a unique randomization at each site. Each soybean plot was bordered by 2 rows of a non-transgenic soybean of similar maturity. The entire trial site was surrounded by a minimum of 20 feet of a non-regulated soybean of similar relative maturity. At each location, all blocks were used for collection of samples for expression and nutrient composition analysis.

Herbicide treatments were designed to replicate maximum label rate commercial practices. 2,4-D (Weedar 64) was applied as 3 broadcast over-the-top applications (seasonal total of 3 lb ae/A). Individual applications were at pre-emergence and approximately V4 and R2 stages. Individual target application rates were 1.0 lb ae/A for Weedar 64 (1120 g ae/ha). Glufosinate (Liberty) was applied as 2 broadcast over-the-top application. Application timing was at approximately V6 and R1 growth stages. The target application rate was 0.33 lb ai/A and 0.41 lb ai/A (374 and 454 g ai/ha).

Sample Collection

Samples were shipped to Dow AgroSciences Regulatory Science and Government Affairs laboratories and maintained frozen until use. Samples of soybean tissues were prepared for expression analysis by coarse grinding, lyophilizing and/or fine-grinding with a Geno/Grinder (Certiprep, Metuchen, New Jersey).

<u>Leaf (V5 and V10)</u>

One leaf sample per plot, each sample containing 8 trifoliate set of leaves collected from separate plants, were collected for each test and control entry. Each leaf sample was the youngest set of fully expanded trifoliate leaves.

<u>Root (R3)</u>

One root sample (representing 3 plants) per plot were collected for each test and control entry at the R3 stage by cutting a circle around the base of the plant. The root ball was removed and cleaned.

<u>Forage (R3)</u>

One forage sample (representing 3 plants) per plot each consisting of the aerial portion (no roots) of 3 whole plants were collected from each test and control entry.

<u>Grain (R8 – Maturity)</u>

One individual sample was collected from each plot of each test and control entry. Each sample contained approximately 500-gram of grain.

Determination of AAD-12 Protein Concentration

The AAD-12 protein was extracted from soybean tissues except grain with a phosphate buffered saline solution with Tween-20 (PBST) and 0.75% ovalbumin (OVA). For grain, the protein was extracted with a PBST buffer containing 0.1% Triton-100. The plant tissue and grain extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analysed using an AAD-12 ELISA kit in a sandwich format. Briefly, an aliquot of the diluted sample and a horseradish

peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate are incubated in the wells of a microtiter plate coated with an immobilized anti-AAD-12 polyclonal antibody. These antibodies bind with AAD-12 protein in the wells and form a "sandwich" with AAD-12 protein bound between soluble and the immobilized antibodies. The unbound samples and conjugate are then removed from the plate by washing with PBST. Subsequent addition of an enzyme substrate generated a coloured product. The reaction was stopped by adding a dilute acid solution. Since the AAD-12 was bound in the antibody sandwich, the level of colour development was related to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower colour development). The absorbance at 450 nm minus 650 nm was measured using a Molecular Devices Spectra Max 190 or Spectra Max M2 plate reader. A calibration curve was generated and the AAD-12 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.

Determination of PAT Protein in Soybean Tissue Samples

The PAT protein was extracted from soybean tissues with a phosphate buffered saline solution with Tween-20 (PBST) and 1% polyvinylpyrrolidone (PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted with PBST/1% PVP, and analysed using a PAT ELISA kit. Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT antibody and anti-PAT antibodies coated in the wells of a 96-well plate in a sandwich ELISA format. 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. The reaction was stopped by adding a dilute acid solution. Since the PAT was bound in the antibody sandwich, the level of colour development was related to the concentration of PAT in the sample (i.e., lower residue concentrations result in lower colour development). The absorbance at 450 minus 650 nm was measured using a Molecular Devices Spectra Max 190 or Spectra max M2 plate reader. A calibration curve was generated and the PAT 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, the N-terminal peptide of the AAD-12 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, 2000; Polevoda and Sherman, 2002).

The C-terminal sequences of the plant- and microbe-derived AAD-12 proteins were determined to be identical to the expected sequences (**Table 9** and **Figure 43**).

e. Novel Protein Silencing

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

f. Novel Protein History of Consumption

Taxonomy and habitat

Lineage (full): Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Delftia

The current taxonomic classification for the bacterial strain that AAD-12 was derived from is *Delftia acidovorans* MC1. This strain was isolated from herbicide-contaminated building rubble (Muller et al. 1999), and shown to degrade a number of phenoxyalkanoic herbicides. This type species was originally classified as *Pseudomonas acidovorans* and then *Comamonas acidovorans*. It was later reclassified as *Delftia acidovorans* based on an analysis of 16S rRNA (Wen *et al.*, 1999). This species is a non glucose-fermenting, gram-negative, non spore-forming rod prevalent in soil and fresh water. Some species have also been isolated from activated sludge and clinical specimens.

History of food use

Delftia acidovorans can be used to transform ferulic acid into vanillin and related flavour metabolites (Yoon *et. al.* 2005). This utility has led to a history of safe use for *D. acidovorans* in the food processing industry. For example, US Patent 5,128,253 “Bioconversion process for the production of vanillin” was issued on July 7, 1992 to Kraft General Foods (Labuda *et al.*, 1992).

This strain also produces polyhydroxyalkanoates that are being developed as biomaterials for medical applications (Sudesh 2004)

Toxicity and Allergenicity

There are limited reports of *D. acidovorans* causing infections in compromised patients (Horowitz et. al. 1990). There are no reports of this strain producing any allergens.

3. Potential Toxicity of the Novel Protein

Part C Section 3 DAS Reports

Larrinua, I.M. & Herman, R.A. (2007) AAD-12 Amino-Acid Homology Search for Similarity to Toxins. Dow AgroSciences LLC Study ID 071035.

Wiescinski, C.M., Golden, R.M. (2008) AAD-12: Acute Oral Toxicity Study in CRL:CD1(1CR) Mice. Dow AgroSciences LLC Study ID 081037.

a. Amino Acid Sequence Comparison to Known Toxins

The AAD-12 protein does not share any amino acid sequence similarities with known toxins that would present any safety concerns. Amino acid homologies with the AAD-12 protein sequence were evaluated using a global sequence similarity search against the GenBank non-redundant protein dataset (posted on February 17, 2007 containing 4,626,804 sequences with 1,596,079,149 amino acids). The only significant homologies identified were with a few major proteins with enzyme activity: 1) taurine dioxegenases that degrade taurine (Eichorn *et al.*, 2007), 2) clavaminic acid synthetases or "CAS-like" (Zhang *et al.*, 2000), 3) tolC proteins which are known efflux pumps (Koronakis *et al.*, 2000), 4) a (S)-2-(2,4-dichlorophenoxy)propionate, 2-oxoglutarate dioxygenase (Schleinitz *et al.*, 2004), 5) a pvcB protein which is a known "CAS-like" protein, 6) an inosine-uridine preferring nucleoside hydrolase (Gopaul *et al.*, 1996), and 7) a hypothetical protein with no functional annotation. None of the similar proteins returned by the search identified any safety concerns that might arise from the expression of AAD-12 protein in plants.

b. Acute Oral Toxicity

An acute oral toxicity study with AAD-12 protein was conducted in mice at a level of 2000 mg AAD-12/kg after adjustment for purity. 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. Therefore the acute oral LD_{50} and NOEL of AAD-12 in male and female mice was greater than 2000 mg/kg based on fact that no mortality was observed and there were no observable effects (adverse or non-adverse effects) with the AAD-12-treated animals. AAD-12 protein displays very low acute toxicity.

4. Potential Allergenicity of Novel Proteins

Part C Section 4 DAS Reports

Schafer, B.W. (2008) Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12). Dow AgroSciences Study ID 080140.

Stagg, N.J. (2010). Endogenous Allergenicity Analysis of DAS-64816-4 Soybean. Dow AgroSciences LLC Study ID 101001.

Herman, R.A. (2007) AAD-12 Amino-Acid Homology Search for Similarity to Allergens. Dow AgroSciences LLC Study ID 071036.

Embrey, S.K., Shafer, B.W. (2008) *In Vitro* simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12 (abbreviation AAD-12). Dow AgroSciences LLC Study ID 080064.

Studies were conducted to ascertain the potential allergenicity of the AAD-12 protein. These studies included: 1) bioinformatics search for amino-acid sequence homology with known allergens, 2) digestive fate in simulated gastric fluid and 3) heat lability. Based on the lack of significant amino acid sequence homology to known allergens, and the lack of enzymatic and heat stability, the AAD-12 protein is considered to have a low risk of allergenic potential.

Endogenous Allergen Analysis

As soybean is one of the top eight important allergenic foods (Sampson 1999, Sicherer and Sampson 2006; Chapman *et al.* 2006), a study was conducted to determine if the genetic modification used to generate DAS-68416-4 soybean altered the endogenous allergen content. (Stagg, N.J., 2010, Study ID: 101001).

IgE binding to extracts of DAS-68416-4 soybean and its non-transgenic control (Maverick) were evaluated with one dimensional (1D) IgE immunoblot (qualitative analysis) and ELISA inhibition (quantitative analysis) using sera from 20 clinically-reactive soy allergic patients [both children and adults with clinical histories of soybean allergy and CAP (Pharmacia Capsulated Hydrophobic Carrier Polymer) scores > 20].

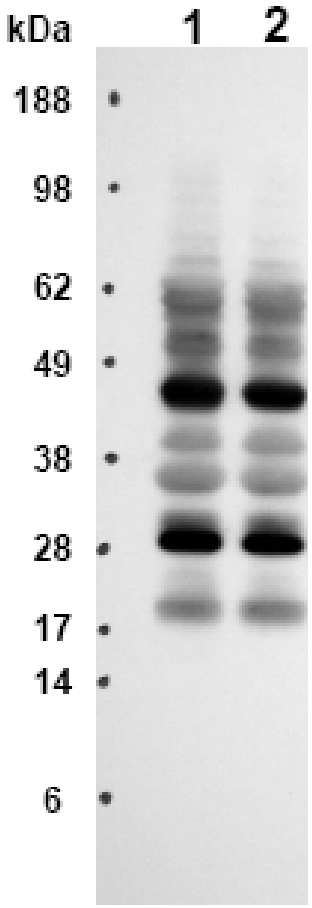
SDS-PAGE with Coomassie Blue Staining and Immunoblot Analysis

Extracts were prepared from the ground seed of DAS-68416-4 and Maverick soybeans. All samples were heated at 70 ºC for 10 min and then run on SDS-PAGE with Coomassie blue to evaluate protein content of the two seed lots. Gel transfer of the proteins to a nitrocellulose membrane was performed with replicated blots, and one blot from each replicate was stained with Ponceau S stain to confirm protein transfer. Unstained blots were blocked in 2% BSA in PBST for at least 1 hour at room temperature followed by overnight incubation in serum from a pool of 20 soy-allergic patients held at 4 ºC. Blots were washed with PBST to remove unbound IgE and then incubated in biotinylated goat IgG-anti-human IgE for 1 hr at room temperature with continuous agitation. Additional washing with PBST before and after adding NeutrAvidin-HRP conjugate was performed. Pierce SuperSignal

chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were covered with Pierce SuperSignal reagent, exposed to Hyperfilm ECL in a darkroom and developed.

The protein profiles between DAS-68416-4 and the non-transgenic control (Maverick) were compared using SDS-PAGE analysis with Coomassie blue staining, which did not reveal any differences in protein banding patterns between the two soybean extracts. The IgE binding profiles of DAS-68416-4 and Maverick were compared in the one-dimensional immunoblot using soy-allergic sera and also showed no difference (**Figure 45**).

Figure 45. Immunoblot of DAS-68416-4 and control (Maverick) soybean extracts with soybean-allergic patient sera. (Stagg, N.J., 2010, Study ID: 101001).



Lane	Contents
1	15 µl of control (Maverick) soybean seed extract (10µg)
2	15 µl of DAS-68416-4 soybean seed extract (10 µg)

ELISA Inhibition

ELISA inhibition of IgE binding from a pooled soybean-allergic (20 patients) serum sample was conducted for DAS-68416-4 and control (Maverick) soybean extracts. Extracts from DAS-68416-4 and Maverick at various concentrations (0.004 to 4000 µg/ml) were incubated with the pooled serum and added to 96-well plates that were previously coated with the non-transgenic control (maverick) extracts. After biotinylated goat IgG-anti-human IgE, NeutraAvidin-HRP conjugate and peroxidase substrate TMB additions with appropriate washing with PBST in between, plates were read on a microplate reader at 450 nm.

The results of the ELISA inhibition experiments were plotted and analysed using GraphPad Prism 4 (GraphPad Software Inc, La Jolla, CA). Data were analysed using a non-linear regression curve fit for a sigmoidal dose-response with a variable slope. This approach uses the following equation, which is identical to the four parameter logistic equation: Y=Bottom + (Top-Bottom)/(1+10^(LogEC50-X)*HillSlope)). X is the logarithm of the protein concentration, and Y is the percent inhibition. Constraints were applied to set the Bottom ≥ 0% and the Top ≤ 100%. The EC50 value from this analysis represents the protein concentration at which the Y value of the curve (% Inhibition) is halfway between the Top and Bottom plateaus of the curve. The EC50 values and their associated 95% confidence intervals are plotted for the Maverick soybean and DAS-68416-4 extracts.

The ELISA inhibition data with the pooled soy-allergic serum showed the same IgE binding between the non-transgenic Maverick soybean and DAS-68416-4 soybean extracts against 2 µg/well of Maverick solid-phase in solute protein concentrations ranging from 0.004 to 4000 µg/ml (**Figure 46**). Furthermore, the associated EC50 values and confidence intervals for Maverick and DAS-68416-4 were similar (**Figure7**).

Figure 46. ELISA inhibition with DAS-68416-4 and control (Maverick) soybean extracts using soybean-allergic patient sera. (Stagg, N.J., 2010, Study ID: 101001).

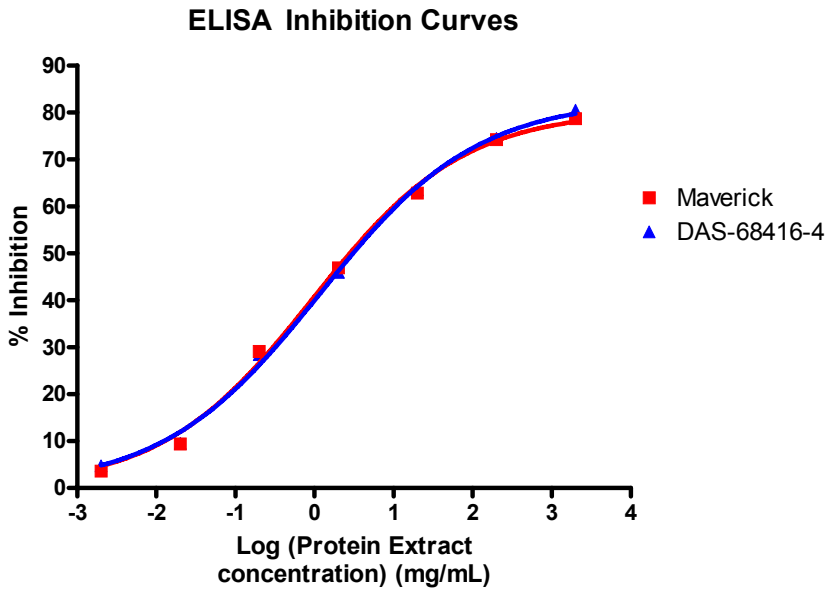
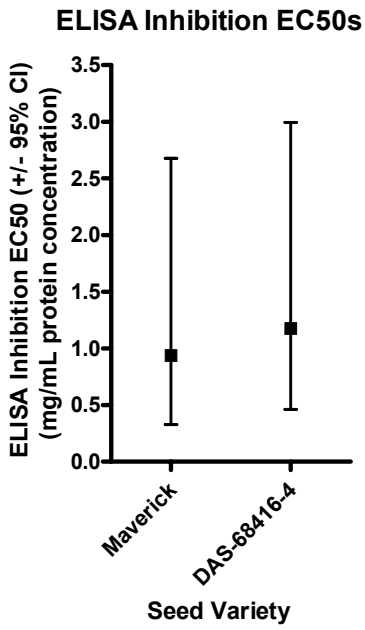


Figure 47. EC50 values from the ELISA inhibition data for DAS-68416-4 and control (Maverick) soybean extracts and their 95% confidence intervals. (Stagg, N.J., 2010, Study ID: 101001). (Note: Confidence limits are asymmetrical after transformation to the natural scale.)



In conclusion, the immunoblot and ELISA inhibition data demonstrate that the genetic modification used to generate DAS-68416-4 soybean did not alter the endogenous allergenicity compared with its non-transgenic control (Maverick).

a. Source of Introduced Protein

The donor organism, *Delftia acidovorans* (formerly designated as *Pseudomonas acidovoran*s and *Comamonas acidovorans*) is a non glucose-fermenting, gram-negative, non spore-forming rod present in soil, fresh water, activated sludge, and clinical specimens (von Gravenitz 1985, Tamaoka et al. 1987, Wen et al., 1999).

Delftia acidovorans can be used to transform ferulic acid into vanillin and related flavour metabolites (Toms and Wood, 1970; Ramachandra Rao and Ravishankar, 2000; Shetty et al., 2006). This utility has led to a history of safe use for Delftia acidovorans in the food processing industry. For example, see US Patent 5,128,253 “Bioconversion process for the production of vanillin” issued on July 7, 1992 to Kraft General Foods (Labuda et al., 1992).

b. Amino Acid Sequence Comparison to Known Allergens

The AAD-12 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 Allergen Database version 9.00, http://www.allergenonline.org).

c. Structural Properties

Please refer to Section C, Part 3, B for information relating to the heat lability of the AAD-12 protein.

d. Serum Screening

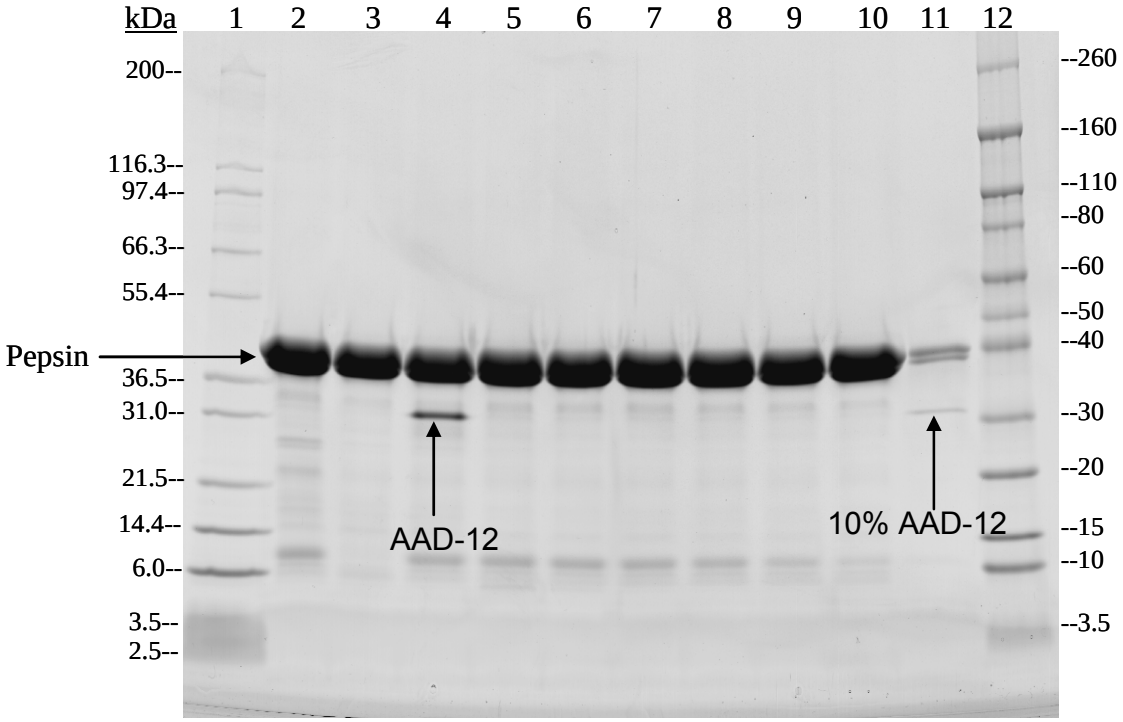
Not applicable.

e. Simulated Gastric Fluid and Heat Lability

Lability in Simulated Gastric Fluid

The digestibility of the AAD-12 protein was tested *in vitro* using simulated gastric fluid (SGF). For the SGF method, the microbially-produced AAD-12 protein was incubated in SGF (0.32% w/v pepsin at pH 1.2; U.S. Pharmacopeia) at a ratio of enzyme to protein of 1.5 mg pepsin to 1.0 nM test substance solution (AAD12: 1.0 nM equals 33 µg). At each time point (0.5, 1, 2, 4, 8, and 16 minutes), 0.1 mL of the reaction mixture was removed and placed into a microcentrifuge tube containing 0.04 mL stop solution (200 mM Na_2CO_3, pH ~11.0). For the zero time point samples, 2.85 mL SGF solution was neutralized with 1.2 mL stop solution and then the AAD-12 protein sample was added. 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 ºC for 5 min. The samples were then analysed via SDS-PAGE and western blot analysis using an antibody specific to AAD-12. The results demonstrated that the AAD-12 protein was readily digested (not detectable at 30 seconds) in SGF (**Figure 48** and **Figure 49**).

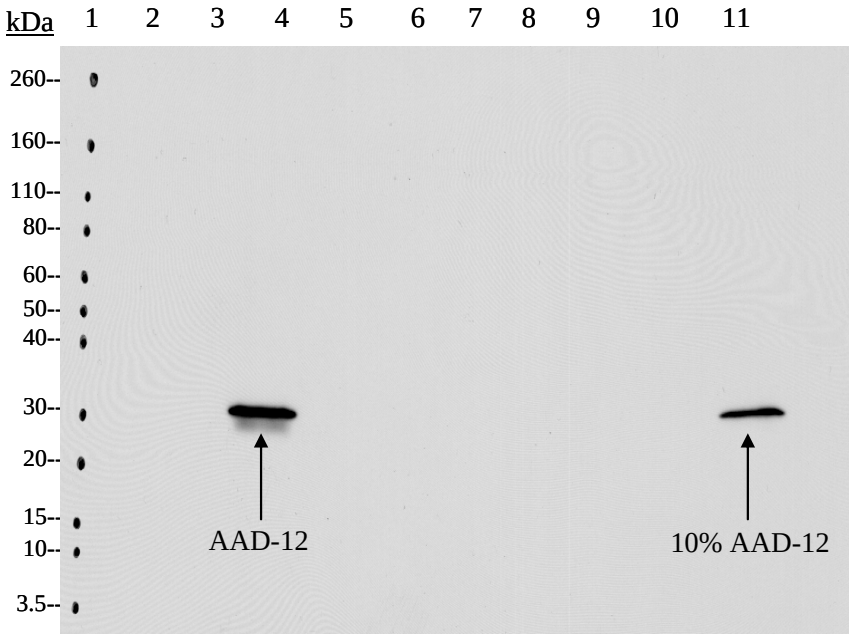
Figure 48. SDS-PAGE analysis of AAD-12 (M.W. ~32 kDa) protein subjected to digestion in simulated gastric fluid. (Embrey et al, 2008, Study ID 080064).



The neutralized and digested AAD-12 samples and SGF controls were held frozen for two days following the digestion. Samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Pierce Chemical. Invitrogen Mark 12 molecular weight markers 3.5 and 2.5 kDa represent Insulin A and B chains which are unresolved when separated on Tris-Glycine buffer systems.

Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW markers	10 µL
2	SGF Reagent Blank, 0 minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~1.67 µg
5	30-second AAD-12 digestion	~1.67 µg
6	1-minute AAD-12 digestion	~1.67 µg
7	2-minute AAD-12 digestion	~1.67 µg
8	4-minute AAD-12 digestion	~1.67 µg
9	8-minute AAD-12 digestion	~1.67 µg
10	16-minute AAD-12 digestion	~1.67 µg
11	10% Neutralized AAD-12 digestion	~0.17 µg
12	Invitrogen Novex Sharp Prestained MW markers	10 µL

Figure 49. Western blot analysis of AAD-12 protein subjected to digestion in simulated gastric fluid. (Embrey et al, 2008, Study ID 080064).



The neutralized and digested AAD-12 samples and SGF controls were held frozen for two days following the digestion. Samples were mixed with equal volumes of Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~95 °C. The samples were loaded into a Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was electro-blotted to a nitrocellulose membrane for 60 minutes under a constant charge of 50 volts. For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2, 4.3 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to film and subsequently developed with a film developer. The molecular weight markers were manually transferred to the film after development.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Prestained MW markers	10 µL
2	SGF Reagent Blank, 0 minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~0.17µg
5	30-second AAD-12 digestion	~0.17µg
6	1-minute AAD-12 digestion	~0.17µg
7	2-minute AAD-12 digestion	~0.17µg
8	4-minute AAD-12 digestion	~0.17µg
9	8-minute AAD-12 digestion	~0.17µg
10	16-minute AAD-12 digestion	~0.17µg
11	10% Neutralized AAD-12 digestion	~0.017µg

Heat Lability

The thermal stability of the AAD-12 protein was evaluated by heating 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. The AAD-12 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 at 510 nm. All heating conditions eliminated the enzymatic activity of the AAD-12 protein.

Conclusions

The aryloxyalkanoate dioxygenase (AAD-12) protein was derived from *Delftia acidovorans*, a gram-negative soil bacterium. AAD-12 is comprised of 293 amino acids and has a molecular weight of ~32 kDa. Detailed biochemical characterization of the AAD-12 protein derived from plant and microbial sources was conducted. Additionally, characterization of AAD-12 protein expression in DAS-68416-4 plants over the growing season was determined by analysing leaf, root, whole plant, and grain tissues from DAS-68416-4 plants sprayed with 2,4-D, glufosinate, both 2,4-D and glufosinate, and non-sprayed.

A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the AAD-12 amino acid sequence. The AAD-12 protein hydrolyses rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-12 protein. Glycosylation analysis revealed no detectable covalently linked carbohydrates in AAD-12 protein expressed in DAS-68416-4 soybean plants. Therefore, the low level expression of the AAD-12 protein presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

5. Compositional Analysis

Part C Section 5 DAS Reports

Smith-Drake, J.K., Dunville, C.M., Phillips, A.M., Herman, R.A. (2009). Field Expression, Nutrient Composition Analysis and Agronomic Characterisation of a Transformed Soybean Cultivar (DAS-68416-4) Containing Aryloxyalkanoate Dioxygenase (AAD-12) and Phosphinotricin Acetyltransferase (PAT). Dow AgroSciences LLC Study ID 080003.

a. Grain and Forage Composition

Compositional analysis was performed on soybean forage and grain to investigate the equivalency between DAS-68416-4 soybean (sprayed with 2,4-D, glufosinate, 2,4-D + glufosinate, or not sprayed with 2,4-D or glufosinate) and conventional soybean.
Trials were conducted at six test sites located within the major soybean-producing regions of the U.S and Canada; with one site each in Iowa (Keokuk county), Illinois (Clinton county), Indiana (Parke county), Nebraska (York county) and two sites in Ontario, Canada.

The herbicide treatments were identical to those used in studies for protein expression analysis (Section C, Part 2, C). Herbicide treatments were applied with a spray volume of approximately 187 L/ha. These applications were designed to replicate maximum label rate for commercial practices. 2,4-D was applied as 3 broadcast over-the-top applications for a seasonal total of 3 lb ae/A. Individual applications of 1.0 lb ae/A (1120 g ae/ha) were made at pre-emergence and approximately V4 and R2 growth stages. Glufosinate was applied as 2 broadcast over-the-top applications for a seasonal total of 0.74 lb ai/A (828 g ai/ha). Individual applications of 0.33 lb ai/A and 0.41 lb ai/A (374 and 454 g ai/ha) were made at approximately V6 and R1 growth stages.

Samples of soybean forage and grain were analysed for nutrient content with a variety of tests (OECD, 2001). The analyses performed for forage included protein, fat, ash, moisture, carbohydrate, acid detergent fiber (ADF), neutral detergent fiber (NDF), calcium and phosphorus. The analyses performed for grain included proximates (ash, total fat, moisture, protein, cholesterol, carbohydrate), fiber, minerals, amino acids, fatty acid, vitamins, anti-nutrients. Samples were shipped to Covance laboratories, Madison, WI and maintained frozen until use. Samples of soybean tissues were prepared for analysis by coarse grinding.

Forage (R3)

One forage sample per plot, each consisting of the aerial portion (no roots) of the soybean plant, was collected from the test and control entries. Sample size was approximately 300 grams.

Grain (R8 – Maturity)

One individual sample was collected from each plot of the test and control entries. Each sample consisted of all remaining grain in the plot.

Additional information on the experimental design and methods of analysis are given further in this section.

The results of the nutritional analysis for soybean forage and grain were compared with values reported in literature. A summary of the compositional data used for comparison can be found in **Tables 19-27.**

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. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between DAS-68416-4 (unsprayed AAD-12), DAS-68416-4 sprayed with glufosinate (AAD-12 + glufosinate), DAS-68416-4 sprayed with 2,4-D (AAD-12 + 2,4-D), and DAS-68416-4 sprayed with both glufosinate and 2,4-D (AAD-12 + both herbicides), and the control entry using t-tests.

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 (75 quantitated analytes), resulting in 300 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%.

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 Analysis of Soybean Forage

An analysis of the protein, fat, ash, moisture, carbohydrate, acid detergent fiber (ADF), neutral detergent fiber (NDF), calcium and phosphorus in soybean forage samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 12** and **Figure 50.**

No statistical differences were observed in the across-site analysis between the control and transgenic entries for protein, fat, ash, moisture, carbohydrates, ADF, NDF, calcium or phosphorus.

Mean ash values across sites for AAD-12 + glufosinate and AAD-12 + both herbicides was outside of the literature range as was the NDF value for AAD-12 + glufosinate and AAD-12 + 2,4-D. ADF values for all treatments including the non-transgenic control were also outside of the literature values. Mean values were not significantly different between the non-transgenic control and any transgenic entry for any proximate, fiber type, or mineral in forage.

Based on these compositional constituents, the forage from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 12. Summary of the proximate, fiber and mineral analysis of soybean forage (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Proximate							
Protein	11.2-24.7	0.805	19.1	19.0 (0.881,0.930)	19.4 (0.666,0.819)	18.9 (0.744,0.860)	18.6 (0.441,0.634)
Fat	1.30-5.1	**0.046**	4.11	4.46 (0.216,0.403)	3.66 (0.107,0.254)	4.17 (0.844,0.908)	3.74 (0.186,0.360)
Ash	6.7-10.8	0.092	10.6	10.1 (0.567,0.767)	**11.1** (0.546,0.741)	10.2 (0.672,0.819)	**12.3** (0.051,0.151)
Moisture (% fresh weight)	73.5-81.6	0.569	77.8	78.5 (0.255,0.444)	78.4 (0.330,0.539)	77.8 (0.960,0.970)	77.8 (0.976,0.979)
Carbohydrates	59.8-74.7	0.675	66.2	66.5 (0.830,0.902)	65.9 (0.739,0.860)	66.7 (0.641,0.808)	65.3 (0.366,0.564)
Fiber							
Acid Detergent Fiber (ADF)	32.0-38.0	0.967	**30.2**	**30.4** (0.904,0.936)	**30.6** (0.797,0.875)	**29.7** (0.746,0.860)	**30.7** (0.740,0.860)
Neutral Detergent Fiber (NDF)	34.0-40.0	0.375	34.4	34.7 (0.877,0.930)	**33.1** (0.397,0.596)	**32.0** (0.135,0.297)	34.5 (0.948,0.962)
Minerals							
Calcium	NR	0.246	1.39	1.36 (0.361,0.560)	1.40 (0.664,0.819)	1.38 (0.842,0.908)	1.43 (0.178,0.352)
Phosphorus	NR	0.957	0.263	0.266 (0.671,0.819)	0.269 (0.442,0.634)	0.266 (0.696,0.831)	0.265 (0.754,0.860)

[a] Combined range from Appendix 6.

[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.

NR = not reported

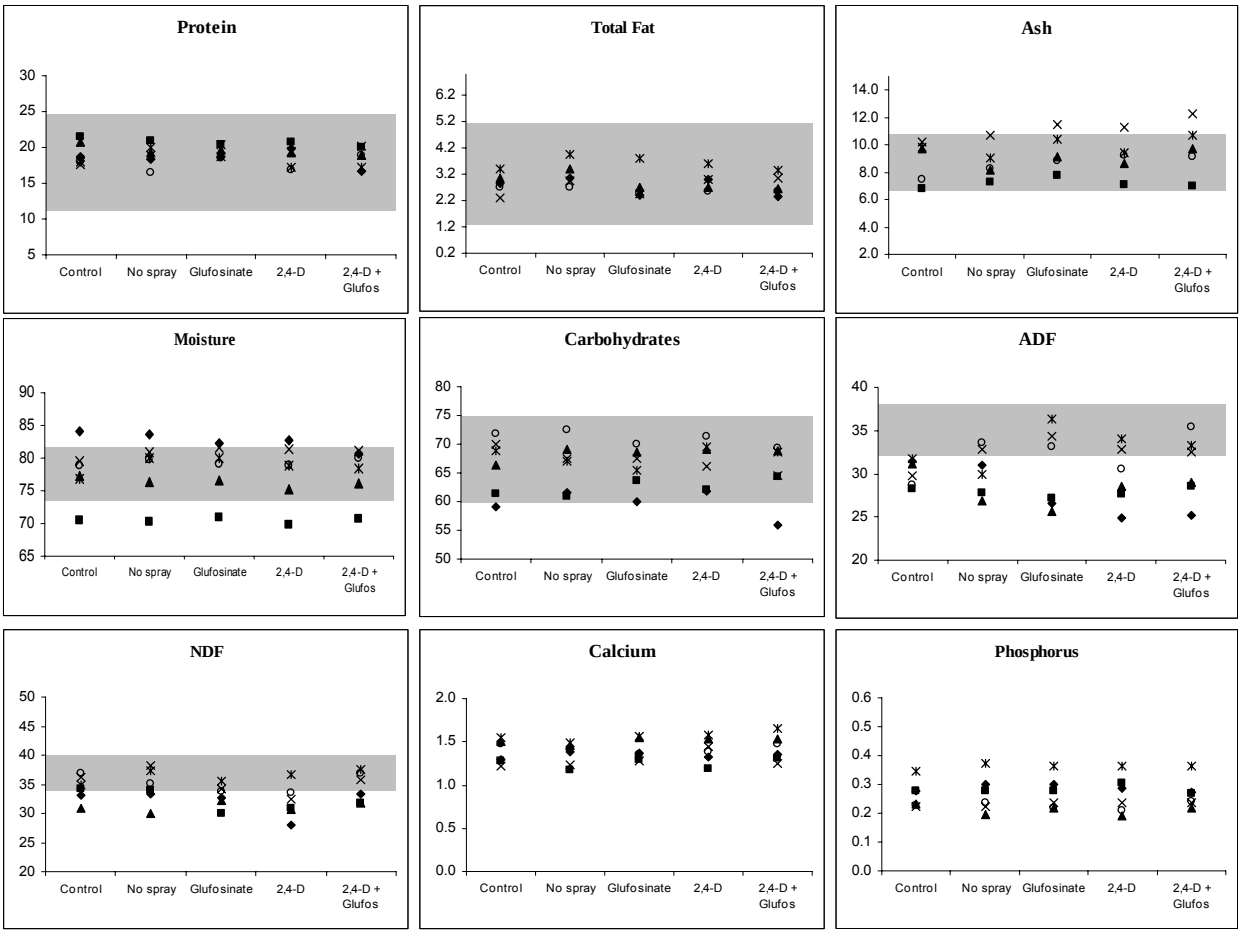
Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 50. Summary of the proximate, fiber and mineral analysis of soybean forage (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Percent dry-weight for all analytes, except moisture which was percent fresh-weight.

Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded.



Compositional Analysis of Soybean Grain

Proximates and Fiber

An analysis of the protein, fat, ash, moisture, cholesterol, carbohydrate, ADF, NDF and total dietary fiber in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 13** and **Figure 51.**

No statistical differences were observed in the across-site analysis between the control and transgenic entries for the fat, ADF or total dietary fiber. However, ADF was slightly higher than the literature range for the AAD-12 + 2,4-D entry.

Protein levels were significantly different in the across-site analysis based on the unadjusted p-value for the unsprayed, AAD-12 + 2,4-D, and AAD-12 + both herbicides compared with the control. However, after FDR adjustment, only the p-value for the AAD-12 + 2,4-D was significant, and overall mean protein values for all treatments were within the reported literature values, indicating that the differences were not biologically meaningful.

A significant unadjusted p-value was observed in the across site analysis of ash between the control and the 2,4-D sprayed AAD-12 treatment, but no overall treatment effect or adjusted p-value was observed. Ash values were also within the reported literature values, indicating that the differences were not biologically meaningful.

Moisture levels were significantly different in the across-site analysis based on the unadjusted p-value for the unsprayed, AAD-12 + 2,4-D, and AAD-12 + both herbicides compared with the control. However, the overall treatment effect was not significant for moisture, only the AAD-12 + 2,4-D treatment had a significant FDR-adjusted p-value, and the mean moisture levels for all treatments were within the literature ranges. This indicated that the differences were not biologically meaningful.

Cholesterol values were all <LOQ and no literature values were reported.

Carbohydrate levels were significantly different in the across-site analysis based on the unadjusted p-value for the unsprayed, AAD-12 + glufosinate, and AAD-12 + 2,4-D compared with the control. However, only the AAD-12 + 2,4-D treatment was significantly different from the control based on the FDR adjusted p-value and all treatment means were within the reported literature values, indicating equivalence to non-transgenic soybean.

NDF levels were significantly different in the across-site analysis based on the unadjusted p-value for AAD-12 + glufosinate compared with the control, but this was not accompanied by a significant adjusted p-value or an overall treatment effect. NDF across-site values were slightly higher than the reported literature values for the AAD-12 + glufosinate and AAD-12 + 2,4-D entries, but the differences were <9% compared with the non-transgenic control.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 13. Summary of the proximate and fiber analysis of soybean grain (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Proximate							
Protein	32.0-45.5	**0.004**	39.2	38.3 (**0.009**,0.051)	38.8 (0.186,0.360)	37.8 (**0.0003**,**0.009**)	38.5 (**0.035**,0.122)
Fat	8.10-24.7	0.105	17.1	17.1 (0.877,0.930)	16.6 (0.059,0.169)	16.7 (0.142,0.305)	17.2 (0.674,0.819)
Ash	3.89-6.99	0.315	4.92	5.04 (0.176,0.351)	5.04 (0.175,0.351)	5.10 (**0.048**,0.145)	5.07 (0.099,0.240)
Moisture % fresh weight	4.70-34.4	0.066	14.9	14.1 (**0.047**,0.143)	14.3 (0.122,0.276)	13.7 (**0.006**,**0.043**)	14.0 (**0.037**,0.124)
Cholesterol	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Carbohydrate	29.6-50.2	**0.010**	38.8	39.6 (**0.046**,0.143)	39.6 (**0.044**,0.138)	40.3 (**0.001**,**0.011**)	39.3 (0.241,0.432)
Fiber							
Acid Detergent Fiber (ADF)	7.81-18.6	0.561	17.8	17.6 (0.772,0.868)	18.0 (0.772,0.868)	**18.8** (0.190,0.362)	18.1 (0.685,0.825)
Neutral Detergent Fiber (NDF)	8.53-21.3	0.184	20.1	20.8 (0.386,0.585)	**21.9** (**0.042**,0.134)	**21.6** (0.090,0.225)	20.3 (0.754,0.860)
Total Dietary Fiber	NR	0.770	31.6	31.7 (0.899,0.936)	31.7 (0.897,0.936)	32.1 (0.466,0.653)	32.5 (0.286,0.482)

[a] Combined range from literature ranges

[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.

NA = statistical analysis was not performed since a majority of the data was < LOQ.

NR = not reported.

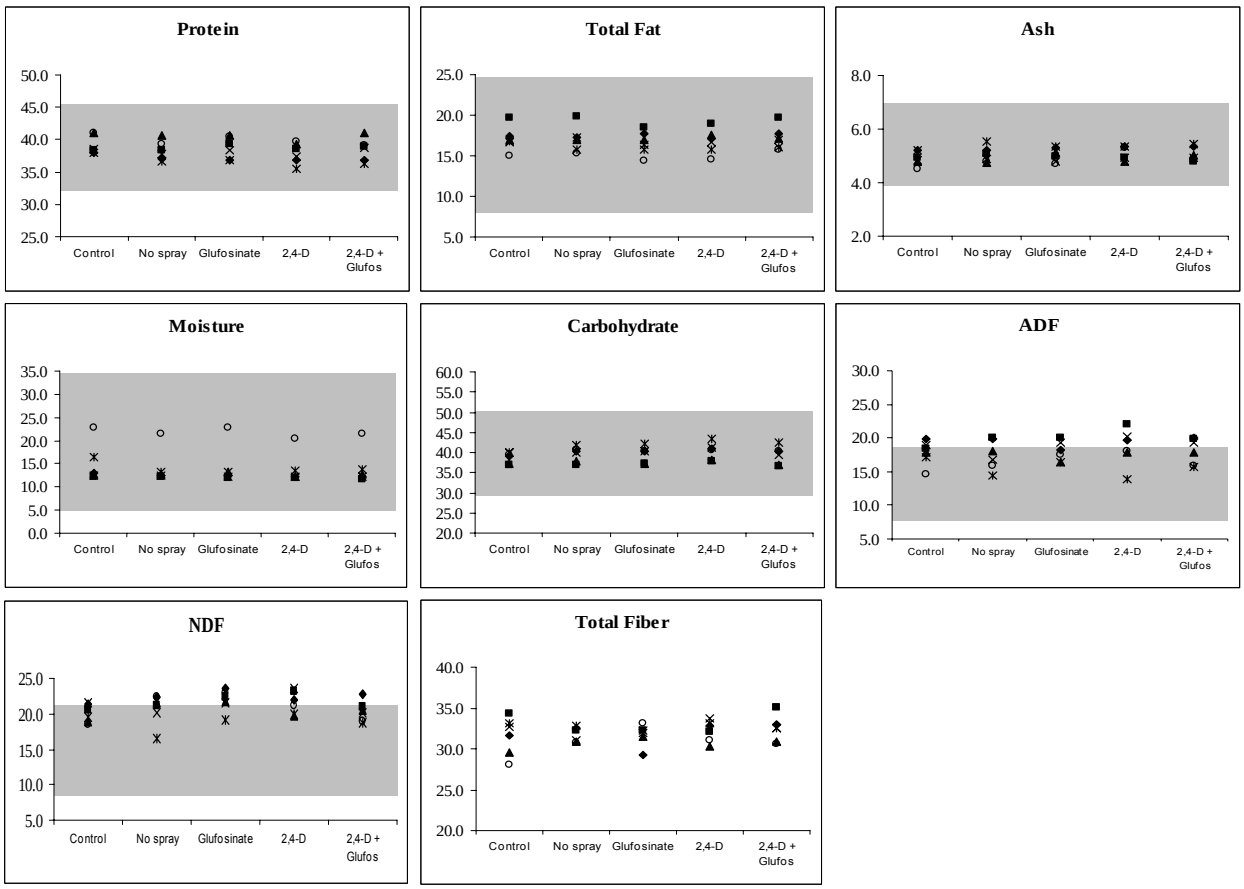
Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 51. Summary of the proximate and fiber analysis of soybean grain. (Smith-Drake et al, 2009, Study ID 080003).

Percent dry-weight for all analytes, except moisture which was percent fresh-weight.

Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded. Grain was also analysed for cholesterol, but results were less than the limit of quantitation.



Minerals

The analysis of the calcium, chromium, copper, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 14** and **Figure 52.**

No statistical differences were observed in the across-site analysis between the control and transgenic entries based on the unadjusted p-value for the chromium, copper, iodine, iron, manganese, molybdenum, phosphorus, selenium and sodium (not detected).

Calcium had a significant difference in the across-site analysis based on the unadjusted p-value for the AAD-12 + 2,4-D, but this was not associated with a significant FDR adjusted p-value or overall treatment effect, and all treatment means fell within the literature range, indicating that the difference was not biologically meaningful.

Magnesium levels were significantly different in the across-site analysis for the AAD12 + both herbicides and AAD-12 + glufosinate compared with the control based on the unadjusted and adjusted p-values, respectively, but the overall treatment effect was not significant. Magnesium across site mean values

were slightly lower than the reported literature values, but the differences were <3% in comparison to the control and all AAD-12 entries were closer to literature values compared with the control.

All AAD-12 entries had significantly higher potassium values compared with the control in the across-site analysis. However, differences were <5% in comparison to the control, and all AAD-12 entries were closer to the literature range compared with the control.

A difference in zinc levels was significant in the across-site analysis based on the unadjusted p-value for AAD-12 + both herbicides, however this was not accompanied by a significant FDR-adjusted p-value or overall treatment effect, and the difference was <4%.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 14. Summary of the mineral analysis of soybean grain (mg/100g dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Calcium	117-307	0.102	256	265 (0.174,0.351)	264 (0.237,0.432)	274 (**0.010**,0.057)	269 (**0.050**,0.148)
Chromium (ppb)	NR	0.775	145	149 (0.912,0.941)	175 (0.468,0.653)	126 (0.613,0.796)	137 (0.855,0.916)
Copper	NR	0.887	1.31	1.28 (0.534,0.728)	1.30 (0.788,0.873)	1.27 (0.367,0.564)	1.28 (0.461,0.649)
Iodine	NR	0.285	0.027	0.023 (0.430,0.632)	0.021 (0.182,0.358)	0.032 (0.348,0.551)	0.023 (0.348,0.551)
Iron	5.54-11.0	0.917	8.15	8.46 (0.719,0.853)	8.95 (0.353,0.552)	8.53 (0.656,0.819)	8.59 (0.608,0.796)
Magnesium	219-313	0.082	**210**	**212** (0.437,0.634)	**215** (**0.020**,0.087)	**213** (0.143,0.305)	**215** (**0.021**,0.088)
Manganese	NR	0.984	2.56	2.60 (0.608,0.796)	2.60 (0.618,0.799)	2.58 (0.781,0.873)	2.59 (0.698,0.831)
Molybdenum (ppb)	NR	0.845	2165	2557 (0.353,0.552)	2462 (0.479,0.665)	2563 (0.346,0.551)	2284 (0.722,0.853)
Phosphorus	507-935	0.675	583	589 (0.630,0.804)	599 (0.191,0.363)	596 (0.272,0.469)	594 (0.349,0.551)
Potassium	1868-2316	**0.0005**	**1801**	1876 (**0.0003**,**0.009**)	1882 (**0.0001**,**0.006**)	1883 (**0.0001**,**0.006**)	**1864** (**0.001**,**0.019**)
Selenium (ppb)	NR	0.490	490	523 (0.626,0.802)	520 (0.659,0.819)	511 (0.758,0.861)	418 (0.280,0.475)
Sodium	NR	NA	<LOQ	< LOQ	<LOQ	<LOQ	<LOQ

Zinc	NR	0.096	5.06	5.07	5.19	5.21	5.25
				(0.868,0.926)	(0.117,0.268)	(0.074,0.197)	(**0.027**,0.105)

[a] Combined range from literature ranges.

[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.

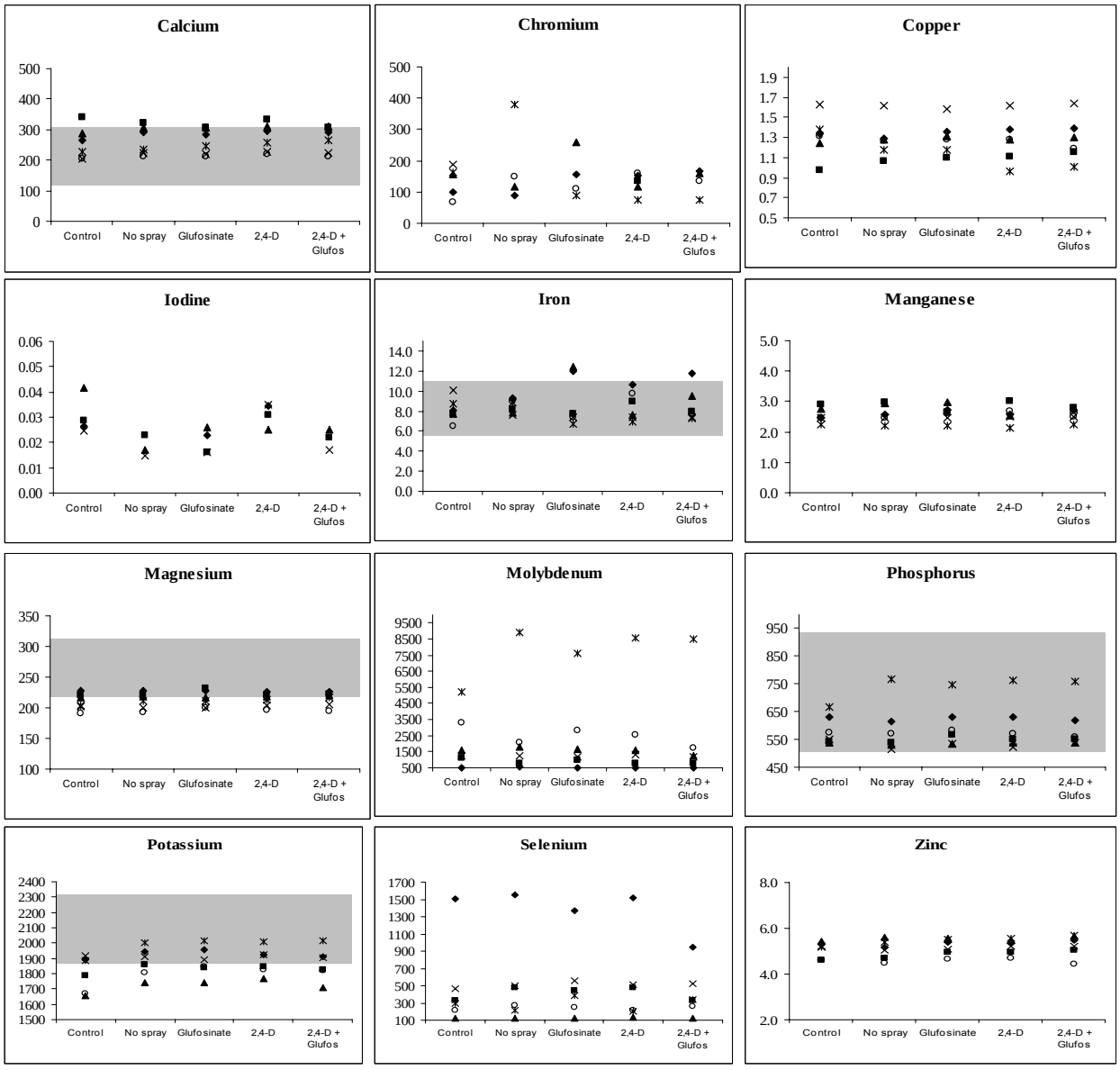
NR = not reported.

NA= statistical analysis was not performed since a majority of the data was < LOQ.

Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 52. Summary of the mineral analysis of soybean grain (mg/100 g dry weight). (Smith-Drake et al, 2009, Study ID 080003).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded. Grain was also analyzed for sodium, but results were less than the limit of quantitation.



Amino Acids

An analysis of the following amino acids: alanine, arginine, aspartic acid, cysteine, glutamic acid glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine; in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 15 and Figure 53**). No statistical differences were observed between the control and transgenic entries for cysteine, methionine, proline, tyrosine or tryptophan. The isoleucine level for AAD-12 + 2,4-D was significantly different from the control based on the unadjusted p-value, but this was not accompanied by a significant FDR-adjusted p-value or a significant overall treatment effect. The levels of the remaining 12 amino acids were slightly lower (<7%) for two or more of the AAD-12 entries compared with the control, but all fell within the literature range for non-transgenic

soybean. All amino acids for all entries were within the literature ranges, indicating that the differences were not biologically meaningful.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 15. Summary of the amino acid analysis of soybean grain (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Alanine	1.51-2.10	**0.003**	1.74	1.70 (**0.001**,**0.017**)	1.70 (**0.004**,**0.033**)	1.69 (**0.0003**,**0.009**)	1.71 (**0.014**,0.067)
Arginine	2.29-3.40	**0.007**	3.15	2.97 (**0.004**,**0.033**)	3.00 (**0.012**,0.066)	2.94 (**0.001**,**0.015**)	2.96 (**0.003**,**0.026**)
Aspartic Acid	3.81-5.12	**0.007**	4.52	4.41 (**0.004**,**0.033**)	4.44 (**0.037**,0.124)	4.38 (**0.0005**,**0.010**)	4.43 (**0.014**,0.067)
Cysteine	0.37-0.81	0.254	0.60	0.60 (0.637,0.808)	0.60 (0.787,0.873)	0.61 (0.900,0.936)	0.61 (0.110,0.260)
Glutamic Acid	5.84-8.20	**0.002**	6.98	6.76 (**0.001**,**0.015**)	6.83 (**0.019**,0.086)	6.70 (**0.0001**,**0.006**)	6.80 (**0.006**,**0.043**)
Glycine	1.46-2.00	**0.001**	1.74	1.69 (**0.0004**,**0.009**)	1.70 (**0.002**,**0.023**)	1.69 (**0.0001**,**0.006**)	1.70 (**0.001**,**0.017**)
Histidine	0.88-1.22	**0.003**	1.09	1.06 (**0.002**,**0.023**)	1.07 (**0.014**,0.067)	1.05 (**0.0002**,**0.007**)	1.07 (**0.013**,0.067)
Isoleucine	1.54-2.08	0.232	1.87	1.83 (0.100,0.241)	1.85 (0.450,0.642)	1.82 (**0.042**,0.134)	1.85 (0.514,0.708)
Leucine	2.20-4.00	**0.010**	3.06	3.00 (**0.007**,**0.046**)	3.02 (0.068,0.186)	2.98 (**0.001**,**0.011**)	3.01 (**0.037**,0.124)
Lysine	2.29-2.84	**0.005**	2.56	2.51 (**0.004**,**0.034**)	2.52 (**0.028**,0.105)	2.49 (**0.0003**,**0.009**)	2.52 (**0.022**,0.093)
Methionine	0.43-0.68	0.433	0.56	0.55 (0.377,0.575)	0.55 (0.245,0.438)	0.55 (0.089,0.225)	0.55 (0.742,0.860)
Phenylalanine	1.60-2.35	**0.008**	2.02	1.97 (**0.014**,0.067)	1.98 (**0.044**,0.138)	1.94 (**0.0004**,**0.009**)	1.97 (**0.027**,0.105)
Proline	1.69-2.28	0.374	1.91	1.85	1.88	1.87	1.87

				(0.059,0.169)	(0.400,0.597)	(0.155,0.324)	(0.240,0.432)
Serine	1.11-2.48	0.063	1.99	1.95	1.95	1.91	1.93
				(0.082,0.210)	(0.115,0.268)	(**0.006**,**0.043**)	(**0.021**,0.088)
Threonine	1.14-1.89	**0.001**	1.62	1.57	1.58	1.55	1.57
				(**0.002**,**0.020**)	(**0.008**,**0.048**)	**<0.0001**,**0.006**	(**0.002**,**0.022**)
Tryptophan	0.36-0.67	0.330	0.43	0.43	0.43	0.43	0.42
				(0.593,0.787)	(0.981,0.981)	(0.904,0.936)	(0.095,0.235)
Tyrosine	1.02-1.61	0.449	1.36	1.34	1.35	1.33	1.33
				(0.275,0.471)	(0.517,0.708)	(0.096,0.235)	(0.153,0.321)
Valine	1.50-2.44	0.159	1.97	1.92	1.94	1.92	1.95
				(**0.032**,0.116)	(0.279,0.475)	(**0.038**,0.124)	(0.346,0.551)

[a] Combined range from literature ranges.

[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.

Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 53. Summary of the amino acid analysis of soybean grain (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded.

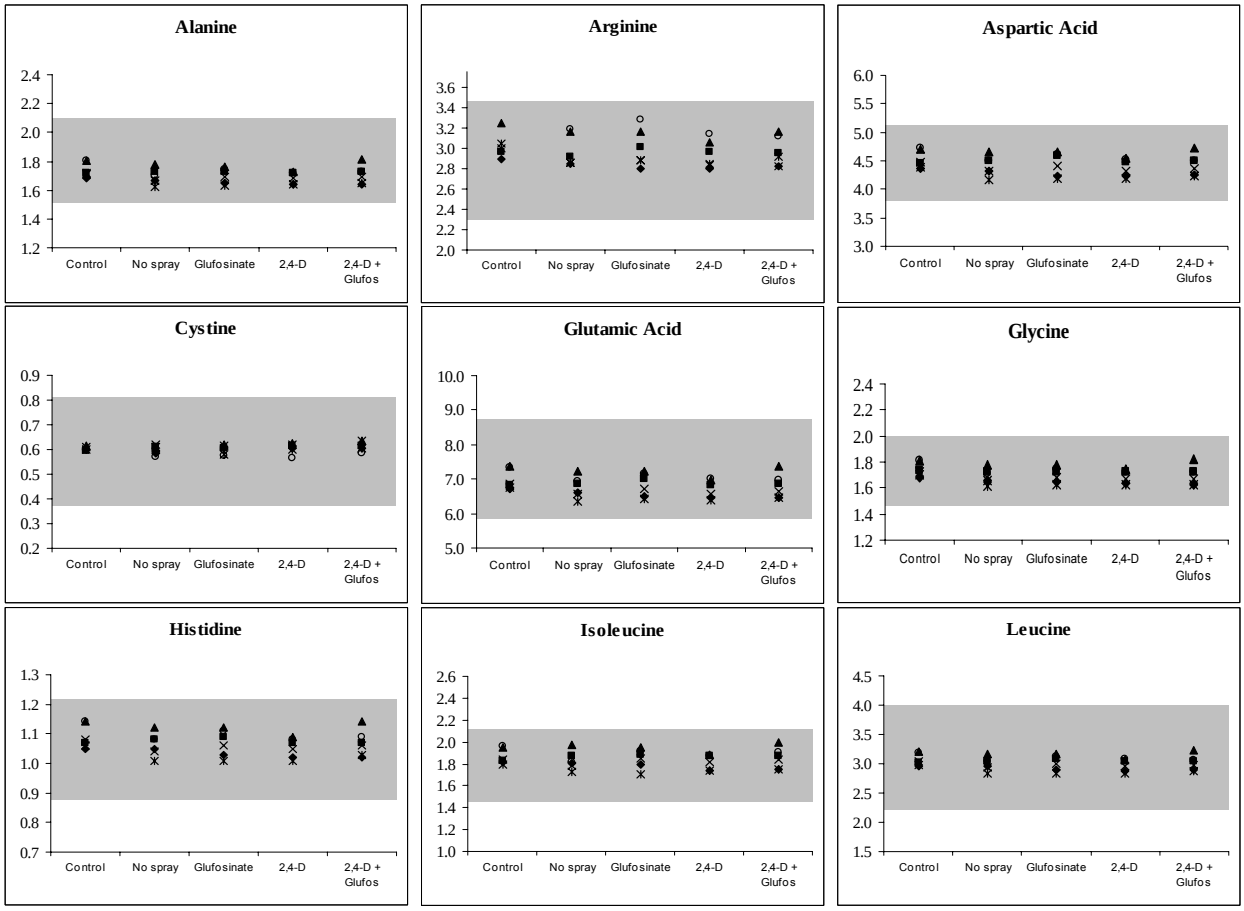
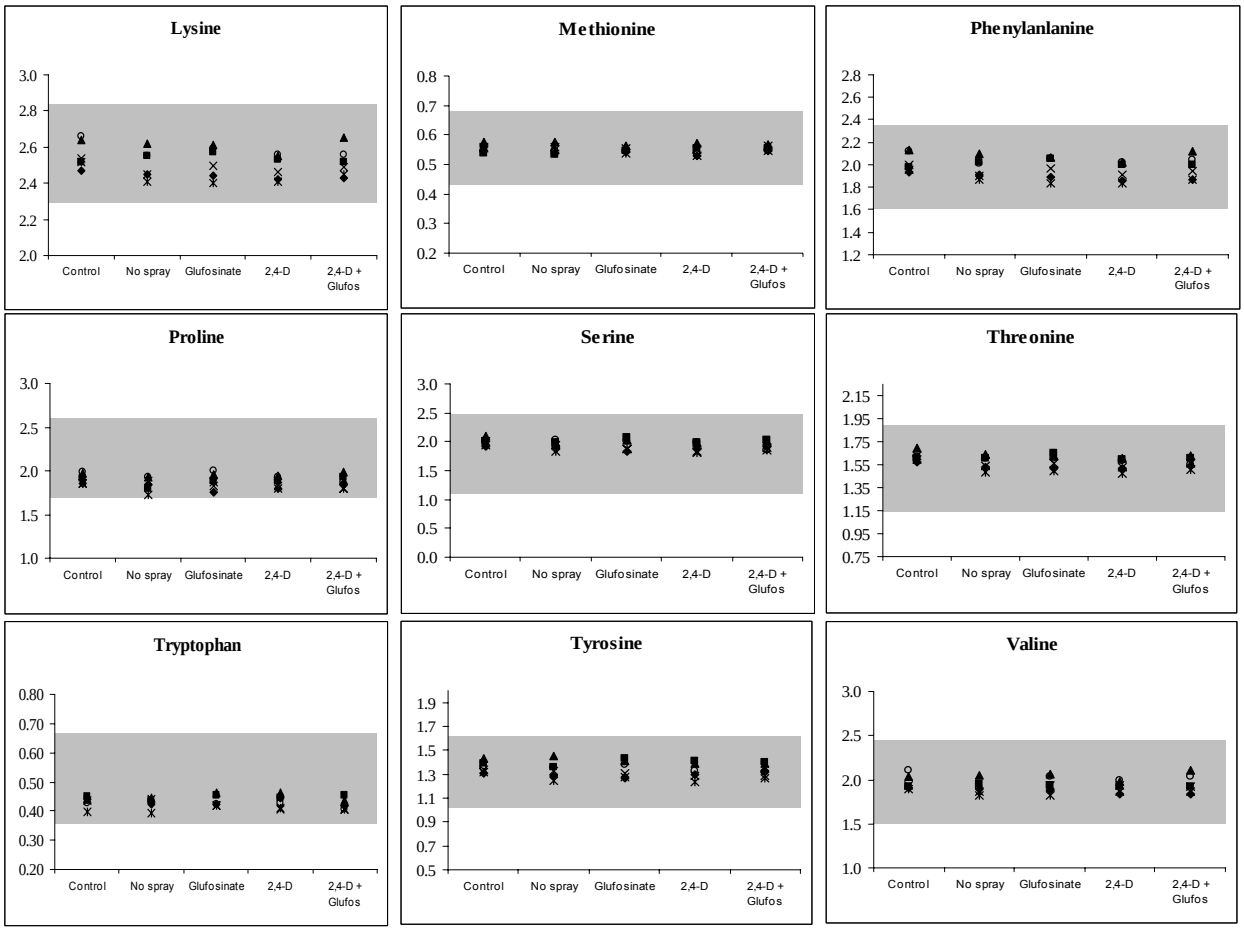


Figure 53. Summary of the amino acid analysis of soybean grain (% dry weight) cont.
(Smith-Drake et al, 2009, Study ID 080003).



Fatty Acids

An analysis of 22 fatty acids in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 16** and **Figure 54**.

The fatty acids 10:0 capric, 15:0 pentadecanoic, 15:1 pentadecenoic, 20:3 eicosatrienoic, 20:4 arachidonic, 8:0 caprylic, 12:0 lauric, 14:0 myristic, 14:1 myristoleic, 17:1 heptadecenoic, 18:3 gamma linolenic, and 20:2 eicosadienoic acids were analysed and the results were <LOQ. The fatty acids 16:0 palmitic, 17:0 heptadecanoic, and 20:1 eicosenoic were not significantly different between the control and the AAD-12 entries, although 20:1 eicosenoic values were lower than the reported literature values for AAD-12 + glufosinate and AAD-12 + both herbicides. However, the differences were <5% in comparison to the control.

The level of 16:1 palmitoleic was significantly different between the control and the unsprayed, AAD-12 + glufosinate, AAD-12 + 2,4-D, and AAD-12 + both herbicides based on unadjusted p-values. However, only the unsprayed AAD-12 entry had a FDR-adjusted p-value that was significant for 16:1 palmitoleic. The 16:1 palmitoleic across-site value was lower for this treatment compared with the reported literature values, but the difference was <13% in comparison to the non-transgenic control.

The level of 18:0 stearic was significantly different between the control and the unsprayed and AAD-12 + glufosinate, based on unadjusted p-values. However, no significant differences were observed based

on the adjusted p-values or the overall treatment effect, and all entries were within the reported literature values, indicating equivalence to non-transgenic soybean.

The level of 18:1 oleic was significantly different between the control and the unsprayed, AAD-12 + glufosinate, AAD-12 + 2,4-D, and AAD-12 + both herbicides. However, 18:1 oleic levels were within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

The level of 18:2 linoleic was significantly different between the control and the unsprayed and AAD-12 + 2,4-D, based on unadjusted p-values.

However, no significant differences were observed in the adjusted p-values or the overall treatment effect, and 18:2 linoleic levels were within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

Levels of 18:3 linolenic were significantly different between each of the AAD-12 entries and the control based on unadjusted p-values, and the adjusted p-values were also significant between the unsprayed AAD-12 and AAD-12 + both herbicide treatment compared with the control. Differences between the AAD-12 and control treatment were <6% and all fell within the literature range.

The level of 20:0 arachidic was significantly different between the control and the unsprayed, AAD-12 + glufosinate, AAD-12 + 2,4-D, and AAD-12 + both herbicides based on unadjusted p-values, and 20:0 arachidic also had significant differences in the across-site analysis in the adjusted p-value for the unsprayed and AAD-12 + glufosinate treatments. However, 20:0 arachidic levels were within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

The level of 22:0 behenic was significantly different between the control and the unsprayed, AAD-12 + glufosinate, AAD-12 + 2,4-D, and AAD-12 + both herbicides based on unadjusted p-values, and the level of 22:0 behenic also had a significant difference in the across-site analysis in the adjusted p-value for the AAD-12 + glufosinate. However, there were no significant overall treatment effect, and 22:0 behenic levels were within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

Of the 22 fatty acids investigated, all four AAD-12 entries were either statistically indistinguishable from the control or within literature values for 21 of the fatty acids. In one case (unsprayed AAD-12; 16:1 palmitoleic), the value was slightly under the minimum literature values and statistically different from the control (<13% lower), however, all three sprayed treatments were within the literature range.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 16. Summary of the fatty acid analysis of soybean grain (% total fatty acids).

(Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
8:0 Caprylic	0.15	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
10:0 Capric	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
12:0 Lauric	0.08-0.13	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
14:0 Myristic	0.07-0.24	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
14:1 Myristoleic	0.12-0.13	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	9.55-15.77	0.607	10.1	10.0 (0.625,0.802)	9.78 (0.148,0.313)	9.94 (0.455,0.644)	9.85 (0.249,0.441)
16:1 Palmitoleic	0.09-0.19	**0.029**	0.097	**0.085** (**0.003**,**0.028**)	0.088 (**0.038**,0.124)	0.087 (**0.027**,0.105)	0.089 (**0.029**,0.109)
17:0 Heptadecanoic	0.09-0.15	0.640	0.111	0.114 (0.162,0.336)	0.113 (0.331,0.539)	0.114 (0.239,0.432)	0.113 (0.296,0.493)
17:1 Heptadecenoic	0.07-0.09	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
18:0 Stearic	2.70-5.88	0.136	4.28	4.03 (**0.048**,0.145)	3.98 (**0.018**,0.081)	4.05 (0.060,0.169)	4.06 (0.073,0.196)
18:1 Oleic	14.3-32.2	**0.010**	21.8	19.8 (**0.004**,**0.033**)	19.5 (**0.001**,**0.017**)	19.9 (**0.006**,**0.043**)	19.9 (**0.006**,**0.043**)
18:2 Linoleic	42.3-58.8	0.145	50.3	52.5 (**0.030**,0.109)	51.9 (0.116,0.268)	52.6 (**0.024**,0.095)	52.0 (0.087,0.222)
18:3 γ-Linolenic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
18:3 Linolenic	3.00-12.52	**0.022**	7.83	8.23 (**0.003**,**0.031**)	8.15 (**0.016**,0.073)	8.10 (**0.034**,0.119)	8.21 (**0.004**,**0.034**)
20:0 Arachidic	0.16-0.48	**0.023**	0.307	0.284 (**0.007**,**0.045**)	0.282 (**0.004**,**0.033**)	0.285 (**0.009**,0.052)	0.287 (**0.014**,0.067)
20:1 Eicosenoic	0.14-0.35	0.683	0.143	0.140 (0.582,0.779)	**0.136** (0.201,0.380)	0.141 (0.794,0.875)	**0.138** (0.327,0.538)
20:2 Eicosadienoic	0.08-0.25	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
20:3 Eicosatrienoic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
20:4 Arachidonic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
22:0 Behenic	0.28-0.60	0.053	0.305	0.288 (**0.023**,0.095)	0.285 (**0.008**,**0.048**)	0.288 (**0.020**,0.087)	0.288 (**0.020**,0.087)

[a] Combined range from literature ranges.

[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.

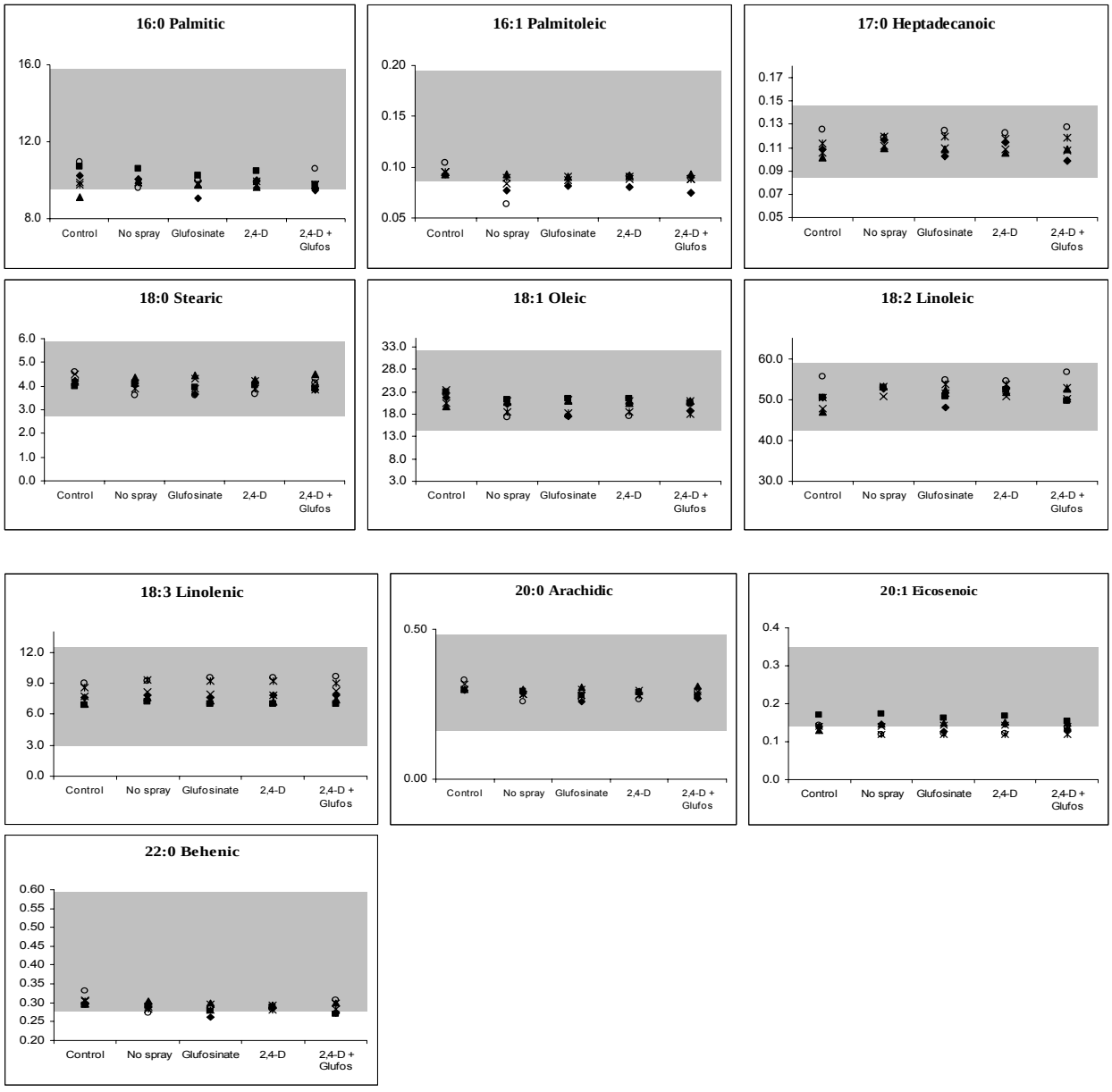
NA = statistical analysis was not performed since a majority of the data was < LOQ.

NR = not reported.

Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 54. Summary of the fatty acid analysis of soybean grain (% total fatty acids). (Smith-Drake et al, 2009, Study ID 080003).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded. Grain was also analyzed for 8:0 Caprylic, 10:0 Capric, 12:0 Lauric, 14:0 Myristic, 14:1 Myristoleic, 15:0 Pentadecanoic, 15:1 Pentadecenoic, 17:1 Heptadecenoic, 18:3 gamma-Linolenic, 20:2 Eicosadienoic, 20:4 Arachidonic, 20:3 Eicosatrienoic, but levels were below the limit of quantitation.



Vitamins

An analysis of vitamins in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 17** and **Figure 55**.

No literature values were found for beta-tocopherol, delta-tocopherol, gamma-tocopherol, Vitamin A, Vitamin B5, Vitamin B6, Vitamin B12, Vitamin C, Vitamin D and niacin in soybean grain. Beta tocopherol, Vitamin A, Vitamin B12 and Vitamin D were all <LOQ. No differences were observed between the control, unsprayed AAD-12 and the treated AAD-12 for Vitamin B1, Vitamin B2, Vitamin B6, Vitamin C, Vitamin E or niacin. Of those vitamins with available literature ranges, all treatments fell within these ranges with the exception of vitamin B2 where values exceeded the range for all treatments including the non-transgenic control.

Delta-tocopherol levels were significantly different between the control and the AAD-12 + glufosinate and AAD-12 + 2,4-D entries based on unadjusted p-values. However this was not accompanied by a significant adjusted p-value or overall treatment effect. Gamma-tocopherol was significantly different between the control and the unsprayed and AAD-12 + 2,4-D entries based on unadjusted and adjusted p-values. However, gamma tocopherol was <11% higher for the AAD-12 treatments compared with the non-transgenic control.

Vitamin B5 levels were significantly different between the control and the AAD-12 + glufosinate entry based on the adjusted p-value. However this was not accompanied by a significant overall treatment effect.

Folic acid was significantly different between the control and the unsprayed, AAD-12 + 2,4-D and AAD-12 + both herbicides based on unadjusted p-values. Folic acid also had significant differences in the adjusted p-values for two of the AAD-12 entries compared with the control. However, folic acid levels were within the reported literature values for all treatments and the AAD-12 entries differed from the non-transgenic control by <9%, indicating equivalence to non-transgenic soybean.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 17. Summary of vitamin analysis of soybean grain (mg/kg dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Beta Carotene (Vitamin A)	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Vitamin B1 (Thiamin)	1.01-2.54	0.560	2.10	2.14 (0.809,0.886)	1.94 (0.312,0.517)	1.97 (0.414,0.615)	2.14 (0.787,0.873)
Vitamin B2 (Riboflavin)	1.90-3.21	0.994	**4.49**	**4.52** (0.933,0.952)	**4.60** (0.677,0.819)	**4.52** (0.922,0.948)	**4.55** (0.817,0.891)
Vitamin B3 (Niacin)	NR	0.211	27.4	25.3 (0.060,0.169)	25.4 (0.076,0.201)	26.9 (0.698,0.831)	26.7 (0.513,0.708)
Vitamin B5 (Pantothenic acid)	NR	0.183	15.1	14.9 (0.601,0.794)	14.2 (**0.041**,0.134)	14.5 (0.170,0.350)	14.3 (0.065,0.178)
Vitamin B6 (Pyridoxine)	NR	0.788	5.50	5.51 (0.929,0.951)	5.40 (0.439,0.634)	5.40 (0.451,0.642)	5.39 (0.420,0.620)
Vitamin B12	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Vitamin C	NR	0.338	84.1	79.6 (0.126,0.281)	85.4 (0.639,0.808)	82.5 (0.580,0.779)	83.5 (0.838,0.907)
Vitamin D	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Vitamin E (Alpha-tocopherol)	1.90-61.7	0.182	14.8	15.1 (0.762,0.863)	14.5 (0.611,0.796)	15.9 (0.137,0.301)	14.3 (0.439,0.634)
Beta-tocopherol	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Delta-tocopherol	NR	0.095	92.6	95.1 (0.142,0.305)	96.5 (**0.030**,0.109)	97.1 (**0.013**,0.067)	94.5 (0.257,0.446)
Gamma-tocopherol	NR	**0.0004**	153	164 (**0.002**,**0.021**)	158 (0.117,0.268)	169 (**0.0005**,**0.006**)	157 (0.174,0.351)
Folic Acid	2.39-4.71	**0.006**	3.70	3.49 (**0.011**,0.060)	3.56 (0.078,0.203)	3.38 (**0.0004**,**0.009**)	3.48 (**0.008**,**0.048**)

[a] Combined range from literature ranges.

[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.

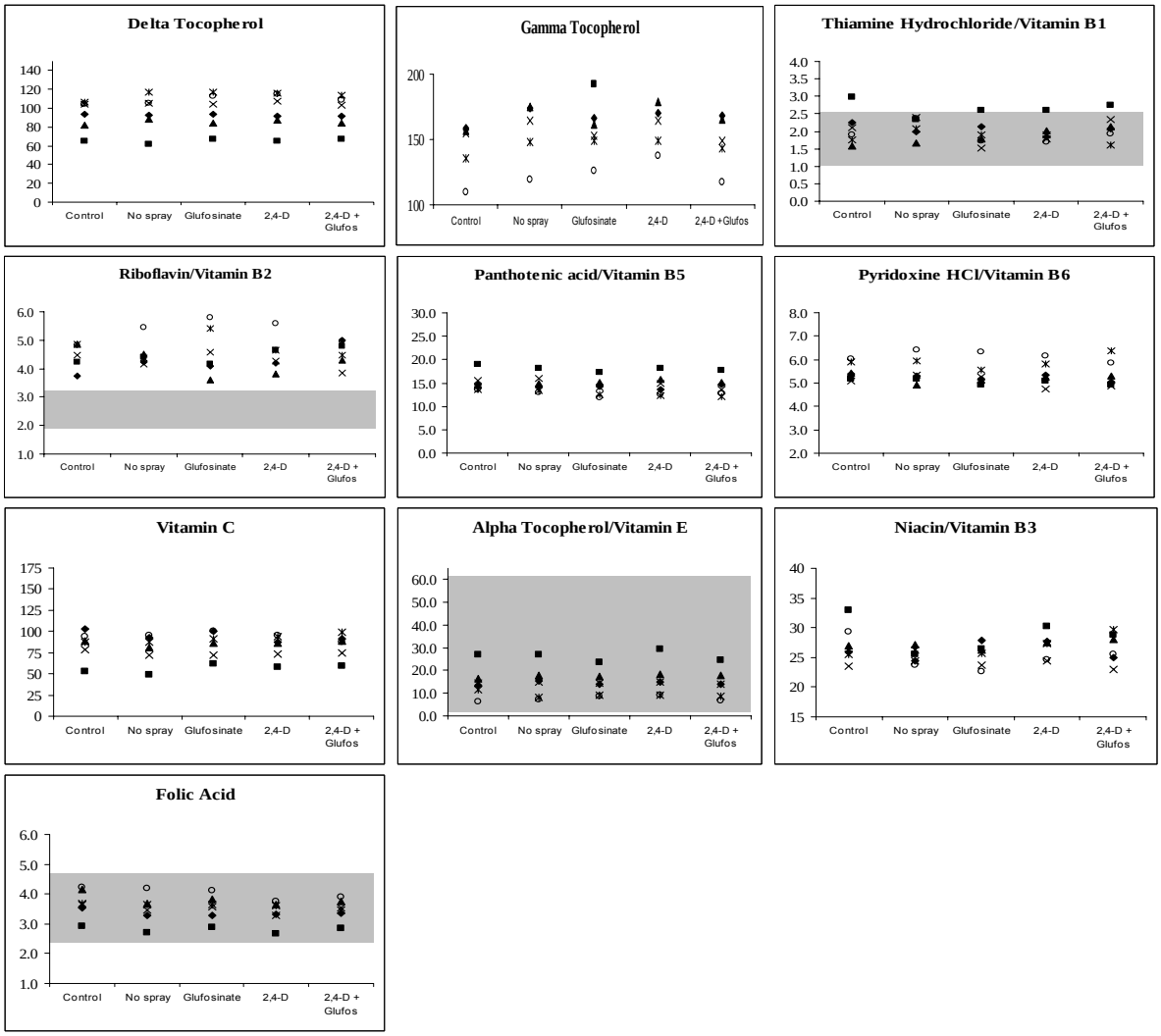
NR = not reported.

NA= statistical analysis was not performed since a majority of the data was < LOQ.

Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 55. Summary of vitamin analysis of soybean grain (mg/kg dry weight). (Smith-Drake et al, 2009, Study ID 080003).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded. Grain was also analysed for Beta-Tocopherol, Vitamin A, Vitamin B12, and Vitamin D, but results were less than the limit of quantitation.



Isoflavones

The analysis of isoflavones in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 18** and **Figure 56**.

The genistein and glycitein results were below the LOQ for the treated samples. Diadzin levels were significantly different between the control and the AAD-12 + both herbicides entries based on unadjusted and adjusted p-values. However, the overall treatment effect was not significant. Although there are no reported literature values, the AAD-12 + both herbicides treatment was <9% different from the non-transgenic control. Genistin levels were significantly different between the control and the AAD-12 + both herbicides entries based on unadjusted and adjusted p-values. However, the overall treatment effect was not significant. Although there are no reported literature values for genistin, the AAD-12 treatments were <9% different compared with the non-transgenic control. Glycitin values were significantly different between the control and the AAD-12 + both herbicides based on unadjusted and adjusted p-values. While there were no reported literature values for glycitin, all AAD-12 entries were <13% different compared with the non-transgenic entry. In addition, all total isoflavone aglycone equivalents were within reported literature ranges.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 18. Summary of isoflavone analysis of soybean grain (µg/g). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Daidzein Daidzin[e]	NR 60.0-2454[f]	NA 0.068	<LOQ 1085	<LOQ 1103 (0.584,0.779)	<LOQ 1112 (0.391,0.589)	<LOQ 1128 (0.187,0.360)	< LOQ 1179 (**0.007**,**0.045**)
Genistein Genistin[e]	NR 144-2837[f]	NA 0.069	<LOQ 1282	< LOQ 1321 (0.292,0.490)	< LOQ 1327 (0.220,0.408)	< LOQ 1357 (0.052,0.152)	< LOQ 1389 (**0.007**,**0.044**)
Glycitein Glycitin[e]	NR 15.3-310[f]	NA **0.032**	< LOQ 253	< LOQ 267 (0.142,0.305)	< LOQ 270 (0.076,0.201)	< LOQ 268 (0.121,0.274)	< LOQ 285 (**0.002,0.021**)

[a] Combined range from literature ranges.

[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] Expressed as aglycone equivalent.

NA= statistical analysis was not performed since a majority of the data was < LOQ.

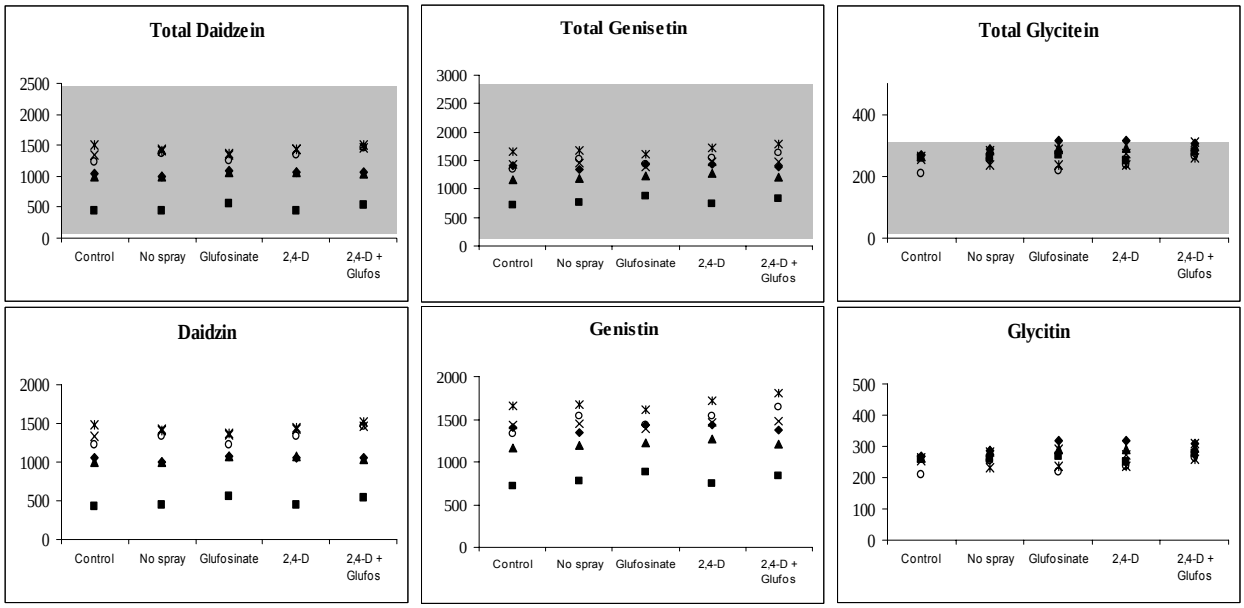
NR = not reported.

Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 56. Summary of isoflavone analysis of soybean grain (µg/g). (Smith-Drake et al, 2009, Study ID 080003).

Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded. Grain was also analysed for genistein and glycitein, but results were less than the limit of quantitation.



Literature Values for Compositional Analysis

Literature values for compositional analysis of soybean grain and forage are from

1) ILSI (International Life Sciences Institute) (2006) ILSI Crop Composition Database. Version 3.0 (http://www.cropcomposition.org/)
2) OECD (2001) Consensus Document on Compositional Considerations for New Varieties of Soybean: Key Food and Fee Nutrients and Anti-Nutrients.

Table 19. Summary of literature values for proximates in soybean forage.

Tissue/Component	OECD	ILSI
Proximate (% DW)		
Moisture (% FW)	NA	73.5-81.6
Protein	11.2-17.3	14.38-24.71
Total Fat	3.1-5.1	1.302-5.132
Ash	8.8-10.5	6.718-10.782
Carbohydrates (calculated)	NA	59.8-74.7
Fiber (% DW)		
Neutral Detergent Fiber (%)	34-40	NA
Acid Detergent Fiber (%)	32-38	NA
Minerals (% DW)		
Calcium	NA	NA
Phosphorus	NA	NA

NA – Literature values not available

FW=Fresh Weight; DW=Dry Weight

Table 20. Summary of literature values for proximates in soybean grain.

Tissue/Component	OECD	ILSI
Proximate (% DW)		
Moisture (% FW)	NA	4.7-34.4
Protein	32-43.6	33.19-45.48
Total Fat	15.5-24.7	8.1-23.56
Ash	4.5-6.4	3.89-6.99
Carbohydrates (calculated)	31.7-31.8	29.6-50.2
Cholesterol	NA	NA
Fiber (% DW)		
Neutral Detergent Fiber	10.0-14.9	8.53-21.25
Acid Detergent Fiber	9-11.1	7.81-18.61
Total Dietary Fiber	NA	NA

NA – Literature values not available

FW=Fresh Weight; DW=Dry Weight

Table 21. Summary of literature values for amino acids in soybean grain.

Amino Acids	OECD (% DW)	ILSI (% DW)
Aspartic Acid	NA	3.81-5.12
Threonine	1.4-1.89	1.14-1.86
Serine	NA	1.11-2.48
Glutamic Acid	NA	5.84-8.20
Proline	NA	1.69-2.28
Glycine	NA	1.46-2.00
Alanine	NA	1.51-2.10
Cysteine	0.45-0.67	0.370-0.808
Valine	1.5-2.44	1.60-2.20
Methionine	0.5-0.67	0.431-0.681
Isoleucine	1.76-1.98	1.54-2.08
Leucine	2.2-4.0	2.59-3.62
Tyrosine	NA	1.02-1.61
Phenylalanine	1.6-2.08	1.63-2.35
Lysine	2.5-2.66	2.29-2.84
Histidine	1.0-1.22	0.88-1.18
Arginine	2.45-3.1	2.29-3.40
Tryptophan	0.51-0.67	0.356-0.502

NA – Literature values not available

DW=Dry Weight

Table 22. Summary of literature values for isoflavones in soybean grain.

Isoflavones (µg/g)	OECD	ILSI
Daidzein	NA	60-2453.5
Glycitein	NA	15.3-310.4
Genistein	NA	144.3-2837.2
Daidzin	NA	NA
Glycitin	NA	NA
Genistin	NA	NA

NA – Literature values not available

Table 23. Summary of literature values for fatty acids in soybean grain.

Fatty Acids	ILSI (% Total FA)
8:0 Caprylic	0.148
10:0 Capric	NA
12:0 Lauric	0.082-0.132
14:0 Myristic	0.071-0.238
14:1 Myristoleic	0.121-0.125
15:0 Pentadecanoic	NA
15:1 Pentadecenoic	NA
16:0 Palmitic	9.55-15.77
16:1 Palmitoleic	0.086-0.194
17:0 Heptadecanoic	0.085-0.146
17:1 Heptadecenoic	0.073-0.087
18:0 Stearic	2.70-5.88
18:1 Oleic	14.3-32.2
18:2 Linoleic	42.3-58.8
18:3 γ-Linolenic	NA
18:3 Linolenic	3.00-12.52
20:0 Arachidic	0.163-0.482
20:1 Eicosenoic	0.140-0.350
20:2 Eicosadienoic	0.077-0.245
20:4 Arachidonic	NA
20:3 Eicosatrienoic	NA
22:0 Behenic	0.277-0.595

NA – Literature values not available

FA – Fatty Acids

Table 24. Summary of literature values for vitamins in soybean grain.

Vitamins (mg/kg)	ILSI
Thiamine Hydrochloride	1.01-2.54
Riboflavin/Vitamin B2	1.90-3.21
Niacin/Vitamin B3	NA
Pyridoxine HCl	NA
Folic Acid	2.39-4.71
Pantothenic acid	NA
Vitamin B12	NA
Vitamin D	NA
Vitamin C	NA
Vitamin A	NA

NA – Literature values not available

Table 25. Summary of literature values for minerals in soybean grain.

Minerals (mg/100g)	ILSI
Calcium	116.55-307.1
Copper	NA
Iron	5.54-10.95
Magnesium	219.4-312.8
Manganese	NA
Phosphorus	506.7-935.2
Potassium	1868.01-2316.14
Sodium	NA
Zinc	NA
Iodine	NA
Minerals (ppb)	
Chromium	NA
Selenium	NA
Molybdenum	NA

NA – Literature values not available

Table 26. Summary of literature values for tocopherols in soybean grain.

	OECD	ILSI
Alpha Tocopherol	NA	1.9-61.7
Beta Tocopherol	NA	NA
Gamma Tocopherol	NA	NA
Delta Tocopherol	NA	NA

NA – Literature values not available

Summary of Grain and Forage Composition

The composition of DAS-68416-4 soybean was either statistically indistinguishable from the non-transgenic control, <13% different from the non-transgenic control, or within the literature range for non-transgenic soybean. Plots of the composition results do not indicate any biologically meaningful treatment-related compositional differences among unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides soybean and the control soybean line.

In conclusion, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides composition results confirm the substantial equivalence of DAS-68416-4 soybean and conventional soybean.

Composition Methods

Acid Detergent Fiber with Ankom

The ANKOM2000 Fiber analyser automated the process of the removal of protein, carbohydrate, and ash. If necessary, the fats and pigments were removed with an acetone wash prior to analysis. A fraction lignin, cellulose and/or insoluble protein complexes was left in the filter bag, and determined gravimetrically. The limit of quantitation for this study was 0.100%.

Reference:

1) *Forage and Fiber Analyses*, Agriculture Handbook No.379, United States Department of Agriculture, Washington, D.C. (1970).
2) Komarek, A.R., Robertson J.B and Van Soest P.J. "A Comparison of Methods for Determining ADF Using the Filter Bag Technique versus Conventional Filtration," *Journal of Dairy Science* Vol. 77 Supplement 1. (1993).

Amino Acid Composition

Total aspartic acid (including asparagine)
Total threonine
Total serine
Total glutamic acid (including glutamine)
Total proline
Total glycine
Total alanine
Total valine
Total isoleucine
Total leucine
Total tyrosine
Total phenylalanine
Total histidine
Total lysine
Total arginine
Total tryptophan

Sulfur-containing amino acids:	Total methionine
	Total cystine (including cysteine)

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolysed, the individual amino acids were then quantitated using an automated amino acid analyser. The limit of quantitation for this study was 0.0100%.

Reference Standards:

Thermo Scientific Amino Acid Standard H, (K18), 2.5 μmol/mL per constituent except cystine (1.25 μmol/mL)

Sigma-Aldrich, L-Tryptophan, 100%

Sigma-Aldrich/BioChemika, L-Cysteic Acid Monohydrate, 99.5% (used as 100%)

Sigma-Aldrich, L-Methionine Sulfone, >99% (used as 100%)

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 982.30, AOAC International, Gaithersburg, Maryland, (2005).

Ash

The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The limit of quantitation for this study was 0.100%.

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 923.03, AOAC International, Gaithersburg, Maryland, (2005).

Beta Carotene (Reported as Vitamin A)

The sample was saponified and extracted with hexane. The sample was then injected on a reverse phase high-performance liquid chromatography system with ultraviolet light detection. Quantitation was achieved with a linear regression analysis. The limit of quantitation was calculated and reported on a fresh weight basis. The limit of quantitation for this study was 0.200 mg/kg.

Reference Standard:

Sigma, Beta Carotene, Type 1, 98.2%, stock standard concentration determined spectrophotometrically

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 941.15, AOAC International, Gaithersburg, Maryland, (2005).

2) Quackenbush, F. W., *Journal of Liquid Chromatography*, 10: 643-653, (1987).

Carbohydrate

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

% carbohydrates = 100 % - (% protein + % fat + % moisture + % ash)

The limit of quantitation for this study was 0.100%.

Reference:

1) United States Department of Agriculture, "Energy Value of Foods", *Agriculture Handbook No. 74,* pp. 2-11, (1973).

Cholesterol

The sample is saponified using ethanolic potassium hydroxide. The unsaponifiable fraction that contains cholesterol and other sterols is extracted with toluene. The toluene is evaporated to dryness and the residue is dissolved in dimethylformamide. The samples are derivatised to form trimethylsilyl ethers. The derivatised cholesterol is quantitatively determined by gas chromatography using 5 α-cholestane as an internal standard. The limit of quantitation for this study was 0.0010%.

Reference Standards:

Sigma, Cholesterol, 99.5%

Chromadex, Campesterol, 97.2%

Sigma, Stigmasterol, 97.0%*

Sigma, Beta-sitosterol, 98.0%

* Present in the standard but not used for cholesterol calculation.

Reference:

1) *Official Methods of Analysis of AOAC International*, 17th Ed., Official Method 994.10. (Modified), AOAC International, Gaithersburg, Maryland, (2000).

Fat by Acid Hydrolysis

The sample was hydrolysed with hydrochloric acid at an elevated temperature. The fat was extracted with ether and hexane. The extract was evaporated on a steambath, re-dissolved in hexane and filtered through a sodium sulfate column. The hexane extract was then evaporated again on a steambath under nitrogen, dried, and weighed. The limit of quantitation for this study was 0.100%.

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 922.06 and 954.02, AOAC International, Gaithersburg, Maryland, (2005).

Fat by Soxhlet Extraction

The sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed. The limit of quantitation for this study was 0.100%.

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 960.39 and 948.22, AOAC International, Gaithersburg, Maryland, (2005)

Fatty Acids

The lipid was extracted and saponified with 0.5N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analysed by gas chromatography using external standards for quantitation. The limit of quantitation was 0.0100-0.0200% depending on percent lipid.

Reference Standards:

Nu Chek Prep GLC Reference Standard Hazelton No. 1, >99%
Nu Chek Prep GLC Reference Standard Hazelton No. 2, >99%
Nu Chek Prep GLC Reference Standard Hazelton No. 3, >99%
Nu Chek Prep GLC Reference Standard Hazelton No. 4, >99%
Nu Chek Prep Methyl Gamma Linolenate, 100%
Nu Chek Prep Methyl Tridecanoate, 100%

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 996.06, AOAC International, Gaithersburg, Maryland, (2005).
2) *Official Methods and Recommended Practices of the AOCS*, 5th Ed., Method Ce 1-62, American Oil Chemists' Society: Champaign, Illinois, (1997).

Folic acid

The sample was hydrolysed in a potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis by autoclaving, the sample was treated with a chicken-pancreas enzyme and incubated approximately 18 hours to liberate the bound folic acid. The amount of folic acid was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus casei*, with the growth response of a folic acid standard. This response was measured turbidimetrically. The limit of quantitation for this study was 0.0600 mg/kg.

Reference Standard:

USP, Folic acid, 98.9%

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 960.46 and 992.05, AOAC International, Gaithersburg, Maryland, (2005).
2) *Methods of Analysis for Infant Formulas*, Infant Formula Council, Atlanta, Georgia, Section C-2, (1985).

Metals by ICP Emission Spectrometry

The sample was dried, precharred, and ashed overnight in a muffle set to maintain 500°C. The ashed sample was re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions.

Inorganic Ventures Reference Standards and Limits of Quantitation:

Mineral	Concentration (µg/ml)	Grain Limit of Quantitation (mg/100g)	Forage Limit of Quantitation (%)
Calcium	200, 1000	2.00	0.00200
Copper	2, 10	0.050	-
Iron	10, 50	0.200	-
Magnesium	50, 250	2.00	-
Manganese	2, 10	0.030	-
Phosphorus	200, 1000	2.00	0.00200
Potassium	200, 1000	10.0	-
Sodium	200, 1000	10.0	-
Zinc	10, 50	0.040	-

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 984.27 and 985.01, AOAC International: Gaithersburg, Maryland, (2005).

Metals by ICP-Mass Spectrometry

The sample was wet-ashed with nitric acid using microwave digestion. Using inductively coupled plasma mass spectrometry, the amount of each element was determined by comparing the counts generated by the unknowns to those generated by standard solutions of known concentrations.

Spex CertiPrep Reference Standards and Limits of Quantitation:

Mineral	Concentration (mg/L)	Limit of Quantitation (ppb)
Selenium	100	50.0
Chromium	100	50.0
Molybdenum	100	50.0

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 993.14, AOAC International: Gaithersburg, Maryland, (2005).
2) EPA Method 200.8, *Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma-Mass Spectrometry*, (1994).
3) Cabrera, C., Gallego, C., Lopez, M. C., Lorenzo, M. L., and Lillo, E., "Determination of Levels of Lead Contamination in Food and Feed Crops", *Journal of AOAC International*, Volume 77(5):1249-1252, (1994).

Iodine

The sample was digested with a combination of alcoholic potassium hydroxide, sodium carbonate, and alcoholic magnesium nitrate, whereby the iodide was converted to potassium iodide. In the case of organic iodides, the conversion was the result of a dehydrohalogenation reaction. After preliminary charring on a hot plate with heat lamps, the sample was placed in a muffle set for 90 minutes to complete the combustion of organic material. The iodide was then extracted from the ash with hot water and filtered. The analysis was completed by colorimetrically measuring the extent of the reaction between arsenic and cerium as catalyzed by the presence of iodide. The greater the amount of iodide present, the greater the rate of reaction as determined by the difference in absorbance for a 15-minute interval. The limit of quantitation for this study was 0.0100 mg/100g.

Reference Standard:

Fisher, Potassium Iodide, 99.9%, Lot Number 061234

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 932.21, AOAC International: Gaithersburg, Maryland, (2005).
2) Binnerts, W. T., "Determination of Iodine in Milk", *Analytica Chimica Acta*, 10:78-80, (1954).
3) Heerspink, W., Op Deweegh, G. J., *Clinica Chimica Acta*, 39:327-338, (1972).

Isoflavones

The samples were extracted at approximately 65°C with a 80/20 methanol:water solution and the extracts were saponified with dilute NaOH solution. The extracts were then acidified, filtered, and then diluted. The samples were analysed on a high-performance liquid chromatography system with ultraviolet spectrophotometric detection and were compared against an external standard curve. The glucosides (diadzin, glycitin and genistin) are calculated as their aglycone equivalents. The limit of quantitation for each individual component for this study was 10 µg/g.

Reference Standards:

Chromadex, Daidzein, 96.5%

Chromadex, Glycitein, 96.3%.

Indofine, Genistein, ≥99% (used as 100%)

Chromadex, Daidzin, 88.5%

Indofine, Glycitin, 98 +% (used as 98%)

Indofine, Genistin, ≥99% (used as 100%)

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Official Methods 2001.10, AOAC International: Gaithersburg, Maryland, (2005).

Moisture

The sample was dried in a vacuum oven at approximately 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture. The limit of quantitation for this study was 0.100%.

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 926.08 and 925.09, AOAC International: Gaithersburg, Maryland, (2005).

Neutral Detergent Fiber, Enzyme Method using Ankom

The ANKOM2000 Fiber Analyzer automated the process of the removal of protein, carbohydrate, and ash. An enzyme treatment of heat stabilized alpha-amylase was used to break down starches. If necessary, fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin and insoluble protein fraction was left in the filter bag and determined gravimetrically. The limit of quantitation for this study was 0.100%.

References:

1) *Approved Methods of the American Association of Cereal Chemists*, 9th Ed., Method 32.20, (1998).

2) *Forage and Fiber Analyses*, Agriculture Handbook No. 379, United States Department of Agriculture, (1970).

3) Komarek, A.R., Robertson J.B and Van Soest P.J. "Comparison of the Filter Bag Technique to Conventional Filtration in the Vn Soest NDF analysis of 21 Feeds," Presented at National Conference on Forage Quality, Evaluation and Utilization Proceedings (University of Nebraska) (1994).

Niacin

The sample was hydrolysed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum,* with the growth response of a niacin standard. This response was measured turbidimetrically. The limit of quantitation for this study was 0.300 mg/kg.

Reference Standard:

USP, Niacin, 99.8%

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 944.13 and 960.46, AOAC International, Gaithersburg, Maryland, (2005).

Pantothenic Acid

The sample was diluted with water or treated with an enzyme mixture to liberate the pantothenic acid from coenzyme A and the pH was adjusted to remove interferences. The amount of pantothenic acid was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum*, with the growth response of a calcium pantothenate standard. This growth response was measured turbidimetrically. The limit of quantitation for this study was 0.400 mg/kg.

Reference Standard:

USP, Calcium pantothenate, 99.0%

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 945.74 and 960.46, AOAC International, Gaithersburg, Maryland, (2005).

Protein

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25. The limit of quantitation for this study was 0.100%.

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 955.04 and 979.09, AOAC International, Gaithersburg, Maryland, (2005).
2) Bradstreet, R. B., *The Kjeldahl Method for Organic Nitrogen*, Academic Press: New York, New York, (1965).
3) Kalthoff, I. M., and Sandell, E. B., *Quantitative Inorganic Analysis*, MacMillan: New York, (1948).

Thiamin Hydrochloride

The sample was autoclaved under weak acid conditions to extract the thiamin. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamin. The solution was purified on a cation-exchange column. An aliquot was reacted with potassium ferricyanide to convert thiamin to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard. The limit of quantitation for this study was 0.10 mg/kg. Results were reported as thiamin hydrochloride.

Reference Standard:

USP, Thiamin hydrochloride, 99.8%, used as 95.9% after correction for moisture

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 942.23, 953.17, and 957.17, AOAC International: Gaithersburg, Maryland, (2005).

Tocopherols, Total

The product was saponified to break down any fat and release vitamin E. The saponified mixture was extracted with an organic solvent, dried down and brought to a suitable volume in hexane. The sample was then quantitated by high-performance liquid chromatography using a silica column. The limit of quantitation for this study was approximately 5.00 mg/kg.

Reference Standard:

USP, Alpha Tocopherol, 100%

Matreya, Beta Tocopherol, stock standard concentration determined spectrophotometrically

Sigma, Gamma Tocopherol, 99%

Sigma, Delta Tocopherol, 95%

References:

1) Speek, A. J., Schijver, J., and Schreurs, W. H. P., "Vitamin E Composition of Some Seed Oils as Determined by High-Performance Liquid Chromatography with Fluorometric Quantitation," *Journal of Food Science,* 50(1):121-124, (1985).
2) Cort, W. M., Vincente, T. S., Waysek, E. H., and Williams, B. D., "Vitamin E Content of Feedstuffs Determined by High-Performance Liquid Chromatographic Fluorescence," *Journal of Agricultural and Food Chemistry,* 31:1330-1333, (1983).

3) McMurray, C. H., Blanchflower, W. J., and Rice, D. A., "Influence of Extraction Techniques on Determination of α-Tocopherol in Animal Feedstuffs," *Journal of the Association of Official Analytical Chemists,* 63(6):1258-1261, (1980).

Total Dietary Fiber

Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber.
The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using the protein and ash values. The limit of quantitation for this study was 1.00%.
Reference:

1) *Official Methods of Analysis of AOAC International*, 18th Ed., Method 985.29, AOAC International, Gaithersburg, Maryland, (2005).

Vitamin B_2 (Riboflavin)

The sample was hydrolysed with dilute hydrochloric acid and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus rhamnosus*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically. The limit of quantitation for this study was 0.200 mg/kg.
Reference Standard:

USP, Riboflavin, 100%

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 940.33 and 960.46, AOAC International, Gaithersburg, Maryland, (2005).
2) *The United States Pharmacopeia,* Twenty-Ninth Revision, p. 1913, United States Pharmacopeial Convention, Inc.: Rockville, Maryland, (2005).

Pyridoxine Hydrochloride

The sample was hydrolysed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the sample, using the yeast *Saccharomyces cerevisiae,* with the growth response of a pyridoxine standard. The response was measured turbidimetrically. Results were reported as pyridoxine hydrochloride. The limit of quantitation for this study was 0.0700 mg/kg.
Reference Standard:

USP, Pyridoxine hydrochloride, 100%

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 961.15, AOAC International: Gaithersburg, Maryland, (2005).
2) Atkins, L., Schultz, A. S., Williams, W. L., and Frey, C. N., "Yeast Microbiological Methods for Determination of Vitamins," *Industrial and Engineering Chemistry, Analytical Edition,* 15:141-144, (1943).

Vitamin B_{12}

Vitamin B_{12} was extracted from the sample into a buffer by heating in an autoclave. Utilizing the bacteria *Lactobacillus delbrueckii*, the amount of vitamin B_{12} was determined turbidimetrically by comparing the growth response of a sample against the growth response of a vitamin B_{12} standard. The limit of quantitation for this study was 0.00300 mg/kg.

Reference Standard:

USP, Cyanocobalamin, 10.7 µg/mg

References:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Methods 952.20 and 960.46, AOAC International: Gaithersburg, Maryland, (2005).
2) *The United States Pharmacopeia,* Twenty-Ninth Revision, pp. 603-4, United States Pharmacopeial Convention, Inc.: Rockville, Maryland, (2005).
3) *Methods of Analysis for Infant Formulas*, Infant Formula Council, Atlanta, Georgia, Section C-2, (1985).

Vitamin C

The vitamin C in the sample was extracted, oxidized, and mixed with o-phenylenediamine to produce a fluorophor having an activation maximum at approximately 350 nm and a fluorescence maximum at 430 nm. Fluorescence was proportional to concentration. Development of the fluorescence compound with the vitamin was prevented by forming a boric acid-dehydroascorbic acid complex prior to addition of the o-phenylenediamine solution. Any remaining fluorescence was due to extraneous material and served as the blank. The limit of quantitation for this study was 10.0 mg/kg.

Reference Standard:

USP, Ascorbic Acid, 100%

Reference:

1) *Official Methods of Analysis of AOAC International,* 18th Ed., Method 967.22, AOAC International: Gaithersburg, Maryland, (2005).

Vitamin D

Vitamin D was extracted with reagent alcohol. After removing any solid particles by centrifuging the extraction solution was saponified by adding KOH solution. The analyte was extracted with hexane, dried down, reconstituted, and injected for LC/MS/MS measurement. The limit of quantitation for this study was 0.005 mg/kg.

Reference Standards:

USP, Cholecalciferol, 100%

USP, Ergocalciferol, 100%

Reference:

1) Huang, M., LaLuzerne P., and Winters, D. "Measurement of Vitamin D in Foods and Nutritional Supplements by Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS)" 2009 (accepted), Journal of AOAC International

b. Secondary Metabolite and Anti-Nutrient Analysis of Grain

An analysis of anti-nutrients in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in **Table 27** and **Figure 57.**

No statistical differences were observed between the control and transgenic entries for lectin, phytic acid, or trypsin inhibitor. These three anti-nutrients were also all within the literature ranges, indicating equivalence to non-transgenic soybean.

Raffinose was significantly lower (<10%) for the AAD-12 + glufosinate treatment compared with the control based on unadjusted p-values. Raffinose was not significantly different in the across-site analysis based on the adjusted p-value or the overall treatment effect. Raffinose levels were also within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

Stachyose was significantly different between the control and the AAD-12 + glufosinate entry based on the unadjusted p-value. Stachyose levels were not significant different in the across-site analysis based on the adjusted p-value or the overall treatment effect. Stachyose levels were also within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

Anti-nutrient analysis for lectin, phytic acid, raffinose, stachyose and trypsin inhibitor were all within the reported literature values, and the two significant differences based on unadjusted p-values had lower levels of anti-nutrients for the AAD-12 treatments compared with the control.

Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 27. Summary of anti-nutrient analysis of soybean grain (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Lectin (H.U./mg)	0.11-9.04	0.552	2.18	2.74 (0.333,0.540)	2.84 (0.254,0.444)	2.98 (0.176,0.351)	3.09 (0.124,0.277)
Phytic Acid	0.63-2.74	0.725	1.20	1.20 (0.949,0.962)	1.22 (0.673,0.819)	1.21 (0.896,0.936)	1.25 (0.253,0.444)
Raffinose	0.212-0.661	0.111	0.344	0.339 (0.753,0.860)	0.310 (**0.033**,0.118)	0.317 (0.082,0.210)	0.315 (0.062,0.173)
Stachyose	1.2-3.5	0.217	2.42	2.34 (0.378,0.575)	2.23 (**0.027**,0.105)	2.28 (0.105,0.253)	2.32 (0.231,0.425)
Trypsin Inhibitor (TIU/mg)	19.6-184	0.435	25.3	27.2 (0.204,0.383)	24.7 (0.657,0.819)	24.9 (0.748,0.860)	25.3 (0.973,0.979)

[a] Combined range from literature ranges.

[b] Overall treatment effect estimated using an F-test.

[c] Comparison of the transgenic treatments to the control using t-tests.

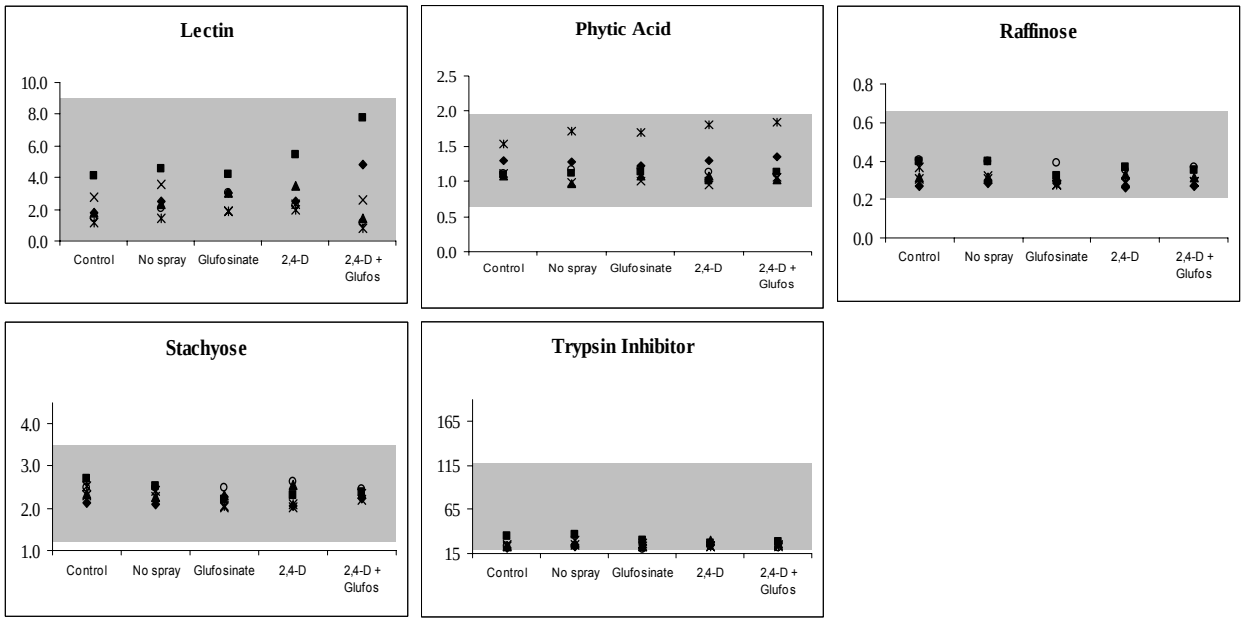
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.

Bolded mean values are outside of the reported literature range.

Bolded P-values are significant (<0.05).

Figure 57. Summary of anti-nutrient analysis of soybean grain (% dry weight). (Smith-Drake et al, 2009, Study ID 080003).

Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges are shaded.



Literature Values

Table 28. Summary of literature values for anti-nutrients in soybean grain.

Anti-Nutrients	OECD	ILSI
Phytic Acid (% DW)	1.0-2.74	0.63-1.960
Raffinose (% DW)	NA	0.212-0.661
Stachyose (% DW)	NA	1.21-3.50
Lectin (H.U./mg)*	NA	0.105-9.038
Trypsin Inhibitor (TIU/mg)**	NA	19.59-118.68

NA – Literature Values Not Available

*H.U. - Hemagglutinating Unit

**TIU - Trypsin Inhibitor Unit

DW – dry weight

Composition Methods

Lectin

The sample was suspended in phosphate buffered saline (PBS), shaken, and filtered. An aliquot of the resulting extract was serially diluted in 10 cuvettes containing PBS. A 10% hematocrit of lyophilized rabbit blood in PBS was added to each dilution. After 2.5 hours, the absorbance of each dilution of the sample and lectin control was measured on a spectrophotometer at 620 nm, using PBS to zero the instrument. One hemagglutinating unit (H.U.) was defined as the level that caused 50% of the standard cell suspension to sediment in 2.5 hours. The limit of quantitation for this study was 0.10 H.U./mg.

References:

1) Klurfeld, D. M. and Kritchevsky, D., "Isolation and Quantitation of Lectins from Vegetable Oils," *Lipids,* 22:667-668, (1987).
2) Klurfeld, D. M., Personal communication.
3) Liener, I. E., "The Photometric Determination of the Hemagglutinating Activity of Soyin and Crude Soybean Extracts," *Archives of Biochemistry and Biophysics,* 54:223-231, (1955).

Phytic Acid

The sample was extracted using 0.5M HCl with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The sample was analysed on a polymer high-performance liquid chromatography column PRP-1, 5μm (150 x 4.1mm) with a refractive index detector. The limit of quantitation for this study was approximately 0.100%.

Reference Standard:

Sigma-Aldrich, Phytic Acid, Dodecasodium Salt Hydrate, 95%

References:

1) Lehrfeld, Jacob, “HPLC Separation and Quantitation of Phytic Acid and Some Inositol Phosphates in Foods: Problem and Solutions,” *Journal of Agricultural and Food Chemistry*, 42:2726-2731, (1994).
2) Lehrfeld, Jacob, “High-Performance Liquid Chromatography Analysis of Phytic Acid on a pH-Stable, Macroporous Polymer Column,” *Cereal Chemistry*, 66(6):510-515, (1989).

Raffinose and Stachyose

The sample was extracted with deionised water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analysed by gas chromatography using a flame ionization detector. The limit of quantitation for this study was 0.100%.

Reference Standards:

Sigma-Aldrich, Raffinose Pentahydrate, 99% (84.0% after correction for degree of hydration)

Sigma-Aldrich, Stachyose, 98% (96.8% after correction for moisture)

References:

1) Brobst, K. M., "Gas-Liquid Chromatography of Trimethylsilyl Derivatives,” *Methods in Carbohydrate Chemistry*, Volume 6, Academic Press: New York, New York, (1972).
2) Mason, B. S., and Slover, H. T., "A Gas Chromatographic Method for the Determination of Sugars in Foods," *Journal of Agricultural and Food Chemistry*, 19(3):551-554, (1971).

Trypsin Inhibitor

The sample was ground and defatted with petroleum ether. A sample of matrix was extracted with 0.01N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride. The sample was allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solution was centrifuged, then the absorbance was determined at 410 nm. Trypsin inhibitor activity was determined by photometrically measuring the inhibition of trypsin’s reaction with benzoyl-DL-arginine~p~nitroanilide hydrochloride. The limit of quantitation for this study was 1.00 Trypsin Inhibitor Units (TIU)/mg.

Reference:

Official Methods and Recommended Practices of the American Oil Chemists’ Society, 5th Ed., Method Ba 12-75, American Oil Chemists’ Society: Champaign, Illinois, (1997).

c. Allergenic Proteins

Please refer to Section C, Part 4 for information regarding the allergenicity of the novel proteins comparative to a non-GM competitor.

D. NUTRITIONAL IMPACT

1. Human Nutritional Impact

Part D Section 1 DAS Reports

Cleveland, C.B., Herman, R.A., Krieger, M.S. (2009) Human and Livestock Exposure Assessment for AAD-12 Protein in DAS 68416-4 Soybeans. Dow AgroSciences LLC Study ID 091141.

Human Dietary Risk Assessment for AAD-12

Protein expression levels of AAD-12 in DAS-68416-4 soybean were used with conservative (i.e. protective) human dietary consumption data for soybean 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-12 protein in DAS-68416-4 soybean, indicating no concern for adverse effects from acute dietary exposure.

The field expression of AAD-12 protein in DAS-68416-4 soybean was measured using a specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean. Protein expression was analysed in leaf, root, forage and grain tissues collected throughout the growing season (V5 to R8 growth stages). Within the six North American field trials, transformed soybean plants were treated either with 2,4-D, glufosinate, both 2,4-D and glufosinate, or not treated with either herbicide; in addition an untreated group of non-transformed Maverick variety served as a control.

In general, the results showed low level expression of the AAD-12 protein with or without 2,4-D or glufosinate herbicide treatments and across environments, indicating a low exposure risk to humans. Only the protein expression in the soybean grain is applicable for human dietary consideration.

In soybean grain collected at growth stage R8, the average value of AAD-12 protein (across treatments) was 16.52 ng/mg tissue on a dry weight basis. The full range of values was narrow with observations from 16.21 to 16.94 ng/mg tissue. Use of the average expression values was used in the human dietary assessment, because grains are a blended commodity, making consumption of single-servings of soybean at the maximum expression-level highly unlikely. Use of these values are conservative and protective estimates for exposure to the AAD-12 protein from soybean; actual dietary exposure to the proteins will be lower because:

1) there will be protein degradation during transport and storage,
2) soybean containing AAD-12 will be mixed with non-transformed soybean,
3) for humans, consumption of soybean 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 soybeans is in forms where the protein concentrations will be reduced by processing, such as in soybean oil which contain very little protein.

A conservative acute consumption (i.e. exposure) estimate was 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 2009). **Table 29** includes 97.5th percentile values for all possible commodities associated with soybean. For DAS-68416-4 soybean, the appropriate maximum consumption value is associated with the "VD541" group with an upper limit for dry soybean reported by Japan. Consumption information for immature seeds is presented here for completeness, but the immature consumption value is lower than for mature seeds and more importantly the seeds cannot be consumed more than once, so the consumption of the mature seeds alone represents a conservative estimate of exposure. Information for soybean oil is presented here for completeness as well, but soybean oil does not contain significant amounts of protein as the protein remains in the meal fraction during processing (OECD 2001). 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 29. Estimates of acute soybean consumption from the GEMS/Food Highest 97.5th percentile "Eater-Only" worldwide. (Cleveland et al, 2009, Study ID 091141).

Commodity[a]	Country with Reported Maximum	Consumption[a] (g/kg/day)	
		General Population	Children ≤6 years
VP 541 soya bean (immature seeds)	Thailand	2.41	3.86
VD 541 soya bean (dry)	Japan	3.03	5.55
OR 541 soya bean oil, refined	USA	1.51	2.36

[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 "VD 541 soya bean (dry)" acute consumption information is coupled to the AAD-12 field expression level of 16.52 ng/mg tissue, an upper limit for acute exposure to the proteins via soybean are estimated as:

0.0500 mg AAD-12 protein/kg bw/day, for general population (i.e. adults)

0.0917 mg AAD-12 protein/kg bw/day, for children of 6 years or younger

Margin of Exposure

Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds which have no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki et al., 2005). Nonetheless, to place the AAD-12 protein exposure estimate in context, a comparison of the exposure information to the lower limit NOEL has been made to provide Margins of Exposure (MOE) for AAD-12 protein where:

$$\text{MOE} = \frac{\text{NOEL}}{\text{Exposure}}$$

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOEL threshold. The calculated MOE values for AAD-12 protein in soybean are extremely large, indicating no concern for adverse effects from acute dietary exposure through soybean.

Table 30. Margin of Exposure for AAD-12 protein in soybean based on WHO 97.5th percentile consumption. (Cleveland et al, 2009, Study ID 091141).

	Protein Exposure[a] (mg/kg bw/day)	NOEL (mg/kg bw)	MOE
General Population	0.0500	>2000	>39960
Children <6 year	0.0917	>2000	>21810

[a] Based on WHO 97.5th percentile consumption of soybean under commodity VD 541.

Livestock Dietary Risk Assessment for AAD-12

Protein expression levels of AAD-12 in DAS-68416-4 soybean were used with conservative (i.e. protective) livestock dietary consumption data for soybean 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-12 protein in DAS-68416-4 soybean, indicating no concern for adverse effects from acute dietary exposure.

An assessment for livestock exposure is presented here based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures of US EPA (2009). Accordingly, several soybean commodity forms are considered potential animal feeds: seed, forage, hay meal, hulls and aspirated grain fractions. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average values of 16.52 ng/mg (or ppm) for AAD-12 protein in DAS-68416-4 soybean seed. This value for soybean seed has also been used to estimate exposure to soybean feeds for which there was no direct expression measurement: the value for the seed is substituted for the meal and hull feeds and a 20X concentration of the seed residue has been assumed for potential aspirated grain exposure. Note however meal and seed are both protein concentrates and are not simultaneously used in a diet. In addition, for cattle, the field expression level of AAD-12 protein in forage (collected at R3) is applicable. The average value of AAD-12 protein in soybean forage (across treatments) was 40.17 ng/mg tissue (dry weight basis) and the maximum value observed was 41.11 ng/mg tissue.

This maximum value in forage was used in the conservative calculation of acute dairy animal feed exposure. No direct measurement for hay was available; however exposure from hay is assumed to be covered by the forage value and if a treated commodity is used as forage it can not be re-eaten as hay. 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. The presence of AAD-12 protein in soybean tissue is not anticipated to have impact for feed ration formulation, because nutrient composition analyses have shown that DAS-68416-4 soybean is substantially equivalent to conventional soybean per the general OECD (2001) and ILSI (2006) guidance. US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (US EPA, 2009):

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% CC), forages (15% R), and protein sources (5% 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: 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). Alternate poultry would be frying and rotisserie chickens weighing 3 to 4 lb, with an average life span of 38 to 42 days. The broiler diet contains 85% CC and 15% 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-12 protein. In addition, the higher values of 41.11 ppm of AAD-12 protein for forage are assumed at 100% DAS transgenic soybean. In reality, exposure via forage will be lower, given the average values in forage are slightly lower and more importantly market adoption of DAS-68416-4 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled in **Table 31.**

Table 31. Intake animal dietary burdens for livestock for AAD-12. (Cleveland et al, 2009, Study ID 091141).

Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig
			Dietary Contribution (%)			
Soybean hulls[1]	R	90	15	20	Nu[3]	Nu
Aspirated grain[2]	CC	85	5	Nu	Nu	Nu
Soybean seed	PC	89	5	10	*Meal used*	15
Soybean forage	R	35	NA	20	Nu	Nu
Soybean meal[1]	PC	NA	*Seed used*	*Seed used*	25	*Seed used*
		AAD-12 (ppm)	Animal Dietary Burden (ppm)			
Soybean hulls[1]		16.52	2.75	3.76	-	-
Aspirated grain[2]		330.4	19.44	-	-	-
Soybean seed		16.52	0.93	1.86	-	2.48
Soybean forage		41.11	-	23.49	-	-
Soybean meal[1]		16.52	-	-	4.13	-
Total			23.12	29.02	4.13	2.48

[1] estimate based on measured value for seed

[2] based on theoretical estimate of 20X the value in soybean seed

[3] Nu = Not used

Because only soybean feeds are considered he nutritional balance of the diets are assumed to be comprised of unmodified feeds. Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal in **Table 32**.

Table 32. Livestock daily dose estimates of AAD-12 protein from soybean feeds. (Cleveland et al, 2009, Study ID 091141).

	Chicken	Dairy	Beef	Pig
Body weight (kg)	1.9	612	544	113
Daily Maximum Feed (kg)	0.052	24	9	3.1
Maximum AAD-12 intake (mg/kg feed)	4.13	29.02	23.12	2.48
Maximum intake (mg/kg bw)	0.11	1.14	0.39	0.07

The highest exposed animal is the dairy cow with 1.14 mg AAD-12/kg bw estimate. When this value is compared to the acute NOEL of >2000 mg/kg bw, there is an adequate margin of safety for livestock. Variations in livestock feed diets elsewhere in the world could result 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-12 protein in DAS-68416-4 soybean.

Summary of AAD-12 Protein Characterization

The aryloxyalkanoate dioxygenase (AAD-12) protein was derived from *Delftia acidovorans*, a gram-negative soil bacterium. AAD-12 is comprised of 293 amino acids and has a molecular weight of ~32 kDa. Detailed biochemical characterization of the AAD-12 protein derived from plant and microbial sources was conducted. Additionally, characterization of AAD-12 protein expression in DAS-68416-4 plants over the growing season was determined by analysing leaf, root, whole plant, and grain tissues from DAS-68416-4 plants sprayed with 2,4-D, glufosinate, both 2,4-D and glufosinate, and non-sprayed. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the AAD-12 amino acid sequence. The AAD-12 protein hydrolyses rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-12 protein. Glycosylation analysis revealed no detectable covalently linked carbohydrates in AAD-12 protein expressed in DAS-68416-4 soybean plants. Therefore, the low level expression of the AAD-12 protein presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

2. Animal Feeding Studies

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

REFERENCES

Dow AgroSciences Internal Studies

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AAD-12 Amino-Acid Homology Search for Similarity to Allergens. Dow AgroSciences LLC Study ID 071036.

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ATTACHMENT 1 – DOW AGROSCIENCES INTERNAL STUDIES