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

OECD Unique Identifier: DAS-444Ø6-6

DAS-444Ø6-6 Soybean

Volume 1 of 49

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And

M.S. Technologies LLC

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GLOSSARY OF ACRONYMS AND SCIENTIFIC TERMS

2,4-D	2,4-Dichlorophenoxyacetic acid
2mepsps	Gene encoding the double mutant 5-enolpyruvylshikimate-3-phosphate synthase (2mEPSPS) from *Zea mays*
2mEPSPS	Mutant 5-enolpyruvylshikimate-3-phosphate synthase
A	Acre
aad-12	Gene from *Delftia acidovorans* which encodes the AAD-12 protein
AAD-12	Aryloxyalkanoate dioxygenase-12 protein
ACCase	Acetyl CoA carboxylase
ADF	Acid detergent fibre
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
ALS	Acetolactate synthase
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
bu	Bushel
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-444Ø6-6	OECD identifier for the soybean event expressing the AAD-12, 2mEPSPS, and PAT proteins
DCP	2,4-Dichlorophenol
dmmg	Same as *2mepsps*
DNA	Deoxyribonucleic acid

ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (US)
epsps	Gene encoding the wild-type 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)
EPSPS	Wild-type 5-enolpyruvylshikimate-3-phosphate synthase
ESA	Endangered Species Act
ESI-LC/MS	Electrospray ionization-liquid chromatography mass spectrometry
FDA	Food and Drug Administration (US)
FDR	False Discovery Rate
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FWS	Fish and Wildlife Service
GS	Glutamine synthetase
ha	Hectare
HRAC	Herbicide Resistance Action Committee
IAA	Indole acetic acid
ILSI	International Life Sciences Institute
IWM	Integrated weed management
Kb	Kilobase pair
kDa	Kilodalton
L	Litre
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 DAS-444Ø6-6 soybean
mEPSPS	Same as 2mEPSPS
MOA	Mode of action
MS Tech	M.S. Technologies LLC
NDF	Neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
pat	Gene from *Streptomyces viridochromogenes* which encodes the PAT protein
PAT	Phosphinothricin *N*-acetyltransferase protein
PBN	US FDA Pre-market Biotechnology Notice
PCR	Polymerase chain reaction
pDAB8264	DNA vector carrying the *aad-12*, *2mepsps* and *pat* expression cassettes
Pf	*Pseudomonas fluorescens*

PPO	Protoporphyrinogen oxidase
PPT	Phosphinothricin
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
UTR	Untranslated region
WSSA	Weed Science Society of America

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GENERAL INFORMATION ON THE APPLICATION

A. The Applicant

This application is submitted by:

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B. Summary

Dow AgroSciences LLC (herein referred to as “DAS”) and M.S. Technologies LLC (herein referred to as “MS Tech”) are submitting an application to amend the Food Standards Code Standard 1.5.2 to approve the use of DAS-444Ø6-6 Soybean; a new food produced using gene technology.

Dow AgroSciences considers this to be a major procedure under the FSANZ assessment procedures. This application is expected to confer and Exclusive Capturable Commercial Benefit.

This submission includes a dossier which addresses all the items identified by FSANZ as necessary to establish food safety and the supporting reports (as per section 3.5.1 of the FSANZ Application Handbook, 1st August, 2011). Only reports produced by Dow Agrosciences or The Dow Chemical Company are provided. All other citations are available but since these are from published literature have not been copied. Any or all of these citations will be forwarded if requested.

The references provided (Attachment 1) are proprietary information which is owned by and has value to Dow Chemical and their subsidiary Companies. These reports may not be used or referenced by any other company or person without our express agreement.

DAS-444Ø6-6 soybean is a transgenic soybean product that provides tolerance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate 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-444Ø6-6 soybean plants have been genetically modified to express the aryloxyalkanoate dioxygenase-12 (AAD-12), double mutant 5-enolpyruvylshikimate-3-phosphate synthase (2mEPSPS), 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 2mEPSPS protein has a decreased sensitivity to the herbicide glyphosate, allowing the enzyme to function in the presence of the herbicide and thereby making the plant tolerant to glyphosate. The 2mEPSPS protein is encoded by a modified version of the *epsps* gene from corn (*Zea mays*). 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*, *2mepsps* and *pat* genes were introduced into DAS-444Ø6-6 soybean using *Agrobacterium*-mediated transformation. Molecular characterization by Southern blot analyses of DAS-444Ø6-6 soybean confirmed that a single, intact DNA insert containing the *aad-12, 2mepsps* and *pat* gene expression cassettes was stably integrated into the soybean genome. Southern blot analyses also confirmed the absence of the plasmid backbone DNA in DAS-444Ø6-6 soybean. The integrity of the inserted DNA was demonstrated in five different breeding generations. Data from segregating generations confirmed the predicted Mendelian inheritance pattern. These data confirmed the stability of DAS-444Ø6-6 soybean during traditional breeding procedures.

The AAD-12, 2mEPSPS and PAT proteins in DAS-444Ø6-6 soybean were characterized biochemically and measured using protein-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-444Ø6-6 soybean plants treated with 2,4-D, glyphosate, glufosinate, all three herbicides in combination, or not treated with any of these herbicides. The results showed a low level of expression of the AAD-12, 2mEPSPS, 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 hydrolysed 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 the AAD-12 protein expressed in DAS-444Ø6-6 soybean plants. 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.

The 2mEPSPS protein was assessed for any potential adverse effects to humans and animals resulting from the environmental release of crops containing the 2mEPSPS protein. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the 2mEPSPS protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the 2mEPSPS amino acid sequence. The 2mEPSPS protein hydrolysed rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 5000 mg/kg body weight of 2mEPSPS protein. Glycosylation analysis revealed no detectable covalently linked carbohydrates in the 2mEPSPS protein expressed in DAS-444Ø6-6 soybean plants. The low level expression of the 2mEPSPS protein presents a low exposure risk to humans and animals, and the results of the overall

safety assessment of the 2mEPSPS protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals. The safety of the 2mEPSPS protein has been assessed previously and it has been approved for use in corn and cotton.

The PAT protein was assessed for any potential adverse effects to humans and animals resulting from the environmental release of crops containing the PAT protein. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the PAT protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence. The PAT protein hydrolysed rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 5000 mg/kg body weight of PAT protein. The low level expression of the PAT protein presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the PAT 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, rice, soybeans, and sugar beets.

Nutrient composition analyses of forage and grain were conducted to compare the composition of DAS-444Ø6-6 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-444Ø6-6 soybean which was treated with 2,4-D, glyphosate, glufosinate, all three herbicides in combination, or not treated with any herbicide. Along with the agronomic data, the compositional analyses indicate that DAS-444Ø6-6 soybean is substantially equivalent to conventional soybean and will not exhibit unexpected or unintended effects with respect to plant pest risk.

Since DAS-444Ø6-6 soybean is agronomically and nutritionally similar to conventional soybean, and the safety of the AAD-12, 2mEPSPS and PAT proteins has been demonstrated, no significant impact is expected on current crop production practices, non-target or endangered species, crop rotation, volunteer management, or commodity food and feed soybean products.

The availability of DAS-444Ø6-6 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 use of DAS-444Ø6-6 soybean will allow growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance in weeds.

In summary, Dow AgroSciences are seeking an amendment of Standard 1.5.2 by inserting: food derived from Herbicide Tolerant DAS-444Ø6-6 soybean line, into column 1 of the Table to clause 2, immediately after the last entry by means of this application.

PART 5 STATUTORY DECLARATION

I, Sarah Russell French, do solemnly and sincerely declare that the information provided in this application fully sets out the matters required and that this information is true to the best of my knowledge and belief and that no information has been withheld which might prejudice this application.

And I make this solemn declaration by virtue of the *Statutory Declarations Act 1959* and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

Declared at Frenchs Forest
the 30 day of March 2012
Signature
before me* Robin James
Title: JP NSW 173911

* a list of persons who may witness statutory declarations under the *Statutory Declarations Act 1959* is contained in the *Statutory Declarations Regulations 1993*, available online at http://scaleplus.law.gov.au/

CHECKLIST FOR GENERAL REQUIREMENTS

This Checklist will assist you in determining if you have met the information requirements as detailed in Section 3.1 – General Requirements. All applications must include this Checklist.

General Requirements (3.1)

3.1.1 Form of application
- ☑ *Executive Summary*
- ☑ *Relevant sections of Part 3 identified*
- ☐ *Pages sequentially numbered*
- ☑ *Electronic + 1 hard copies*
- ☑ *Electronic and hard copies identical*
- ☑ *Hard copies capable of being laid flat*
- ☐ *All references provided*

3.1.2 Applicant details

3.1.3 Purpose of the application

3.1.4 Justification for the application

3.2.5 Information to support the application

3.1.6 Assessment procedure
- ☐ *General*
- ☑ *Major*
- ☐ *Minor*

☑ 3.1.7 Confidential Commercial Information
- ☐ *Confidential material separated in both electronic and hard copy*
- ☐ *Justification provided*

☐ 3.1.8 Exclusive Capturable Commercial Benefit

☑ 3.1.9 International and Other National standards

☑ 3.1.10 Statutory Declaration

☑ 3.1.11 Checklist/s provided with Application
- ☑ *3.1 Checklist*
- ☐ *Any other relevant checklists for Sections 3.2-3.7*

CHECKLIST FOR STANDARDS RELATED TO NEW FOODS

This Checklist is in addition to the Checklist for Section 3.1 and will assist you in determining if you have met the information requirements as specified in Sections 3.5.1-3.5.3.

Foods Produced using Gene Technology (3.5.1)

- ☑ A.1 Nature and identity of GM food
- ☑ A.2 History of use of host and donor organisms
- ☑ A.3 Nature of genetic modification
- ☑ A.4 Labelling information on GM food
- ☑ B.1 Equivalence studies
- ☑ B.2 Antibiotic resistance marker genes (if used)
- ☑ B.3 Characterisation of novel protein(s)/substances
- ☑ B.4 Toxicity of novel protein(s)/substances
- ☑ B.5 Potential allergenicity of novel protein(s)
- ☑ B.6 Toxicity of novel herbicide metabolites
- ☑ B.7 Compositional Analyses
- ☑ C.1 Nutritional impact of GM food
- ☑ C.2 Animal feeding studies (if available)

Novel Foods (3.5.2)

- ☐ A. Exclusive use
- ☐ B.1 Type of novel food
- ☐ B.2 Information on potential beneficial outcomes
- ☐ B.3 Chemical and physical properties
- ☐ B.4 Impurity profile
- ☐ B.5 Manufacturing process
- ☐ B.6 Specification for identity and purity
- ☐ B.7 Analytical detection method

C – Information on the safety of the novel food

(I) Plant or animal extracts

- ☐ 1. Extraction and composition
- ☐ 2. Effects of food processing or preparation
- ☐ 3. Current use
- ☐ 4. Potential adverse effects

(II) Plant and animal extracts

- ☐ 1. Method or extraction and composition of extract
- ☐ 2. Use as a food in other countries
- ☐ 3. Toxicity studies
- ☐ 4. Safety assessments from other agencies

(III) Herbs (both non-culinary and culinary) including extracts

- ☐ 1. History of use
- ☐ 2. Composition
- ☐ 3. Method of extraction and composition of extract
- ☐ 5. Potential allergenicity
- ☐ 6. Toxicity studies
- ☐ 7. Safety assessments from other agencies

☐ 4. Use in other countries

(IV & V) Single chemical entities & Dietary macrocomponents

☐ 1. Toxicokinetics and metabolism
☐ 2. Toxicity studies
☐ 3. Safety assessments from other agencies

(VI) Microorganisms (including probiotics)

☐ 1. Potential pathogenicity
☐ 2. Effects on gut microflora
☐ 3. Use as a food in other countries
☐ 4. Human toleration studies

(VII) Food ingredients derived from a new source

☐ 1. Safety of the source organism, including allergen statement
☐ 2. Composition
☐ 3. Toxicity studies
☐ 4. Overseas safety reports

(VIII) Foods produced by a process not previously applied to food

☐ 1. Details of the new process
☐ 2. Toxicity studies
☐ 3. Overseas safety reports

☐ D.1 List of foods likely to contain the novel food or novel food ingredient
☐ D.2 Proposed levels in foods
☐ D.3 Information on levels of consumption
☐ D.4 Percentage of food group or market
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☐ D.6 Information to show whether the food or ingredient will replace another food
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☐ E.1 Nutritional impact information
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☐ F.1 Demonstrated consumer awareness and understanding
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☐ A.1 Nature of the food or food ingredient to be irradiated
☐ A.2 Technological need
☐ A.3 Food products likely to contain irradiated food
☐ B. Safety Information
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C. Purpose of the application

Dow AgroSciences LLC (herein referred to as “DAS”) and M.S. Technologies LLC (herein referred to as “MS Tech”) have developed a transgenic soybean plant product, DAS-444Ø6-6 soybean, that provides tolerance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate and glufosinate. DAS-444Ø6-6 is the unique identifier of these plants, in accordance with the Organisation for Economic Co-operation and Development’s (OECD) “Guidance for the Desingation 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 DAS-444Ø6-6soybean line, into column 1 of the Table to clause 2, immediately after the last entry.

D. Justification for application

i. Advantage of the genetically modified food

The availability of DAS-444Ø6-6 soybean is expected to have a beneficial impact on weed control practices by providing growers with an advanced tool to address their weed control needs. The availability of DAS-444Ø6-6 soybean will allow growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance, in weeds.

With the introduction of genetically engineered, glyphosate-tolerant crops in the mid-1990’s, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds that was unparalleled in agriculture. Consequently, producers were quick to adopt glyphosate-tolerant crops, and in many instances, abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, and incorporation of mechanical with chemical and cultural weed control. Currently glyphosate-tolerant soybean, cotton, corn, alfalfa, 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.*, 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) (Information Systems for Biotechnology 2011). 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 2011). 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 halepense* (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), *Urochola 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% and 54% of U.S. and Canadian soybean acres, respectively, >60% of U.S. and Canadian corn and >60% US cotton acres (USDA ERS 2011). 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 glyphosate-resistant (or highly tolerant and shifted) broadleaf 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 with a glyphosate-tolerance trait 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-444Ø6-6 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. 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.

The commercial introduction of transgenic soybean exhibiting tolerance to 2,4-D, glyphosate and glufosinate in key cultivation countries such as the USA, will bring new weed control alternatives to growers. This new weed management tool will allow for the improved control of key broadleaf and grassy weeds which affect the vigour and yield of the crop, allow an increased herbicide application window for effective weed control, and provide an effective resistance management/prevention solution to the increased incidence of glyphosate- and acetolactate synthase (ALS)-resistant weeds.

ii. Safety of the genetically modified organism

AAD-12
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 Graevenitz 1985; Tamaoka, Ha et al. 1987; Wen, Fegan et al. 1999).

Delftia acidovorans can be used to transform ferulic acid into vanillin and related flavour metabolites (Toms and Wood 1970; Rao and Ravishankar 2000; Shetty, Paliyath 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, Goers et al. 1992)

2MEPSPS
The donor organism, *Zea mays*, (commonly referred to as corn or maize) is a major cereal crop grown for food and feed. The 2mEPSPS contains two amino acid substitutions compared with the wild-type EPSPS (Herouet-Guicheneyet al. 2009).

The 2mEPSPS protein is expressed in other events and crops that have previously been approved by FSANZ, for example:

- GA21 corn (OECD Unique Identifier MON-ØØØ21-9) was approved by FSANZ in 2000 (A362).
- GHB614 cotton (OECD Unique Identifier BCS-GHØØ2-5) was approved by FSANZ in 2009 (A614).

PAT

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium. PAT is comprised of 183 amino acids and has a molecular weight of ~21 kDa (OECD, 1999, 2002).

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.

iii. 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-444Ø6-6 in the USA will be significantly hampered. DAS and MS Tech intend to submit dossiers to the regulatory authorities of trade partners for import clearance and production approval, including but not limited to, the USA, Japan, Korea, Taiwan, European Union, South Africa, Canada, Brazil, Argentina and Mexico. 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.

iv. Cost 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-444Ø6-6. The trade implications however are clear since non-approval by FSANZ would impose a trade restriction on DAS-444Ø6-6 and the products derived from these lines.

3.5.1 FOODS PRODUCED USING GENE TECHNOLOGY

A. TECHNICAL INFORMATION ON THE GM FOOD

1. Nature and identity of the genetically modified food

a. Description of the GM organism

DAS-444Ø6-6 soybean is a transgenic soybean product that provides tolerance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate and glufosinate.

DAS-444Ø6-6 soybean plants have been genetically modified to express the aryloxyalkanoate dioxygenase-12 (AAD-12), double mutant 5-enolpyruvylshikimate-3-phosphate synthase (2mEPSPS), 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 2mEPSPS protein has a decreased sensitivity to the herbicide glyphosate, allowing the enzyme to function in the presence of the herbicide and thereby making the plant tolerant to glyphosate. The 2mEPSPS protein is encoded by a modified version of the *epsps* gene from corn (*Zea mays*). 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*, *2mepsps* and *pat* genes were introduced into DAS-444Ø6-6 soybean using *Agrobacterium*-mediated transformation. Molecular characterization by Southern blot analyses of DAS-444Ø6-6 soybean confirmed that a single, intact DNA insert containing the *aad-12, 2mepsps* and *pat* gene expression cassettes was stably integrated into the soybean genome. Southern blot analyses also confirmed the absence of the plasmid backbone DNA in DAS-444Ø6-6 soybean. The integrity of the inserted DNA was demonstrated in five different breeding generations. Data from segregating generations confirmed the predicted Mendelian inheritance pattern. These data confirmed the stability of DAS-444Ø6-6 soybean during traditional breeding procedures.

The AAD-12, 2mEPSPS and PAT proteins in DAS-444Ø6-6 soybean were characterized biochemically and measured using protein-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-444Ø6-6 soybean plants treated with 2,4-D, glyphosate, glufosinate, all three herbicides in combination, or not treated with any of these herbicides. The results showed a low level of expression of the AAD-12, 2mEPSPS, and PAT proteins across herbicide treatments and environments, indicating a low exposure to humans and animals.

DAS-444Ø6-6 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-444Ø6-6. No commercial name has yet been identified.

c. Food Identity

There is no intention to market food items containing soybean derived from DAS-444Ø6-6 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*

The vegetative and reproductive stages of a soybean plant are described using the following nomenclature (Pedersen, 2004; Gaska, 2006):

Vegetative Stages		Reproductive Stages	
VE	Emergence	R1	Beginning bloom
VC	Unrolled unifoliate leaves	R2	Full bloom
V1	First-trifoliate	R3	Beginning pod
V2	Second-trifoliate	R4	Full pod
V3	Third-trifoliate	R5	Beginning seed
V(n)	n^{th}-trifoliate	R6	Full seed
		R7	Beginning maturity
		R8	Full maturity

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 of Use of the Host and Donor Organisms

Part B Section 2 DAS Reports

Zhuang, M., Pareddy, D., 2011. Transformation information for plasmid pDAB8264. Study ID 101880. Dow AgroSciences LLC. Indianapolis, IN.

a. Donor Organism

DAS-444Ø6-6 soybean was generated by *Agrobacterium*-mediated transformation using the plasmid pDAB8264 (Figure 1). The T-DNA insert in the plasmid contains the *2mepsps* (double mutant 5-enolpyruvylshikimate-3-phosphate synthase) gene from *Zea mays*, a synthetic, plant-optimized sequence of the *aad-12* gene from *Delftia acidovorans,* and the *pat* gene from *Streptomyces viridochromogenes* (Figure 2). A summary of the genetic elements is given in Table 1.

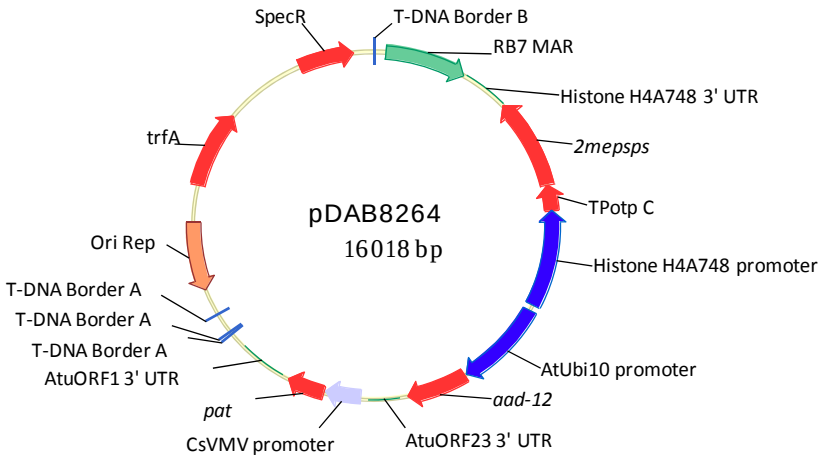


Figure 1. Plasmid map of pDAB8264

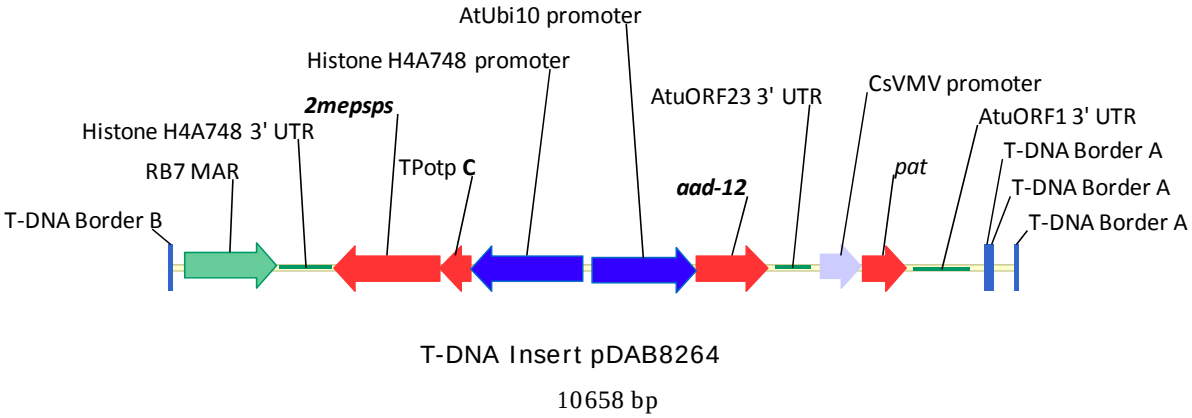


Figure 2. Diagram of T-DNA insert in plasmid pDAB8264.

Table 1. Genetic elements of the T-DNA insert from plasmid pDAB8264.

Feature Name	Feature Start	Feature Stop	Feature Length	Description
T-DNA Border B	1	24	24	Required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)
Intervening sequence	25	160	136	Non-specific DNA sequences necessary for cloning
RB7 MAR	161	1326	1166	Matrix attachment region from the *Nicotiana tabacum* rb-7-5A gene (Hall *et al.*, 1991)
Intervening sequence	1327	1365	39	Non-specific DNA sequences necessary for cloning
Histone H4A748 3′ UTR	1366	2026	661	3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of the histone H4A748 gene from *Arabidopsis thaliana* (Chaboute *et al.*, 1987)
Intervening sequence	2027	2049	23	Non-specific DNA sequences necessary for cloning
2mepsps	2050	3387	1338	Native 5-enolpyruvylshikimate-3-phosphate synthase gene from *Zea mays* with two mutations providing glyphosate tolerance (Lebrun *et al.*, 1996; Lebrun *et al.*, 2003)
TPotp C	3388	3759	372	Optimized chloroplast transit peptide derived from maize and sunflower RuBisCO (Lebrun *et al.*, 1996; Lebrun *et al.*, 2003)
Intervening sequence	3760	3763	4	Non-specific DNA sequences necessary for cloning
Histone H4A748 promoter	3764	5193	1430	Promoter along with the 5′ untranslated region of the Histone H4A748 gene from *Arabidopsis thaliana* including an intron from the Histone 3 gene from *Arabidopsis thaliana* (Chaboute *et al.*, 1987)
Intervening sequence	5194	5285	92	Non-specific DNA sequences necessary for cloning
AtUbi10 promoter	5286	6607	1322	Promoter along with the 5' untranslated region and intron from the *Arabidopsis thaliana* polyubiquitin 10 (UBQ10) gene (Norris *et al.*, 1993)
Intervening sequence	6608	6615	8	Non-specific DNA sequences necessary for cloning

Feature Name	Feature Start	Feature Stop	Feature Length	Description
aad-12	6616	7497	882	Plant-optimized version of an aryloxyalkanoate dioxygenase gene from *Delftia acidovorans* encoding an enzyme with an alpha ketoglutarate-dependent dioxygenase activity which results in metabolic inactivation of the herbicide(s) (Wright *et al.*, 2009; Wright *et al.*, 2010)
Intervening sequence	7498	7599	102	Non-specific DNA sequences necessary for cloning
AtuORF23 3′ UTR	7600	8056	457	3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 23 (ORF23) of plasmid pTi15955 from *Agrobacterium tumefaciens* (Barker *et al.*, 1983)
Intervening sequence	8057	8170	114	Non-specific DNA sequences necessary for cloning
CsVMV promoter	8171	8687	517	Promoter along with the 5′ untranslated region derived from the Cassava Vein Mosaic virus (Verdaguer *et al.*, 1996)
Intervening sequence	8688	8694	7	Non-specific DNA sequences necessary for cloning
pat	8695	9246	552	Plant-optimized version of phosphinothricin acetyltransferase (PAT) gene, isolated *from Streptomyces viridochromogenes*, encoding a protein that confers tolerance to glufosinate (Wohlleben *et al.*, 1988)
Intervening sequence	9247	9348	102	Non-specific DNA sequences necessary for cloning
AtuORF1 3′ UTR	9349	10052	704	3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of plasmid pTi15955 from *Agrobacterium tumefaciens* (Barker *et al.*, 1983)
Intervening sequence	10053	10280	228	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
T-DNA Border A	10281	10304	24	Required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)

Feature Name	Feature Start	Feature Stop	Feature Length	Description
Intervening sequence	10305	10323	19	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
T-DNA Border A	10324	10347	24	Required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells, aiming to prevent vector DNA being transferred into plant genome (Barker *et al.*, 1983)
Intervening sequence	10348	10634	287	Sequence from Ti plasmid pTi15955 (Barker *et al.*, 1983)
T-DNA Border A	10635	10658	24	Required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells, aiming to prevent vector DNA being transferred into plant genome (Barker *et al.*, 1983)

Three gene expression cassettes were present in the pDAB8264 vector for insertion into soybeans.

The *2mepsps* expression cassette is designed to express a double mutant maize 5-enolpyruvylshikimate-3-phosphate synthase gene that encodes the 2mEPSPS protein. The *2mepsps* gene was originally isolated from *Zea mays* and fused with an optimized chloroplast transit peptide derived from maize and sunflower ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) at its N-terminus (Lebrun, Leroux et al. 1996; Lebrun, Sailland et al. 2003). The *2mepsps* gene (also referred to as *dmmg*, *mEPSPS*) has been introduced as the source of glyphosate tolerance in the maize transgenic event GA21(OECD unique identifier MON-ØØØ21-9), which has been approved by different agencies worldwide for environment, food, and feed (FSANZ (A362) 2000, USDA 1997) and in GlyTol™ cotton (OECD unique identifier BCS-GHØØ2-5), which was approved by FSANZ in and deregulated by USDA APHIS in 2009 It is also present in soybean event FG-72 (OECD unique identifier MST-FGØ72-3) which is currently in review at FSANZ, CFIA and at USDA. The *2mepsps* gene encodes a protein of 445 amino acids that has a molecular weight of approximately 47.5 kDa. The encoded protein is insensitive to glyphosate, thus providing tolerance to glyphosate in plants expressing the 2mEPSPS protein.

Expression of *2mepsps* in the T-DNA insert of pDAB8264 is controlled by the Histone H4A748 promoter from *Arabidopsis thaliana* and Histone H4A748 3′ UTR sequence from *Arabidopsis thaliana*. The Histone H4A748 promoter is known to drive constitutive expression of the genes it controls (Chaboute, Chaubet et al. 1987).

The *aad-12* expression cassette 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) (Approved by FSANZ 2011-A1046 and US FDA 2011 – BNF124).

Delftia acidovorans, which has previously been identified 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 Graevenitz 1985; Tamaoka, Ha et al. 1987; Wen, Fegan et al. 1999). *Delftia acidovorans* can be used to transform ferulic acid into vanillin and related flavour metabolites (Rao and Ravishankar 2000; Shetty, Paliyath et al. 2006). This utility has led to a history of safe use for *Delftia 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, Goers et al. 1992).

Expression of *aad-12* in the T-DNA insert of pDAB8264 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, Meyer et al. 1993). The function of AtuORF23 (GenBank Accession Number: CAA25184) in pTi15955 (GenBank Accession Number: X00493) was not identified (Barker, Idler et al. 1983). A search of its translated amino acid sequence returned no significant similarity with known functional proteins.

The *pat* expression cassette 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 the soybean genome confers tolerance to glufosinate and was used as a selectable marker during the soybean transformation. The *pat* gene has been widely used both as a selectable marker and herbicide tolerance trait in previously approved products (FSANZ A1046, A481, A446, A543 andA518).

Expression of the *pat* gene 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 circular 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, de Kochko et al. 1996). The function of AtuORF1 (GenBank Accession Number: CAA25163) in pTi15955 (GenBank Accession Number: X00493) was not identified (Barker, Idler et al. 1983), but its translated

amino acid sequence has a significant similarity with an indole-3-lactate synthase (GenBank Accession Number: AAK90967) from *Agrobacterium tumefaciens* str. C58.

A matrix attachment region (MAR) of RB7 from *Nicotiana tabacum* was included adjacent to the Histone H4A748 3' UTR in the *2mepsps* PTU to potentially facilitate transgene expression in the plant. Matrix attachment 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 (Han, Ma et al. 1997; Abranches, Shultz et al. 2005; Verma, Verma et al. 2005).

For information on the potential toxicity or allergenicity of the proteins see section C part 3 and 5 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-444Ø6-6 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

Han, L., Hoffman, T., 2011. Event Sorting and Selection Process for the Development of DAS-444Ø6-6. Study ID 110325. Dow AgroSciences LLC. Indianapolis, IN.

Mo, J., 2011. Molecular characterization of DAS-44406-6 soybean within a single segregating generation. Study ID 102097. Dow AgroSciences LLC. Indianapolis, IN.

Poorbaugh, J., 2011. Molecular characterization of DAS-44406-6 soybean. Study ID 101947. Dow AgroScience LLC. Indianapolis, IN.

Zhuang, M., Pareddy, D., 2011. Transformation information for plasmid pDAB8264. Study ID 101880. Dow AgroSciences LLC. Indianapolis, IN.

a. Transformation Method

DAS-444Ø6-6 soybean was generated through *Agrobacterium*-mediated transformation of soybean (*Glycine max*) cotyledonary node explants (Zhuang and Pareddy, 2011). The disarmed *Agrobacterium tumefaciens* strain EHA101 (Hall *et al.*, 1991), carrying the binary vector with three genes of interest (*aad-12*, *2mepsps* and *pat*) within the T-DNA region, 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 to inhibit the growth 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 painted with glufosinate to screen for putative transformants. The glufosinate-resistant plantlets were transferred to the greenhouse, allowed to acclimate and then painted with glufosinate to reconfirm tolerance. Surviving plantlets were deemed to be putative transformants. The screened plants were sampled and analysed at the molecular level for the presence of the genes of interest and the absence of the vector backbone DNA. Specifically, for T0 plants, PCR

analysis was performed to verify the absence of the spectinomycin resistance gene in the vector backbone as well as the presence of the *aad-12* coding region and *2mepsps* plant transcription unit (PTU). A PCR-based zygosity assay was conducted for copy number detection for *pat*, *aad-12*, and *2mepsps* genes. Selected T0 plants were allowed to self-fertilize in the greenhouse to give rise to T1 seed. For T1 plants, PCR analysis, zygosity assay, and Southern blot analysis were performed to detect copy number, number of integration sites, and PTU integrity.

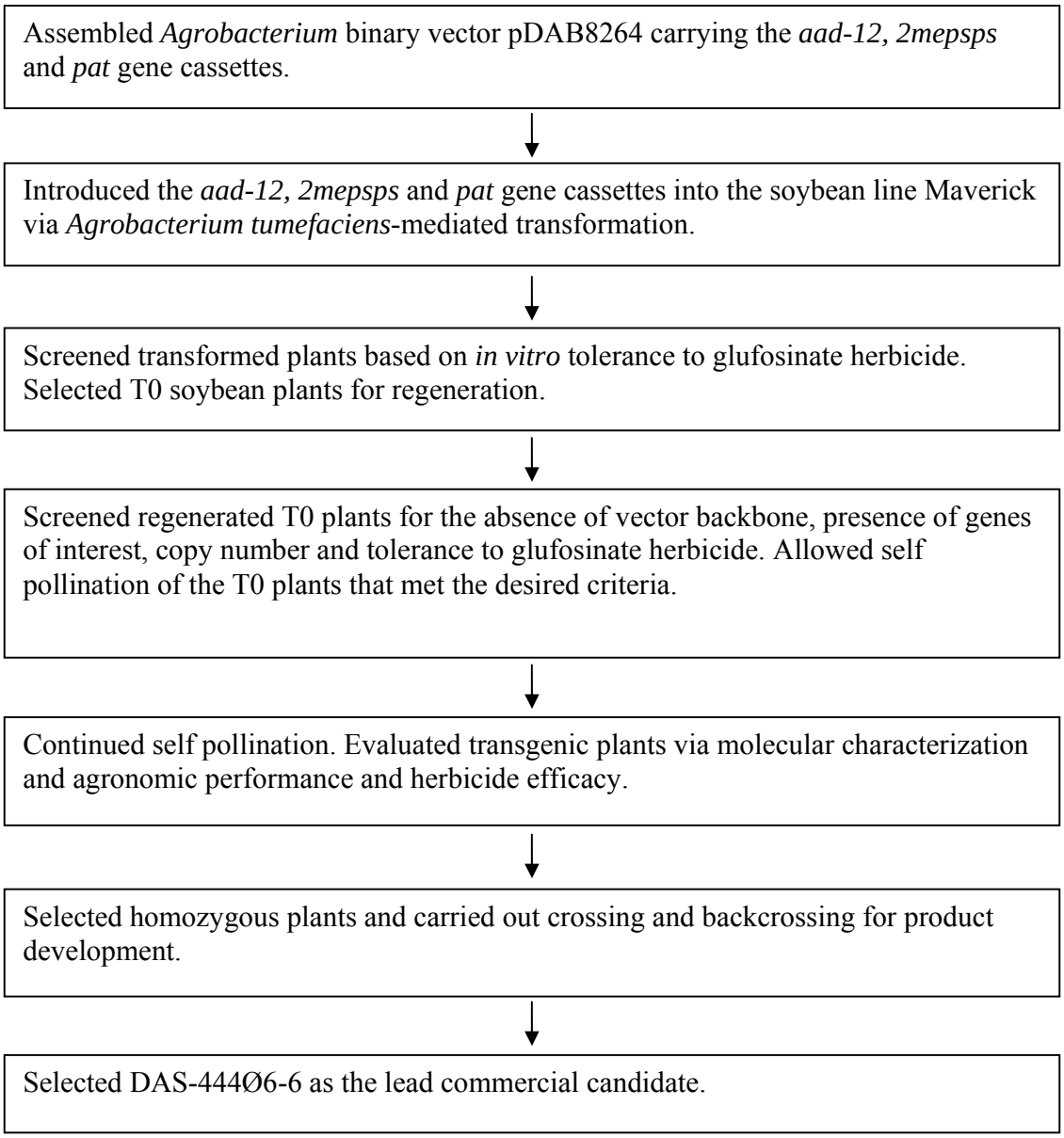
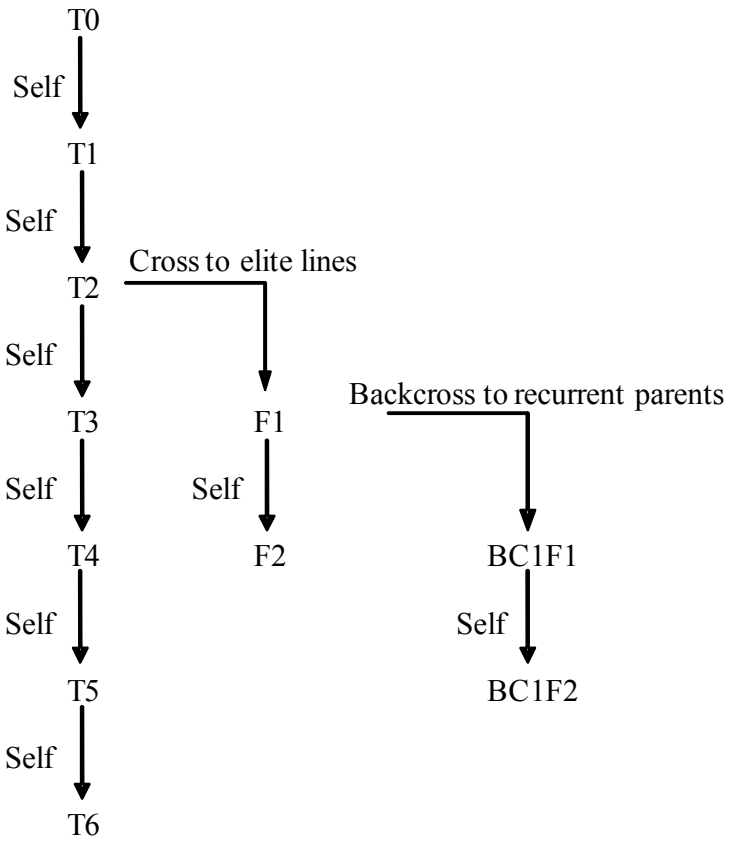


Figure 3. Development of DAS-444Ø6-6 soybean.

(Han and Hoffman, 2011)



Analysis	DAS-444Ø6-6 Soybean Generations Used	Control
Molecular Analysis	T2, T3, T4, T6, F2	Maverick
Segregation Analysis	F2, BC1F2	Maverick
Protein Characterization	T4	Maverick
Protein Expression	T4	Maverick
Composition	T4	Maverick

Figure 4. Breeding diagram of DAS-444Ø6-6 soybean.

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 (pDAB8264) that was used for the transformation.

c. Gene Construct and Vectors

DAS-444Ø6-6 soybean was generated by *Agrobacterium*-mediated transformation using the plasmid pDAB8264 (Figure 1). The T-DNA insert in the plasmid contains the *2mepsps* (double mutant 5-enolpyruvylshikimate-3-phosphate synthase) gene from *Zea mays*, a synthetic, plant-optimized sequence of the *aad-12* gene from *Delftia acidovorans,* and the *pat* gene from *Streptomyces viridochromogenes* (Figure 2).

d. Molecular Characterization

The results demonstrate that the transgene insert in DAS-444Ø6-6 soybean occurred as a simple integration of the T-DNA insert from plasmid pDAB8264, including a single, intact copy of each of the *2mepsps, aad-12,* and *pat* expression cassettes. The insert is stably integrated and inherited across and within breeding generations, and no plasmid backbone sequences are present in DAS-444Ø6-6 soybean.

Detailed Southern blot analysis was conducted using probes specific to the gene coding sequences, promoters, terminators, and other regulatory elements contained in the pDAB8264 plasmid. The locations of the probes on the pDAB8264 plasmid are described in Table 2 and shown in Figure 5. The expected and observed fragment sizes with specific digest and probe combinations, based on the known restriction enzyme recognition sites of the pDAB8264 plasmid are shown in

Table 3 and Figure 7, respectively. The Southern blot analyses described here made use of two types of restriction fragments: a) internal fragments in which known restriction enzyme recognition sites are completely contained within the T-DNA insert of pDAB8264 and b) border fragments in which one known restriction enzyme recognition site is located within the T-DNA insert and another site is located in the soybean genome flanking the insert. Border fragment sizes vary by event because they rely on the location of the restriction enzyme recognition sites in 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.

Genomic DNA for Southern blot analysis was prepared from leaf material of individual DAS-444Ø6-6 soybean plants from five distinct breeding generations. Genomic DNA from leaves of non-transgenic variety Maverick was used as the control material. Plasmid DNA of pDAB8264 added to genomic DNA from the conventional control served as the positive control for the Southern blot analysis.

The expected restriction fragments of the inserted DNA are shown in

Table 3 and Figure 7 through Figure 25. Southern blot analysis showed that DAS-444Ø6-6 soybean contains a single intact copy of the *2mepsps, aad-12,* and *pat* expression cassettes integrated at a single locus (Section 0). No vector backbone sequences were detected in DAS-444Ø6-6 soybean (Section 0). The hybridization patterns across five generations of DAS-444Ø6-6 soybean (Poorbaugh, 2011) (T2, T3, T4, T6, and F2) were identical, indicating that the insertion is stably integrated in the soybean genome. The inheritance of DAS-444Ø6-6 soybean insert in segregating generations was investigated using event-specific PCR, detection of the PAT and/or AAD-12 protein, and detection of the *aad-12* gene (Mo, 2011). All results confirmed the predicted inheritance pattern of the transgenes at a single locus.

Table 2. List of probes and their positions in plasmid pDAB8264.

Probe Name	Position in pDAB8264	Size (bp)
RB7	306-1315	1010
Histone H4A748 UTR	1356-1907	552
2mepsps	2048-3759	1712
Histone H4A748 Promoter	3682-5197	1516
AtUbi10 Promoter	5347-6659	1313
aad-12	6616-7497	882
AtuORF23 UTR	7637-8049	413
CsVMV	8172-8703	532
pat	8676-9284	609
AtuORF1 UTR	9257-10055	799
Backbone 3	10670-10990	321
Ori-Rep	10971-12057	1087
Backbone 2	12038-13751	1714
Backbone 1	13721-14974	1254
SpecR	14955-15749	795
Backbone 4	15724-16015	292

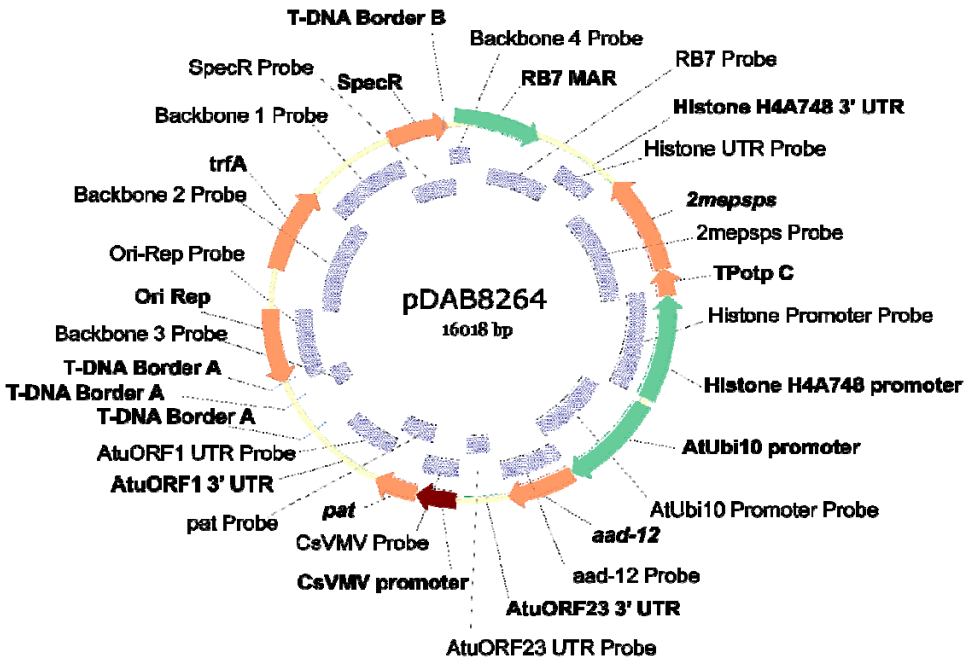


Figure 5. Location of probes on pDAB8264 used in Southern blot analysis of DAS-444Ø6-6 soybean.

Table 3. Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-444Ø6-6 soybean.

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
RB7	*Hind*III	pDAB8264	9322	~9300	8A
		Maverick	None	None	
		DAS-444Ø6-6	>4261	~4700	
	*Msc*I	pDAB8264	5929	~5900	8B
		Maverick	None	None	
		DAS-444Ø6-6	>1330	~3400	
Histone H4A748 UTR	*Hind*III	pDAB8264	9322	~9300	9
		Maverick	None	None	
		DAS-444Ø6-6	>4261	~4700	
	*Msc*I	pDAB8264	10089	~10100	9
		Maverick	None	None	
		DAS-444Ø6-6	>9328	~15000	
Histone H4A748 Promoter	*Xho*I	pDAB8264	16018	~16000	10A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Msc*I	pDAB8264	10089	~10100	10B
		Maverick	None	None	
		DAS-444Ø6-6	>9328	~15000	
AtUbi10 Promoter	*Xho*I	pDAB8264	16018	~16000	11A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Msc*I	pDAB8264	10089	~10100	11B
		Maverick	None	None	
		DAS-444Ø6-6	>9328	~15000	
AtuORF23 UTR	*Xho*I	pDAB8264	16018	~16000	12A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Hind*III	pDAB8264	4731	~4700	12B
		Maverick	None	None	
		DAS-444Ø6-6	>4432	~7000	
CsVMV	*Xho*I	pDAB8264	16018	~16000	13A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
AtuORF1 UTR	*Hind*III	pDAB8264	4731	~4700	13B
		Maverick	None	None	
		DAS-444Ø6-6	>4432	~7000	
	*Xho*I	pDAB8264	16018	~16000	14A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Hind*III	pDAB8264	4731	~4700	14B
		Maverick	None	None	
		DAS-444Ø6-6	>4432	~7000	
2mepsps	*Xho*I	pDAB8264	16018	~16000	15A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Hind*III	pDAB8264	9322	~9300	15B
		Maverick	None	None	
		DAS-444Ø6-6	>4261	~4700	
	*Msc*I	pDAB8264	10089	~10100	15C
		Maverick	None	None	
		DAS-444Ø6-6	>9328	~15000	
aad-12	*Xho*I	pDAB8264	16018	~16000	16A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Hind*III	pDAB8264	4731	~4700	16B
		Maverick	None	None	
		DAS-444Ø6-6	>4432	~7000	
	*Msc*I	pDAB8264	10089	~10100	16C
		Maverick	None	None	
		DAS-444Ø6-6	>9328	~15000	
pat	*Xho*I	pDAB8264	16018	~16000	17A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Hind*III	pDAB8264	4731	~4700	17B
		Maverick	None	None	
		DAS-444Ø6-6	>4432	~7000	
	*Msc*I	pDAB8264	10089	~10100	17C
		Maverick	None	None	

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
		DAS-444Ø6-6	>9328	~15000	
Histone H4A748 Promoter	*Msc*I/*Eco*RI (Release PTU)	pDAB8264	4469	~4500	18A
		Maverick	None	None	
		DAS-444Ø6-6	4469	~4500	
2mepsps		pDAB8264	4469	~4500	18B
		Maverick	None	None	
		DAS-444Ø6-6	4469	~4500	
Histone H4A748 UTR		pDAB8264	4469	~4500	18C
		Maverick	None	None	
		DAS-444Ø6-6	4469	~4500	
AtUbi10 Promoter	*Pst*I/*Xho*I (Release PTU)	pDAB8264	2868	~2900	19A
		Maverick	None	None	
		DAS-444Ø6-6	2868	~2900	
aad-12		pDAB8264	2868	~2900	19B
		Maverick	None	None	
		DAS-444Ø6-6	2868	~2900	
AtuORF23 UTR		pDAB8264	2868	~2900	19C
		Maverick	None	None	
		DAS-444Ø6-6	2868	~2900	
CsVMV	*Pst*I/*Xho*I (Release PTU)	pDAB8264	1928	~1900	20A
		Maverick	None	None	
		DAS-444Ø6-6	1928	~1900	
pat		pDAB8264	1928	~1900	20B
		Maverick	None	None	
		DAS-444Ø6-6	1928	~1900	
AtuORF1 UTR		pDAB8264	1928	~1900	20C
		Maverick	None	None	
		DAS-444Ø6-6	1928	~1900	
Backbone 3/ Backbone 4	*Msc*I/*Eco*RI	pDAB8264	1049, 5929	~1000, ~5900	21A
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
	*Hind*III	pDAB8264	4731, 9322	~4700, ~9300	21B
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
Ori-Rep	*Hin*dIII	pDAB8264	9322	~9300	22A
		Maverick	None	None	

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
Backbone 1	*Pst*I/*Xho*I	DAS-444Ø6-6	None	None	22B
		pDAB8264	9288	~9300	
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
	*Hind*III	pDAB8264	9322	~9300	23A
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
	*Pst*I/*Xho*I	pDAB8264	9288	~9300	23B
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
Backbone 2	*Hind*III	pDAB8264	9322	~9300	24A
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
	*Pst*I/*Xho*I	pDAB8264	9288	~9300	24B
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
SpecR	*Hind*III	pDAB8264	9322	~9300	25A
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
	*Pst*I/*Xho*I	pDAB8264	9288	~9300	25B
		Maverick	None	None	
		DAS-444Ø6-6	None	None	

1. Expected fragment sizes are based on the plasmid map of the pDAB8264 (Figure 6) and the linearized T-DNA map (Figure 7).
2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labelled DNA Molecular Weight Marker 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.

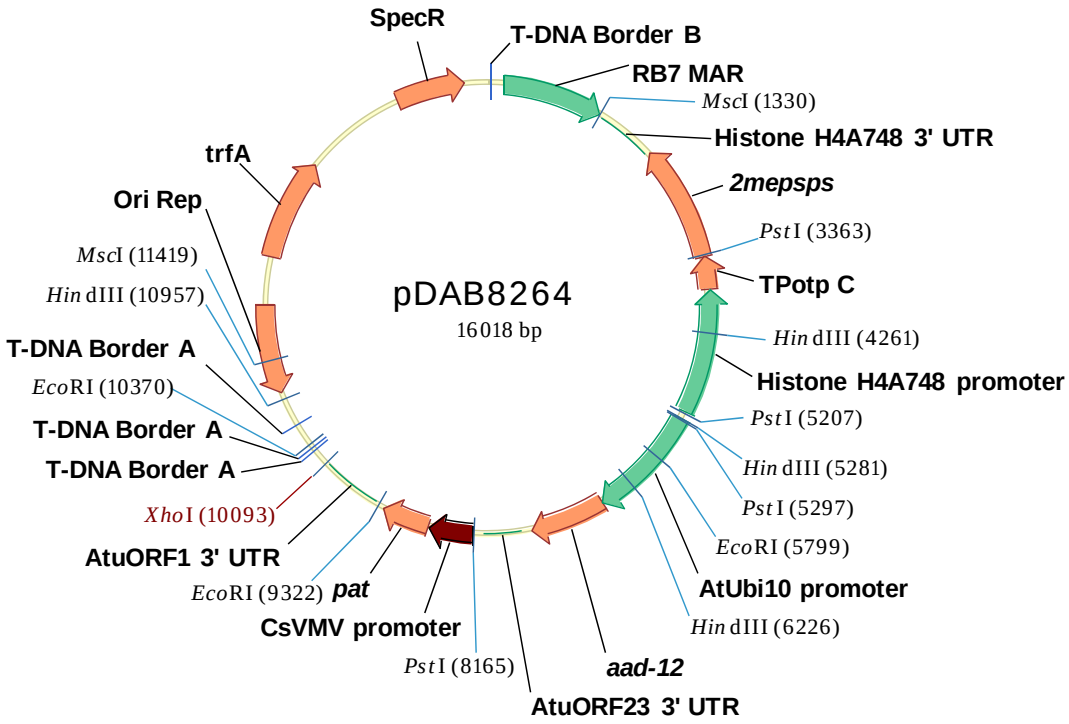


Figure 6. Plasmid map of pDAB8264 with selected restriction enzyme sites used for Southern analysis.

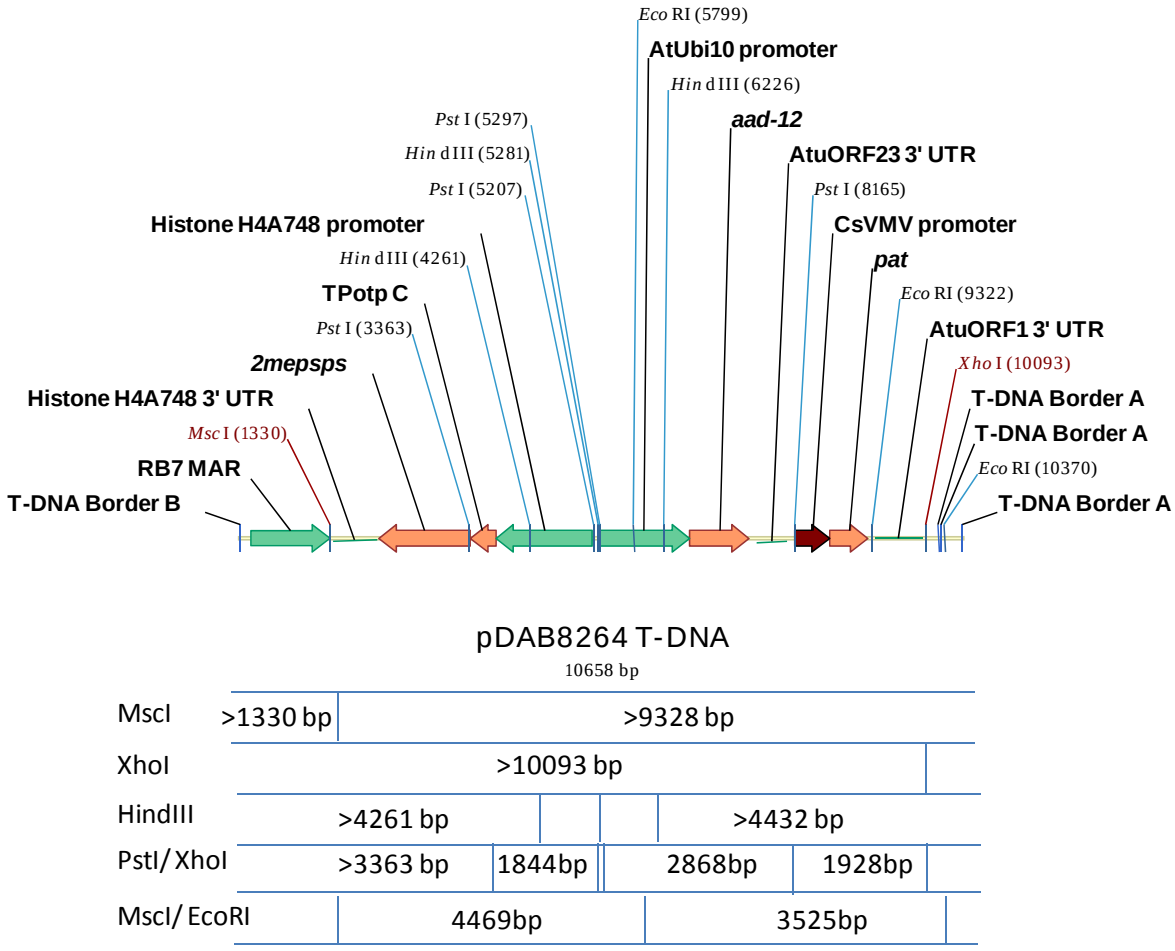


Figure 7. Linearized intended T-DNA insert from pDAB8264 with restriction enzymes used for DNA digestion and the expected hybridization bands.

Analysis of the Insert and Its Genetic Elements

Number of Insertion Sites

The restriction enzymes *Msc*I, *Xho*I and *Hind*III were chosen to determine the number of insertions in DAS-444Ø6-6 soybean (

Table 3 and Figure 6). Probes derived from the DNA sequences for *2mepsps*, Histone H4A748 Promoter, Histone H4A748 UTR, *aad-12*, *pat*, RB7, AtUbi10 Promoter, AtuORF23 UTR, CsVMV, and AtuORF1 UTR were then hybridized to the digested genomic DNA to determine the number of insertion sites in DAS-444Ø6-6 soybean.

When digested with the *Msc*I restriction enzyme and independently probed with the Histone H4A748 UTR, Histone H4A748 Promoter, AtUbi10 Promoter, 2mepsps, aad-12 and pat probes, a single hybridization band of ~15000 bp was observed in all DAS-444Ø6-6 samples, consistent with the predicted size of >9328 bp for the *Msc*I fragment as indicated in Figure 7 (Figure 9B, Figure 10B, Figure 11B, Figure 15C, Figure 16C and Figure 17C respectively). The same enzyme digestion was also used for characterization of the RB7 MAR feature. The resulting Southern analysis indicated a single band of ~3400 bp in DAS-444Ø6-6 samples which is consistent with the expected size of >1330 bp (Figure 8B).

For additional characterization of the T-DNA insert, genomic DNA samples was digested with the *Xho*I restriction enzyme and independently probed with the Histone H4A748 Promoter, AtUbi10 Promoter, AtuORF23 UTR, CsVMV, AtuORF1 UTR, 2mepsps, aad-12, and pat probes. In each case, a single hybridization band of ~12000 bp was observed in all DAS-444Ø6-6 samples, consistent with the predicted size of >10093 bp as indicated in Figure 7 (Figure 10A, Figure 11A, Figure 12A, Figure 13A, Figure 14A, Figure 15A, Figure 16A and Figure 17a respectively.

Moreover, the restriction enzyme *Hind*III was also used to provide further characterization of DAS-444Ø6-6 soybean. Digestion of the genomic DNA with this enzyme followed by independently probing with the RB7, Histone H4A748 UTR, or 2mepsps probe resulted in a single hybridization band of ~4700 bp across all DAS-444Ø6-6 samples, which is consistent with the expected size of >4261 bp as indicated in Figure 7 (Figure 8A, Figure 9A and Figure 15B respectively). Digestion of the genomic DNA with *Hind*III and probed independently with the AtuORF23 UTR, CsVMV, AtuORF1 UTR, aad-12, or pat probe resulted in a single hybridization band of ~7000 bp for all DAS-444Ø6-6 samples. This hybridization pattern is consistent with the expected size of >4432 bp for all probe combinations (Figure 12B, Figure 13B, Figure 14B, Figure 16B and Figure 17B respectively).

All these data indicate that there is a single insertion of the T-DNA containing all the expected elements in DAS-444Ø6-6 soybean genome.

Structure of the Insert and Genetic Elements

According to the restriction map of the T-DNA insert in pDAB8264 in Figure 6 and Figure 7, the plant transcription unit (PTU) for *aad-12* and *pat* could be released by restriction digestion with *Pst*I/*Xho*I, while the *2mepsps* PTU could be released with *Msc*I/*Eco*RI digestion. These digestions were performed to verify the presence of intact PTUs in DAS-444Ø6-6 soybean.

When digested with *Msc*I/*Eco*RI and hybridized with the 2mepsps probe, each individual DAS-444Ø6-6 plant across the five generations along with the pDAB8264 positive control resulted in a single hybridization band of ~4500 bp, which is consistent with the predicted size of 4469 bp for the *2mepsps* PTU (Figure 18B). When the same genomic DNA samples were probed with the Histone H4A748 Promoter or Histone H4A748 UTR probe, the same hybridization band of ~4500 bp was detected in the same sample set (Figure 18A and Figure 18C). These data indicate that an intact *2mepsps* PTU is present in all generations of DAS-444Ø6-6 soybean tested.

When digested with *Pst*I/*Xho*I and hybridized with the aad-12 probe, each individual DAS-444Ø6-6 plant across the five generations along with the pDAB8264 positive control resulted in a single hybridization band of ~2900 bp, which is consistent with the predicted size of 2868 for the *aad-12* PTU (Figure 18B). When the same genomic DNA samples were hybridized with the AtUbi10 Promoter or AtuORF23 UTR probes, the same hybridization band of ~2900 bp was also detected in the same sample set (Figure 18A and Figure 18C) These data indicate that an intact *aad-12* PTU is present in all generations of DAS-444Ø6-6 soybean tested.

When digested with *Pst*I/*Xho*I and hybridized with the pat probe, each individual DAS-444Ø6-6 plant across the five generation along with the pDAB8264 positive control resulted in a single hybridization band of ~1900 bp, which is consistent with the predicted size of 1928 bp for the *pat* PTU (Figure 20B). When the same genomic DNA samples were hybridized with the CsVMV or AtuORF1 UTR probes, the same hybridization band of ~1900 bp was detected in the same sample set (Figure 20a and 20C). These data indicate that an intact *pat* PTU is present in all generations of DAS-444Ø6-6 soybean tested.
As expected, no specific hybridization bands were detected in the negative control samples in any of the restriction enzyme and probe combinations.

Taken together, the Southern blot analyses indicate that the single insert in DAS-444Ø6-6 soybean contains an intact single copy of each of the PTUs for *2mepsps, aad-12,* and *pat*.

Absence of Vector Backbone DNA

To confirm that no plasmid vector backbone sequences exist in DAS-444Ø6-6 soybean, six probes covering nearly the entire backbone region of pDAB8264 were used to hybridize the membrane blots containing genomic DNA samples digested with *Msc*I/*Eco*RI, *Hind*III, and *Pst*I/*Xho*I (Figure 21 - Figure 25). Based on the expected fragment sizes of *Msc*I/*Eco*RI and *Hind*III digestion, the Backbone 3 and Backbone 4 probes were mixed at an approximate 1:1 molar ratio for hybridization, while the Ori-Rep, Backbone 1, Backbone 2, and SpecR probes were hybridized independently on separate blots of *Hind*III and *Pst*I/*Xho*I digestion. The results demonstrated that no specific hybridization bands were detected in any samples tested, except for the positive controls, as expected (Figure 21 - Figure 25). When hybridized with the Backbone 4 and Backbone 3 probes, the *Msc*I/*Eco*RI digested positive control sample had an observed hybridization band of ~5900 bp for Backbone 4 and ~1000 bp for backbone 3 probes, respectively, which is consistent with the expected bands of 5929 bp and 1049 bp, respectively (Figure 21A) When hybridized with the same two probes, the *Hind*III digested positive control samples had an observed hybridization band of ~9300 bp for the Backbone 4 probe and an observed band of ~4700 bp for the Backbone 3 probe, which is consistent with the expected bands of 9322 bp and 4731 bp, respectively (Figure 21B). When hybridized with the individual Ori-Rep, Backbone 1, Backbone 2, and SpecR probes, the *Hind*III and *Pst*I/*Xho*I digested positive control sample resulted in a single hybridization band of ~9300 bp and ~9300 bp, respectively, which is consistent with the expected bands of 9322 bp and 9288 bp, respectively (Figure 22 – Figure 25). Taken together, these data confirm that no vector backbone sequences from pDAB8264 were integrated into DAS-444Ø6-6 soybean.

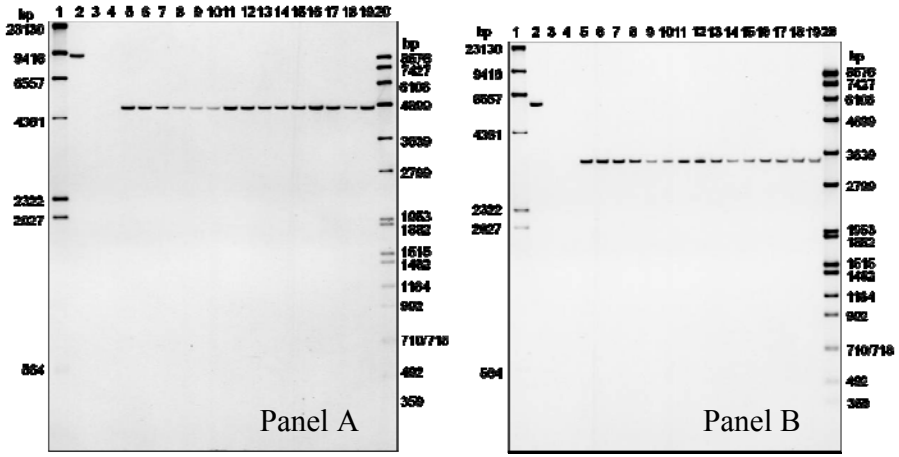


Figure 8. Southern blot analysis of *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: RB7 probe.

Approximately 10 μg of genomic DNA was digested with *Hind*III (Panel A) and *Msc*I (Panel B) and hybridized with the RB7 probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image; 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

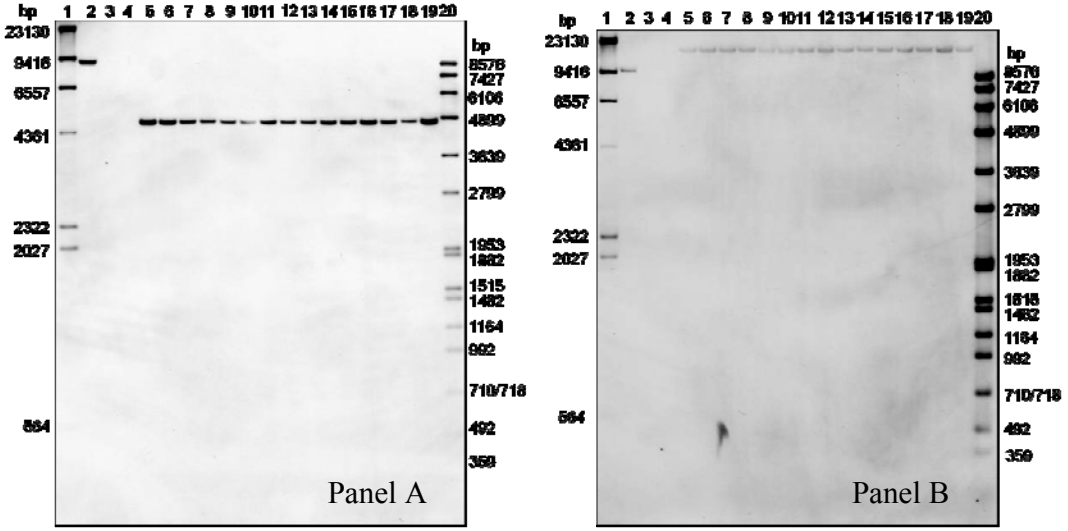


Figure 9. Southern blot analysis of *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: Histone H4A748 UTR probe.

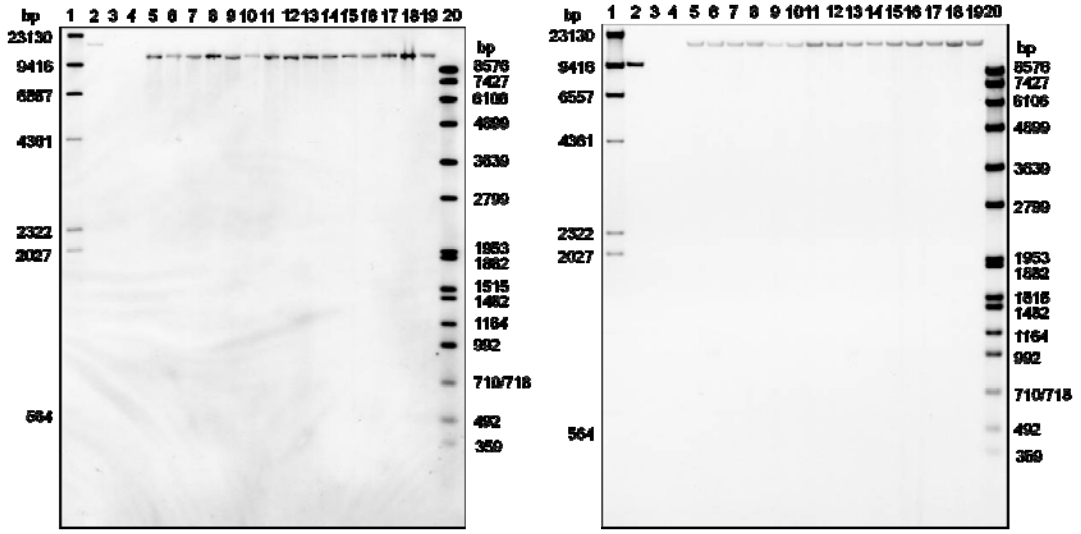
Approximately 10 μg of DAS-444Ø6-6 genomic DNA was digested with *Hind*III (Panel A) and *Msc*I (Panel B) and hybridized with the Histone H4A748 UTR probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3

10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

Panel A Panel B

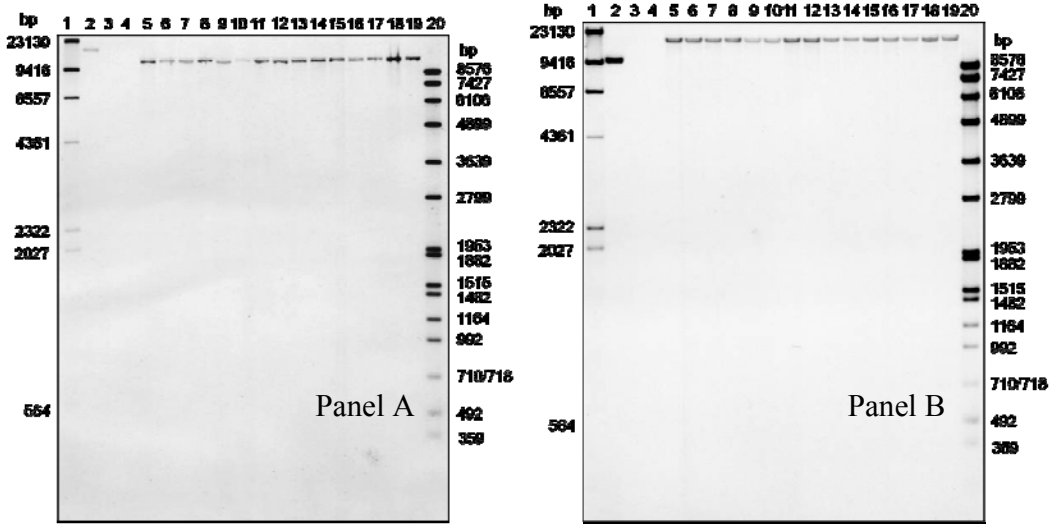
Figure 10. Southern blot analysis of *Xho*I and *Msc*I digested DAS-444Ø6-6 soybean: Histone H4A748 Promoter probe.



Approximately 10 μg of genomic DNA was digested with *Xho*I (Panel A) and *Msc*I (Panel B) and hybridized with the Histone H4A748 Promoter probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

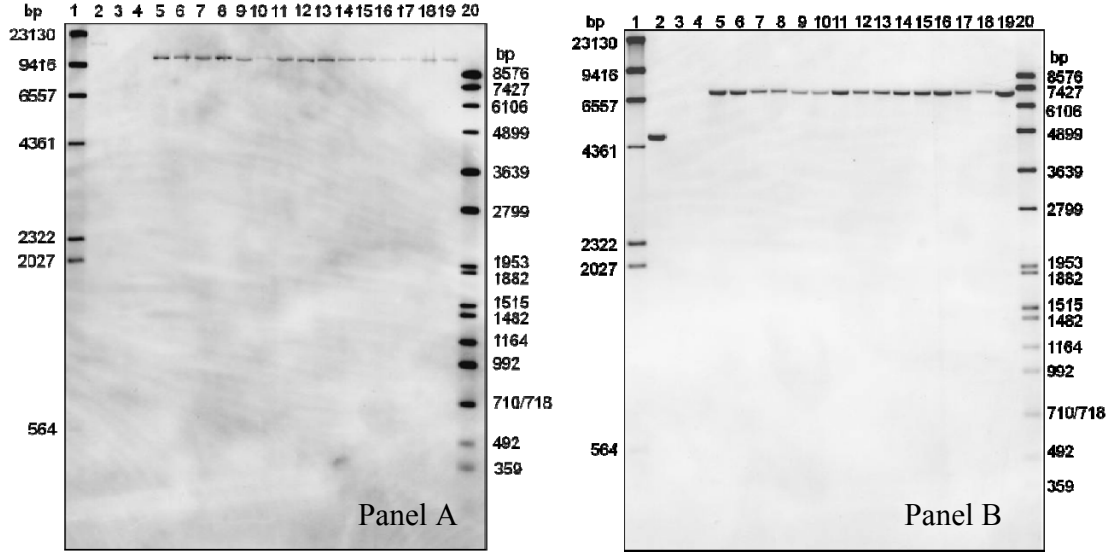
Figure 11. Southern blot analysis of *Xho*I and *Msc*I digested DAS-444Ø6-6 soybean: AtUbi10 Promoter probe.



Approximately 10 μg of genomic DNA was digested with *Xho*I (Panel A) and *Msc*I (Panel B) and hybridized with the AtUbi10 Promoter probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

Figure 12. Southern blot analysis of *Xho*I and *Hind*III digested DAS-444Ø6-6 soybean: AtuORF23 UTR probe.



Approximately 10 μg of genomic DNA was digested with *Xho*I (Panel A) and *Hin*dIII (Panel B) and hybridized with the AtuORF23 UTR probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

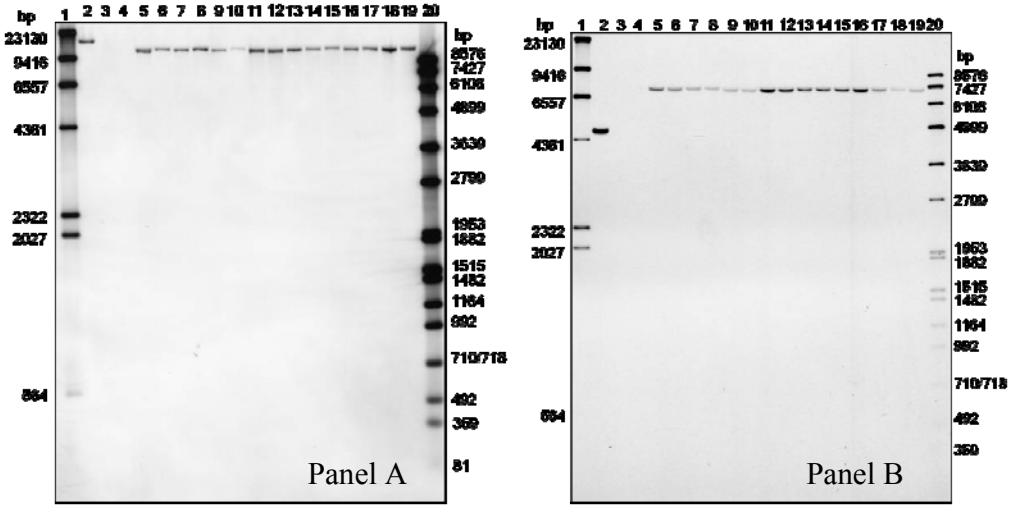
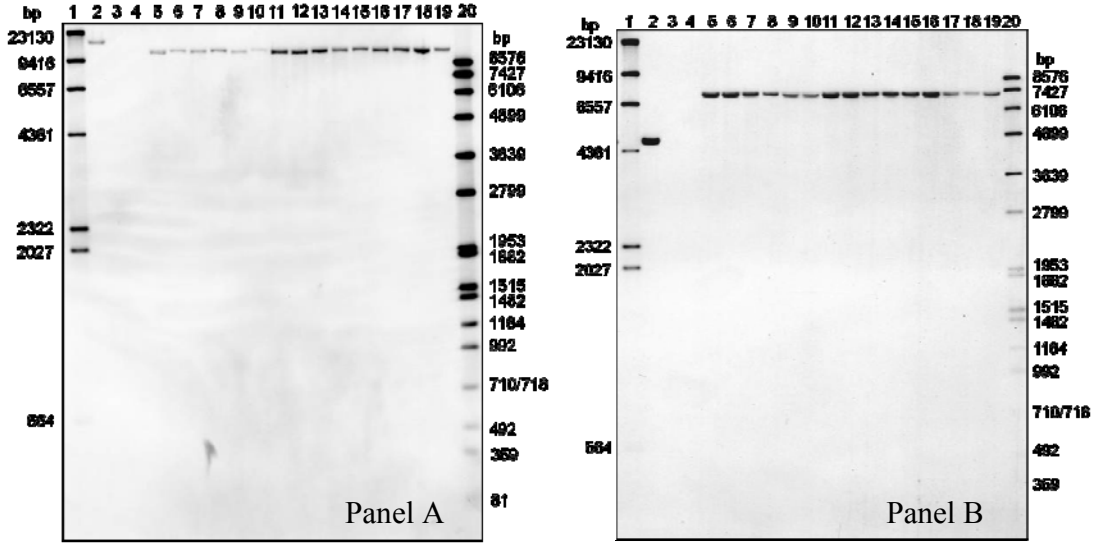


Figure 13. Southern blot analysis of *Xho*I and *Hind*III digested DAS-444Ø6-6 soybean: CsVMV probe.

Approximately 10 μg genomic DNA was digested with *Xho*I (Panel A) and *Hind*III (Panel B) and hybridized with the CsVMV probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII


bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23130
9416
6557
4361
2322
2027
564
bp
8576
7427
6106
4899
3639
2799
1953
1882
1515
1482
1164
992
710/718
492
369
81
Panel A
bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23130
9416
6557
4361
2322
2027
564
bp
8576
7427
6106
4899
3639
2799
1953
1882
1515
1482
1164
992
710/718
492
369
Panel B

Approximately 10 μg of genomic DNA was digested with *Xho*I (Panel A) and *Hind*III (Panel B) and hybridized with the AtuORF1 UTR probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

Figure 14. Southern blot analysis of *Xho*I and *Hind*III digested DAS-444Ø6-6 soybean: AtuORF1 UTR probe.

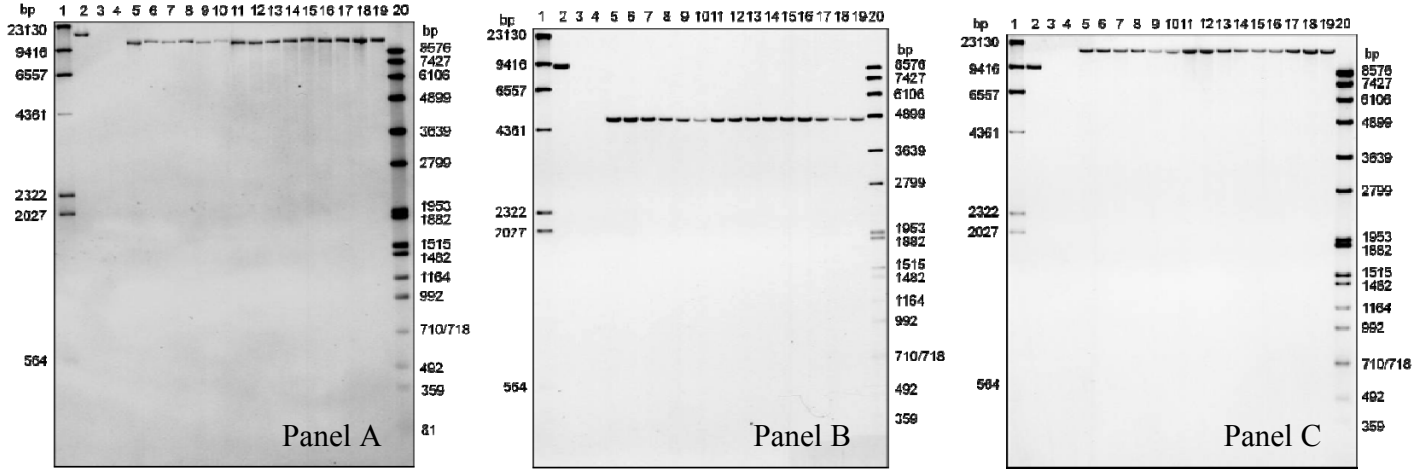


Figure 15. Southern blot analysis of *Xho* I, *Hind*III and *Msc* I digested DAS-444Ø6-6 soybean: 2mepsps probe.

Approximately 10 μg of genomic DNA was digested with *Xho*I (Panel A), *Hind*III (Panel B) and *Msc*I (Panel C) followed by hybridization with the 2mepsps probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**				**Panel C**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

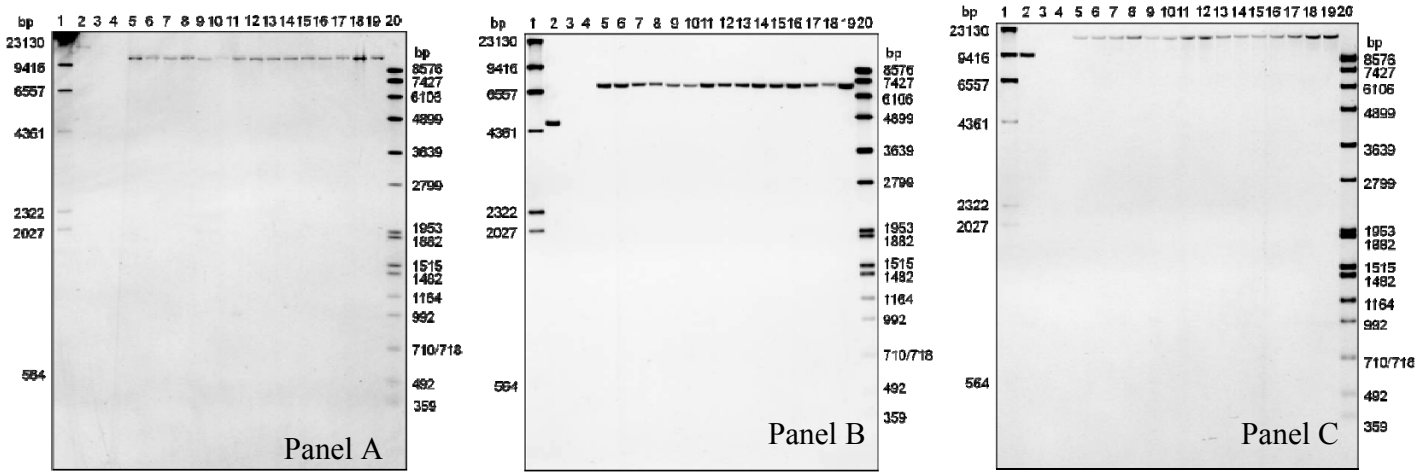


Figure 16. Southern blot analysis of *Xho*I, *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: aad-12 probe. Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A), *Hind*III (Panel B) and *Msc*I (Panel C) followed by hybridization with the aad-12 probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**				**Panel C**			
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3

10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

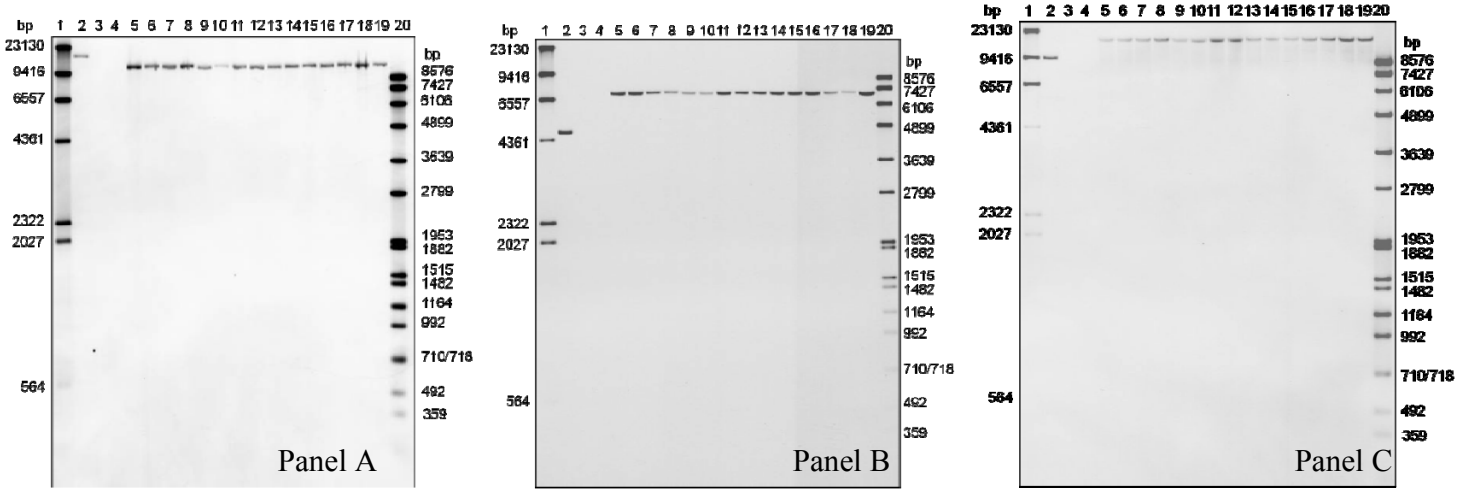


Figure 17. Southern blot analysis of *Xho*I, *Hind*III, and *Msc*I digested DAS-444Ø6-6 soybean: pat probe.

Approximately 10 μg of genomic DNA was digested with *Xho*I (Panel A), *Hind*III (Panel B) and *Msc*I (Panel C) followed by hybridization with the pat probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**				**Panel C**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3

10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

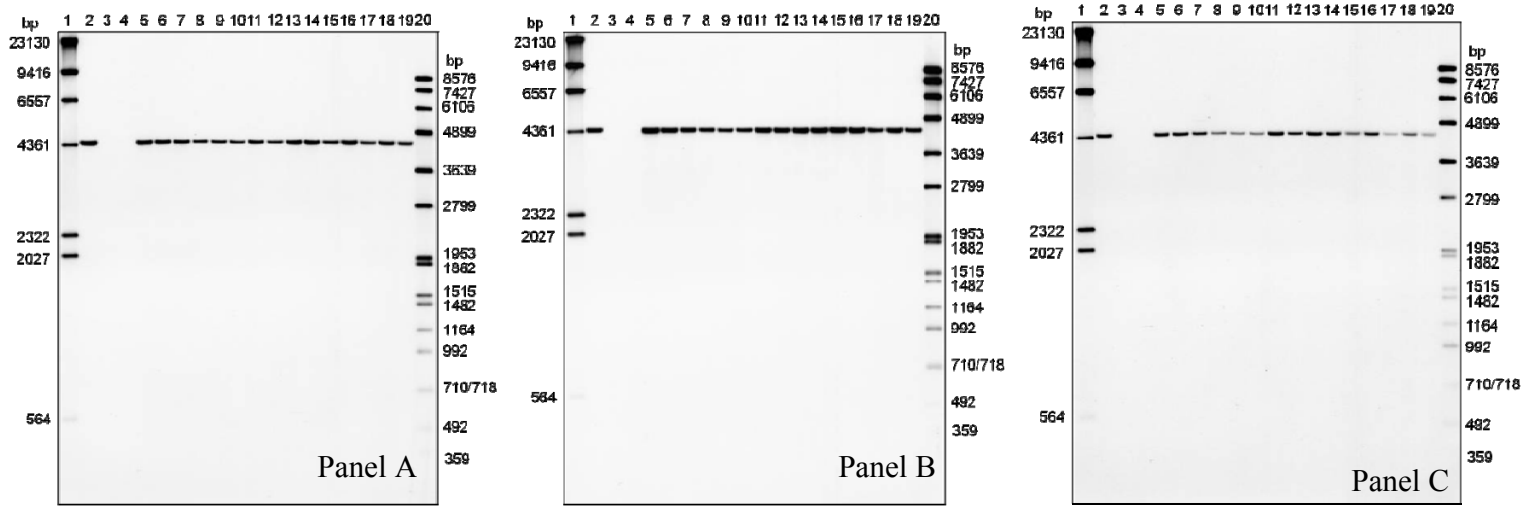


Figure 18. Southern blot analysis of *Msc*I/*Eco*RI digested DAS-444Ø6-6 soybean: Histone H4A748 Promoter, 2mepsps and Histone H4A748 UTR probes.

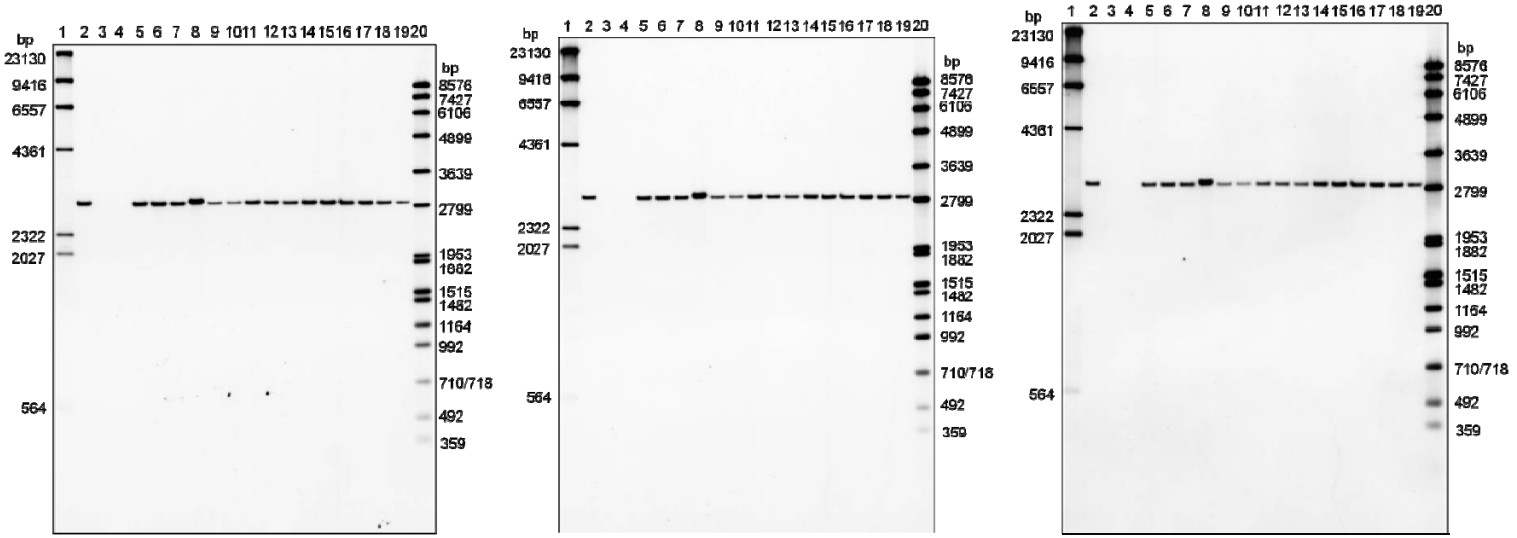
Approximately 10 μg of genomic DNA was digested *Msc*I/*Eco*RI followed by hybridization with the Histone H4A748 Promoter (Panel A), 2mepsps (Panel B) and Histone H4A748 UTR (Panel C) probes. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**				**Panel C**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-3 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2	2	Maverick-3 + pDAB8264	12	4406-T3-2
3	Maverick-3	13	4406-T3-3	3	Maverick-1	13	4406-T3-3	3	Maverick-3	13	4406-T3-3
4	Maverick-2	14	4406-T4-1	4	Maverick-2	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3

10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

Panel A Panel B Panel C

Figure 19. Southern blot analysis of *PstI*/*XhoI* digested DAS-444Ø6-6 soybean: AtUbi10 Promoter, aad-12, and AtuORF23 UTR probes.

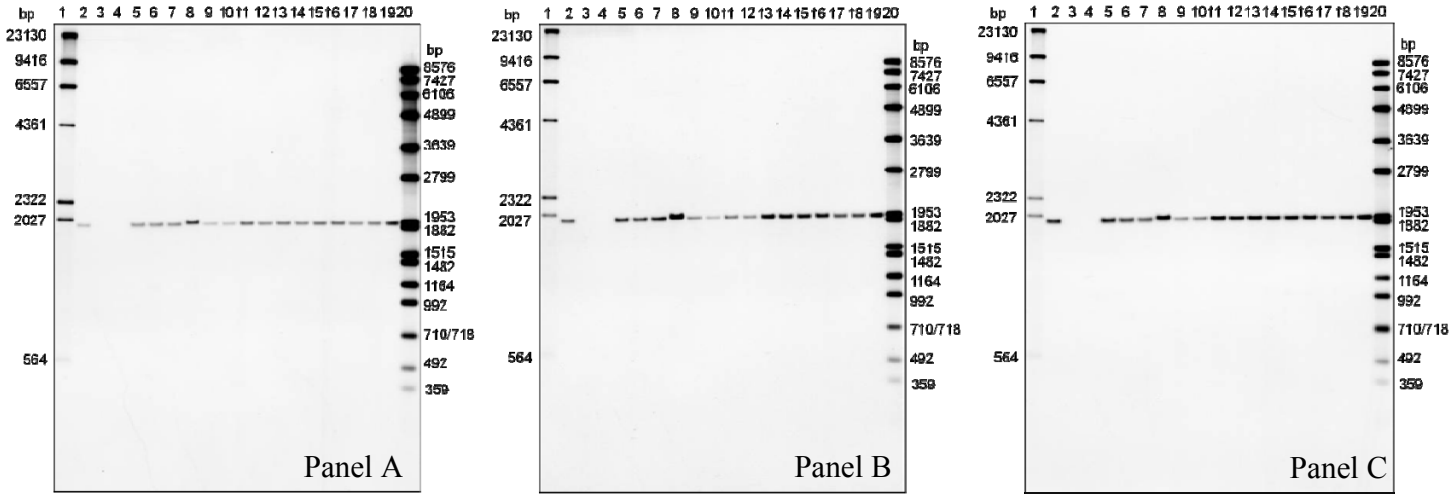


Approximately 10 μg of genomic DNA was digested *Pst*I/*Xho*I followed by hybridization with the AtUbi10 Promoter (Panel A), aad-12 (Panel B) and AtuORF23 UTR (Panel C) probes. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**				**Panel C**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-1 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2
3	Maverick-1	13	4406-T3-3	3	Maverick-1	13	4406-T3-3	3	Maverick-1	13	4406-T3-3
4	Maverick-2	14	4406-T4-1	4	Maverick-2	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3

Figure 20. Southern blot analysis of *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: CsVMV, pat, and AtuORF1 UTR probes.

10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII



Approximately 10 μg of genomic DNA was digested *Pst*I/*Xho*I followed by hybridization with the CsVMV (Panel A), pat (Panel B) and AtuORF1 UTR (Panel C) probes. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**				**Panel C**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-1 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2
3	Maverick-1	13	4406-T3-3	3	Maverick-1	13	4406-T3-3	3	Maverick-1	13	4406-T3-3
4	Maverick-2	14	4406-T4-1	4	Maverick-2	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

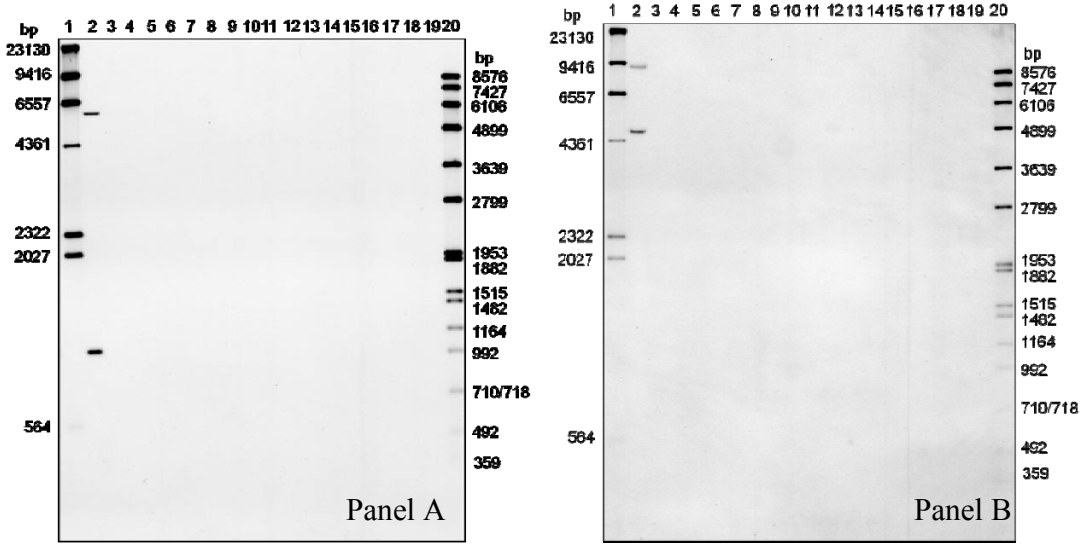


Figure 21. Southern blot analysis of *Msc*I/*Eco*RI and *Hind*III digested DAS-444Ø6-6 soybean: Backbone 3 and Backbone 4 probes.

Approximately 10 μg of genomic DNA was digested with *Msc*I/*Eco*RI (Panel A) and *Hind*III (Panel B) and hybridized with the Backbone 3 and Backbone 4 probes. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-1 + pDAB8264	12	4406-T3-2	2	Maverick-5 + pDAB8264	12	4406-T3-2
3	Maverick-1	13	4406-T3-3	3	Maverick-5	13	4406-T3-3
4	Maverick-2	14	4406-T4-1	4	Maverick-4	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

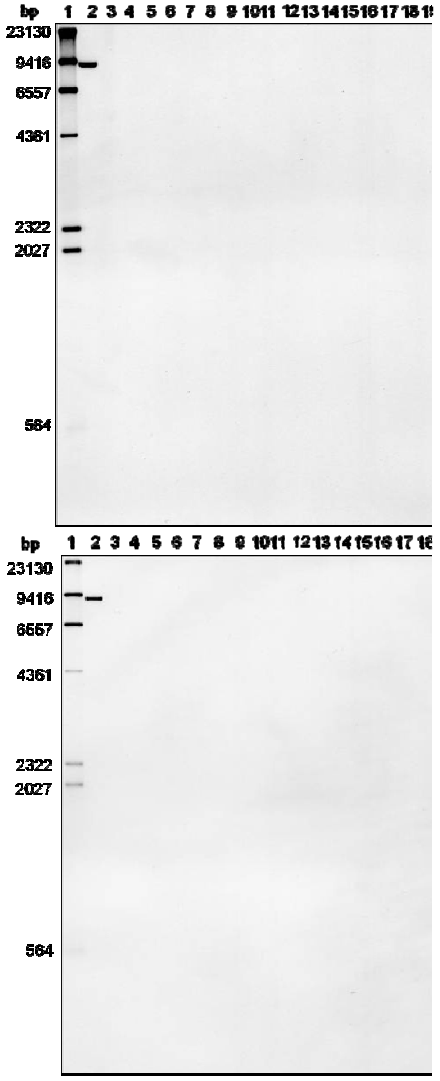
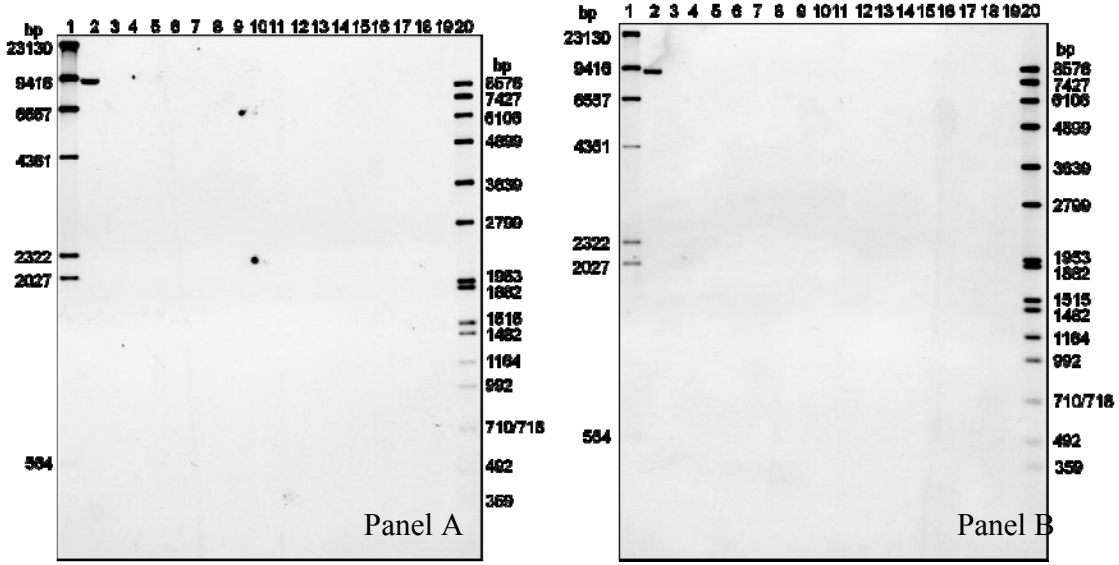


Figure 22. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: Ori-Rep probe.

Approximately 10 μg of genomic DNA was digested with *Hind*III (Panel A) *Pst*I/*Xho*I and (Panel B) and hybridized with the Ori-Rep probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

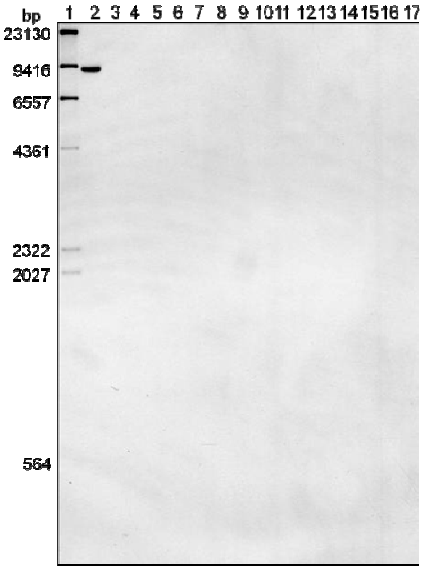
	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-1	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII



Approximately 10 μg of genomic DNA was digested with *Hind*III (Panel A) *Pst*I/*Xho*I and (Panel B) and hybridized with the Backbone 1 probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-1	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

Figure 23. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: Backbone 1 probe.



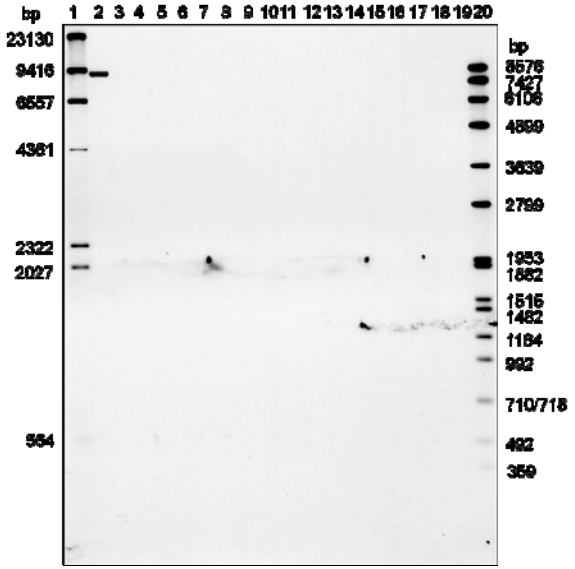


Figure 24. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: Backbone 2 probe.

Approximately 10 μg of genomic DNA was digested with *Hind*III (Panel A) *Pst*I/*Xho*I and (Panel B) and hybridized with the Backbone 2 probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6.

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-1	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

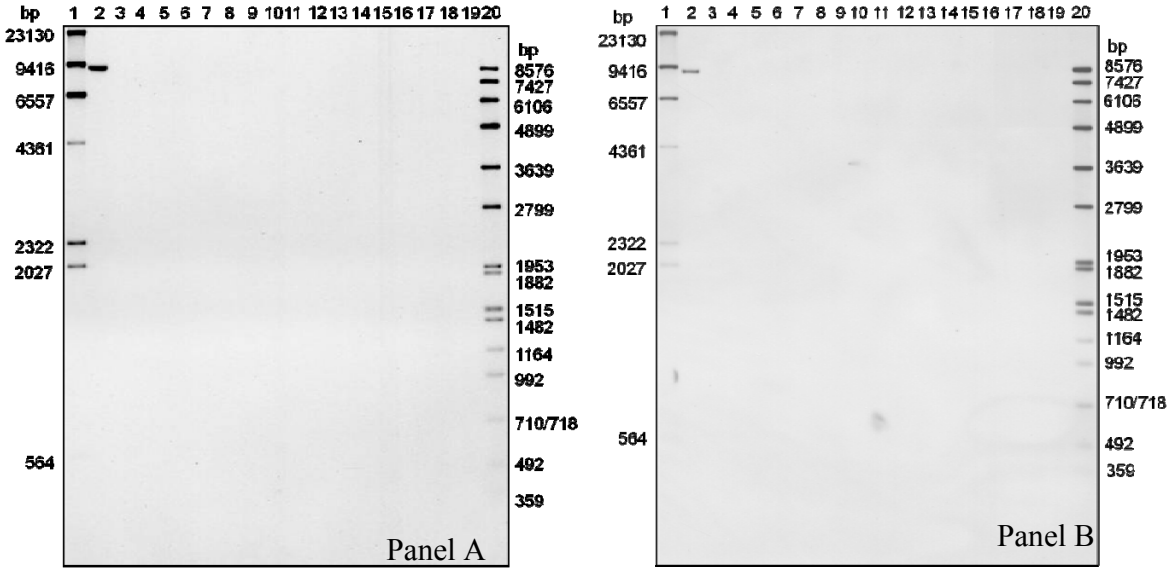


Figure 25. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: SpecR probe.

Approximately 10 μg of genomic DNA was digested with *Hind*III (Panel A) *Pst* I/*Xho* I and (Panel B) and hybridized with the SpecR probe. Plasmid pDAB8264 served as the positive control at approximately one copy per soybean genome. Fragment sizes of the DIG-labeled DNA Molecular Weight Marker II and VII are indicated adjacent to image. 4406= DAS-444Ø6-6. *Note: The splotch at ~4000 bp between lanes 9 and 10 in panel B is non-specific background signal since it falls between the lanes.*

	Panel A				**Panel B**		
Lane	Sample	Lane	Sample	Lane	Sample	Lane	Sample
1	DIG MWM II	11	4406-T3-1	1	DIG MWM II	11	4406-T3-1
2	Maverick-5 + pDAB8264	12	4406-T3-2	2	Maverick-1 + pDAB8264	12	4406-T3-2
3	Maverick-5	13	4406-T3-3	3	Maverick-1	13	4406-T3-3
4	Maverick-4	14	4406-T4-1	4	Maverick-2	14	4406-T4-1
5	4406-T2-1	15	4406-T4-4	5	4406-T2-1	15	4406-T4-4
6	4406-T2-2	16	4406-T4-5	6	4406-T2-2	16	4406-T4-5
7	4406-T2-3	17	4406-T6-1	7	4406-T2-3	17	4406-T6-1
8	4406-F2-1	18	4406-T6-2	8	4406-F2-1	18	4406-T6-2
9	4406-F2-3	19	4406-T6-3	9	4406-F2-3	19	4406-T6-3
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

Genetic and Molecular Analysis of a Segregating Generation

The inheritance pattern of the transgene insert within a segregating generation was demonstrated with event-specific PCR analysis of individual plants from a F2 population of DAS-444Ø6-6 soybean. The F1 generation was generated by crossing T2 plants of DAS-444Ø6-6 soybean with a conventional soybean line. The F1 plants were self pollinated to produce the F2 seeds.

A total of 119 F2 plants were tested by event-specific PCR to determine the presence or absence of the DAS-444Ø6-6 transgene insert. Genomic DNA samples from each of the 119 plants, along with DNA samples from the non-transgenic control, were analysed by DAS-444Ø6-6 soybean event-specific PCR. Of the 119 plants tested, 96 plants were positive for the presence of DAS-444Ø6-6 transgene insert, and the remaining 23 plants were negative (segregated null). In addition, leaves of the individual plants were tested for the presence or absence of the PAT protein using a PAT-specific lateral flow strip test kit. All of the plants that tested positive for DAS-444Ø6-6 transgene insert displayed positive result for PAT protein expression, and all plants that were negative for DAS-444Ø6-6 transgene insert displayed negative result for PAT protein expression (Table 4), confirming that the phenotypic segregation matched the genotypic makeup of the tested F2 population. Statistical analysis using a χ^2 goodness of fit test indicated the genotypic segregation ratio of the plants with positive transgene insert versus negative is consistent with the 3:1 segregation ratio characteristic of the Mendelian inheritance pattern of a single dominant trait (Mo, 2011).

Table 4. Results of F2 individual plants tested for PAT expression and event specific PCR within a single segregating generation.

Tested Method	Total plants tested	Positive	Negative	Expected ratio	P-value[a]
Event-Specific PCR	119	96	23	3:1	0.153
PAT Expression	119	96	23	3:1	

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

Segregation Analysis of Breeding Generations

Chi-square analysis of trait inheritance data from three populations of the BC1F2 breeding generation was conducted to determine the Mendelian inheritance of *aad-12* and *2mepsps* in DAS-444Ø6-6 soybeans. The presence or absence of *aad-12* and *2mepsps* was determined using a herbicide spray (2,4-D + glyphosate), which is specific for AAD-12 and 2mEPSPS protein expressing soybeans. The expected segregation ratio of 3:1 for plants expressing AAD-12 and 2mEPSPS versus plants that do not express AAD-12 nor 2mEPSPS proteins was observed (Table 5).

Table 5. Results of BC1F2 individual plants from event DAS-444Ø6-6 tested for AAD-12 and 2mEPSPS protein expression within a single segregating generation.

Generation/ Source ID	Total plants tested	*aad-12* and *2mepsps* gene positive (resistant to 2,4-D + glyphosate)	*aad-12* and *2mepsps* gene negative (susceptible to 2,4-D + glyphosate)	Expected ratio	P-value[a]
BC1F2	39	27	12	3:1	0.4054
BC1F2	58	46	12	3:1	0.4484
BC1F2	31	26	5	3:1	0.2540

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

Summary of the Genetic Characterization

DAS-444Ø6-6 soybean was produced using *Agrobacterium*-mediated transformation with the plasmid pDAB8264. The T-DNA insert of pDAB8264 consists of 1) the *2mepsps* gene, controlled by the Histone H4A748 promoter and Histone H4A748 3′ UTR regulatory sequences; 2) the *aad-12* gene, controlled by the AtUbi10 promoter and AtuORF23 3′ UTR regulatory sequences; 3) the *pat* gene, controlled by the CsVMV promoter and AtuORF1 3′ UTR regulatory sequences. In addition, a RB7 MAR element is located at the 5′ end of the T-DNA insert. Various breeding generations were developed and used to examine the integrity, stability, and inheritance of the transgenic insert in DAS-444Ø6-6 soybean.

Molecular characterization of DAS-444Ø6-6 soybean by Southern blot analysis confirmed the single insertion of the T-DNA insert from pDAB8264 containing a single intact copy of each of the *2mepsps*, *aad-12* and *pat* PTUs. No additional DNA fragments from the *2mepsps*, *aad-12* and *pat* expression cassettes were identified in DAS-444Ø6-6 and no plasmid backbone sequences were present. The T-DNA insert for DAS-444Ø6-6 was shown to be stably integrated across five breeding generations (T2, T3, T4, T6, and F2) tested. Moreover, the T-DNA insert displayed the expected Mendelian inheritance pattern for a single independent insert/locus in a segregating generation (F2).

Methods for Molecular Characterization of DAS-444Ø6-6 Soybean

DAS-444Ø6-6 Soybean Material

Transgenic soybean seeds from five distinct generations of soybean containing event DAS-444Ø6-6 were planted in the greenhouse. After at least one week of growth, leaf punches were taken from each plant and were tested for PAT 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 PAT protein.

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 *2mepsps, aad-12,* and *pat* genes.

Reference Materials

DNA of the plasmid pDAB8264 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 for the Southern hybridization.

DNA Probe Preparation

DNA probes specific to the genetic elements in the T-DNA insert of pDAB8264 and the vector backbone sequences were produced via polymerase chain reaction (PCR) amplification using pDAB8264 plasmid DNA as a template, followed by purification from agarose gel.

Sample Collection and DNA Extraction

Labeled leaf samples were collected from green house for DNA extraction or stored in -80°C freezer for future use. Genomic DNA was extracted with 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). 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 (Sigma). DNA was precipitated by mixing the supernatant

with equal volume of precipitation buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA). The precipitated DNA was dissolved in high salt TE buffer (1 × TE pH 8.0, 1.0 M NaCl) followed by precipitation with isopropyl alcohol. The precipitated DNA was rinsed with 70% ethanol, air-dried, then dissolved in appropriate volume of 1 × TE buffer (pH 8.0).
To determine 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 1× 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) using a spectrofluorometer (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 10 µg of genomic DNA with approximately 5-10 units of the selected restriction enzyme per µg of DNA in the corresponding reaction buffer. Each sample was incubated at 37^{o}C overnight. The positive control sample was prepared by combining pDAB8264 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 test 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 (89 mM Tris, 89 mM Boric acid, 2 mM 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 (3 M NaCl, 0.3 M 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, into the DNA fragments generated by primers specific to the genetic elements and other regions from plasmid pDAB8264. The PCR synthesis of the probes was performed using a PCR DIG Probe Synthesis Kit (Roche Diagnostics) 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 and VII were used to determine the size of the hybridizing fragments 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 at 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 bound probe in a solution containing 0.2 M NaOH and 1.0% SDS. The alkali-based stripping procedure successfully removes the labelled probes from the membranes, allowing them to be re-hybridized with a different DNA probe.

e. Breeding Pedigree

The publicly available cultivar 'Maverick' was used as the recipient line for the generation of DAS-444Ø6-6 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-4406-6.

Figure 4 shows a breeding diagram for DAS-444Ø6-6 soybean including identification of the generations used in the various safety assessment studies.

f. Genetic Stability

Stability of the Insert Across Generations

Southern blot hybridizations were conducted with samples across five distinct generations (T2, T3, T4, T6, and F2) of DAS-444Ø6-6 soybean. Prior to initiation of Southern blot analysis, all plants were tested for PAT protein expression using a lateral flow strip test kit to allow for confirmation of PAT expression-positive plants. All of the genetic element probes: 2mepsps, Histone H4A748 promoter, Histone H4A748 UTR, aad-12, AtUbi10 promoter, AtuORF23 UTR, CsVMV promoter, pat, AtuORF1 UTR, and RB7 MAR, as well as the probes covering the vector backbone sequences of plasmid pDAB8264, were hybridized with the DAS-444Ø6-6 soybean samples. Results across all five generations of DAS-444Ø6-6 soybean were consistent with what was expected (

Table 3, Figure 8 - Figure 25), indicating stable integration and inheritance of the intact, single copy transgene insert across multiple generations of DAS-444Ø6-6 soybean.

4. Analytical Method for Detection

Part B section 4 DAS Reports

Maldonado, P.M., 2011a. Field expression of a transformed soybean cultivar containing aryloxyalkanoate dioxygenase (AAD-12), double mutant EPSPS gene (2mEPSPS), and phosphinothricin acetyltransferase (PAT) - event DAS-44406-6. Study ID 101104.02. Dow AgroSciences LLC. Indiandpolis, IN.

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-444Ø6-6 . Please specifically refer to the results found in Table 24 for data pertaining to soybean food products.

b. Detection Methodology

DAS Reports

Maldonado, P. M. (2011). Method Validation for the Determination of 5-Enol-Pyruvylshikimat 3-Phosphate Synthase (2mEPSPS) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA). Indianapolis, IN, Dow AgroSciences LLC.

Smith-Drake, J. S., M. J. Sosa, et al. (2009). Method Validation for the Determination of Phosphinotricin Acetyltransferase (PAT) in Soybean Tissues Using an Enzyme-linked Immunosorbent Assay (ELISA). Study 081022. Indianapolis, IN, Dow AgroSciences LLC.

Determination of AAD-12 Protein Concentration in Soybean Tissue Samples

ELISA method GRM08.04 was used to determine AAD-12 protein concentration in soybean tissue

samples (Smith-Drake, Sosa et al. 2009). The AAD-12 protein was extracted from soybean tissues except grain with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 0.75% ovalbumin (OVA). For grain, the protein was extracted with a PBST buffer containing 0.1% (v/v) Triton-100. The plant tissue and grain extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analyzed using an AAD-12 ELISA kit. Briefly, an aliquot of the diluted sample and a horseradish peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate were 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 excess conjugate were 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 color development, determined by measuring the absorbance of the solution, was related to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm with a background subtraction at 650 or 620 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the AAD-12 concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

Determination of 2mEPSPS Protein in Soybean Tissue Samples

ELISA method 101768 was used to determine 2mEPSPS protein concentration in soybean tissue samples (Maldonado 2011). The 2mEPSPS protein was extracted from soybean tissues with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 2X Casein (PBST/Casein). The plant tissue extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analyzed using a 2mEPSPS ELISA kit. Briefly, an aliquot of the diluted sample is incubated in the wells of a microtiter plate coated with an immobilized anti-2mEPSPS polyclonal antibody. After a washing step, an enzyme-conjugated monoclonal antibody specific to the 2mEPSPS protein is added to the microtiter plate. These antibodies bind with 2mEPSPS protein in the wells and form a "sandwich" with 2mEPSPS protein bound between soluble and the immobilized antibodies. At the end of an incubation period, the unbound reagents were 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 2mEPSPS was bound in the antibody sandwich, the level of color development, determined by measuring the absorbance of the solution, was related to the concentration of 2mEPSPS in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm with a background subtraction at 650 or 620 nm was measured using a Molecular

Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the 2mEPSPS concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

Determination of PAT Protein in Soybean Tissue Samples

ELISA method GRM08.05 was used to determine PAT protein concentration in soybean tissue samples (Smith-Drake, Sosa et al. 2009). The PAT protein was extracted from soybean tissues with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 1% polyvinylpyrrolidone (PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted with PBST/1% PVP, and analyzed using a PAT ELISA kit. Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT monoclonal antibody and anti-PAT polyclonal 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 color development, determined by measuring the absorbance of the solution, was related to the concentration of PAT in the sample (i.e., lower residue concentrations result in lower color development). The absorbance at 450 nm with a background subtraction at 650 or 620 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the PAT concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

B. SAFETY OF THE GENETICALLY MODIFIED FOOD

1. Antibiotic Resistance Marker Genes

As described in Section B, Part 3 D of this dossier, Southern blot analysis confirms that the PTU insertion into DAS-444Ø6-6 soybean does not contain any vector backbone from the plasmid pDAB8264. Therefore the spectinomycin resistance gene (*SpecR,* **Figure 1**) in plasmid pDAB8264 was not inserted into the soybean genome in event DAS-444Ø6-6.

a. Clinical Relevance

Not applicable

b. Therapeutic Efficacy

Not applicable

c. Safety of the Gene Product

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

d. End Use Viability (micro-organisms)

Not applicable

2. Characterization of the Novel Proteins

Part C Section 2 DAS Reports

Embrey, S.K., 2011a. Certificate of analysis of the test/reference/control substances: Aryloxyalkanoate dioxygenase-12 (AAD-12) – TSN030732. BIOT10-227507. Dow AgroSciences LLC. Indianapolis, IN.

Embrey, S.K., Schafer, B.W., 2009. Certificate of analysis of the test/reference/control substance: Phosphinothricin acetyltransferase (PAT - TSN031116-0001). BIOT09-203839. Dow AgroSciences LLC. Indianapolis, IN.

Guttikonda, S.K., 2011c. Cloning and characterization of the DNA sequence for the insert and its flanking border regions of DAS-44406-6 soybean. Study ID 102117. Dow AgroSciences LLC. Indianapolis, IN.

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a. Biochemical function and phenotypic effect of novel protein

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 99.6% 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 26).

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

Figure 26. Amino acid sequence of the AAD-12 protein.

Mode of Action of the AAD-12 Protein

Expression of the AAD-12 protein in transgenic crops has been shown to provide tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) by catalysing the conversion of 2,4-D to 2,4-dichlorophenol (DCP) a herbicidally inactive compound (Figure 27) (Müller *et al.*, 1999; Westendorf *et al.*, 2002; Westendorf *et al.*, 2003; Wright *et al.*, 2009; Wright *et al.*, 2010). 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 (Figure 28).

AAD-12 has selectivity for (S)-enantiomers of the chiral phenoxy acid herbicides (*e.g.*, dichlorprop and mecoprop), but does not catalyse degradation of the (R)-enantiomers (Kohler, 1999; Schleinitz *et al.*, 2004). The R-enantiomers are herbicidally active; therefore, AAD-12 does not provide tolerance to commercially-available chiral phenoxy acid herbicides.

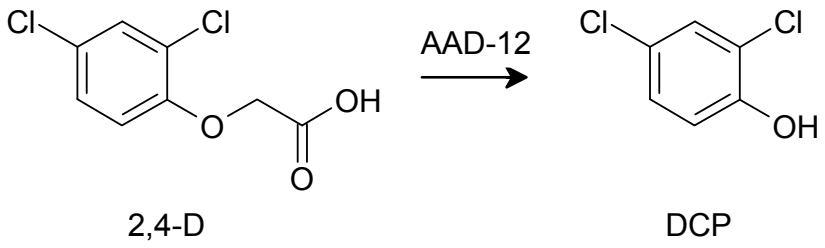


Figure 27. Degradation reaction of 2,4-D catalyzed by AAD-12.

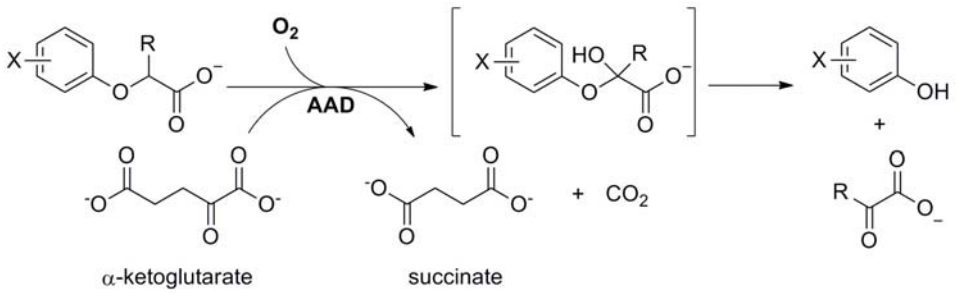


Figure 28. General reaction catalyzed by AAD-12 (R=H or CH_3).

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 in *Pseudomonas fluorescens*. Characterization studies were performed to confirm the equivalency of the AAD-12 protein produced in *P. fluorescens* with the AAD-12 protein produced *in planta* in DAS-444Ø6-6 soybean. 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/mass spectrometry (MALDI-TOF MS/MS) were used to characterize the biochemical properties of the protein (Karnoup and Kuppannan 2008; Schafer, Juba et al. 2011). Using these methods, the AAD-12 protein isolated from *P. fluorescens* and DAS-444Ø6-6 soybean were shown to be biochemically equivalent, thereby supporting the use of the microbe-derived protein in safety assessment studies.

The methods and results of the biochemical characterization of the DAS-444Ø6-6 soybean- and microbe-derived AAD-12 proteins are described in detail following the characterisation summary and in (Karnoup and Kuppannan 2008; Schafer, Juba et al. 2011). Briefly, both the plant and *P. fluorescens*-derived AAD-12 proteins were observed at the expected molecular weight of ~32 kDa by SDS-PAGE and were immunoreactive to AAD-12 protein-specific antibodies by western blot analysis. There was no evidence of glycosylation of the DAS-444Ø6-6 soybean-derived AAD-12 protein. Greater than 84% of the soybean-derived protein amino acid sequence was confirmed by either enzymatic peptide mass fingerprinting or MS/MS sequence analysis by MALDI-TOF MS/MS. The N-terminal methionine was found to be cleaved from both protein sources and a portion of the N-terminal peptide of the plant-derived AAD-12 was determined to be acetylated after the N-terminal methionine was cleaved [two forms of the N-terminal peptide were detected (both acetylated and non-acetylated forms)]. These two post-translational processes, cleavage of the N-terminal methionine residue and N-terminal acetylation, are common modifications that have been found to occur on the vast majority (~85%) of eukaryotic proteins (Polevoda

and Sherman 2003). The C-terminal peptides from DAS-444Ø6-6 soybean and *P. fluorescens* were intact and determined to be identical.

Identity of the 2mEPSPS Protein

The double mutant 5-enolpyruvylshikimate-3-phosphate synthase (2mEPSPS) protein is encoded by a modified *epsps* gene from corn (*Zea mays*) (Herouet-Guicheney *et al.*, 2009). The *2mepsps* transgene in DAS-444Ø6-6 encodes a protein sequence that is the wild-type EPSPS carrying two substitutions at amino acids 102 and 106 (Figure 29: UniProt Accession number: O24566). The mutations are known as the TIPS mutation where threonine was substituted by isoleucine at amino acid 102 and proline was substituted by serine at amino acid 106. The 2mEPSPS protein is comprised of 445 amino acids and has a molecular weight of ~47.5 kDa. The N-terminal methionine is cleaved from the mature protein *in vivo* and is not included in **Figure 29**.

```
  1 AGAEEIVLQPIKEISGTVKLPGSKSLSNRI 30
 31 LLLAALSEGTTVVDNLLNSEDVHYMLGALR 60
 61 TLGLSVEADKAAKRAVVVGCGGKFPVEDAK 90
 91 EEVQLFLGNAGIAMRSLTAAVTAAGGNATY 120
121 VLDGVPRMRERPIGDLVVGLKQLGADVDCF 150
151 LGTDCPPVRVNGIGGLPGGKVKLSGSISSQ 180
181 YLSALLMAAPLALGDVEIEIIDKLISIPYV 210
211 EMTLRLMERFGVKAEHSDSWDRFYIKGGQK 240
241 YKSPKNAYVEGDASSASYFLAGAAITGGTV 270
271 TVEGCGTTSLQGDVKFAEVLEMMGAKVTWT 300
301 ETSVTVTGPPREPFGRKHLKAIDVNMNKMP 330
331 DVAMTLAVVALFADGPTAIRDVASWRVKET 360
361 ERMVAIRTELTKLGASVEEGPDYCIITPPE 390
391 KLNVTAIDTYDDHRMAMAFSLAACAEVPVT 420
421 IRDPGCTRKTFPDYFDVLSTFVKN       444
```

Figure 29. Amino acid sequence of the 2mEPSPS protein.

* Note: The N-terminal Met is not shown, as it is cleaved from the mature protein.

Mode of Action of the 2mEPSPS Protein

Glyphosate normally exerts herbicidal activity by binding and inactivating EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), an essential enzyme in the shikimic acid pathway which is found only in plants and certain microorganisms (Sikorski and Gruys 1997).

Soybean (*Glycine max*) lines have been genetically modified for tolerance to glyphosate herbicides by expressing in the plant a modified *epsps* gene from corn (*Zea mays*), *2mepsps*, which introduced two amino acid changes in the enzyme. The amino acids changed in the 2mEPSPS protein significantly lower the sensitivity to glyphosate, allowing the enzyme to continue to function in the presence of the herbicide (Herouet-Guicheney, Rouquié et al. 2009).

Biochemical Characterization of the 2mEPSPS Protein

Large quantities of purified 2mEPSPS protein are required to perform safety assessment studies. As it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans 2004), the 2mEPSPS protein was produced in *Pseudomonas fluorescens*. Characterization studies were performed to confirm the equivalency of the 2mEPSPS protein expressed in DAS-444Ø6-6 soybean with the *P. fluorescens*-derived 2mEPSPS 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), MALDI-TOF MS/MS, and electrospray ionization-liquid chromatography mass spectrometry (ESI-LC/MS) were used to characterize the biochemical properties of the proteins (Karnoup and Kuppannan 2010; Schafer 2010; Schafer, Juba et al. 2011) . Using these methods, the 2mEPSPS protein from *P. fluorescens* and DAS-444Ø6-6 soybean were shown to be biochemically equivalent, thereby supporting the use of the microbe-derived protein in safety assessment studies (Schafer, Juba et al. 2011).

The methods and results of the biochemical characterization of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS proteins are described in detail following the characterisation summary. Briefly, both the plant and *P. fluorescens*-derived 2mEPSPS proteins showed the expected molecular weight of ~47 kDa by SDS-PAGE and were immunoreactive to 2mEPSPS protein-specific antibodies by western blot analysis. There was no evidence of any post-translational modifications (i.e. glycosylation) of the DAS-444Ø6-6 soybean-derived 2mEPSPS protein. The amino acid sequence (including the N- and C-termini) was confirmed by enzymatic peptide mass fingerprinting using MALDI-TOF MS and MALDI-TOF MS/MS and was shown to be as expected and was identical to the protein expressed in *P. fluorescens*. The 2mEPSPS protein of both sources did not contain the methionine residue at its N terminus. The result is

consistent with those for the 2mEPSPS protein expressed in other systems (Herouet-Guicheney, Rouquié et al. 2009).

Identity of the PAT Protein

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium (Strauch *et al.*, 1988; OECD, 1999). The *pat* transgene in DAS-444Ø6-6 encodes a protein sequence that is identical to the native PAT protein (UniProt Accession Number: Q57146). PAT is comprised of 183 amino acids and has a molecular weight of ~21 kDa (Figure 30. Amino Acid sequence of the PAT protein).

001 MSPERRPVEIRPATAADMAAVCDIVNHYIETSTVNFRTEPQTPQEWIDDL
051 ERLQDRYPWLVAEVEGVVAGIAYAGPWKARNAYDWTVESTVYVSHRHQRL
101 GLGSTLYTHLLKSMEAQGFKSVVAVIGLPNDPSVRLHEALGYTARGTLRA
151 AGYKHGGWHDVGFWQRDFELPAPPRPVRPVTQI

Figure 30. Amino acid sequence of the PAT protein.

Mode of Action of the PAT Protein

The L-isomer of phosphinothricin (PPT) is a potent inhibitor of glutamine synthetase (GS) in plants and is used as a non-selective herbicide (OECD, 1999). Inhibition of GS by PPT causes rapid accumulation of intracellular ammonia which leads to cessation of photorespiration and results in the death of the plant cell (Duan *et al.*, 2009). The *pat* gene which encodes phosphinothricin acetyltransferase (PAT) acetylates the free NH_2 group of PPT (in the presence of acetyl coenzyme A) and thereby prevents autotoxicity in the producing organism (Figure 31, (Duke, 1996)).

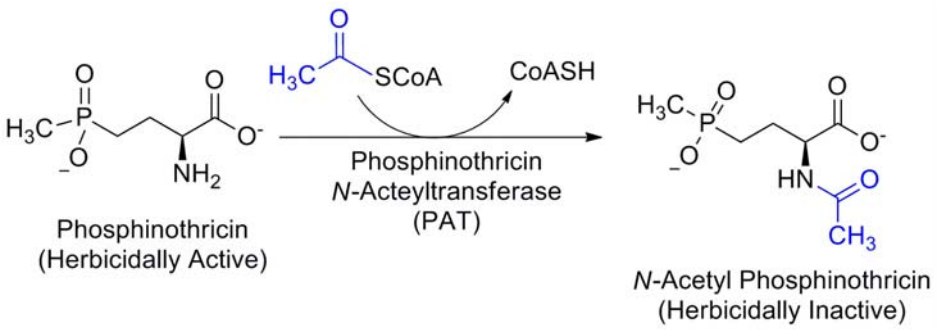


Figure 31. Mode of action of the PAT protein.

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), ELISA and western blot analysis. The methods and results are described in detail following the characterisation summary. Using these methods the PAT protein produced in DAS-444Ø6-6 soybean was shown to be equivalent to that produced in other transgenic crops (USDA 1996; USDA 2001; USDA 2004; USDA 2005; Schafer and Juba 2011).

Summary of AAD-12, 2mEPSPS and PAT Protein Characterization

AAD-12 Summary

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 DAS-444Ø6-6 soybean and *P. fluorescens* was conducted.

2mEPSPS Summary

The 2*mepsps* coding sequence was produced by introducing two point mutations to the wild-type *epsps* gene cloned from corn (*Zea mays*) through *in vitro* DNA technologies. The resultant 2mEPSPS protein has a lower binding affinity for glyphosate, thus allowing sufficient enzyme activity for the plants to grow in the presence of glyphosate herbicide. 2mEPSPS is comprised of 445 amino acids and has a molecular weight of ~47.5 kDa. Detailed biochemical characterization of the 2mEPSPS protein derived from DAS-444Ø6-6 soybean and *P. fluorescens* were conducted.

PAT Summary

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium. PAT is comprised of 183 amino acids and has a molecular weight of ~21 kDa (OECD 1999; OECD 2002). ~~Detailed b~~Biochemical characterization of the PAT protein derived from DAS-444Ø6-6 soybean was conducted. Western blot analysis demonstrated that the PAT protein expressed in DAS-444Ø6-6 soybean had the same molecular weight and immunoreactivity as the native protein.

Methods and Results for Characterization of AAD-12 Protein

DAS-444Ø6-6 Transgenic Soybean Material

Greenhouse-grown DAS-444Ø6-6 soybean plants (T4 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 an immunospecific rapid lateral flow test strip according to the manufacturer's instructions. Tissues 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-444Ø6-6 soybean plants, but did not contain the *aad-12* gene. Absence of AAD-12 expression in the control plants was confirmed using the same AAD-12 specific rapid lateral flow test strip as previously mentioned. Tissues of control plants were harvested, lyophilized, ground and stored under the same conditions as the DAS-444Ø6-6 soybean.

Reference Material

Recombinant AAD-12 protein was produced in *Pseudomonas fluorescens* and purified to a lyophilized powder (Lin, Allen et al. 2006). The microbe-derived AAD-12 protein preparation was stored dry and resuspended in a Tris-based buffer to maintain activity prior to use.

Protein Purification from DAS-444Ø6-6 Soybean Plant Tissue

A soybean crude protein extract containing the AAD-12 protein was extracted from lyophilized root tissue in a Tris-based buffer, pH 8.0 with added stabilizers, filtered though cheesecloth and the soluble proteins were separated from the insoluble plant material by centrifugation. The supernatant was slowly adjusted to 1 M ammonium sulfate and insoluble proteins were removed by centrifugation. The remaining soluble protein fraction was loaded onto a 5 mL Phenyl Sepharose HP Hi-Trap Column (GE Healthcare) and the unbound proteins were washed from the column with 50 mM Tris (pH 8.0), 1 M ammonium sulfate. Proteins retained on the column were eluted with a decreasing gradient of ammonium sulfate and fractions containing AAD-12 protein (as determined by ELISA) were pooled. The pooled fraction was then incubated with an anti-AAD-12 immunoaffinity resin which had been conjugated with an AAD-12 specific monoclonal antibody using a crosslinked immuno-precipitation kit (Thermo-Pierce). Bound proteins were eluted according to the manufacturer's protocols. The unbound fraction was re-incubated with the resin and a second pool of protein was collected. Eluted fractions were analyzed by SDS-PAGE (stained with

GelCode Blue total protein stain from Thermo-Pierce), western blot, and ELISA. Fractions containing the soybean-derived AAD-12 protein were pooled, desalted into 50 mM ammonium bicarbonate utilizing a PD-10 desalting column (GE Healthcare), aliquoted, and lyophilized.

SDS-PAGE and Western Blot Analysis of Crude Soybean Leaf Extracts

Fresh leaf tissue from event DAS-444Ø6-6 and Maverick was mixed with a Tris-based buffer containing ~3% protease inhibitor cocktail (Sigma) and the soluble proteins were extracted by grinding with ball bearings in a Geno/Grinder (Certiprep, Metuchen, NJ) for 3 minutes. The samples were centrifuged and the supernatants were mixed with Laemmli sample buffer, heated at 95°C for 5 minutes, and briefly centrifuged. The positive and negative reference standards, microbe-derived AAD-12 and bovine serum albumin, respectively, were also mixed with sample buffer and prepared the same as the plant extracts. The samples were loaded directly on to two separate Bio-Rad Criterion SDS-PAGE gels and the proteins were electrophoresed with MES running buffer (Bio-Rad) for ~ 60 minutes at 150 volts. Following electrophoresis, one gel was stained with Thermo Pierce GelCode Blue protein stain for total protein and the other gel was electro-blotted onto a nitrocellulose membrane for western blot analysis. After transfer, the nitrocellulose membrane was blocked with 5% dry milk in PBST and probed with an AAD-12 specific polyclonal antibody. A chemiluminescent substrate (GE Healthcare) was used to visualize the immunoreactive bands on X-ray films (Thermo Pierce).

Detection of Post-Translational Glycosylation

The immunoaffinity-purified, plant-derived AAD-12 protein was analyzed for evidence of glycosylation by SDS-PAGE along with microbe-derived AAD-12 protein, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase used as reference controls. The reference protein samples were adjusted to concentrations approximately equal to that of the plant-derived AAD-12 protein and mixed with Laemmli buffer. The proteins were heated at 95°C for 5 minutes, 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 Thermo Pierce GelCode Blue stain for total protein. The remaining half of the gel was stained with GelCode Glycoprotein Stain to visualize glycoproteins. The glycoproteins present on the gel were visualized as magenta bands on a light pink background.

Mass Spectrometry Peptide Mass Fingerprinting and Sequencing of Plant- and Microbe-Derived AAD-12 Protein

The immunoaffinity purified AAD-12 plant-derived protein was subjected to in-gel digestion by trypsin and chymotrypsin followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF MS-MS. The peptide fragments of the plant-derived AAD-12 protein

(including the N- and C-termini) were analyzed and compared with the sequence of the microbe-derived protein (Embrey 2011; Schafer, Juba et al. 2011).

Results of the SDS-PAGE and Western Blot Analysis

In the microbe-derived AAD-12, the major protein band, as visualized on the Coomassie stained SDS-PAGE gel, was approximately 32 kDa (Figure 32). As expected, the corresponding plant-derived AAD-12 protein was identical in size to the microbe-derived protein. Predictably, the protein fractions purified from DAS-444Ø6-6 tissue 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; Williams *et al.*, 2006).

The microbe-derived AAD-12 and DAS-444Ø6-6 plant tissue extract showed a positive signal of the expected size on the western blot using an anti-AAD-12 polyclonal antibody (Figure 33). In the AAD-12 western blot analysis, no immunoreactive proteins were detected in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were detected in the samples from the transgenic plant. Lack of alternate size proteins in the AAD-12 sample derived from DAS-444Ø6-6 soybean tissue in the western analysis indicates the absence of any glycosylated form of AAD-12. This result is consistent with the outcome of the glycosylation study on the AAD-12 protein of DAS-444Ø6-6 soybean.

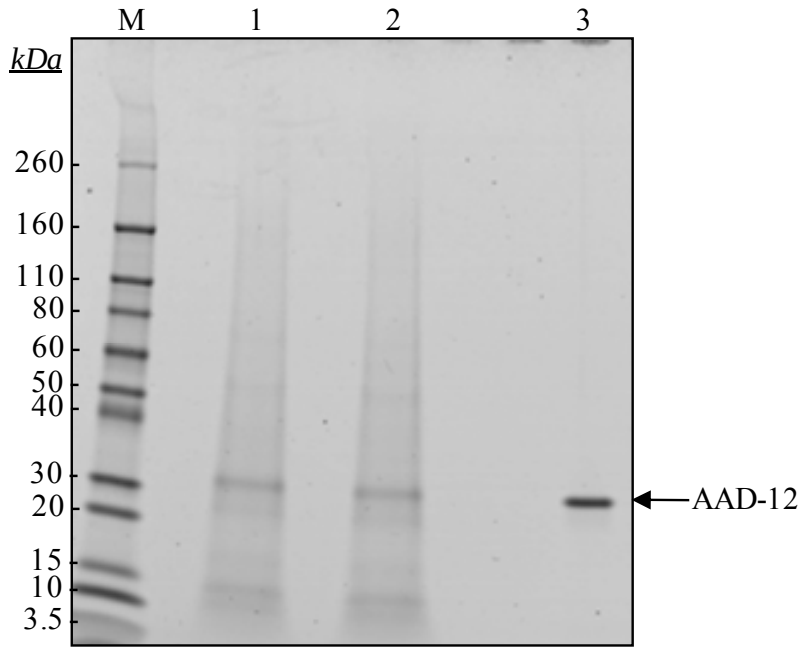


Figure 32. SDS-PAGE of DAS-444Ø6-6 soybean- and microbe-derived AAD-12.

Note: Affinity-purified soybean-derived AAD-12 and microbe-derived AAD-12 were separated by SDS-PAGE. Following electrophoresis, the gel was stained with Thermo-Pierce GelCode Blue stain for total protein according to the manufacturer's protocol. The protein fractions purified from DAS-444Ø6-6 tissue 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.

Lane	Sample	Amount Loaded
M	Novex Sharp MW Markers	5 µL
1	DAS-444Ø6-6 derived AAD-12	20 µL
2	DAS-444Ø6-6 derived AAD-12	20 µL
3	Microbe-derived AAD-12	780 ng

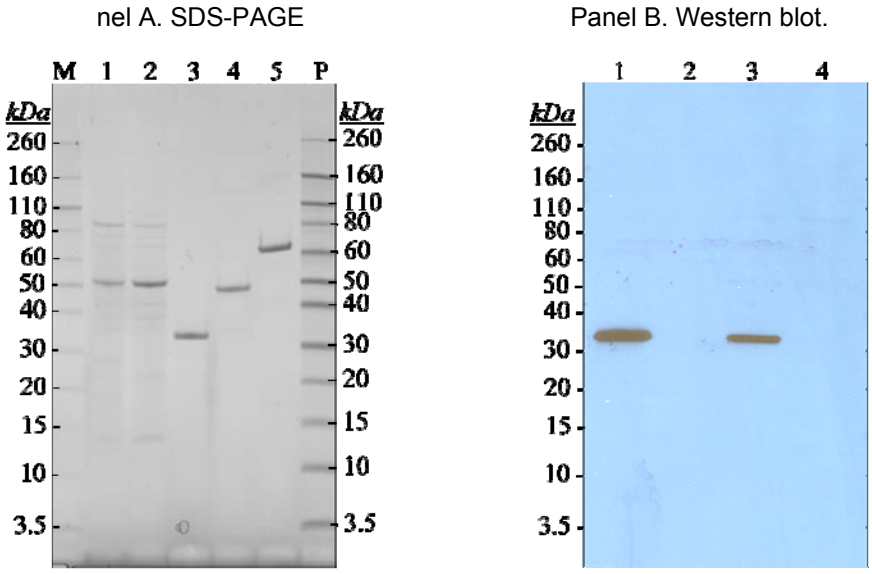


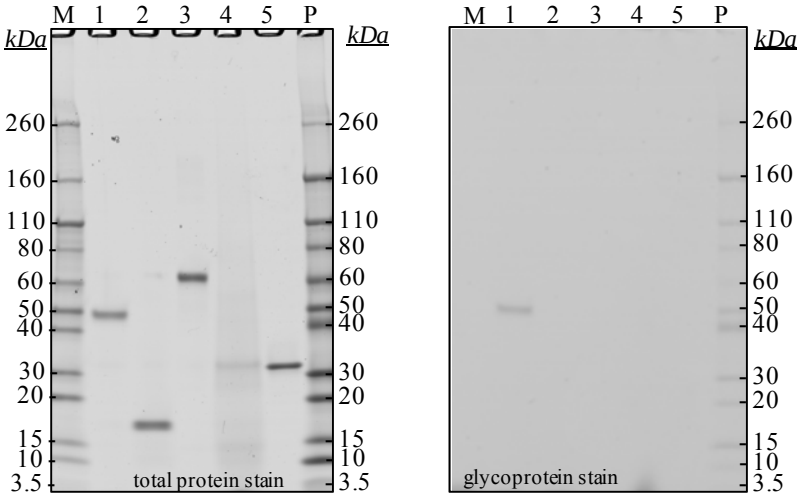
Figure 33. SDS-PAGE and western blot analysis of DAS-444Ø6-6 soybean- and microbe-derived AAD-12 protein.

Note: Crude extracts from fresh leaf tissue of event DAS-444Ø6-6 and Maverick were separated by SDS-PAGE along with microbe-derived AAD-12 and bovine serum albumin. Following electrophoresis the gel was cut in half, one half was stained with Thermo Pierce GelCode Blue stain for total protein and the remaining half was electro-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was then probed with AAD-12 specific polyclonal antibodies and detected with HRP-labelled antibodies. A chemiluminescent substrate was used to visualize the immunoreactive bands.

Panel A.			Panel B.		
Lane	Sample	Amount Loaded	Lane	Sample	Amount Loaded
M	Novex Sharp Unstained MW Marker	10 µL	*kDa*	Novex Pre-stained MW Marker	
1	Non-transgenic (Maverick) Leaf Extract	30 µL	1	Microbe-derived AAD-12 (TSN030732)	1 ng
2	DAS-444Ø6-6 Soybean Leaf Extract	30 µL	2	Bovine Serum Albumin (BSA)	1 ng
3	Microbe-derived AAD-12 (TSN030732)	1.1 µg	3	DAS-444Ø6-6 Soybean Leaf Extract	10 µL
4	Microbe-derived 2mEPSPS (TSN033171)	1.0 µg	4	Non-transgenic (Maverick) Leaf Extract	10 µL
5	Bovine Serum Albumin (BSA)	1.1 µg			
P	Novex Pre-stained MW Marker	10 µL			

Results of Detection of Glycosylation of AAD-12 Protein

Glycoproteins were not detected in the DAS-444Ø6-6 soybean-derived or microbe-derived AAD-12 samples (Figure 34). The result indicates that the AAD-12 protein was not modified with covalently bound carbohydrate moieties. In this study horseradish peroxidase, a glycoprotein, was used as a positive indicator for glycoprotein staining. Soybean trypsin inhibitor and bovine serum albumin, both non-glycoproteins, served as negative controls.



Note: The Novex Sharp protein standard does not appear on the glycoprotein stained gels as they do not contain glycoproteins.

Figure 34. Glycosylation analysis of DAS-444Ø6-6 soybean- and microbe-derived AAD-12 proteins.

Note: The affinity-purified AAD-12 derived from event DAS-444Ø6-6, microbe-derived AAD-12, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted to a similar concentration prior to separation by SDS-PAGE. After electrophoresis, the gel was cut in half and one half was stained with Thermo Pierce GelCode Blue stain for total protein, and the other half of the gel was stained with a Thermo Pierce GelCode Glycoprotein Staining Kit to visualize the glycoproteins.

Lane	Sample	Amount Loaded
M	Novex Sharp Protein Standard	10 µL
1	Horseradish peroxidase (+ control)	~500 ng
2	Soybean trypsin inhibitor (- control)	~500 ng
3	Bovine serum albumin (- control)	~500 ng
4	DAS-444Ø6-6 derived AAD-12	~200 ng
5	*P. fluorescens* derived AAD-12	~500 ng
P	Novex Pre-stained MW Marker	10 µL

Results of MALDI-TOF MS and MALDI-TOF MS/MS tryptic and chymotryptic peptide mass fingerprints of DAS-444Ø6-6-derived AAD-12 protein

Following digestion of the DAS-444Ø6-6-derived AAD-12 protein by trypsin and chymotrypsin, 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 35 illustrates the theoretical peptide cleavage which was generated *in silico* using PAWs software (Proteometrics LLC).

In the trypsin and chymotrypsin digestions of soybean-derived AAD-12 protein, the peptide sequence coverage was excellent (84.3%) and 76.1% of the peptide primary sequence was confirmed by MS/MS analysis (Figure 36). The detected peptide fragments covered nearly the entire protein sequence lacking only four peptide fragments (Figure 36), one near the N-terminus (L^{40} to L^{45}), two in the middle of the protein (Q^{89} to Y^{114} and S^{125} to F^{138}), and one near the C-terminus (I^{211} to R^{213}). The peptide sequence that was missed did not contain sequence motif that are typically required for glycosylation (Asn-Xxx-Ser/Thr (Hamby and Hirst, 2008)).

Digestion at K (lysine) and R (arginine)

Digestion at F (phenylalanine), L (leucine), W (tryptophan), and Y (tyrosine)

Figure 35. Theoretical trypsin (top panel) and chymotrypsin (bottom panel) cleavage of the AAD-12 protein.

Note: Alternating blocks of upper (black) and lower (red) case letters within the amino acid sequence are used to differentiate the potential peptides after protease digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
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	30	27
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	24	20
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	28	22
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	6	0
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	16	16
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	30	30
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	30	30
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	30	27
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	30	28
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	23	23
																															Tot. a.a. covered =	*247*	*223*

A = Tryptic MS coverage
A = Tryptic MS-MS data
A = Chymotryptic MS coverage
A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 84.3 %
MALDI MS-MS sequence coverage = 76.1 %

Figure 36. Overall sequence coverage of trypsin and chymotrypsin digests for DAS-444Ø6-6-derived AAD-12 protein by MALDI-TOF MS and MALDI TOF-TOF.

Cys residues were alkylated with iodoacetamide. Overall sequence coverage was 84.3% with peptide mass fingerprint data and 76.1% by tandem MS data. Two forms of the N-terminus were detected. The N-terminal peptide was detected with Met[1] intact and removed by an aminopeptidase. The peptide with Met[1] missing was acetylated at Ala[2].

Results of MALDI-TOF MS/MS N-and C-terminal sequence analysis of AAD-12

The amino acid residues at the N-and C-termini of the soybean-derived AAD-12 protein (immunoaffinity purified from DAS-444Ø6-6 soybean) were determined and compared with the sequence of the microbe-derived protein. The soybean-derived AAD-12 protein sequences were determined by MALDI-TOF MS/MS. The chymotrypsin and trypsin digestions were performed on the soybean-derived AAD-12 protein followed by mass spectrometry analysis and two forms of the N-terminus were determined (Table 6).

Table 6. Summary of N-terminal sequence data of AAD-12 soybean- and microbe-derived proteins.

Source	Expected N-terminal Sequence[a]
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^{27}
Soybean Event DAS-444Ø6-6	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^{27}

Source	Detected N-terminal Sequence[b,c,d]
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^{27}
Soybean Event DAS-444Ø6-6	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^{27}
Soybean Event DAS-444Ø6-6[4]	$^{N\text{-}Ac}A^2$ Q T T L Q I T P T G A T L G A T V T G V H L A T^{27}

Notes:

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

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

[c]Numbers in superscript (R^x) indicate the amino acid residue number in the sequence. The N-terminal amino acid sequence was confirmed by peptide mass fingerprinting and MS/MS sequencing.

[d]The MALDI-TOF MS/MS data for the N-terminal peptide revealed that the soybean-derived AAD-12 protein had a portion of the peptide that was acetylated (*N-Acetyl*-AQTTL).

Amino acid residue abbreviations:

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

These results demonstrate that the N-terminus of the AAD-12 protein was intact and the amino acid sequence was as predicted (Table 6 and Figure 36). In addition, a portion of the protein extracts was missing the N-terminal methionine and the second amino acid, alanine, was acetylated (Table 6). This result is encountered frequently with eukaryotic (plant) expressed proteins as approximately 80-90% of the N-terminal residues are modified in such a way (Wellner, Panneerselvam et al. 1990; Polevoda and Sherman 2003). This result indicates that during or after translation in soybean and *P. fluorescens*, the N-terminal methionine was cleaved by a methionine aminopeptidase (MAP). MAPs cleave methionyl residues rapidly when the second residue on the protein is small, such as Gly, Ala, Ser, Cys, Thr, Pro, and Val (Walsh 2005). Also, it has been shown that proteins with serine and alanine at the N-termini are most frequently acetylated (Polevoda and Sherman 2002). The two co-translational processes, cleavage of N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman 2002). However, examples demonstrating biological significance associated with N-terminal acetylation are rare (Polevoda and Sherman 2000).

The C-terminal sequence of the soybean- and microbe-derived AAD-12 proteins were determined essentially as described above and compared with the expected amino acid sequences. The results indicated the detected sequences were identical to the expected sequences, and both the soybean- and microbe-derived AAD-12 proteins were identical and unaltered at the C-terminus (Table 7, Figure 36, and Figure 37).

Table 7. Summary of C-terminal sequence data of DAS-444Ø6-6- and P. fluorescens-derived proteins.

Source	Expected C-terminal Sequence[a]
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-444Ø6-6	^{281}L A G R P E T E G A A L V^{293}

Source	Detected C-terminal Sequence[b]
P. fluorescens	^{281}L A G R P E T E G A A L V^{293}
Soybean Event DAS-444Ø6-6	^{281}L A G R P E T E G A A L V^{293}

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

[b]Detected C-terminal sequences of *P. fluorescens*- and DAS-444Ø6-6-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		

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
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	29	29
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	30	21
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	30	30
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	30	30
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	30	30
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	30	22
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	30	30
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	30	30
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	30	30
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	23	23
																															Tot. a.a. covered =	*292*	*275*

A = PMF MS coverage (MS)
A = ISD Coverage (MS/MS)

MALDI PMF sequence coverage = 100.0 %
MALDI MS-MS sequence coverage = 94.2 %

Figure 37. Overall sequence coverage of trypsin, chymotrypsin, Arg-C, Asp-N, and Glu-C digests for *P. fluorescens*-derived AAD-12 protein by MALDI-TOF MS and ISD MS/MS.

Cys residues were alkylated with iodoacetamide. Overall sequence coverage was 100% (taking into account the post-translational removal of Met[1]) with peptide mass fingerprint data and 94.2% by in-source decay MS/MS data. The N-terminal Met[1] was removed by an aminopeptidase.

Methods and Results for Characterization of 2mEPSPS Protein

DAS-444Ø6-6 transgenic soybean material

Greenhouse-grown DAS-444Ø6-6 soybean plants (T4 generation) were used as the plant source of the 2mEPSPS protein. Prior to use, individual plants were analyzed by a lateral flow strip assay (American Bionostica Inc.) to confirm the presence of the AAD-12 protein (through inference, the plants were considered to contain 2mEPSPS as it is part of the molecular stack) using a rapid lateral flow test strip according to the manufacturer's instructions (Schafer *et al.*, 2011). Tissues from 2mEPSPS 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-444Ø6-6 soybean plants, but did not contain the *2mepsps* gene. Seeds of the Maverick soybean line were planted, grown, harvested, tested, processed, and stored under the same conditions as the transgenic plants described above.

Reference material

Recombinant 2mEPSPS protein was produced in *Pseudomonas fluorescens* and purified to a lyophilized powder (Lin *et al.*, 2011). The microbe-derived 2mEPSPS protein preparation (Lot Number: DMMG_033110) was stored dry as a lyophilized powder and resuspended in a HEPES based buffer to maintain activity prior to use.

Protein purification of 2mEPSPS from DAS-444Ø6-6 soybean plant tissue

The 2mEPSPS protein was extracted from lyophilized leaf tissue with a HEPES-based buffer (see Table 88 for buffer components). The tissue was blended and the extract was filtered through cheesecloth and the filtrate was collected and clarified by centrifugation at 10,000 ×g. Ammonium sulfate was added to the clarified extract to a final concentration of 2 M and the solution was then centrifuged, filtered and loaded onto a 1 mL Phenyl HP Hi-trap column (GE Healthcare) equilibrated with 50 mM HEPES, 2.0 M ammonium sulfate, pH 7.0. After loading, the column was washed with the same buffer and the bound proteins were eluted with a gradient to 100% 50 mM HEPES, pH 7.0. The eluted proteins were collected and assayed for 2mEPSPS content by western blot using a mouse monoclonal antibody raised against the microbe-derived 2mEPSPS protein. Fractions containing 2mEPSPS were pooled and desalted into 50 mM HEPES, pH 7.0 buffer using a PD-10 desalting column (GE Healthcare) according to manufacturer's protocol. The Phenyl HP Pool was then loaded onto a 1 mL Q Sepharose FF Hi-Trap Column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.0. After loading, the column was washed and eluted with a gradient to 100% 50 mM HEPES, 500 mM NaCl, pH 7.0. The eluted proteins were collected and the fractions were assayed for 2mEPSPS content by western blot as described above. Fractions containing 2mEPSPS were pooled and desalted into 50 mM HEPES, pH 7.0 buffer using a PD-10 desalting column. The desalted Q-pool was then loaded onto a 5 mL Blue Sepharose HP Hi-Trap column (GE Healthcare) equilibrated with 50 mM HEPES, pH 7.0 buffer. The column was washed and the bound protein was eluted with a gradient to 100% 50 mM HEPES, 500 mM NaCl, pH 7.0. Fractions were collected and assayed for 2mEPSPS content by Coomassie staining a SDS-PAGE gel. The fraction containing the highest level of 2mEPSPS by visual inspection was concentrated to ~130 µL using a 10 kDa MWCO centrifugal filter device (Amicon) according to the manufacture's protocol. The final purified sample was held at 4°C until used for subsequent analyses.

Table 88. Composition of extraction buffer for soybean-derived 2mEPSPS.

Ingredient	Supplier	Final Concentration
HEPES	Fisher	50 mM
Sodium Ascorbate	Sigma	20 mM
Sodium Metabisulfite	Sigma	10 mM
Polyvinylpyrrolidone	Sigma	1.0%
Protease Inhibitor Cocktail	Sigma	0.5%

Notes:

a. The buffer pH was adjusted to 7.0 before bringing buffer up to final volume.

b. The buffer was made fresh the day of use.

SDS-PAGE and polyclonal antibody western blot analysis of crude soybean leaf extracts

The soybean leaf tissues of the transgenic DAS-444Ø6-6 soybean and nontransgenic isoline were harvested fresh from the greenhouse on the day of testing. Extracts were prepared by grinding the tissue with steel ball bearings in a Tris-based buffer (Table 9) using a Geno/Grinder (Certiprep, Metuchen, NJ). The supernatants collected were mixed with Laemmli sample buffer (containing □-mercaptoethanol) heated/denatured and loaded directly on the gel with a positive reference standard (microbe-derived 2mEPSPS - TSN033171-0001), and control standard BSA (Thermo-Pierce). SDS-PAGE separation of the recombinant 2mEPSPS, BSA, non-transgenic Maverick, and DAS-444Ø6-6 soybean extracts was performed with Bio-Rad Criterion gels fitted in a Criterion Cell gel module with XT-MES running buffer (Bio-Rad). Two identical gels were prepared and the electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes. After separation, one gel was stained with Thermo Scientific GelCode Blue protein stain and scanned with a densitometer (GE Healthcare) to obtain a permanent record of the image. The remaining gel was electro-blotted to a nitrocellulose membrane (Bio-Rad) and probed with a 2mEPSPS-specific rabbit polyclonal antibody (Lot #: G2874, 3.3 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) antibody and horseradish peroxidase (Thermo-Pierce) was used as the secondary detection antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to CL-XPosure detection film (Thermo Scientific) for various time points and subsequently developed with an All-Pro 100 film developer.

Table 9. Western blot extraction buffer.

Ingredient	Supplier	Final Concentration
1M Tris-HCl	Sigma	50 mM
Sodium Ascorbate	Sigma	20 mM
Sodium Metabisulfite	Sigma	10 mM
NaCl	N/A	250 mM
PVP	Sigma	0.7%
2-mercaptoethanol	Bio-Rad	0.2%
Protease Inhibitor Cocktail	Sigma	3.3%

Notes:

a. The buffer pH was adjusted to 8.0 before bringing buffer up to final volume.

b. The buffer was made fresh the day of use.

SDS-PAGE and monoclonal antibody western blot analysis of the 2mEPSPS protein

The soybean leaf tissues of the transgenic event and non-transgenic isoline were harvested fresh from the greenhouse and stored at -80°C until used for testing. On the day of analysis, soybean leaf material was ground in liquid nitrogen, transferred to a micro-centrifuge tube, and the soluble proteins were extracted using a Geno/Grinder (Certiprep, Metuchen, NJ). The supernatants were clarified by centrifugation, mixed with Laemmli sample buffer (Bio-Rad, containing freshly added □ -mercaptoethanol) and heated at 95°C for 5 minutes. After a brief centrifugation, the resulting supernatants were loaded directly on the gel with a reference standard, 2mEPSPS (TSN033171-0001), and control standard, BSA (Thermo-Pierce). SDS-PAGE and western blot analysis of the recombinant 2mEPSPS, BSA, non-transgenic Maverick, and DAS-444Ø6-6 soybean extracts were performed with Bio-Rad Criterion gels fitted in a Criterion Cell gel module with XT-MES running buffer (Bio-Rad). One gel was prepared and the electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes. After separation the gel was cut in half and one half of the gel was stained with Thermo Scientific GelCode Blue protein stain and scanned with a densitometer (GE Healthcare) to obtain a permanent record of the image. The remaining gel half was transferred to a nitrocellulose membrane and probed with a 2mEPSPS-specific mouse monoclonal antibody (Lot #: 609.48A-2-4, 2.1 mg.mL). A conjugate of goat anti-mouse IgG (H+L) antibody and horseradish peroxidase (Thermo-Pierce) was used as the secondary detection antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to CL-XPosure detection film (Thermo Scientific) for various time points and subsequently developed with an All-Pro 100 film developer.

Detection of post-translational glycosylation

The purified, plant-derived 2mEPSPS protein was analyzed for evidence of glycosylation by SDS-PAGE along with microbe-derived 2mEPSPS protein. Soybean trypsin inhibitor, bovine serum albumin, and

horseradish peroxidase were added as reference controls. The reference protein samples were adjusted to concentrations approximately equal to that of the plant-derived 2mEPSPS protein and mixed with Laemmli buffer. The proteins were heated at 95°C for 5 minutes, centrifuged at 20,000×g for 2 minutes, and applied directly to a Bio-Rad Criterion SDS-PAGE gel and electrophoresed as described above. Following electrophoresis, the gel was cut in half and one half of the gel was stained with Thermo Pierce GelCode Blue stain for total protein. The remaining half was stained with GelCode Glycoprotein Stain to visualize glycoproteins according to the manufacture's protocol. 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 2mEPSPS proteins

The purified plant-derived 2mEPSPS protein was subjected to in-gel and in-solution digestion by trypsin and chymotrypsin followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF MS-MS (Schafer *et al.*, 2011). The peptide fragments of the plant-derived 2mEPSPS protein (including the N- and C-termini) were analyzed and compared with the sequence of the microbe-derived protein (Karnoup and Kuppannan, 2010; Schafer *et al.*, 2011).

Results of the SDS-PAGE and western blot analysis

SDS-PAGE

In the microbe-derived 2mEPSPS sample, the major protein band, as visualized on the Coomassie stained SDS-PAGE gel, was approximately 47 kDa (Figure38). As expected, the corresponding plant-derived 2mEPSPS 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 2mEPSPS 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; Williams *et al.*, 2006).

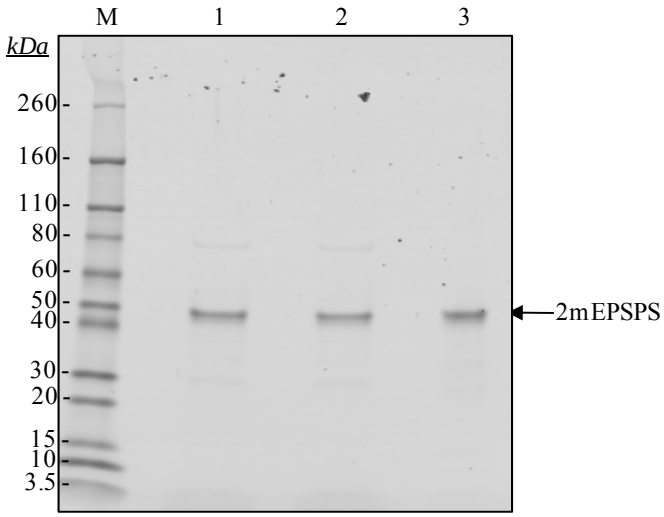


Figure 38. SDS-PAGE of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS.

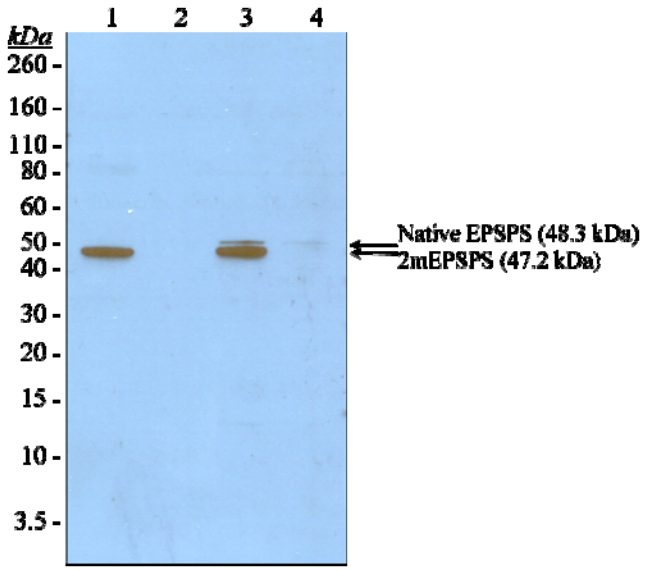
Note: The purified soybean-derived 2mEPSPS and microbe-derived 2mEPSPS were separated by SDS-PAGE. Following electrophoresis, the gel was stained with Thermo-Pierce GelCode Blue stain for total protein according to the manufacturer's protocol.

Lane	Sample	Amount Loaded
M	Novex Sharp Protein Standard	10 µL
1	DAS-444Ø6-6 derived 2mEPSPS	~500 ng
2	DAS-444Ø6-6 derived 2mEPSPS	~500 ng
3	*P. fluorescens* derived 2mEPSPS	520 ng

Western blot

The microbe-derived 2mEPSPS and DAS-444Ø6-6 plant tissue extract showed a positive signal of the expected size on the western blot using an anti-2mEPSPS polyclonal antibody (Figure 393839, Panel A). In the 2mEPSPS western blot analysis, the native soybean EPSPS protein (48.3 kDa) was also observed in the control Maverick extract as well as in the transgenic event DAS-444Ø6-6 extract. This result was expected as the native soybean endogenous EPSPS has 76% homology to the 2mEPSPS protein and likely cross-reacted with the polyclonal antibody. To prove this hypothesis, a monoclonal antibody (lot #: 609.48A-2-4) was used to probe the soybean leaf extracts. As a result, only the recombinant 2mEPSPS protein was detected in the microbe-derived 2mEPSPS preparations and DAS-444Ø6-6 tissue, with no immunoreactive proteins observed in the Maverick control extract (Figure 393839, Panel B). This result indicated that the polyclonal antibody was reacting to the native EPSPS protein at the expected molecular weight (48.3 kDa). In addition, in both the polyclonal and monoclonal western blot analyses, no alternate size proteins (aggregates or degradation products) were seen in the transgenic samples. These results add to the evidence that the protein expressed in soybean is not glycosylated or post-translationally modified which would add to the overall protein molecular weight.

Panel A. Polyclonal antibody western blot



Panel B. Monoclonal antibody western blot

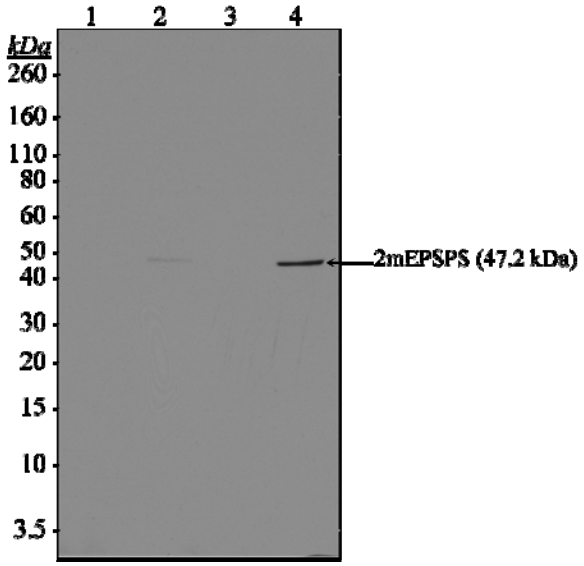


Figure 3938. Western blot of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS protein.

Note: Crude extracts from leaf tissue of DAS-444Ø6-6 soybean and Maverick were separated by SDS-PAGE along with microbe-derived 2mEPSPS and bovine serum albumin. The nitrocellulose membranes were then probed with 2mEPSPS specific polyclonal and monoclonal antibodies and detected with HRP-labeled antibodies. A chemiluminescent substrate was used to visualize the immunoreactive bands.

Panel A.			Panel B.		
Lane	Sample	Amount Loaded	Lane	Sample	Amount Loaded
kDa	Novex Pre-stained MW Marker		*kDa*	Novex Pre-stained MW Marker	
1	Microbe-derived 2mEPSPS	~1 ng	1	Bovine Serum Albumin (BSA)	~1 ng

	(TSN033171)				
2	Bovine Serum Albumin (BSA)	~1 ng	2	Microbe-derived 2mEPSPS (TSN033171)	~1 ng
3	DAS-444Ø6-6 Soybean Leaf Extract	10 µL	3	Non-transgenic (Maverick) Leaf Extract	10 µL
4	Non-transgenic (Maverick) Leaf Extract	10 µL	4	DAS-444Ø6-6 Soybean Leaf Extract	10 µL

Results of detection of glycosylation of 2mEPSPS protein

Detection of carbohydrates, possibly covalently linked to soybean-derived 2mEPSPS proteins, was assessed by the GelCode Glycoprotein Staining Kit from Thermo-Pierce. The purified soybean-derived 2mEPSPS protein was electrophoresed simultaneously with a set of control and reference protein standards. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation, and non-glycoproteins; microbe-derived 2mEPSPS, soybean trypsin inhibitor, and bovine serum albumin, were employed as negative reference controls. The results showed that the soybean- and microbe-derived 2mEPSPS proteins had no detectable covalently linked carbohydrates (Figure 4039).

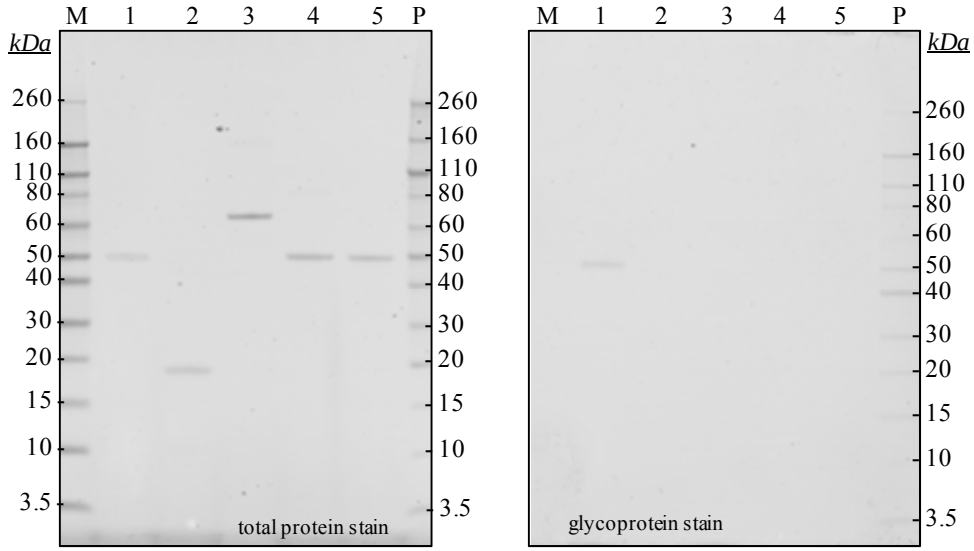


Figure 4039. Glycosylation analysis of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS proteins.

Note: The Novex Sharp protein standard does not appear on the glycoprotein stained gels as they do not contain glycoproteins.

Note: The purified DAS-444Ø6-6-derived 2mEPSPS, microbe-derived 2mEPSPS, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted to a similar concentration prior to separation by SDS-PAGE. After electrophoresis, the gel was cut in half and one half was stained with Thermo Pierce GelCode Blue stain for total protein, and the other half of the gel was stained with a Thermo Pierce GelCode Glycoprotein Staining Kit to visualize the glycoproteins.

Lane	Sample	Amount Loaded
M	Novex Sharp Protein Standard	10 µL
1	Horseradish peroxidase (+ control)	~500 ng
2	Soybean trypsin inhibitor (- control)	~500 ng

3	Bovine serum albumin (- control)	~500 ng
4	DAS-444Ø6-6 derived 2mEPSPS	~500 ng
5	*P. fluorescens* derived 2mEPSPS	~500 ng
P	Novex Pre-stained MW Marker	10 µL

Results of MALDI-TOF MS and MALDI-TOF MS/MS tryptic and chymotryptic peptide mass fingerprints of DAS-444Ø6-6-derived 2mEPSPS protein

The 2mEPSPS protein derived from the tissue of the transgenic soybean event DAS-444Ø6-6 was separated by SDS-PAGE (Figure). The band corresponding to the size of 2mEPSPS was excised and subjected to in-gel digestion by trypsin and chymotrypsin. In addition, the protein was subject to in solution digestion with the same enzymes. The resulting peptide mixture was analyzed by MALDI-TOF and MALDI-TOF MS/MS to determine the peptide sequences and protein identity. The masses of the detected peptides were compared to those deduced based on potential trypsin or chymotrypsin cleavage sites in the sequence of the soybean-derived 2mEPSPS protein. Figure 440. Theoretical trypsin (top panel) and chumotrypsin (bottom panel) cleavage of the 2mEPSPS protein

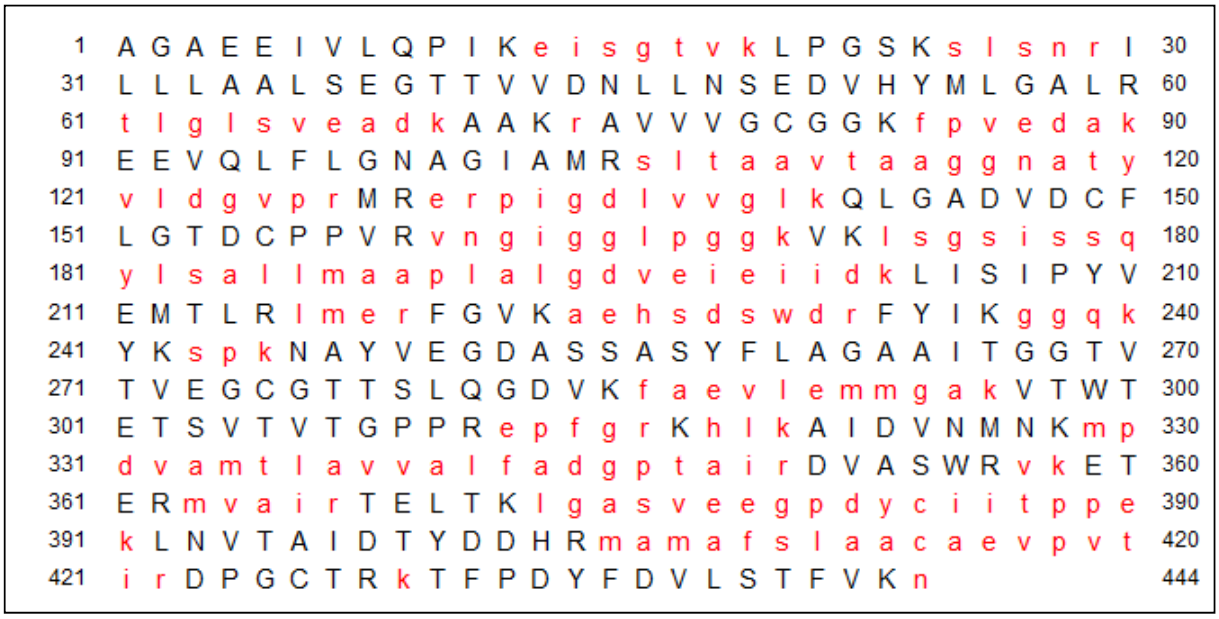
41 illustrates the theoretical cleavage of the 2mEPSPS protein when subjected to endoprotease digestion *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC.

The theoretical and observed amino acid digest (and molecular weights) of the soybean-derived 2mEPSPS protein are also described in (Schafer *et al.*, 2011). The 2mEPSPS protein, once denatured, is readily digested by endoproteases and will generate numerous peptide peaks.

In the endoproteinase digest of the transgenic-soybean-derived 2mEPSPS protein, the peptide sequence coverage was excellent (86.3%) and 70.0% of the peptide primary sequence was confirmed by MS/MS analysis (Figure 42). The detected peptide fragments covered nearly the entire protein sequence lacking only six peptide fragments (Figure), two near the N-terminus (S^{65} to K^{70} and A^{75} to K^{83}), three in the middle of the protein (V^{249} to Y^{258}, A^{287} to K^{296}, and A^{321} to K^{328}), and one near the C-terminus (M^{405} to R^{422}). The peptide fragments that were not detected in this study did not contain sequence motifs that are typically required for glycosylation (Asn-Xxx-Ser/Thr, (Hamby and Hirst, 2008)).

This analysis confirmed the soybean-derived protein amino acid sequence matched that of the microbe-derived 2mEPSPS protein (Figure 4341) at both the N- and C-terminus as well as a major portion of the internal sequence (Karnoup and Kuppannan, 2010). In the MS chromatograms, there were several unidentified peptides detected in the enzyme digest preparations (as discussed earlier). Results of these analyses indicate that the amino acid sequence of the soybean-derived 2mEPSPS protein was equivalent to the *P. fluorescens*-expressed protein characterized earlier (Karnoup and Kuppannan, 2010; Schafer, 2010).

Digestion at K (lysine) and R (arginine)



Digestion at F (phenylalanine), L (leucine), W (tryptophan), and Y (tyrosine)

Figure 440. Theoretical trypsin (top panel) and chumotrypsin (bottom panel) cleavage of the 2mEPSPS protein

Note: Alternating blocks of upper (black) and lower (red) case letters within the amino acid sequence are used to differentiate the potential peptides after protease digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	A	G	A	E	E	I	V	L	Q	P	I	K	E	I	S	G	T	V	K	L	P	G	S	K	S	L	S	N	R	I	30	30	30
31	L	L	L	A	A	L	S	E	G	T	T	V	V	D	N	L	L	N	S	E	D	V	H	Y	M	L	G	A	L	R	60	30	29
61	T	L	G	L	S	V	E	A	D	K	A	A	K	R	A	V	V	V	G	C	G	G	K	F	P	V	E	D	A	K	90	15	15
91	E	E	V	Q	L	F	L	G	N	A	G	I	A	M	R	S	L	T	A	A	V	T	A	A	G	G	N	A	T	Y	120	30	30
121	V	L	D	G	V	P	R	M	R	E	R	P	I	G	D	L	V	V	G	L	K	Q	L	G	A	D	V	D	C	F	150	30	30
151	L	G	T	D	C	P	P	V	R	V	N	G	I	G	G	L	P	G	G	K	V	K	L	S	G	S	I	S	S	Q	180	30	27
181	Y	L	S	A	L	L	M	A	A	P	L	A	L	G	D	V	E	I	E	I	I	D	K	L	I	S	I	P	Y	V	210	30	30
211	E	M	T	L	R	L	M	E	R	F	G	V	K	A	E	H	S	D	S	W	D	R	F	Y	I	K	G	G	Q	K	240	30	30
241	Y	K	S	P	K	N	A	Y	V	E	G	D	A	S	S	A	S	Y	F	L	A	G	A	A	I	T	G	G	T	V	270	20	8
271	T	V	E	G	C	G	T	T	S	L	Q	G	D	V	K	F	A	E	V	L	E	M	M	G	A	K	V	T	W	T	300	20	7
301	E	T	S	V	T	V	T	G	P	P	R	E	P	F	G	R	K	H	L	K	A	I	D	V	N	M	N	K	M	P	330	22	20
331	D	V	A	M	T	L	A	V	V	A	L	F	A	D	G	P	T	A	I	R	D	V	A	S	W	R	V	K	E	T	360	30	14
361	E	R	M	V	A	I	R	T	E	L	T	K	L	G	A	S	V	E	E	G	P	D	Y	C	I	I	T	P	P	E	390	30	23
391	K	L	N	V	T	A	I	D	T	Y	D	D	H	R	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420	14	2
421	I	R	D	P	G	C	T	R	K	T	F	P	D	Y	F	D	V	L	S	T	F	V	K	N							444	22	16
																															Tot. a.a. covered =	***383***	***311***

A = Tryptic MS coverage

A = Tryptic MS-MS data

A = Chymotryptic MS coverage

A = Chymotryptic MS-MS data

MALDI PMF sequence coverage = 86.3 %

MALDI MS-MS sequence coverage = 70.0 %

Figure 42. Sequence coverage of trypsin and chymotrypsin digests for DAS-444Ø6-6-derived 2mEPSPS protein by MALDI-TOF MS and MALDI TOF-TOF.

Cys residues were alkylated with iodoacetamide. Overall sequence coverage was 86.3% with peptide mass fingerprint data and 70.0% by tandem MS data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		Peptides covered by PMF	Peptides covered by MS-MS
1	A	G	A	E	E	I	V	L	Q	P	I	K	E	I	S	G	T	V	K	L	P	G	S	K	S	L	S	N	R	I	30	30	30
31	L	L	L	A	A	L	S	E	G	T	T	V	V	D	N	L	L	N	S	E	D	V	H	Y	M	L	G	A	L	R	60	30	30
61	T	L	G	L	S	V	E	A	D	K	A	A	K	R	A	V	V	V	G	C	G	G	K	F	P	V	E	D	A	K	90	27	27
91	E	E	V	Q	L	F	L	G	N	A	G	I	A	M	R	S	L	T	A	A	V	T	A	A	G	G	N	A	T	Y	120	30	30
121	V	L	D	G	V	P	R	M	R	E	R	P	I	G	D	L	V	V	G	L	K	Q	L	G	A	D	V	D	C	F	150	30	30
151	L	G	T	D	C	P	P	V	R	V	N	G	I	G	G	L	P	G	G	K	V	K	L	S	G	S	I	S	S	Q	180	28	28
181	Y	L	S	A	L	L	M	A	A	P	L	A	L	G	D	V	E	I	E	I	I	D	K	L	I	S	I	P	Y	V	210	30	30
211	E	M	T	L	R	L	M	E	R	F	G	V	K	A	E	H	S	D	S	W	D	R	F	Y	I	K	G	G	Q	K	240	26	26
241	Y	K	S	P	K	N	A	Y	V	E	G	D	A	S	S	A	S	Y	F	L	A	G	A	A	I	T	G	G	T	V	270	25	6
271	T	V	E	G	C	G	T	T	S	L	Q	G	D	V	K	F	A	E	V	L	E	M	M	G	A	K	V	T	W	T	300	30	27
301	E	T	S	V	T	V	T	G	P	P	R	E	P	F	G	R	K	H	L	K	A	I	D	V	N	M	N	K	M	P	330	30	30
331	D	V	A	M	T	L	A	V	V	A	L	F	A	D	G	P	T	A	I	R	D	V	A	S	W	R	V	K	E	T	360	30	30
361	E	R	M	V	A	I	R	T	E	L	T	K	L	G	A	S	V	E	E	G	P	D	Y	C	I	I	T	P	P	E	390	30	30
391	K	L	N	V	T	A	I	D	T	Y	D	D	H	R	M	A	M	A	F	S	L	A	A	C	A	E	V	P	V	T	420	30	30
421	I	R	D	P	G	C	T	R	K	T	F	P	D	Y	F	D	V	L	S	T	F	V	K	N							444	30	30
																															Tot. a.a. covered =	*436*	*414*

A = PMF MS coverage (MS)
A = ISD Coverage (MS/MS)

MALDI PMF sequence coverage = 98.2 %
MALDI MS-MS sequence coverage = 93.2 %

Figure 4341. Sequence coverage of trypsin, chymotrypsin, Arg-C, Asp-N, and Glu-C digests for *P. fluorescens*-derived 2mEPSPS protein by MALDI-TOF MS and ISD MS/MS.

Cys residues were alkylated with iodoacetamide. Overall sequence coverage was 98.2% with peptide mass fingerprint data and 93.2% by tandem MS data.

Results of MALDI-TOF MS/MS N- and C-terminal sequence analysis of 2mEPSPS

The amino acid residues at the N-and C-termini of the soybean-derived 2mEPSPS protein (purified from DAS-444Ø6-6 soybean) were determined and compared with the sequence of the previously characterized microbe-derived protein (Karnoup and Kuppannan, 2010). The soybean-derived 2mEPSPS protein sequences were determined by MALDI-TOF MS/MS. The chymotrypsin and trypsin digestions were performed on the soybean-derived 2mEPSPS protein followed by mass spectrometry analysis and the N-terminus was determined to be identical to the expected sequences, and both the soybean- and microbe-derived 2mEPSPS proteins were indistinguishable and unaltered (Table 10 and Figure 440.

Theoretical trypsin (top panel) and chumotrypsin (bottom panel) cleavage of the 2mEPSPS protein

).

Table 10. Summary of N-terminal sequence data of DAS-444Ø6-6 soybean- and P. fluorescens-derived 2mEPSPS.

Source	Expected N-terminal Sequence[a]
P. fluorescens	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}
Soybean Event DAS-444Ø6-6	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}

Source	Detected N-terminal Sequence[b]
P. fluorescens	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}
Soybean Event DAS-444Ø6-6	A^{1} G A E E I V L Q P I K E I S G T V K L P G S K S L S^{27}

Notes:

[a]Expected N-terminal sequence of the first 27 amino acid residues of *P. fluorescens*- and soybean-derived 2mEPSPS.

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

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

Amino acid residue abbreviations:

A:	alanine	E:	glutamic acid	G:	glycine
I:	isoleucine	K:	lysine	L:	leucine
P:	proline	Q:	glutamine	S:	serine
T:	threonine	V:	valine		

The C-terminal sequence of the soybean- and microbe-derived 2mEPSPS proteins was determined essentially as described above and compared with the expected amino acid sequences. The results indicated the measured sequences were identical to the expected sequences, and both the soybean- and microbe-derived 2mEPSPS proteins were indistinguishable and unaltered at the C-terminus (Table and Figure).

Table 11. Summary of C-terminal sequence data of 2mEPSPS soybean- and microbe-derived proteins.

Source	Expected C-terminal Sequence[a]
P. fluorescens	K^{429} T F P D Y F D V L S T F V K N^{444}
Soybean Event DAS-444Ø6-6	K^{429} T F P D Y F D V L S T F V K N^{444}
Source	**Detected C-terminal Sequence[b]**
P. fluorescens	K^{429} T F P D Y F D V L S T F V K N^{444}
Soybean Event DAS-444Ø6-6	K^{429} T F P D Y F D V L S T F V K N^{444}

Notes:

[a]Expected C-terminal sequence of the last 16 amino acid residues of *P. fluorescens*- and soybean-derived 2mEPSPS.

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

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

Amino acid residue abbreviations:

D:	aspartic acid	F:	phenylalanine	K:	lysine
L:	leucine	N:	asparagine	P:	proline
S:	serine	T:	threonine	V:	valine
Y:	tyrosine				

Conclusions

The biochemical analyses confirmed that the 2mEPSPS derived from the leaf tissue of DAS-444Ø6-6 soybean was equivalent to 2mEPSPS purified from *P. fluorescens*. The plant- and microbe-derived 2mEPSPS proteins showed the expected molecular weight of ~47 kDa by SDS-PAGE and were immunoreactive to 2mEPSPS protein specific antibodies by western blot analysis. The amino acid sequence of both proteins was confirmed by enzymatic peptide mass fingerprinting by MALDI-TOF MS. The N- and C-terminus of the protein from the 2 different sources were shown to be identical via MALDI-TOF MS/MS and ESI-LC/MS. In addition, the lack of glycosylation of the plant-derived 2mEPSPS protein

provided additional evidence that the 2mEPSPS protein produced by *P. fluorescens* and DAS-444Ø6-6 soybean are biochemically equivalent.

Methods and Results for Characterization of PAT Protein

DAS-444Ø6-6 Transgenic Soybean Material

Greenhouse-grown DAS-444Ø6-6 soybean plants (T4) were used as the source of the PAT protein. Prior to use, the plant tissue was tested to confirm expression of the PAT protein using a commercially available ELISA kit according to the manufacturer's instructions (EnviroLogix Inc.). Leaves (and some stems) from the PAT expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed (Schafer and Juba, 2011).

Control Soybean Material

The control soybean line (Maverick) had a genetic background representative of the DAS-444Ø6-6 soybean plants, but did not contain the *pat* gene. Prior to use, the absence of the PAT protein in the control plants was confirmed by immunoassay using a commercially available PAT specific ELISA kit. Leaves (and some stems) of control plants were grown, harvested, lyophilized, ground, and stored under the same conditions as the DAS-444Ø6-6 soybean (Schafer and Juba, 2011).

Reference Material

Recombinant PAT protein was produced in *Escherichia coli* and purified to homogeneity by GeneScript (Piscataway, NJ – Identification number: 55238). The *E. coli*-derived PAT protein preparation was aliquoted and stored at -80 ºC to maintain activity (Embrey and Schafer, 2009).

SDS-PAGE and Western Blot Analysis of Crude Extracts

Lyophilized tissue from event DAS-444Ø6-6 and Maverick was mixed with a PBST based buffer containing ~2.0% protease inhibitor cocktail (Sigma) and the soluble proteins were extracted by grinding with ball bearings in a Geno/Grinder (Certiprep, Metuchen, NJ). The samples were centrifuged at 4°C for 5 minutes at 20,000×g and the supernatants were mixed with Laemmli sample buffer, heated at 100°C for 5 minutes, and briefly centrifuged (20,000×g for 2 minutes at 4°C). The positive reference standard (*E. coli*-derived PAT) and negative reference standard (BSA) were also mixed with sample buffer and the resulting supernatants were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel and electrophoresis was conducted with Tris/glycine/SDS buffer. Following separation, the gel was cut in half, with one half stained with Thermo Scientific 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 half probed with a PAT specific polyclonal rabbit antibody and the remaining half probed with a PAT specific monoclonal

antibody. The antibodies were detected with HRP-labeled secondary antibodies. A chemiluminescent substrate (GE Healthcare) was used to visualize the immunoreactive bands.

Results of the SDS-PAGE and Western Blot Analysis

The extracts of lyophilized soybean tissue, microbe-derived PAT protein, and bovine serum albumin (BSA) were separated by SDS-PAGE and visualized by Coomassie stain (Figure). The soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal antibodies and showed the expected band at approximately 21 kDa (Figure). 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. The monoclonal antibody did detect a small amount of a dimer in the microbe-derived PAT preparation. These results add to the evidence that the PAT protein expressed in soybean is not post-translationally modified which would have added to or subtracted from the overall protein molecular weight.

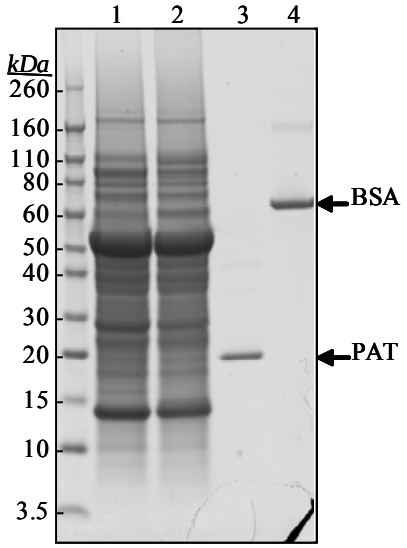


Figure 44. SDS-PAGE of DAS-444Ø6-6 and non-transgenic Maverick soybean extracts, microbe-derived PAT protein.

Lane	Sample	Amount Loaded
M	Novex Prestained MW Markers	10 µL
1	Non-transgenic soybean Maverick	40 µL
2	DAS-444Ø6-6 soybean	40 µL
3	PAT protein standard (TSN031116-0001)	1.2 µg
4	Bovine serum albumin (BSA)	1.5 µg

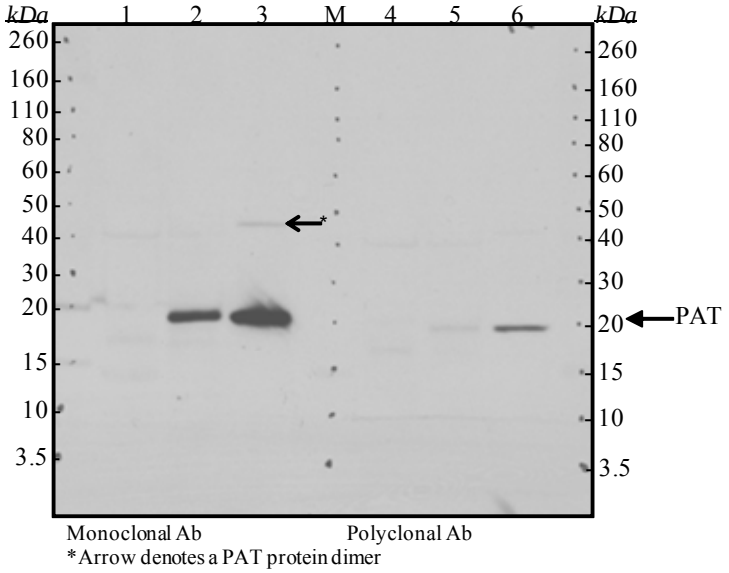


Figure 45. Western blot of DAS-444Ø6-6 and Maverick soybean extracts.

Note: Crude extracts from lyophilized leaf tissue of event DAS-444Ø6-6 and Maverick were separated by SDS-PAGE along with microbe-derived PAT and bovine serum albumin. Following electrophoresis the gel was cut in half, one half was stained with Thermo Scientific GelCode Blue stain for total protein (Figure) and the remaining half was electro-blotted onto a nitrocellulose membrane (Figure). The nitrocellulose membrane was then probed with PAT specific polyclonal and monoclonal antibodies and detected with HRP-labeled antibodies. A chemiluminescent substrate (GE Healthcare) was used to visualize the immunoreactive bands.

Lane	Sample	Amount Loaded
Probed with Monoclonal Antibody		
kDa	Novex Pre-stained MW Markers	10 µL
1	Non-transgenic soybean Maverick	40 µL
2	DAS-444Ø6-6 soybean	40 µL
3	PAT protein standard (TSN031116-0001)	12.1 ng
Probed with Polyclonal Antibody		
M	Novex Prestained MW Markers (not labeled)	10 µL
4	Non-transgenic soybean Maverick	40 µL
5	DAS-444Ø6-6 soybean	40 µL
6	PAT protein standard (TSN031116-0001)	12.1 ng
kDa	Novex Pre-stained MW Markers	10 µL

Conclusions

The PAT protein produced in DAS-444Ø6-6 soybean was shown to be equivalent to that produced in *E. coli* and the characterization results are consistent with the protein expressed in other transgenic crops (USDA, 1996, 2001, 2004, 2005; Schafer and Juba, 2011).

b. Identification of Other Novel Substances

No other novel substances have been identified.

c. Potential Novel Protein Expression

To determine if any novel open reading frames (>45 amino acids) were created and endogenous gene or regulatory element were disrupted by the integration of the *aad-12, 2mepsps* and *pat* expression cassettes into the soybean genome, DNA sequences of the insert and its flanking border regions were cloned and analyzed (Guttikonda 2011). In total, 13659 bp of DAS-444Ø6-6 soybean genomic sequence were confirmed, comprising 1494 bp of the 5′ flanking border sequence, 1885 bp of the 3′ flanking border sequence, and 10280 bp of the DNA insert. In addition, 7762 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 (>45 amino acids) resulted from the DNA insertion in DAS-444Ø6-6 soybean. Sequence comparison of the flanking border with the parental locus indicated that a 4383-bp fragment was deleted in DAS-444Ø6-6 soybean. According to the currently available sequence information, no genes and regulatory elements have been identified in the deleted regions, and there is no evidence to indicate disruption of an endogenous gene or regulatory element due to the integration of DAS-444Ø6-6 insert.

Expression of AAD-12 Protein in Plant Tissues

A field expression study was conducted in the U.S. during 2010. Ten sites (Georgia, Iowa (2 sites), Illinois (2 sites), Indiana, Michigan, Missouri, and Nebraska (2 sites)) were planted with DAS-444Ø6-6 soybean and the conventional control (Maverick). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Five treatments of the DAS-444Ø6-6 soybean (unsprayed, sprayed with 2,4-D, sprayed with glufosinate, sprayed with glyphosate, or sprayed with 2,4-D, glufosinate, and glyphosate) were tested. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at the V5 and V10-12 stages, while root and forage were collected at the R3 stage. Grain was collected at the R8 stage of development (Gaska 2006).

The soluble, extractable AAD-12 protein was measured using a validated enzyme-linked immunosorbent assay (ELISA) method (Smith-Drake, Sosa et al. 2009). 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 following this section.

A summary of the AAD-12 protein concentrations (averaged across sites) in the various soybean matrices is shown in Table . Average expression values ranged from 23.52 ng/mg dry weight in R3 stage root to 121.22 ng/mg dry weight in V10-12 stage leaf tissue. Expression levels were comparable for all treatments. No AAD-12 protein was detected in the control tissues across the ten locations.

Table 12. Expression of AAD-12 in DAS-444Ø6-6 soybean.

Tissue	Treatment	AAD-12 ng/mg Tissue Dry Weight		
		Mean	Std. Dev. (n=10)	Min/Max Range
Leaf V5	DAS-444Ø6-6	112.61	34.05	42.00 – 179.50
	DAS-444Ø6-6 w/ 2,4-D	111.32	27.48	58.62 – 190.50
	DAS-444Ø6-6 w/ Gluf	107.75	29.91	60.00 – 179.50
	DAS-444Ø6-6 w/ Glyp	101.93	29.54	36.58 – 179.50
	DAS-444Ø6-6 w/ All	103.67	34.25	52.50 – 196.50
Leaf V10-12	DAS-444Ø6-6	118.57	36.34	68.00 – 312.00
	DAS-444Ø6-6 w/ 2,4-D	121.22	36.61	58.40 – 279.00
	DAS-444Ø6-6 w/ Gluf	109.29	25.94	64.50 – 170.00
	DAS-444Ø6-6 w/ Glyp	114.73	27.75	62.82 – 193.50
	DAS-444Ø6-6 w/ All	119.83	46.45	54.00 – 240.00
Forage R3	DAS-444Ø6-6	73.47	20.77	35.00 – 122.00
	DAS-444Ø6-6 w/ 2,4-D	72.53	22.59	37.00 – 117.50
	DAS-444Ø6-6 w/ Gluf	73.75	20.39	37.00 – 123.50
	DAS-444Ø6-6 w/ Glyp	76.04	19.36	40.00 – 121.00
	DAS-444Ø6-6 w/ All	70.73	21.88	38.50 – 118.00
Root R3	DAS-444Ø6-6	23.52	10.81	0.77 – 52.80
	DAS-444Ø6-6 w/ 2,4-D	24.62	10.16	0.67 – 67.60
	DAS-444Ø6-6 w/ Gluf	24.35	11.12	ND – 67.60
	DAS-444Ø6-6 w/ Glyp	29.03	7.86	2.19 – 67.40
	DAS-444Ø6-6 w/ All	27.21	9.44	6.00 – 50.60
Grain	DAS-444Ø6-6	27.37	9.70	6.99 – 45.40
	DAS-444Ø6-6 w/ 2,4-D	27.34	10.35	8.03 – 43.00
	DAS-444Ø6-6 w/ Gluf	27.34	10.02	9.77 – 47.20
	DAS-444Ø6-6 w/ Glyp	25.77	6.79	10.04 – 46.60
	DAS-444Ø6-6 w/ All	25.83	6.51	12.60 – 42.00

Gluf = glufosinate; Glyp = glyphosate; All = 2,4-D + glufosinate + glyphosate

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

	AAD-12 (ng/mg sample dry weight)	
Matrix	LOD	LOQ
Leaf V5	0.5	1.0
Leaf V10-12	0.5	1.0
Root	0.5	1.0
Forage	0.5	1.0
Grain	0.5	1.0

Expression of 2mEPSPS in Plant Tissues

A field expression study was conducted in the U.S. during 2010. Ten sites [Georgia, Iowa (2 sites), Illinois (2 sites), Indiana, Michigan, Missouri, and Nebraska (2 sites)] were planted with DAS-444Ø6-6 soybean and the conventional control (Maverick). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Five treatments of the DAS-444Ø6-6 soybean (unsprayed, sprayed with 2,4-D, sprayed with glufosinate, sprayed with glyphosate, or sprayed with 2,4-D, glufosinate, and glyphosate) were tested. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at the V5 and V10-12 stages, while root and forage were collected at the R3 stage. Grain was collected at the R8 stage of development (Gaska 2006).

The soluble, extractable 2mEPSPS protein was measured using a validated enzyme-linked immunosorbent assay (ELISA) method (Maldonado 2011). 2mEPSPS 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 following this section.

A summary of the 2mEPSPS protein concentrations (averaged across sites) in the various soybean matrices is shown in Table . Average expression values ranged from 21.86 ng/mg dry weight in grain to 2583.46 ng/mg dry weight in V10-12 stage leaf tissue. Expression levels were comparable for all treatments. No 2mEPSPS protein was detected in the control tissues across the ten locations.

Table 13. Expression of 2mEPSPS in DAS-444Ø6-6 soybean.

Tissue	Description	2mEPSPS ng/mg Tissue Dry Weight		
		Mean	Std. Dev. (n=10)	Min/Max Range
Leaf V5	DAS-444Ø6-6	2368.16	973.22	585.00 – 7250.00
	DAS-444Ø6-6 w/ 2,4-D	2261.10	1009.75	850.00 – 7400.00
	DAS-444Ø6-6 w/ Gluf	2062.07	962.71	262.00 – 5150.00
	DAS-444Ø6-6 w/ Glyp	1846.04	975.50	353.00 – 4715.00
	DAS-444Ø6-6 w/ All	2100.96	784.83	680.00 – 4860.00
Leaf V10-12	DAS-444Ø6-6	2583.46	825.47	961.40 – 4999.85
	DAS-444Ø6-6 w/ 2,4-D	2203.83	584.92	256.57 – 3600.00
	DAS-444Ø6-6 w/ Gluf	2188.12	543.24	1335.25 – 3405.00
	DAS-444Ø6-6 w/ Glyp	2512.58	1259.06	511.74 – 8650.00
	DAS-444Ø6-6 w/ All	2131.73	726.92	412.94 – 3210.00
Forage R3	DAS-444Ø6-6	357.09	146.12	182.40 – 862.22
	DAS-444Ø6-6 w/ 2,4-D	330.02	109.78	189.20 – 680.15
	DAS-444Ø6-6 w/ Gluf	321.92	74.69	173.46 – 539.08
	DAS-444Ø6-6 w/ Glyp	400.47	140.66	167.21 – 1150.00
	DAS-444Ø6-6 w/ All	367.32	125.39	154.04 – 1196.00
Root R3	DAS-444Ø6-6	89.71	32.33	ND – 200.4
	DAS-444Ø6-6 w/ 2,4-D	93.54	20.51	4.96 – 174.40
	DAS-444Ø6-6 w/ Gluf	103.48	47.88	ND – 200.40
	DAS-444Ø6-6 w/ Glyp	112.27	30.26	7.10 – 233.60
	DAS-444Ø6-6 w/ All	104.97	43.24	16.59 – 195.60
Grain	DAS-444Ø6-6	21.97	6.28	8.68 – 35.80
	DAS-444Ø6-6 w/ 2,4-D	22.17	6.95	8.94 – 34.90
	DAS-444Ø6-6 w/ Gluf	22.22	7.43	8.52 – 35.02
	DAS-444Ø6-6 w/ Glyp	22.80	6.87	8.24 – 46.80
	DAS-444Ø6-6 w/ All	21.86	6.81	8.66 – 39.85

Gluf = glufosinate; Glyp = glyphosate; All = 2,4-D + glufosinate + glyphosate

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the 2mEPSPS ELISA in the tissue matrices were as follows:

	2mEPSPS (ng/mg sample dry weight)	
Matrix	LOD	LOQ
Leaf V5	4.0	8.0
Leaf V10-12	4.0	8.0
Root	4.0	8.0
Forage	4.0	8.0
Grain	4.0	8.0

Expression of the PAT Protein in Plant Tissues

A field expression study was conducted in the U.S. during 2010. Ten sites (Georgia, Iowa (2 sites), Illinois (2 sites), Indiana, Michigan, Missouri, and Nebraska (2 sites)) were planted with DAS-444Ø6-6 soybean and the conventional control (Maverick). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Five treatments of the DAS-444Ø6-6 soybean (unsprayed, sprayed with 2, 4-D, sprayed with glufosinate, sprayed with glyphosate, or sprayed with 2,4-D, glufosinate, and glyphosate) were tested. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at the V5 and V10-12 stages, while root and forage were collected at the R3 stage. 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 (Smith-Drake, Sosa et al. 2009). 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 in following this section.

A summary of the PAT protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 94. Average expression values ranged from 1.56 ng/mg dry weight in R3 stage root to 10.59 ng/mg dry weight in V10-12 stage leaf tissue. Expression levels were comparable for all treatments. No PAT protein was detected in the control tissues across the ten locations.

Table 94. Expression of PAT in DAS-444Ø6-6 soybean.

Tissue	Description	PAT ng/mg Tissue Dry Weight		
		Mean	Std. Dev. (n=10)	Min/Max Range
Leaf V5	DAS-444Ø6-6	8.98	4.03	3.00 – 19.70
	DAS-444Ø6-6 w/ 2,4-D	9.20	3.24	4.07 – 15.80
	DAS-444Ø6-6 w/ Gluf	8.46	4.01	0.42 – 21.10
	DAS-444Ø6-6 w/ Glyp	8.14	3.58	0.44 – 17.60
	DAS-444Ø6-6 w/ All	8.47	3.23	4.29 – 17.60
Leaf V10-12	DAS-444Ø6-6	10.59	2.86	5.80 – 17.23
	DAS-444Ø6-6 w/ 2,4-D	9.95	3.75	2.18 – 21.20
	DAS-444Ø6-6 w/ Gluf	10.42	2.74	3.10 – 17.60
	DAS-444Ø6-6 w/ Glyp	9.64	3.16	0.59 – 19.40
	DAS-444Ø6-6 w/ All	10.49	3.09	3.88 – 16.80
Forage R3	DAS-444Ø6-6	6.19	1.79	3.55 – 10.45
	DAS-444Ø6-6 w/ 2,4-D	5.90	1.40	3.50 – 9.65
	DAS-444Ø6-6 w/ Gluf	6.72	1.67	2.90 – 11.20
	DAS-444Ø6-6 w/ Glyp	6.48	1.87	3.65 – 10.35
	DAS-444Ø6-6 w/ All	6.33	1.54	4.25 – 9.55
Root R3	DAS-444Ø6-6	1.56	0.68	ND – 3.04
	DAS-444Ø6-6 w/ 2,4-D	1.71	0.67	0.37 – 3.34
	DAS-444Ø6-6 w/ Gluf	1.77	0.77	ND – 3.10
	DAS-444Ø6-6 w/ Glyp	1.80	0.45	0.10 – 2.94
	DAS-444Ø6-6 w/ All	1.86	0.60	0.62 – 3.60
Grain	DAS-444Ø6-6	2.12	0.49	1.36 – 3.19
	DAS-444Ø6-6 w/ 2,4-D	2.13	0.36	1.38 – 2.82
	DAS-444Ø6-6 w/ Gluf	2.11	0.44	1.21 – 3.23
	DAS-444Ø6-6 w/ Glyp	2.15	0.39	1.30 – 3.05
	DAS-444Ø6-6 w/ All	2.11	0.38	1.26 – 3.04

Gluf = glufosinate; Glyp = glyphosate; All = 2,4-D + glufosinate + glyphosate

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

	PAT (ng/mg sample dry weight)	
Matrix	LOD	LOQ
Leaf V5	0.06	0.12
Leaf V10-12	0.06	0.12
Root	0.06	0.12
Forage	0.06	0.12
Grain	0.06	0.12

Methods for AAD-12, 2mEPSPS and PAT Protein Expression Analysis

Experimental Design

Samples were collected from a field study conducted in the US in 2010 that included ten (10) field sites; Georgia, Iowa (2 sites), Illinois (2 sites), Indiana, Michigan, Missouri, and Nebraska (2 sites) (referred to as GA, IA1, IA2, IL1, IL2, IN, MI, MO, NE1, and NE2). Each site consisted of one plot of each treatment per block, with 4 blocks per location (Maldonado 2011). Plot size was 4 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 10 feet of a non-regulated soybean of similar relative maturity. At each location, all blocks were used for collection of samples for protein expression, agronomic properties, and nutrient composition analysis.

Herbicide treatments were designed to replicate maximum label rates:

2,4-D only Treatment: 2,4-D (Weedar 64) was applied as three broadcast applications to DAS-444Ø6-6. Application timing was at planting / pre-emergence, and approximately V3 and R2 stages. Individual target application rates were 1.0 lb ae (acid equivalent)/A for Weedar 64, or 1120 g ae/ha.

Glufosinate only Treatment: Glufosinate (Liberty) was applied as two broadcast applications to DAS-444Ø6-6. Application timing was at approximately V5 and R1 stages. The target application rate at V5 was 0.33 lb ai/A for Liberty, or 374 g ai/ha. The target application rate at R1 was 0.41 lb ai/A for Liberty, or 454 g ai/ha.

Glyphosate only Treatment: Glyphosate (Durango DMA) was applied as three broadcast applications to DAS-444Ø6-6. Individual applications were at planting / pre-emergence, and approximately V3 and R2 stages. Individual target application rates were 1.1 lb ae/A for Durango DMA, or 1260 g ae/ha.

2,4-D + Glufosinate + Glyphosate Treatment: 2,4-D (Weedar 64) + Glyphosate (Durango DMA) as a tank mixture was applied as three broadcast applications to DAS-444Ø6-6. Individual applications were at planting / pre-emergence, and approximately V3 and R2 stages. Individual target application rates were 1.0 lb ae/A for Weedar 64, or 1120 g ae/ha. Individual target application rates were 1.1 lb ae/A for Durango DMA, or 1260 g ae/ha. Glufosinate (Liberty) was also applied as two broadcast applications to DAS-444Ø6-6. Application timing was at approximately V5 and R1 stages. The target application rate was 0.33 lb ai/A for Liberty, or 374 g ai/ha.

Sample Collection

Samples were shipped to Dow AgroSciences Regulatory Sciences 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, NJ).

a) Leaf (V5 and V10-12)

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

b) Root (R3)

One root sample (representing 3 plants) per plot was 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.

c) Forage (R3)

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

d) Grain (R8 – Maturity)

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

Determination of AAD-12 Protein Concentration in Soybean Tissue Samples

ELISA method GRM08.04 was used to determine AAD-12 protein concentration in soybean tissue samples (Smith-Drake, Sosa et al. 2009). The AAD-12 protein was extracted from soybean tissues except grain with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 0.75% ovalbumin (OVA). For grain, the protein was extracted with a PBST buffer containing 0.1% (v/v) Triton-100. The plant tissue and grain extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analyzed using an AAD-12 ELISA kit. Briefly, an aliquot of the diluted sample and a horseradish peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate were 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 excess conjugate were 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 color development, determined by measuring the absorbance of the solution, was related to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm with a background subtraction at 650 or 620 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the AAD-12 concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

Determination of 2mEPSPS Protein in Soybean Tissue Samples

ELISA method 101768 was used to determine 2mEPSPS protein concentration in soybean tissue samples (Maldonado 2011). The 2mEPSPS protein was extracted from soybean tissues with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 2X Casein (PBST/Casein). The plant tissue extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analyzed using a 2mEPSPS ELISA kit. Briefly, an aliquot of the diluted sample is incubated in the wells of a microtiter plate coated with an immobilized anti-2mEPSPS polyclonal antibody. After a washing step, an enzyme-conjugated monoclonal antibody specific to the 2mEPSPS protein is added to the microtiter plate. These antibodies bind with 2mEPSPS protein in the wells and form a "sandwich" with 2mEPSPS protein bound between soluble and the immobilized antibodies. At the end of an incubation period, the unbound reagents were 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 2mEPSPS was bound in the antibody sandwich, the level of color development, determined by measuring the absorbance of the solution, was related to the concentration of 2mEPSPS in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm with a background subtraction at 650 or 620 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the 2mEPSPS concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

Determination of PAT Protein in Soybean Tissue Samples

ELISA method GRM08.05 was used to determine PAT protein concentration in soybean tissue samples (Smith-Drake, Sosa et al. 2009). The PAT protein was extracted from soybean tissues with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 1% polyvinylpyrrolidone (PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted with PBST/1% PVP, and analyzed using a PAT ELISA kit. Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT monoclonal antibody and anti-PAT polyclonal 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 color development, determined by measuring the absorbance of the solution, was related to the concentration of PAT in the sample (i.e., lower residue concentrations result in lower color development). The absorbance at 450 nm with a background subtraction at 650 or 620 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the PAT concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

d. Post-Translational Modification in the New Host

The amino acid residues at the N-and C-termini of the soybean-derived AAD-12 protein (immunoaffinity purified from DAS-444Ø6-6 soybean) were determined and compared with the sequence of the microbe-derived protein (Karnoup and Kuppannan, 2008). The soybean-derived AAD-12 protein sequences were determined by MALDI-TOF MS/MS (Harpham, 2011). The chymotrypsin and trypsin digestions were performed on the soybean-derived AAD-12 protein followed by mass spectrometry analysis and two forms of the N-terminus were determined (Table 6).

The result indicates that during or after translation in soybean and *P. fluorescens*, the N-terminal methionine was cleaved by a methionine aminopeptidase (MAP). The two co-translational processes, cleavage of N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman, 2002).

The C-terminal sequence of the soybean- and microbe-derived AAD-12 proteins results indicated the detected sequences were identical to the expected sequences, and both the soybean- and microbe-derived AAD-12 proteins were identical and unaltered at the C-terminus (Table 7, Figure 36, and Figure 37).

The methods and results of the biochemical characterization of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS proteins are described in detail above. Briefly, both the plant and *P. fluorescens*-derived 2mEPSPS proteins showed the expected molecular weight of ~47 kDa by SDS-PAGE and were immunoreactive to 2mEPSPS protein-specific antibodies by western blot analysis. There was no evidence of any post-translational modifications (i.e. glycosylation) of the DAS-444Ø6-6 soybean-derived 2mEPSPS protein. The amino acid sequence (including the N- and C-termini) was confirmed by enzymatic peptide mass fingerprinting using MALDI-TOF MS and MALDI-TOF MS/MS and was shown to be as expected and was identical to the protein expressed in *P. fluorescens*. The 2mEPSPS protein of both sources did not contain the methionine residue at its N terminus. The result is consistent with those for the 2mEPSPS protein expressed in other systems (Herouet-Guicheney *et al.*, 2009).

The PAT protein is rapidly degraded in simulated digestive fluid (US EPA, 1997; OECD, 1999) and is readily denatured by heat (US EPA, 1997; OECD, 1999). Biochemical characterization of the PAT protein in DAS-444Ø6-6 soybean indicated that the protein did not undergo post-translational modifications such as glycosylation.

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

AAD-12

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.

2mEPSPS

Taxonomy and habitat

Lineage (full): Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACMAD clade; Panicoideae; Andropogoneae; Zea

History of food use

The donor organism, *Zea mays*, (commonly referred to as corn or maize) is a major cereal crop grown for food and feed. The 2mEPSPS contains two point mutations compared with the wild-type *epsps* gene (Herouet-Guicheney *et al.*, 2009).

Toxicity and Allergenicity

The low level expression of the 2mEPSPS protein presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the 2mEPSPS protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

PAT

Taxonomy and habitat

Lineage (full): Bacteria; Actinobateria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces

Streptomyces viridochromogenes is an aerobic soil bacterium. The PPT-acetyltransferase (PAT) gene from S. viridochromogenes normally acts to inhibit glutamine synthetase, causing a fatal accumulation of ammonia.

History of food use

The PAT protein produced in DAS-444Ø6-6 soybean was shown to be equivalent to that produced in other transgenic crops that have been previously approved by FSANZ (A1046, A481, A446, A543 and A518). The food and feed safety of PAT was assessed in these products and shown to present no significant food or feed safety risk.

Additionally, the US EPA concluded that there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the PAT protein and the genetic material necessary for its introduction (US EPA, 1997). US EPA has consequently established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for PAT and the genetic material necessary for its production in all plants.

Toxicity and Allergenicity

The low level expression of the PAT protein in DAS-444Ø6-6 soybean presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the PAT protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

3. Potential Toxicity of the Novel Protein

Part C Section 3 DAS Reports

Jeong, Y.C., Golden, R.M., 2011. 2mEPSPS: Acute oral toxicity study in Crl:CD1(ICR) mice. Study ID 101168. The Dow Chemical Company. Midland, MI.

Guttikonda, S., 2011b. Similarity assessment of 2mEPSPS protein to known toxins by bioinformatics analysis (updated March, 2011). Study ID 110329. Dow AgroSciences LLC. Indianapolis, IN.

Song, P., 2011d. Sequence similarity assessment of PAT protein to known toxins by bioinformatics analysis (updated February, 2011). Study ID 110331. Dow AgroSciences LLC. Indianapolis, IN.

Song, P., 2011b. Sequence similarity assessment of AAD-12 protein to known toxins by bioinformatics analysis (updated February, 2011). Study ID 110327. Dow AgroSciences LLC. Indianapolis, IN.

Wiescinski, C.M., Golden, R.M., 2008. AAD-12: Acute oral toxicity study in Crl:CD1(ICR) mice. Study ID 081037. The Dow Chemical Company. Midland, MI.

a. Assessment of Toxicity Potential of AAD-12

Amino Acid Sequence Comparison of AAD-12 to Known Toxins

The AAD-12 protein does not share meaningful amino acid sequence similarities with known toxins. Amino acid homologies with the AAD-12 protein sequence were evaluated using BLASTp search algorithm against the GenBank non-redundant protein sequences (up to date as of February 18, 2011 containing 13,473,798 sequences with 4,621,495,809 amino acids). By their annotations, the proteins returned by BLASTp search can be grouped into the following 11 categories: 2,4-D/alpha-ketoglutarate dioxygenase, alkylsulfatase AtsK, alpha-ketoglutarate (dependent) dioxygenase, alpha-ketoglutarate-

dependent sulfonate dioxygenase, ketoglutarate dehyronase, taurine catabolism dioxygenase, taurine dioxygenase, dioxygenase, oxidoreductase, pyoverdine biosynthesis protein, and hypothetical (putative) or unnamed proteins. AAD-12 (aryloxyalkanoate dioxygenase-12) itself is an alpha-ketoglutarate dependent dioxygenase. Hypothetical and unnamed proteins are derived from conceptual translation of DNA sequences generated from massive genome sequencing projects of various fungi and bacteria. Those proteins have functional annotations such as “probable taurine catabolism dioxygenase”, “clavaminic acid synthetase (CAS) –like”, and “putative alpha-ketoglutarate dependend dioxygenase”. None of those proteins are associated with protein toxins that are harmful to humans or animals (Song 2011).

Acute Oral Toxicity of AAD-12

An acute oral toxicity study with the *P. fluorescens*-derived AAD-12 protein was conducted in mice administered 2000 mg AAD-12/kg body weight after adjustment for purity (5666 mg/kg of test substance at 35.3% purity) (Wiescinski and Golden 2008). The study was conducted following OECD Guideline 423, and used a total of 10 mice (5 male and 5 female). 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 LD50 and no observed effect level (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 potential.

b. Assessment of Toxicity Potential of 2mEPSPS

Amino Acid Sequence Comparison of 2mEPSPS to Known Toxins

The 2mEPSPS protein does not share meaningful amino-acid sequence similarities with known toxins that would present any safety concerns. Amino acid homologies with the 2mEPSPS protein sequence were evaluated using BLASTp search algorithm against the GenBank non-redundant protein sequences (up to date as of March 29, 2011 containing 13,254,464 sequences with 4,535,100,774 amino acids). By their annotations, the majority of proteins returned by BLASTp with statistically significant alignments are related to shikimate pathway associated proteins including 3-phosphoshikimate 1-carboxyvinyltransferase enzyme 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) enzyme, and UDP-N-acetylglucosamine 1-carboxyvinyltransferase enzyme, and dehydroquinate synthase. None of those proteins is associated with known protein toxins that are harmful to humans and animals (Guttikonda 2011).

Acute Oral Toxicity of 2mEPSPS

An acute oral toxicity study with the *P. fluorescens*-produced 2mEPSPS protein was conducted in mice administered 5000 mg 2mEPSPS/kg after adjustment for purity (7519 mg/kg test substance at 66.5% purity) (Jeong and Golden 2011). The study was conducted following OECD Guideline 423, and used a total of 10 mice (5 male and 5 female). 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 LD50 and NOEL of 2mEPSPS protein in male and female mice was greater than 5000 mg/kg based on the fact that no mortality was observed and there were no observable effects (adverse or non-adverse effects) with the 2mEPSPS-treated animals. 2mEPSPS protein displays very low acute toxicity potential.

c. Assessment of Toxicity Potential of PAT

The PAT protein does not share any amino acid sequence similarity with known toxins that would present any safety concerns. Amino acid homologies with the PAT protein sequence were evaluated using BLASTp search algorithm against the GenBank non-redundant protein sequences (up to date of February 18, 2011). By their annotations, the majority of proteins returned by BLASTp with statistically significant alignments are phosphinothricin acetyltransferase, other acetyltransferases, and hypothetical proteins without assigned function. None of these proteins is associated with known protein toxins that are harmful to humans and animals (Song 2011).

There is no evidence available indicating that the PAT protein is toxic to either humans or animals. In acute toxicity studies mice gavaged with high levels of PAT protein showed no treatment-related significant toxic effects (US EPA 1997; OECD 1999).

4. Potential Allergenicity of the Novel Protein

Part C Section 4 DAS Reports

Embrey, S.K., 2011. Heat lability of Double Mutant 5-Enol Pyruvylshikamate-3-Phosphate Synthase (2mEPSPS) Protein. Study ID 110461. Dow AgroSciences LLC. Indianapolis, IN.

Embrey, S.K., 2011b. In vitro simulated gastric fluid digestibility study of double mutant 5-enol pyruvylshikimate-3-phosphate synthase (2mEPSPS) protein. Study ID 102106. Dow AgroSciences LLC. Indianapolis, IN.

Guttikonda, S., 2011a. Similarity assessment of 2mEPSPS protein to known allergens by bioinformatics analysis (updated March, 2011). Study ID 110328. Dow AgroSciences LLC. Indianapolis, IN.

Harpham, N.V.J., Ma, E., Stagg, N.J., 2011. Endogenous Allergen Analysis of DAS-444Ø6-6 Soybean. Study ID 110512. Dow AgroSciences LLC. Indianapolis, IN.

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

Schafer, B.W., Embrey, S.K., 2008. *In vitro* Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12 (abbreviation AAD-12). Study ID 080064. Dow AgroSciences LLC. Indianapolis, IN.

Schafer, B.W., Embrey, S.K., 2011. *In vitro* simulated gastric fluid digestibility of aryloxyalkanoate dioxygenase-12 (AAD-12) in soybean leaf extracts. Study ID 110265. Dow AgroSciences LLC. Indianapolis, IN.

Song, P., 2011a. Sequence similarity assessment of AAD-12 protein to known allergens by bioinformatics analysis (updated February, 2011). Study ID 110326. Dow AgroSciences LLC. Indianapolis, IN.

Song, P., 2011c. Sequence similarity assessment of PAT protein to known allergens by bioinformatics analysis (updated February, 2011). Study ID 110330. Dow AgroSciences LLC. Indianapolis, IN.

a. Allergenicity Considerations

Lack of Allergenicity and Toxicity of AAD-12

A detailed safety assessment of the AAD-12 protein was conducted to assess any potential adverse effects to humans or animals resulting from the environmental release of crops containing the AAD-12 protein (Codex Alimentarius Commission 2009). The conclusion from that assessment is that the AAD-12 protein is unlikely to cause allergic reaction in humans or be a toxin to humans or animals. The AAD-12 protein has been previously deregulated by FDA and approved by FSANZ.

Assessment of AAD-12 Allergenicity Potential

Studies were conducted to ascertain the potential allergenicity of the AAD-12 protein. These studies included: 1) bioinformatic 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.

Lack of Allergenicity and Toxicity of 2mEPSPS

A detailed safety assessment of the 2mEPSPS protein was conducted to assess any potential adverse effects to humans or animals resulting from the environmental release of crops containing the 2mEPSPS protein (Codex Alimentarius Commission 2009). The conclusion from that assessment is that the 2mEPSPS protein is unlikely to cause allergic reactions in humans or to be toxic to humans or animals.

Safety of Donor Organism for 2mEPSPS

The donor organism, *Zea mays*, (commonly referred to as corn or maize) is a major cereal crop grown for food and feed. The 2mEPSPS contains two amino acid substitutions compared with the wild-type EPSPS (Herouet-Guicheney, Rouquié et al. 2009).

The 2mEPSPS protein is expressed in other events and crops that have previously been deregulated by the USDA, CFIA and FSANZ, for example:

- GA21 corn (OECD Unique Identifier MON-ØØØ21-9) was approved by FSANZ in 2000 (A362).
- GHB614 cotton (OECD Unique Identifier BCS-GHØØ2-5) was approved by FSANZ in 2009 (A614).

Assessment of 2mEPSPS Allergenicity Potential

Studies were conducted to ascertain the potential allergenicity of the 2mEPSPS protein. These studies included: 1) bioinformatic 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 2mEPSPS protein is considered to have a low risk of allergenic potential.

Lack of Allergenicity and Toxicity of PAT

Safety evaluation of the PAT protein was conducted to assess any potential adverse effects to humans or animals resulting from the environmental release of crops containing the PAT protein(Codex Alimentarius Commission 2009). The conclusion from the assessment is that the PAT protein is unlikely to cause allergic reactions in humans or to be toxic to humans or animals. The PAT protein has been previously deregulated in a variety of products, including corn, soybean, cotton, and canola.

Assessment of PAT Allergenicity Potential

Studies were conducted to ascertain the potential allergenicity of the PAT protein, including 1) an updated bioinformatic search for amino acid sequence homology with known allergens, 2) previously conducted digestive fate in simulated gastric fluid and heat lability. Based on the lack of significant amino acid sequence homology to known allergens, and the lack of enzymatic and heat stability, the PAT protein is considered to have a low risk of allergenic potential.

b. Endogenous Allergen Analysis

Soybean is listed as one of the eight most common allergenic foods in the United States of America and one of the 12 most common allergic foods in Europe (FDA 2004; EFSA 2007). Therefore, a study was conducted to determine if the genetic modification used to generate DAS-444Ø6-6 soybean altered the endogenous allergen content compared to the non-transgenic counterpart (Maverick).
IgE binding to extracts of DAS-444Ø6-6 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 10 clinically-reactive soy allergic patients(Harpham, Ma et al. 2011).

The PAT protein has no biologically meaningful sequence similarities 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) using an allergen database (FARRP Allergen Database version 11 containing 1491 allergens, Released in January 2011, http://www.allergenonline.org) and no such matches were found (Song 2011).
The PAT protein is rapidly degraded in simulated digestive fluid (US EPA 1997; OECD 1999) and is readily denatured by heat (US EPA 1997; OECD 1999). Biochemical characterization of the PAT protein in DAS-444Ø6-6 soybean indicated that the protein did not undergo post-translational modifications such as glycosylation.

SDS-PAGE with Coomassie Blue Staining and Immunoblot Analysis

Extracts were prepared from the ground seed of DAS-444Ø6-6 and Maverick soybeans. To extract the soluble protein from the both Maverick and DAS-444Ø6-6 soybean seeds, the soybean seed powders were removed from -80ºC and approximately 12.5 g was weighed into appropriate containers and 125 mL (1:10 w/v) of extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 8.5) was added to each sample. The samples were covered with foil and the samples were incubated overnight at 4ºC. Samples were removed from 4 ºC and homogenized at room temperature for 3 minutes using a Cuisinart Smart Stick homogenizer. Samples were then centrifuged at 3,000 x g for 30 minutes and the resulting supernatants were vacuum filtered through P8 grade filter paper. Extracts were further clarified by centrifugation at 3,000 x g for 30 minutes at 4ºC. The supernatants were then filtered through a 0.22 µm sterile filter and collected into sterile 50mL centrifuge tubes. The resulting sample was aliquoted into 1-mL sub-aliquots and stored in a -80ºC freezer. All samples were mixed with Laemmli buffer and heated at 95ºC for 5 min and then subjected to SDS-PAGE with Coomassie blue staining to evaluate protein content of the two seed lots. Gel transfer of the proteins to a nitrocellulose membrane was performed with blots replicating the SDS-PAGE. Transfer was confirmed with the use of pre-stained molecular weight markers. The blotted membranes were blocked with 5% non-fat milk in PBST for at least 1 hour at room temperature followed by overnight incubation at 4ºC in serum (1:20 dilution) from a pool of 10 soy-allergic patients. The blots were washed with PBST to remove unbound IgE and then incubated in biotinylated goat anti-human IgE antibody for 1 hour at room temperature with continuous agitation. Additional washing with PBST was carried out and then the blots were incubated with NeutrAvidin-HRP (horse radish peroxidase) conjugate for 30 minutes at room temperature. GE Healthcare ECL Plus chemiluminescent substrate was used for development and visualization of the immunoreactive protein

bands. The membranes were covered with ECL Plus reagent for 5 minutes, excess solution removed and exposed to Thermo Scientific CLX-Posure film in a darkroom and developed.

The protein profiles between DAS-444Ø6-6 and the non-transgenic soybean line, 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-444Ø6-6 and Maverick were compared in the one-dimensional immunoblot using soy-allergic sera and also showed no difference (Figure).

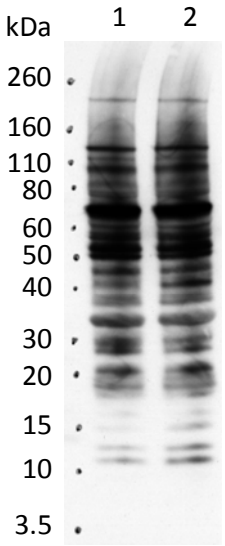


Figure 46. Immunoblot of DAS-444Ø6-6 and control (Maverick) soybean extracts with soybean-allergic patient sera.

Lane	Sample	Amount Loaded
1	15 µl of control (Maverick) soybean seed extract	15 µg
2	15 µl of DAS-444Ø6-6 soybean seed extract	15 µg

ELISA Inhibition

ELISA inhibition of IgE binding from a pooled soybean-allergic serum sample was conducted for DAS-444Ø6-6 and control (Maverick) soybean extracts. Extracts from DAS-444Ø6-6 and Maverick (at various concentrations: 0.0075 to 7500 □g/ml of total soluble protein) were pre-incubated with the pooled serum and transferred to 96-well plates previously coated with non-transgenic control (Maverick) extracts at 25 µg/mL (100 □L/well). After a washing step with PBST, biotinylated goat-anti-human IgE antibody and NeutraAvidin-HRP conjugate were sequentially incubated on the plates with a PBST washing step after each incubation. The plates were lastly incubated with peroxidase substrate TMB and the reaction was terminated with 1 N HCl. A microtiter plate reader was used to measure the absorbance of the samples in the wells at 450 nm with 650 nm background subtraction.

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^(LogEC$_{50}$-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 EC_{50} 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 EC_{50} values and their associated 95% confidence intervals are plotted for the Maverick soybean and DAS-444Ø6-6 extracts.

The ELISA inhibition data with the pooled soy-allergic serum showed the same IgE binding between the non-transgenic Maverick soybean and DAS-444Ø6-6 soybean extracts against 2.5 µg/well of immobilized Maverick on the plate (Figure)**.** Furthermore, the associated EC_{50} values and 95% confidence intervals for Maverick and DAS-444Ø6-6 were similar (Figure 42).

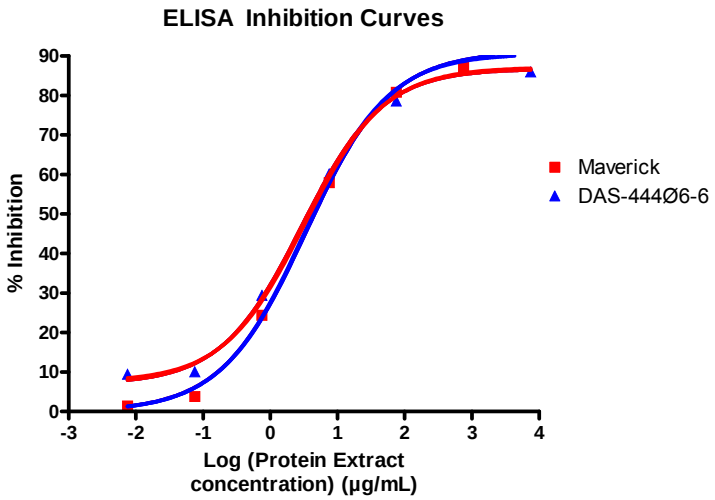
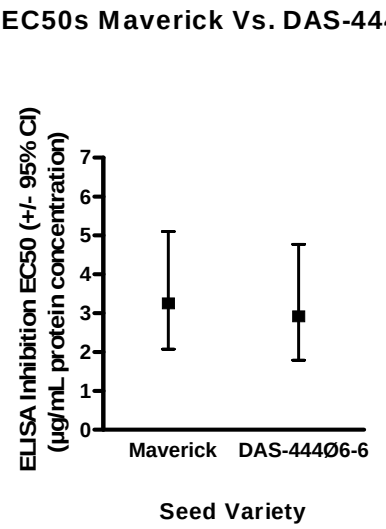


Figure 47. ELISA inhibition with DAS-444Ø6-6 and control (Maverick) soybean extracts using soybean-allergic patient sera.

Figure 428. EC50 values from the ELISA inhibition data for DAS-444Ø6-6 and control (Maverick) soybean extracts and their 95% confidence intervals.



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-444Ø6-6 soybean did not alter the endogenous allergenicity compared with its non-transgenic control, Maverick.

c. Source of Introduced Protein

AAD-12

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 Graevenitz 1985; Tamaoka, Ha et al. 1987; Wen, Fegan et al. 1999).

Delftia acidovorans can be used to transform ferulic acid into vanillin and related flavour metabolites (Toms and Wood 1970; Rao and Ravishankar 2000; Shetty, Paliyath 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, Goers et al. 1992)

2MEPSPS

The donor organism, *Zea mays*, (commonly referred to as corn or maize) is a major cereal crop grown for food and feed. The 2mEPSPS contains two amino acid substitutions compared with the wild-type EPSPS (Herouet-Guicheney, Rouquié et al. 2009).

The 2mEPSPS protein is expressed in other events and crops that have previously been approved by FSANZ, for example:

- GA21 corn (OECD Unique Identifier MON-ØØØ21-9) was approved by FSANZ in 2000 (A362).
- GHB614 cotton (OECD Unique Identifier BCS-GHØØ2-5) was approved by FSANZ in 2009 (A614).

PAT

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium. PAT is comprised of 183 amino acids and has a molecular weight of ~21 kDa (OECD, 1999, 2002).

d. Amino Acid Sequence Comparison

Amino Acid Sequence Comparison of AAD-12 to Known Allergens

The step-wise, weight-of-evidence approach (Codex Alimentarius Commission 2009) was used to assess the allergenic potential of the AAD-12 protein. The AAD-12 protein does not share meaningful amino acid sequence similarities with known allergens. No significant homology was identified when the AAD-12

protein sequence was compared with known allergens in the FARRP (Food Allergy Research and Resource Program) version 11.00 allergen database (Released in February, 2011), using the search criteria of either a match of eight or more contiguous identical amino acids, or >35% identity over 80 amino acid residues (Song 2011).

Amino Acid Sequence Comparison of 2mEPSPS to Known Allergens

The step-wise, weight-of-evidence approach was used to assess the allergenic potential of the 2mEPSPS protein (Codex Alimentarius Commission 2009). The 2mEPSPS protein does not share meaningful amino-acid sequence similarities with known allergens. No significant homology was identified when the 2mEPSPS protein sequence was compared with known allergens in the FARRP (Food Allergy Research and Resource Program) version 11.00 allergen database (Released in February, 2011), using the search criteria of either a match of eight or more contiguous identical amino acids, or >35% identity over 80 amino-acids residues (Guttikonda 2011).

Amino Acid Sequence Comparison of PAT to Known Allergens

A step-wise, weight-of-evidence approach was used to assess the allergenic potential effects from the PAT protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence.The analyses was conducted 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) using an allergen database (FARRP Allergen Database version 11 containing 1491 allergens, Released in January 2011, http://www.allergenonline.org) and no such matches were found (Song 2011).

e. Simulated Gastric Fluid and Heat Lability

Lability of AAD-12 in Simulated Gastric Fluid

The digestibility of the the *P. fluorescens*-derived AAD-12 protein was tested *in vitro* using simulated gastric fluid (SGF). The AAD-12 protein (3.7 µM) was incubated in SGF (0.32% w/v pepsin at pH 1.2; U.S Pharmacopeia) for various periods of time. 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 Na2CO3, 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 95ºC for 5 min. The samples were then analysed via SDS-PAGE and western blot analysis using a polyclonal antibody specific to AAD-12. The results demonstrated that the AAD-12 protein was readily digested (not detectable at 30 seconds) in SGF (Figure 43 and Figure (Schafer and Embrey 2008; Schafer and Embrey 2011)).

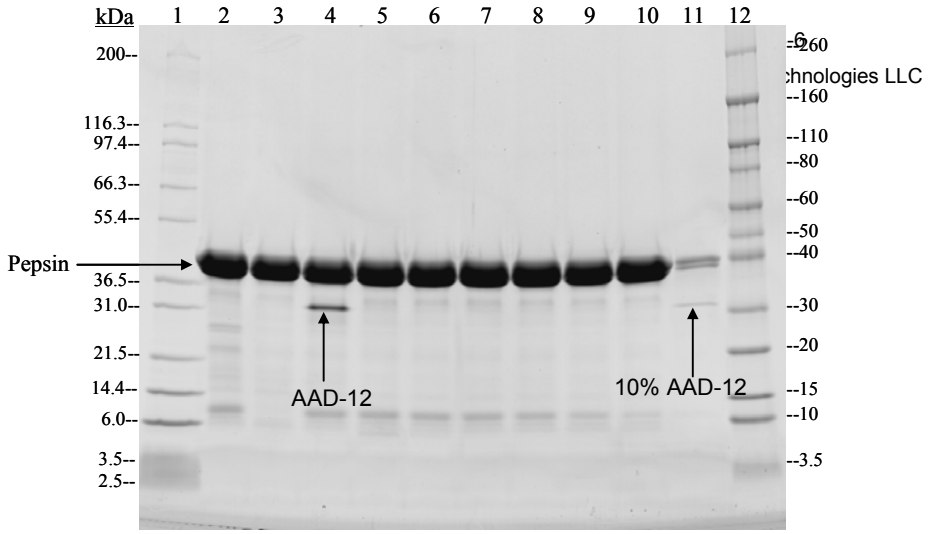


Figure 439. SDS-PAGE analysis of AAD-12 protein subjected to digestion in simulated gastric fluid.

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 per gel 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 run on Tris-Glycine buffer systems.

Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW Markers	10 µL
2	SGF Reagent Blank, zero minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~1.68 µg
5	30-second AAD-12 digestion	~1.68 µg
6	1-minute AAD-12 digestion	~1.68 µg
7	2- minute AAD-12 digestion	~1.68 µg
8	4-minute AAD-12 digestion	~1.68 µg
9	8-minute AAD-12 digestion	~1.68 µg
10	16-minute AAD-12 digestion	~1.68 µg
11	10% Neutralized AAD-12 digestion	~0.17 µg
12	Invitrogen Novex Sharp Prestained MW Markers	10 µL

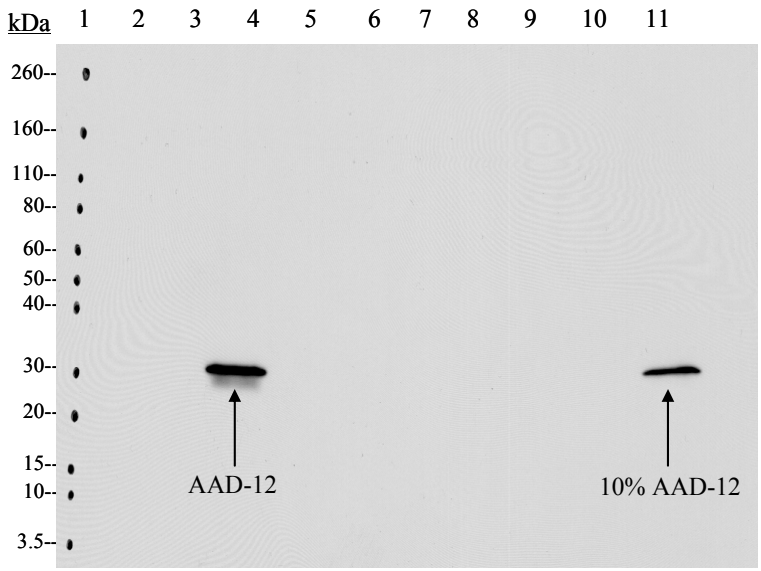


Figure 50. Western blot analysis of AAD-12 protein subjected to digestion in simulated gastric fluid.

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 two separate Bio-Rad 4-20% Tris-HCl Criterion gel and electrophoresed at a constant voltage of 180 V per gel for ~45 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, one gel was stained with GelCode Blue stain from Pierce and the other 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, zero minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~0.17 µg
5	30-second AAD-12 digestion	~0.17 µg
6	1-minute AAD-12 digestion	~0.17 µg
7	2- minute AAD-12 digestion	~0.17 µg
8	4-minute AAD-12 digestion	~0.17 µg
9	8-minute AAD-12 digestion	~0.17 µg
10	16-minute AAD-12 digestion	~0.17 µg
11	10% Neutralized AAD-12 digestion	~0.017 µg

Lability of 2mEPSPS in Simulated Gastric Fluid

The digestibility of the *P. fluorescens*-produced 2mEPSPS protein was tested *in vitro* using simulated gastric fluid (SGF). The 2mEPSPS protein (3.7 µM) was incubated in SGF (0.32% w/v pepsin at pH 1.2; U.S Pharmacopeia) for various periods of time. At each time point (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 of 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 2mEPSPS 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°C for 5 min. The samples were then analysed via SDS-PAGE and western blot analysis using a polyclonal antibody specific to 2mEPSPS. The results demonstrated that the 2mEPSPS protein was readily digested (not detectable at 1 minute) in SGF (Figure and Figure (Embrey 2011)).

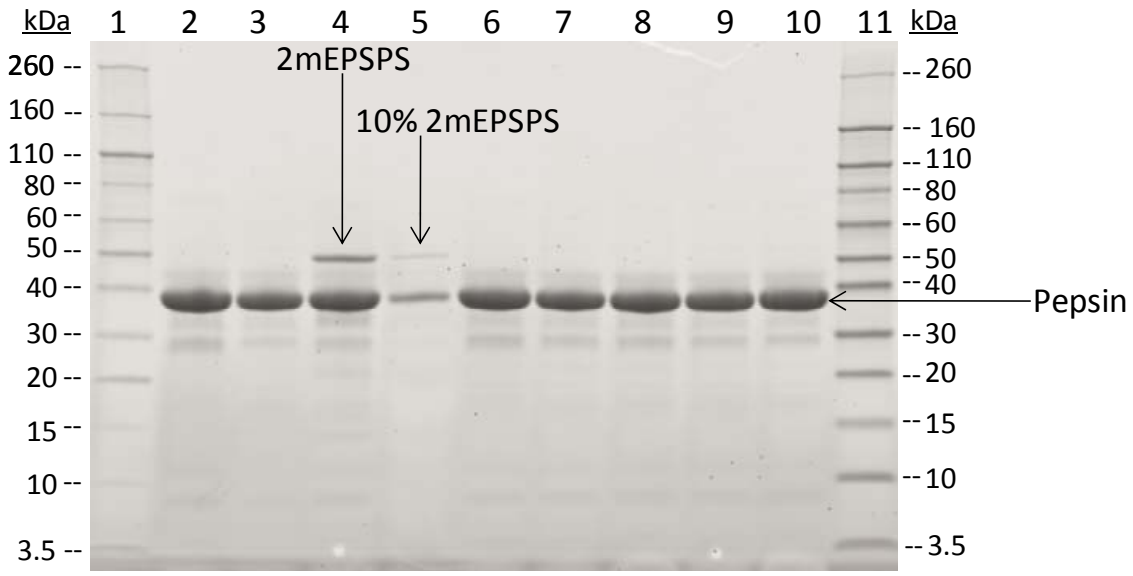


Figure 51. SDS-PAGE analysis of 2mEPSPS (M.W. ~47 kDa) protein subjected to digestion in simulated gastric fluid.

The neutralized and digested 2mEPSPS samples and SGF controls were held frozen 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% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V for ~60 minutes using Tris/Glycine/SDS buffer from Bio-Rad. After separation, the gel was stained with GelCode Blue stain from Thermo-Pierce.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Unstained MW Markers	10 µL
2	SGF Reagent Blank, zero minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized 2mEPSPS digestion	~1.24 µg
5	10% Neutralized 2mEPSPS digestion	~0.124 µg
6	1-minute 2mEPSPS digestion	~1.24 µg
7	2- minute 2mEPSPS digestion	~1.24 µg
8	4-minute 2mEPSPS digestion	~1.24 µg
9	8-minute 2mEPSPS digestion	~1.24 µg
10	16-minute 2mEPSPS digestion	~1.24 µg
11	Invitrogen Novex Sharp Prestained MW Markers	10 µL

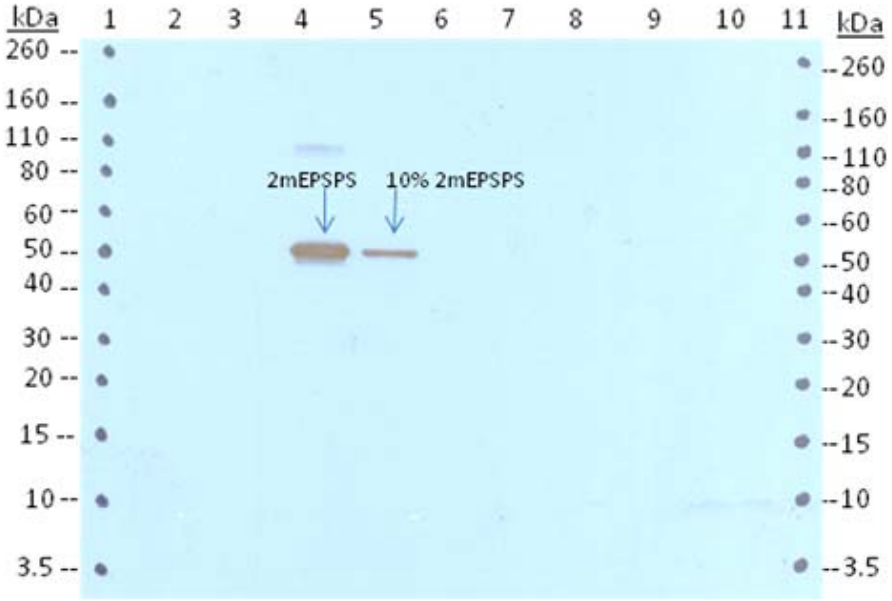


Figure 52. Western blot analysis of 2mEPSPS protein subjected to digestion in simulated gastric fluid.

The neutralized and digested 2mEPSPS samples and SGF controls were held frozen 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% Bis-Tris Criterion gel and electrophoresed at a constant voltage of 150 V for ~60 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 100 volts. For immunodetection, the membrane was probed with an 2mEPSPS specific polyclonal rabbit antibody (Lot: G2874, 3.3 mg/mL). A conjugate of goat anti-rabbit IgG 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. The high molecular weight band in lane 4 is a 2mEPSPS dimer that is not fully denatured.

Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Prestained MW Markers	10 µL
2	SGF Reagent Blank, zero minute incubation	20 µL
3	SGF Reagent Blank, >16 minute incubation	20 µL
4	Neutralized 2mEPSPS digestion	~0.124 µg
5	10% Neutralized 2mEPSPS digestion	~0.0124 µg
6	1-minute 2mEPSPS digestion	~0.124 µg
7	2- minute 2mEPSPS digestion	~0.124 µg
8	4-minute 2mEPSPS digestion	~0.124 µg
9	8-minute 2mEPSPS digestion	~0.124 µg
10	16-minute 2mEPSPS digestion	~0.124 µg
11	Invitrogen Novex Sharp Prestained MW Markers	10 µL

Lability of PAT in Simulated Gastric Fluid

The PAT protein is rapidly degraded in simulated digestive fluid (US EPA 1997; OECD 1999)

Heat Lability of AAD-12

The thermal stability of the *P. fluorescens*-derived AAD-12 protein was evaluated by heating protein solutions for 30 min at 50°C, 70°C and 95°C and 20 min in an autoclave (120°C at ~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-12 protein catalyzes the conversion of dichlorphenoxyacetate to 2,4-dichlorophenol and glyoxylate concomitant with the decomposition of α-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 color 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 (Schafer 2008).

Heat Lability of 2mEPSPS

The thermal stability of the *P. fluorescens*-produced 2mEPSPS protein was evaluated by comparing heated 2mEPSPS protein solutions at 25°C, 37°C, 55°C, 75°C, and 95°C for 30 minutes with a sample held on ice. After heat treatment, all samples were immediately placed on ice and assayed by colorimetric enzymatic assay (Lanzetta, Alvarez et al. 1979), ELISA and SDS-PAGE. At temperatures at or above 55°C, the enzymatic activity of the 2mEPSPS protein was reduced by ≥73%. At temperatures at or above 55°C, the 2mEPSPS protein lost ≥ 90% of its immunoreactivity as measured by a polyclonal antibody sandwich ELISA. SDS-PAGE analysis indicated that the molecular mass of the 2mEPSPS protein (approximately 47 kDa) was unchanged. These data indicate that industrial processing of the soybean grain would significantly degrade the tertiary structure of the 2mEPSPS protein, reduce its immunoreactivity, and significantly diminish its enzymatic activity (Embrey, 2011).

Heat Lability of PAT

The PAT protein is readily denatured by heat (US EPA 1997; OECD 1999).

Metabolic Profile and Residue Levels

The metabolic profiles and residue levels of 2,4-D and glyphosate from the expression of AAD-12 and 2mEPSPS in soybeans have already been established.

The AAD-12 protein in DAS-44406-6 soybean is identical to that produced in DAS-68416-4 soybean. MRLs associated with 2,4-D in soybeans / A1046 are also be relevant for DAS-44406-6 soybean.

The 2mEPSPS protein in DAS-44406-6 soybean is identical to that produced in other products (A1051), and functionally identical to the CP4 EPSPS protein (A614, A362). No revision to current MRLs for glyphosate in soybean are necessary.

Summary of AAD-12, 2mEPSPS and PAT Toxicity and Allergenicity

AAD-12 Summary

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 hydrolyzed 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-444Ø6-6 soybean. 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.

2mEPSPS Summary

A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the 2mEPSPS protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the 2mEPSPS amino acid sequence. The 2mEPSPS protein hydrolyzed rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 5000 mg/kg body weight of 2mEPSPS protein. Glycosylation analysis revealed no detectable covalently linked carbohydrates in 2mEPSPS protein expressed in DAS-444Ø6-6 soybean plants. Therefore, the low level expression of the 2mEPSPS protein presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the 2mEPSPS protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

PAT Summary

A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the PAT protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence. The PAT protein hydrolyzed rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 5000 mg/kg body weight of PAT protein. The low level expression of the PAT protein in DAS-444Ø6-6 soybean presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the PAT 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

Cleveland, C.B., 2011a. Global Dietary and Livestock Assessment of 2mEPSPS Protein for DAS Soybean Cultivar Based on Event DAS-444Ø6-6. Study ID 110600. Dow AgroSciences LLC. Indianapolis, IN.

Cleveland, C.B., 2011b. Global Dietary and Livestock Assessment of PAT Protein for DAS Soybean Cultivar Based on Event DAS-444Ø6-6. Study ID 110599. Dow AgroSciences LLC. Indianapolis, IN.

Cleveland, C.B., Stagg, N.J., 2011. Global Dietary and Livestock Assessment of AAD-12 Protein for DAS Soybean Cultivar Based on Event DAS-444Ø6-6. Study ID 110598. Dow AgroSciences LLC. Indianapolis, IN.

Fletcher, D.W., 2011. DAS-68416-4 (AAD-12) Soybean Feeding Study in the Broiler Chicken. Study ID 101088. Dow AgroSciences LLC. Indianapolis, IN.

Lepping, M., 2011. Nutrient Composition of a Transformed Soybean Cultivar Containing Aryloxyalkanoate Dioxygenase-12 (AAD-12), Double Mutant Maize EPSPS Gene (2mEPSPS), and Phosphinothricin Acetyltransferase (PAT) – Event DAS-444Ø6-6. Study ID 101104.03. Dow AgroSciences LLC. Indianapolis, IN.

Moldonado,P.M., 2011. Field Expression of a Transformed Soybean Cultivar Containing Aryloxyalkanoate Dioxygenase (AAD-12), Double Mutant Maize EPSPS Gene (2mEPSPS) and Phosphinothricin Acetyltransferase (PAT) – Event DAS-444Ø6-6. Study ID 101104.02. Dow AgroSciences LLC. Indianapolis, IN.

a. Grain and Forage Composition

Field trials with DAS-444Ø6-6 soybean, a non-transgenic control, and reference lines were conducted in 2010 at ten sites located in the U.S (Lepping, 2011). This study used the same plots that were used for protein expression (Chapter 3) and agronomic characterization (Chapter 6) studies. No biologically meaningful unintended compositional differences were observed between the non-transgenic near-isogenic control and DAS-444Ø6-6 soybean plots. Results from this study demonstrate compositional equivalence between event DAS-444Ø6-6 (unsprayed and sprayed) and non-transgenic soybean.

Field Study Design (Lepping, 2011; DAS report 101104.03)

A crop composition study with DAS-444Ø6-6 soybean, a near-isogenic non-transgenic control (Maverick) and six non-transgenic commercial reference lines (Dairyland Seed (DSR) 75213-72, 98860-71, 99914N, and 99915; Porter 75148; Williams 82) was conducted in 2010. The ten sites were located in Sycamore, Georgia; Richland, Iowa; Bagley, Iowa; Carlyle, Illinois; Wyoming, Illinois; Sheridan, Indiana; Deerfield, Michigan; Fisk, Missouri; Brunswick, Nebraska; and York, Nebraska. Reference lines were of a similar maturity to the control and test substance and were randomized across sites in a balanced incomplete-block (BIB) design with three references at each site and each reference line present at five sites.

Unsprayed DAS-444Ø6-6 soybean plots and DAS-444Ø6-6 soybean plots treated with various herbicide regimes were included as separate entries. Herbicides were applied in a spray volume of approximately 20 gallons per acre (187 L/ha). Herbicide applications included approximately 2% v/v Ammonium sulfate (AMS) for Weedar 64, Durango DMA, and Liberty.

2,4-D only Treatment: 2,4-D (Weedar 64) was applied as three broadcast applications to DAS-444Ø6-6. Application timing was at planting / pre-emergence, and approximately V3 and R2 stages. Individual target application rates were 1.0 lb ae (acid equivalent)/A for Weedar 64, or 1120 g ae/ha.

Glufosinate only Treatment: Glufosinate (Liberty) was applied as two broadcast applications to DAS-444Ø6-6. Application timing was at approximately V5 and R1 stages. The target application rate at V5 was 0.33 lb ai/A for Liberty, or 374 g ai/ha. The target application rate at R1 was 0.41 lb ai/A for Liberty, or 454 g ai/ha.

Glyphosate only Treatment: Glyphosate (Durango DMA) was applied as three broadcast applications to DAS-444Ø6-6. Individual applications were at planting / pre-emergence, and approximately V3 and R2 stages. Individual target application rates were 1.1 lb ae/A for Durango DMA, or 1260 g ae/ha.

2,4-D + Glufosinate + Glyphosate Treatment: 2,4-D (Weedar 64) + Glyphosate (Durango DMA) as a tank mixture was applied as three broadcast applications to DAS-444Ø6-6. Individual applications were at planting / pre-emergence, and approximately V3 and R2 stages. Individual target application rates were

1.0 lb ae/A for Weedar 64, or 1120 g ae/ha. Individual target application rates were 1.1 lb ae/A for Durango DMA, or 1260 g ae/ha. Glufosinate (Liberty) was also applied as two broadcast applications to DAS-444Ø6-6. Application timing was at approximately V5 and R1 stages. The target application rate was 0.33 lb ai/A for Liberty, or 374 g ai/ha.

The test, control, and reference soybean seed was planted at a seeding rate of approximately 125 seeds per row with seed spacing within each row of approximately 2.4 inches (6 cm). At each site, 4 replicate plots of each entry were established, with each plot consisting of four 25 ft rows. Each soybean plot was bordered by 2 rows of a non-transgenic soybean cultivar of similar maturity. Plots were arranged in a randomized complete block (RCB) design, with a unique randomization at each site. The entire trial site was surrounded by a minimum of 4 rows (or 10 ft) of a non-transgenic soybean cultivar of similar maturity. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop.

Compositional Analyses of Soybean Forage

Samples of soybean forage and seed were analysed at Covance Laboratories Inc. for nutrient content (Lepping, 2011) based on methods detailed in Appendix 6. The analytes examined are presented in Table). The results of the compositional analysis for soybean forage and seed were compared with values reported in literature (Iskander 1987; Hartwig and Kilen 1991; Padgette, Taylor et al. 1996; Taylor, Fuchs et al. 1999; OECD 2001; McCann, Liu et al. 2005; Harrigan, Ridley et al. 2007; Bilyeu, Zeng et al. 2008; Lundry, Ridley et al. 2008; Berman, Harrigan et al. 2009; Berman, Harrigan et al. 2010; Harrigan, Glenna et al. 2010; ILSI 2011). A summary of the compositional data used for comparison can be found following this section.

Table 15. Composition analytes.

A. Forage	
Proximates and Fiber	Minerals
Protein	Calcium
Fat	Phosphorus
Ash	
Moisture	
Carbohydrates	
Acid Detergent Fiber (ADF)	
Neutral Detergent Fiber (NDF)	

B. Seed					
Proximates and Fiber	Minerals	Amino Acids		Fatty Acids	
Protein	Calcium	Alanine	Lysine	8:0 Caprylic	18:0 Stearic
Fat	Copper	Arginine	Methionine	10:0 Capric	18:1 Oleic
Ash	Iron	Aspartic acid	Phenylalanine	12:0 Lauric	18:2 Linoleic
Moisture	Magnesium	Cystine	Proline	14:0 Myristic	18:3 Linolenic
Carbohydrates	Manganese	Glutamic acid	Serine	14:1 Myristoleic	18:3 γ-Linolenic
Acid Detergent Fiber (ADF)	Phosphorus	Glycine	Threonine	15:0 Pentadecanoic	20:0 Arachidic
Neutral Detergent Fiber (NDF)	Potassium	Histidine	Tryptophan	15:1 Pentadecenoic	20:1 Eicosenoic
Total Dietary Fiber	Selenium	Isoleucine	Tyrosine	16:0 Palmitic	20:2 Eicosadienoic
	Sodium	Leucine	Valine	16:1 Palmitoleic	20:3 Eicosatrienoic
	Zinc			17:0 Heptadecanoic	20:4 Arachidonic
				17:1 Heptadecenoic	22:0 Behenic

Vitamins		Bioactives	
Vitamin A (β-Carotene)	Vitamin C (Ascorbic acid)	Total Daidzein Equivalent	Lectin
Vitamin B_1 (Thiamine HCl)	Vitamin E (α-Tocopherol)	Total Genistein Equivalent	Phytic acid
Vitamin B_2 (Riboflavin)	β-Tocopherol	Total Glycitein Equivalent	Raffinose
Vitamin B_3 (Niacin)	γ-Tocopherol		Stachyose
Vitamin B_5 (Pantothenic acid)	δ-Tocopherol		Trypsin Inhibitor
Vitamin B_6 (Pyridoxine HCl)	Total Tocopherol		
Vitamin B_9 (Folic acid)			

Analysis of variance was conducted across field sites (combined-site analysis) for composition data using a mixed model (SAS Institute Inc. 2009). Entry was considered a fixed effect, and location, block within location, and location-by-entry, were designated as random effects. Significant differences were declared at the 95% confidence level. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between DAS-444Ø6-6 (sprayed or unsprayed) entries and the control entry using t-tests.

Due to the large number of contrasts made in this study, multiplicity was an issue. Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise P-values is

very high ($1-0.95^{\text{number of comparisons}}$). In this study there were five comparisons per analyte (71 analysed analytes for composition), resulting in 355 comparisons made in the combined-site composition analysis. Therefore, the probability of declaring one or more false differences based on unadjusted P-values was >99.99% ($1-0.95^{355}$).

One method to account for multiplicity is to adjust P-values to control the experiment-wise error rate, 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 a False Discovery Rate (FDR) control procedure (Benjamini and Hochberg 1995); FDR methods are commonly applied in studies examining transgenic crops (Herman, Storer et al. 2007; Coll, Nadal et al. 2008; Huls, Erickson et al. 2008; Jacobs, Utterback et al. 2008; Stein, Rice et al. 2009; Herman, Phillips et al. 2010). Therefore, the P-values from the composition contrasts were each adjusted using the FDR method to improve discrimination of true differences among treatments from random effects (false positives). Differences were considered significant if the FDR-adjusted P-value was less than 0.05.

Compositional Analyses Results

A statistical analysis of composition data from the non-transgenic near-isogenic, unsprayed DAS-444Ø6-6 and sprayed DAS-444Ø6-6 entries was conducted. A summary of the compositional results across locations is presented in Table to Table 13. For each analyte and entry, the least square means, standard error, and minimum and maximum sample values are reported. Also for comparison, the minimum and maximum values from reference lines across all sites (reference ranges) and literature ranges for each analyte are reported. Each minimum and maximum value is an individual data point reported for a single test plot, and applies to literature ranges except where noted. Arithmetic means for each analyte from each field site are plotted for the non-transgenic control, DAS-444Ø6-6 (sprayed and unsprayed), and reference line entries (Figure 53 to Figure). Literature ranges reported as not detected (ND) or less than the limit of quantitation (<LOQ) were plotted as zeros.

Proximate, Fibre and Mineral Analysis of Forage

Soybean forage samples from the control, reference, and DAS-444Ø6-6 entries were analysed for proximate content (protein, fat, ash, moisture, and carbohydrates), fibre (acid detergent fibre (ADF), neutral detergent fibre (NDF)), and minerals (calcium and phosphorus). A summary of the results across all locations is presented in Table , 17, Figure 53 and Figure . All mean values were within literature ranges (when available) and within ranges for reference lines included in the study. No statistical differences were observed in the combined-site analysis between the control and DAS-444Ø6-6 entries for protein, fat, ash, carbohydrates, ADF, NDF, calcium, and phosphorus. Statistically significant

differences were observed between the control and DAS-444Ø6-6 entries for moisture, where mean differences were negligible and not biologically meaningful as means were within literature ranges and within ranges for reference lines included in the study.

Table 16. Summary of the proximate and fiber analysis of DAS-444Ø6-6 soybean forage from all sites, and associated literature range.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Proximate									
Protein		19.7 ± 0.5	19.7 ± 0.5	19.4 ± 0.5	19.2 ± 0.5	19.6 ± 0.5	19.7 ± 0.5		
		13.7 - 23.4	16 - 25.3	15.5 - 23.4	14.8 - 24.1	14.5 - 23.9	15.2 - 25.2	13 - 29.1	11.2 - 24.71
(% DW)	0.425		(0.890, 0.958)	(0.227, 0.440)	(0.103, 0.275)	(0.655, 0.806)	(0.990, 0.996)		
Fat		2.87 ± 0.1	2.75 ± 0.1	2.81 ± 0.1	2.85 ± 0.1	2.68 ± 0.1	2.68 ± 0.1		
		1.95 - 4.11	0.769 - 4.01	1.6 - 3.88	1.14 - 3.79	1.91 - 3.52	1.47 - 3.69	1.69 - 4.63	1.01 - 9.87
(% DW)	0.443		(0.337, 0.549)	(0.648, 0.804)	(0.886, 0.956)	(0.122, 0.297)	(0.121, 0.297)		
Ash		9.4 ± 0.8	9.2 ± 0.8	8.9 ± 0.8	9.7 ± 0.8	8.9 ± 0.8	8.9 ± 0.8		
		7.13 - 28.3	5.96 - 31	6.14 - 19	6.57 - 24	6.42 - 21.4	6.85 - 18.7	5.86 - 36.6	4.68 - 10.782
(% DW)	0.523		(0.766, 0.894)	(0.388, 0.604)	(0.542, 0.726)	(0.367, 0.582)	(0.301, 0.514)		
Moisture		78.7 ± 0.6	77.6 ± 0.6	77.6 ± 0.6	77.6 ± 0.6	77.2 ± 0.6	77.4 ± 0.6		
		75.6 - 82.3	71.8 - 80.5	71 - 81	69.9 - 81.1	69.1 - 80.7	69.8 - 81.1	70.9 - 81.4	32.05 - 84.60
(% FW)	**0.002**		(**0.003**, **0.023**)	(**0.003**, **0.019**)	(**0.003**, **0.020**)	(**<0.001**, **0.002**)	(**<0.001**, **0.005**)		
Carbohydrates[e]		68.0 ± 1	68.4 ± 1	68.9 ± 1	68.2 ± 1	68.8 ± 1	68.8 ± 1		
		55.8 - 74.3	49.8 - 76.3	59.1 - 73	53.8 - 74.4	56.3 - 74.4	59.5 - 73.8	48.8 - 74.7	59.8 - 80.18
(% DW)	0.534		(0.548, 0.729)	(0.128, 0.303)	(0.763, 0.894)	(0.174, 0.372)	(0.196, 0.400)		
Fiber									
Acid Detergent		31.6 ± 0.9	31.5 ± 0.9	31.7 ± 0.9	31.7 ± 0.9	32.3 ± 0.9	30.6 ± 0.9		
Fiber (ADF)		22.9 - 38.7	24.3 - 44.2	23 - 42.2	25 - 44.7	26 - 43.6	23.7 - 39.3	21.5 - 57.2	22.72 - 59.03
(% DW)	0.653		(0.933, 0.976)	(0.882, 0.956)	(0.903, 0.963)	(0.478, 0.692)	(0.301, 0.514)		
Neutral Detergent		37.6 ± 1.2	37.4 ± 1.2	38.1 ± 1.2	37.3 ± 1.2	37.5 ± 1.2	37.6 ± 1.2		
Fiber (NDF)		29.1 - 46.5	27.2 - 51.3	27.3 - 50	28.4 - 50.4	24.7 - 50	21.8 - 52.3	24.9 - 63.1	19.61 - 73.05
(% DW)	0.974		(0.840, 0.941)	(0.609, 0.778)	(0.759, 0.894)	(0.924, 0.970)	(0.951, 0.976)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

[e] % Carbohydrates = 100 % - (% Protein + % Fat + % Ash + % Moisture)

Table 17. Summary of the mineral analysis of DAS-444Ø6-6 soybean forage from all sites, and associated literature range.

Component (Units)[a]	Effect (Pr > F)[b]	Min - Max	Min - Max (P-value, Adj.P)[c]	Min - Max (P-value, Adj.P)[c]	Min - Max (P-value, Adj.P)[c]	Min - Max (P-value, Adj.P)[c]	Min - Max (P-value, Adj.P)[c]	Min - Max	Min - Max
Mineral									
Calcium		1240 ± 63	1236 ± 63	1208 ± 63	1211 ± 63	1227 ± 63	1263 ± 63		
		880 - 1770	652 - 1590	817 - 1560	650 - 1540	858 - 1600	762 - 1760	695 - 1860	NR
Phosphorus		271 ± 13	266 ± 13	266 ± 13	264 ± 13	265 ± 13	265 ± 13		
		190 - 384	177 - 381	197 - 374	186 - 385	197 - 399	170 - 394	175 - 427	NR
(mg/100g dry wt.)	0.690		(0.253, 0.458)	(0.239, 0.449)	(0.118, 0.294)	(0.182, 0.378)	(0.231, 0.443)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was converted from % dry wt. to mg/100g dry wt. prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

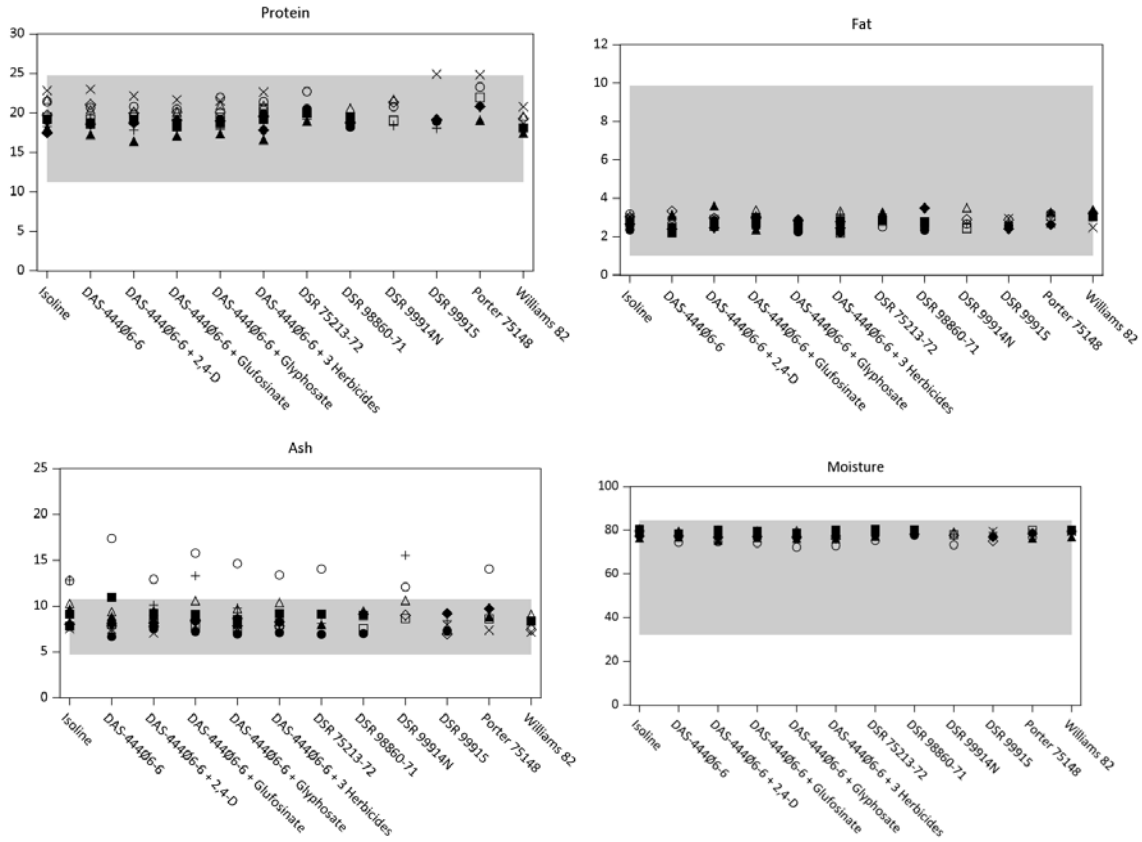


Figure 53. Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean forage.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

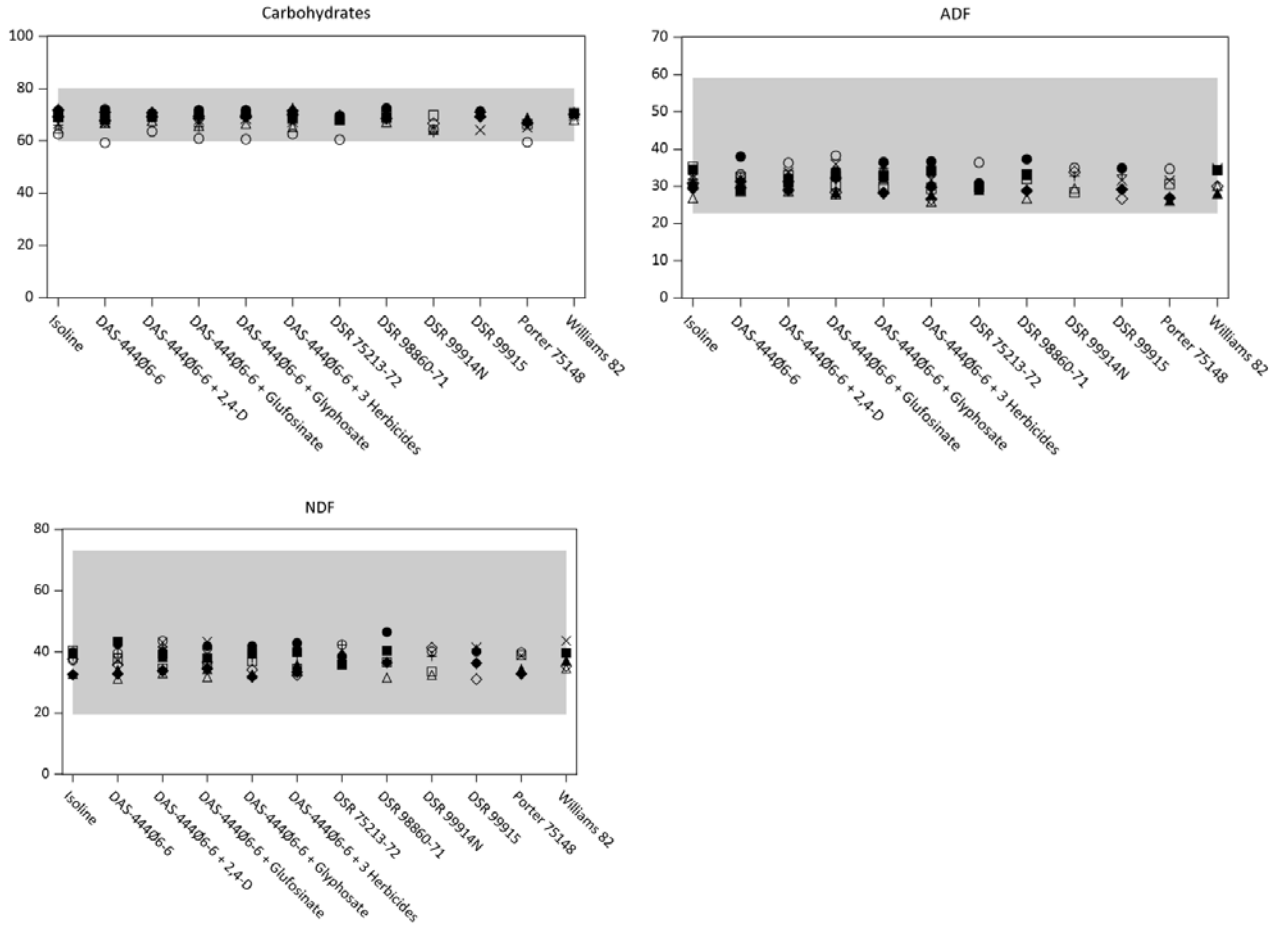


Figure 53. (Cont). Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean forage. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

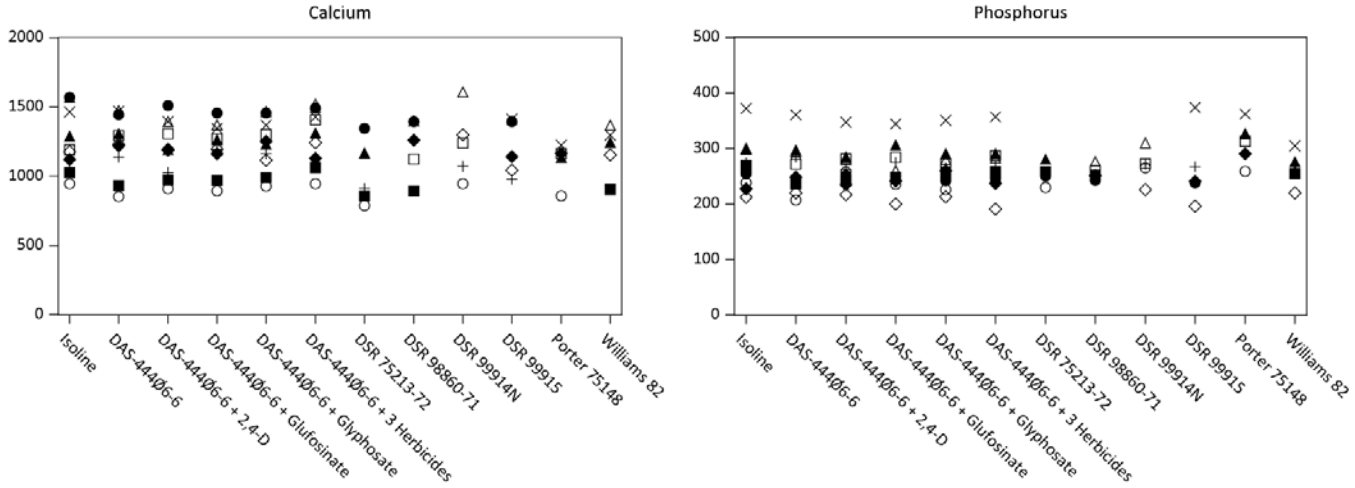


Figure 54. Minerals (mg/100g dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean forage.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

Proximate and Fibre Analysis of Seed

Soybean seed samples from the control, reference, and DAS-444Ø6-6 entries were analysed for proximate content (protein, fat, ash, moisture, and carbohydrates) and fibre (acid detergent fibre (ADF), neutral detergent fibre (NDF), and total dietary fibre). A summary of the results across all locations is presented in Table 10 and Figure . All mean results were within literature ranges (when available) and within ranges for reference lines included in the study. Statistically significant overall treatment effects were found for protein and carbohydrates, where some DAS-444Ø6-6 entries contained more protein and less carbohydrate than the control. Similarly, variations in fat, ash, and moisture were also observed for some pair-wise contrasts between DAS-444Ø6-6 entries and the control. Carbohydrate composition is calculated from values for protein, fat, ash, and moisture (Table 108). Therefore, an increase in protein and related proximate components is expected to result in a partial decrease in carbohydrates. Statistical differences were also found for neutral detergent fibre (NDF) and total dietary fibre, where values were slightly lower in some DAS-444Ø6-6 entries compared with the control. No biologically meaningful differences were detected as all results for proximate content and fibre were within literature ranges and within ranges for reference lines included in the study.

Table 108. Summary of the proximate and fiber analysis of DAS-444Ø6-6 soybean seed from all sites, and associated literature range.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Proximate									
Protein		37.8 ± 0.3	38.0 ± 0.3	38.5 ± 0.3	38.2 ± 0.3	38.2 ± 0.3	38.6 ± 0.3		
		29.7 - 40.3	35.6 - 39.7	37.1 - 40.4	34 - 40.6	36 - 40.9	36.1 - 42.5	35.1 - 44.9	32 - 48.4
(% DW)	**0.039**		(0.323, 0.537)	(**0.012**, 0.065)	(0.106, 0.279)	(0.086, 0.246)	(**0.002**, **0.019**)		
Fat		18.9 ± 0.6	19.5 ± 0.6	19.2 ± 0.6	19.3 ± 0.6	19.2 ± 0.6	19.1 ± 0.6		
		13.6 - 23.5	16.9 - 23.5	15.8 - 23.4	16.7 - 23.4	16.5 - 23.1	16.2 - 22.6	15.3 - 22.9	8.104 - 24.7
(% DW)	0.069		(**0.003**, **0.023**)	(0.152, 0.346)	(**0.028**, 0.109)	(0.144, 0.333)	(0.246, 0.453)		
Ash		5.15 ± 0.09	5.23 ± 0.09	5.24 ± 0.09	5.24 ± 0.09	5.21 ± 0.09	5.22 ± 0.09		
		4.49 - 5.86	4.66 - 6.34	4.59 - 5.99	4.55 - 6.87	4.49 - 5.78	4.48 - 6.42	4.45 - 6.3	3.885 - 6.994
(% DW)	0.278		(0.060, 0.188)	(**0.035**, 0.128)	(**0.041**, 0.140)	(0.158, 0.357)	(0.128, 0.303)		
Moisture		10.6 ± 0.7	10.2 ± 0.7	10.0 ± 0.7	9.9 ± 0.7	9.9 ± 0.7	9.9 ± 0.7		
		7.58 - 20.4	7.1 - 22.1	7.19 - 13.8	6.54 - 14.1	7.13 - 14.5	6.87 - 12.9	7.26 - 17.2	4.7 - 34.4
(% FW)	0.072		(0.160, 0.358)	(**0.026**, 0.104)	(**0.018**, 0.085)	(**0.010**, 0.059)	(**0.010**, 0.060)		
Carbohydrates[e]		38.13 ± 0.75	37.22 ± 0.76	37.11 ± 0.75	37.21 ± 0.75	37.38 ± 0.75	37.04 ± 0.75		
		32.6 - 47.7	32.5 - 40.8	31.3 - 41.3	31.2 - 40.6	32.1 - 41.5	32.2 - 40.7	28.7 - 43	29.3 - 50.2
(% DW)	**0.002**		(**0.002**, **0.014**)	(**<0.001**, **0.005**)	(**0.001**, **0.012**)	(**0.008**, **0.049**)	(**<0.001**, **0.002**)		
Fiber									
Acid Detergent		15.5 ± 0.5	15.2 ± 0.5	15.5 ± 0.5	15.0 ± 0.5	15.6 ± 0.5	14.9 ± 0.5		
Fiber (ADF)		9.84 - 24.1	7.68 - 20.7	8.71 - 18.7	10.3 - 18.2	11.7 - 19.6	11.3 - 20.3	8.02 - 20.9	7.81 - 26.26
(% DW)	0.577		(0.435, 0.648)	(0.911, 0.966)	(0.284, 0.491)	(0.940, 0.976)	(0.174, 0.372)		
Neutral Detergent		17.7 ± 0.3	17.0 ± 0.3	17.1 ± 0.3	16.7 ± 0.3	17.3 ± 0.3	16.5 ± 0.3		
Fiber (NDF)		14.6 - 24.1	9.41 - 20.9	10.9 - 21.4	13.1 - 20.1	13.2 - 22	13.4 - 19.7	13.3 - 22.2	8.53 - 23.90
(% DW)	**0.030**		(0.088, 0.249)	(0.111, 0.284)	(**0.012**, 0.065)	(0.317, 0.535)	(**0.002**, **0.014**)		
Total Dietary		22.4 ± 0.5	21.6 ± 0.5	21.7 ± 0.5	21.5 ± 0.5	21.9 ± 0.5	21.4 ± 0.5		
Fiber		16.7 - 27.8	18 - 25.4	17.9 - 26.1	17.3 - 26.5	16.9 - 25.8	16 - 25.6	16.2 - 27.7	NR
(% DW)	0.144		(**0.048**, 0.159)	(0.092, 0.256)	(**0.033**, 0.120)	(0.252, 0.458)	(**0.012**, 0.065)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

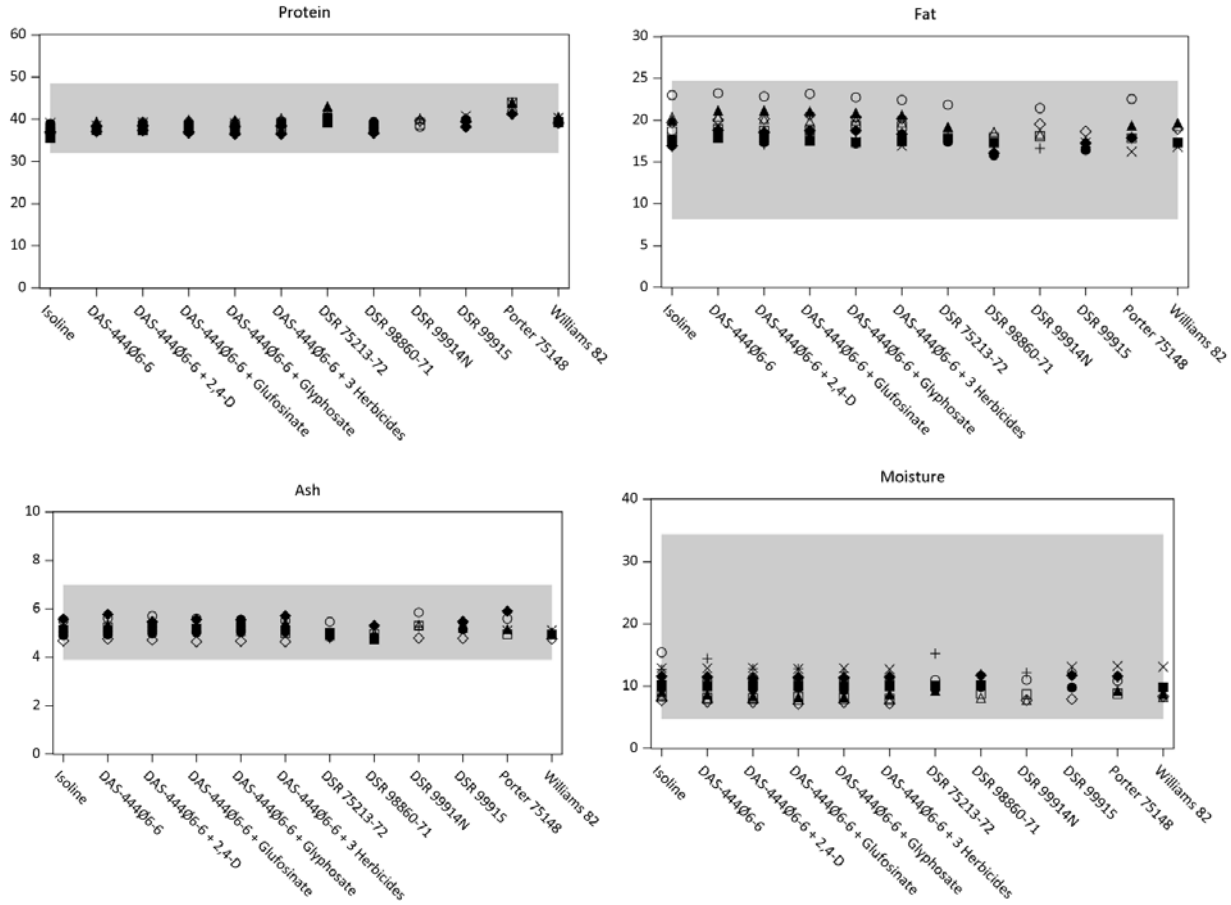
[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

[e] % Carbohydrates = 100 % - (% Protein + % Fat + % Ash + % Moisture)


Protein
Fat
Ash
Moisture
Isoline
DAS-444Ø6-6
DAS-444Ø6-6 + 2,4-D
DAS-444Ø6-6 + Glufosinate
DAS-444Ø6-6 + Glyphosate
DAS-444Ø6-6 + 3 Herbicides
DSR 75213-72
DSR 98860-71
DSR 99914N
DSR 99915
Porter 75148
Williams 82

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

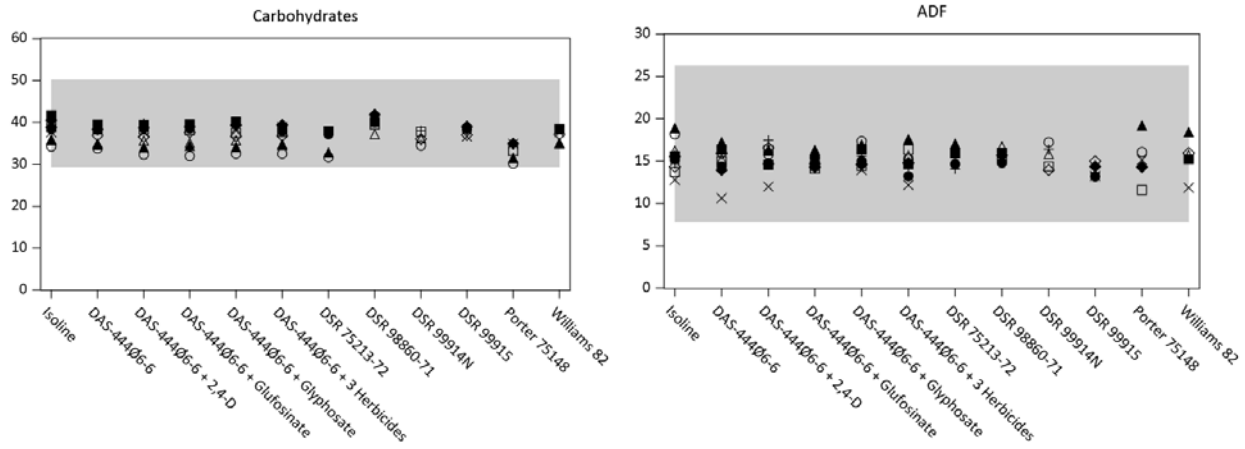


Figure 55. Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

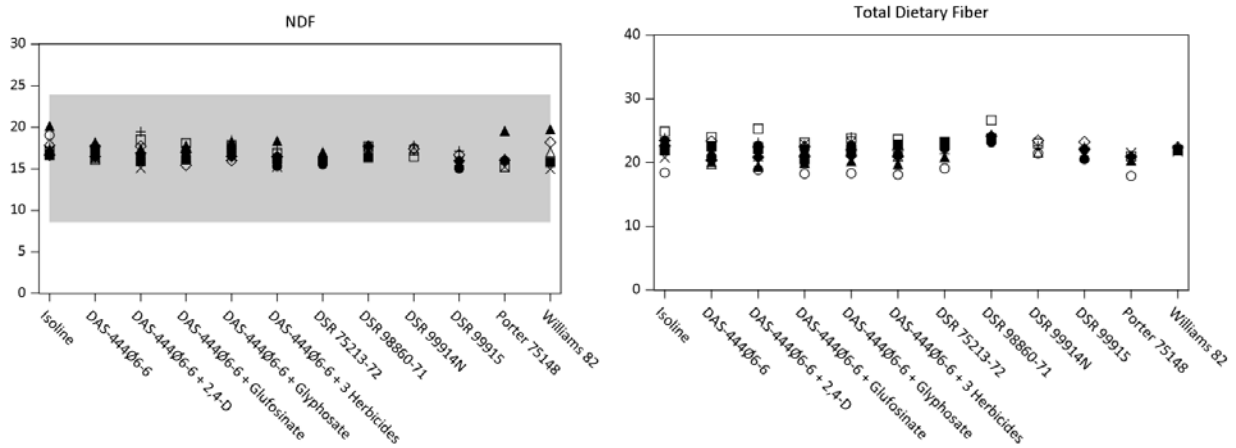


Figure (Cont). Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

Mineral Analysis of Seed

Soybean seed samples from the control, reference, and DAS-444Ø6-6 entries were analysed for minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium, and zinc). A summary of the results across all locations is presented in Table 11 and Figure 44. All mean results were within literature ranges (when available) and/or within ranges for reference lines included in the study. For sodium, statistical analysis was not performed since greater than 50% of the samples were found to be below the LOQ. No statistical differences were observed in the combined-site analysis between the control and DAS-444Ø6-6 entries for copper, iron, magnesium, manganese, phosphorus, and selenium. Statistically significant differences were observed for calcium, potassium, and zinc for some DAS-444Ø6-6 entries compared with the control, where mean differences were negligible and not biologically meaningful as means were within literature ranges and/or within ranges for reference lines included in the study.

Table 11. Summary of the mineral analysis of DAS-444Ø6-6 soybean seed from all sites, and associated literature range.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Mineral									
Calcium		301 ± 13	324 ± 13	318 ± 13	304 ± 13	320 ± 13	306 ± 13		
		235 - 403	261 - 425	252 - 404	241 - 398	249 - 413	243 - 404	174 - 383	116.55 - 510
(mg/100 g DW)	**<0.001**		(**<0.001**, **<0.001**)	(**<0.001**, **0.001**)	(0.407, 0.623)	(**<0.001**, **<0.001**)	(0.184, 0.381)		
Copper		1.32 ± 0.05	1.34 ± 0.05	1.35 ± 0.05	1.32 ± 0.05	1.35 ± 0.05	1.33 ± 0.05		
		0.995 - 1.68	1.01 - 1.71	1.03 - 1.93	1.04 - 1.74	1.15 - 1.7	1.09 - 1.71	0.91 - 1.77	0.632 - 1.092
(mg/100 g DW)	0.310		(0.222, 0.436)	(0.057, 0.182)	(0.780, 0.901)	(0.082, 0.242)	(0.324, 0.537)		
Iron		8.2 ± 0.5	8.3 ± 0.6	7.7 ± 0.5	8.6 ± 0.5	7.8 ± 0.5	8.5 ± 0.5		
		6.51 - 14.2	6.33 - 26.2	6.18 - 9.64	6.55 - 41.9	6.42 - 12.3	6.54 - 24.5	5.35 - 87.9	3.734 - 10.954
(mg/100 g DW)	0.650		(0.906, 0.963)	(0.395, 0.609)	(0.547, 0.729)	(0.537, 0.725)	(0.674, 0.822)		
Magnesium		229 ± 6	231 ± 6	230 ± 6	227 ± 6	230 ± 6	229 ± 6		
		207 - 279	205 - 283	206 - 287	202 - 276	203 - 279	200 - 284	195 - 317	219.40 - 312.84
(mg/100 g DW)	0.226		(0.281, 0.490)	(0.331, 0.543)	(0.285, 0.491)	(0.347, 0.554)	(0.974, 0.985)		
Manganese		2.99 ± 0.53	3.10 ± 0.54	3.27 ± 0.53	3.09 ± 0.53	3.18 ± 0.53	3.14 ± 0.53		
		2.11 - 7.83	1.69 - 8.27	1.78 - 10.4	2.03 - 8.57	1.89 - 10.8	2.08 - 9.46	1.9 - 9.53	2.52 - 3.876
(mg/100 g DW)	0.620		(0.483, 0.695)	(0.084, 0.243)	(0.532, 0.725)	(0.231, 0.443)	(0.335, 0.548)		
Phosphorus		557 ± 21	561 ± 21	558 ± 21	554 ± 21	558 ± 21	557 ± 21		
		400 - 640	394 - 661	384 - 681	377 - 657	403 - 645	388 - 660	360 - 659	506.74 - 935.24
(mg/100 g DW)	0.856		(0.474, 0.690)	(0.905, 0.963)	(0.526, 0.723)	(0.935, 0.976)	(0.866, 0.949)		
Potassium		1730 ± 20	1780 ± 20	1790 ± 20	1770 ± 20	1770 ± 20	1770 ± 20		
		1580 - 1850	1610 - 1930	1640 - 1940	1630 - 1930	1620 - 1890	1610 - 1940	1530 - 2030	1868.01 - 2510
(mg/100 g DW)	**<0.001**		(**<0.001**, **<0.001**)	(**<0.001**, **<0.001**)	(**<0.001**, **0.002**)	(**<0.001**, **0.005**)	(**0.001**, **0.011**)		
Selenium		451 ± 108	438 ± 108	489 ± 108	389 ± 108	420 ± 108	469 ± 108		
		66.7 - 1980	77.7 - 1770	63.8 - 2320	77 - 1770	84 - 1670	72.3 - 2360	59.5 - 3380	NR
(ppb DW)	0.552		(0.831, 0.934)	(0.496, 0.707)	(0.278, 0.488)	(0.592, 0.767)	(0.741, 0.885)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Mineral									
Sodium		NA < LOQ	NA < LOQ - 15.7	NA < LOQ - 15	NA < LOQ - 16.7	NA < LOQ - 13.4	NA < LOQ - 11.7	< LOQ - 18.5	4.05 - 30
(mg/100 g DW)	NA								
Zinc		4.17 ± 0.12 3.62 - 4.7	4.34 ± 0.12 3.45 - 5.02	4.34 ± 0.12 3.53 - 5.59	4.25 ± 0.12 3.62 - 5.01	4.3 ± 0.12 3.56 - 5.05	4.3 ± 0.12 3.67 - 6.01	3.34 - 5.82	4.98 - 7.578
(mg/100 g DW)	0.077		(**0.010**, 0.058)	(**0.010**, 0.058)	(0.205, 0.414)	(**0.036**, 0.128)	(**0.042**, 0.143)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

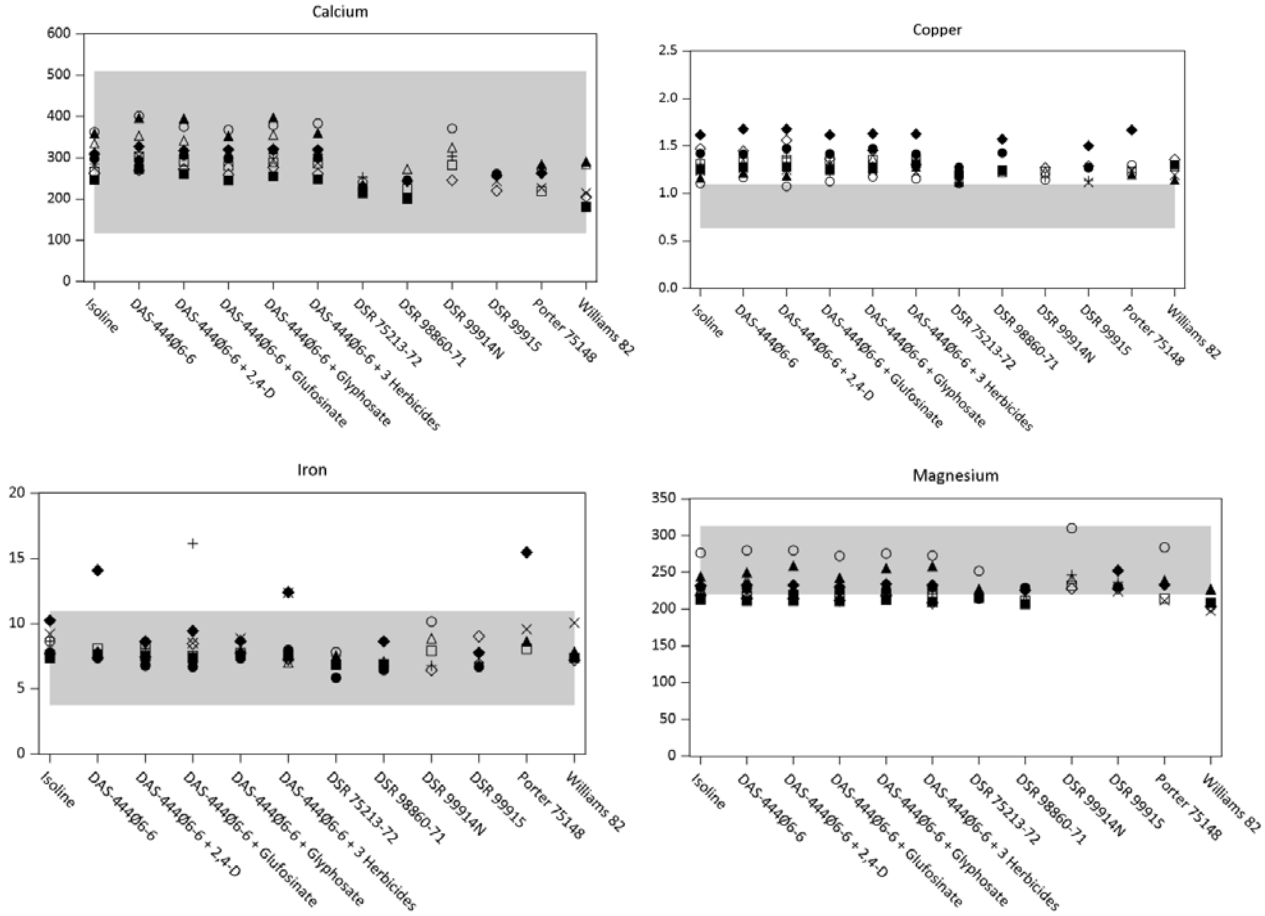


Figure 44. Minerals (mg/100g dry weight for all minerals except selenium (ppb dry weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

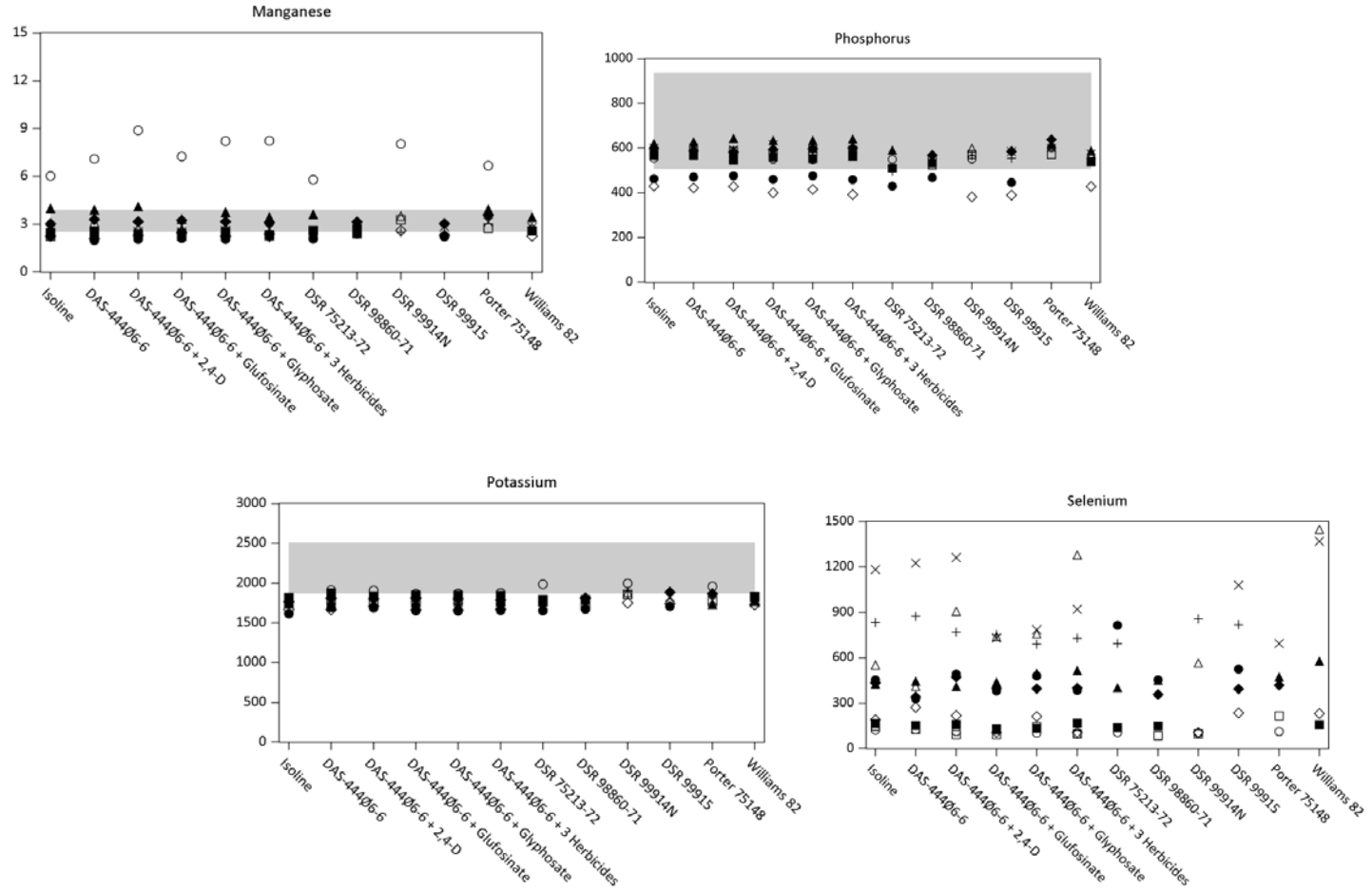


Figure 44 (Cont). Minerals (mg/100g dry weight for all minerals except selenium (ppb dry weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open **square** = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

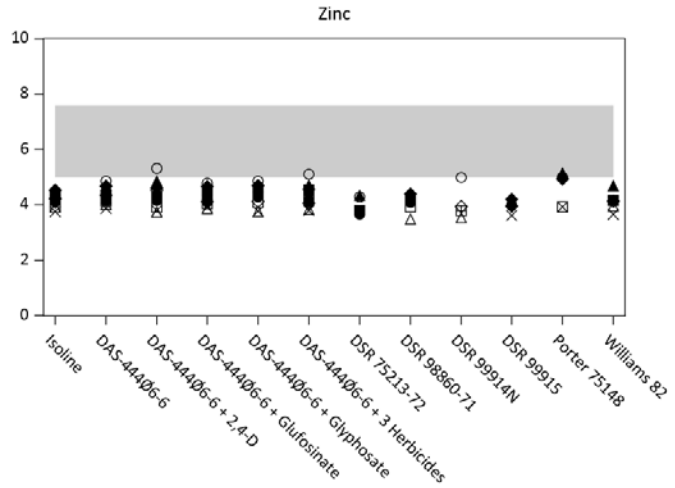


Figure 44 (Cont). Minerals (mg/100g dry weight for all minerals except selenium (ppb dry weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

Amino Acid Analysis of Seed

Soybean seed samples from the control, reference, and DAS-444Ø6-6 entries were analysed for amino acid content. A summary of the results across all locations is presented in Table 20 and Figure 57. All mean results were within literature ranges and within ranges for reference lines included in the study. No statistical differences were observed in the combined-site analysis between the control and DAS-444Ø6-6 entries for alanine, arginine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine. Statistically significant differences were observed for cystine, histidine, lysine, tryptophan, and tyrosine for some DAS-444Ø6-6 entries compared with the control, where mean differences were negligible and not biologically meaningful as means were within literature ranges and within ranges for reference lines included in the study.

Table 20. Summary of the amino acid analysis of DAS-444Ø6-6 soybean seed from all sites, and associated literature range.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Amino Acid									
Alanine		1.68 ± 0.01	1.67 ± 0.01	1.67 ± 0.01	1.68 ± 0.01	1.67 ± 0.01	1.68 ± 0.01		
		1.56 - 1.76	1.58 - 1.76	1.6 - 1.74	1.6 - 1.74	1.6 - 1.81	1.59 - 1.88	1.55 - 1.9	1.43 - 2.10
(% DW)	0.734		(0.282, 0.490)	(0.453, 0.667)	(0.980, 0.988)	(0.616, 0.784)	(0.763, 0.894)		
Arginine		2.74 ± 0.02	2.71 ± 0.02	2.75 ± 0.02	2.74 ± 0.02	2.74 ± 0.02	2.75 ± 0.02		
		2.55 - 2.94	2.46 - 2.88	2.6 - 2.95	2.6 - 2.99	2.55 - 3	2.58 - 3.16	2.59 - 3.45	2.15 - 3.46
(% DW)	0.440		(0.182, 0.378)	(0.620, 0.786)	(0.856, 0.949)	(0.762, 0.894)	(0.603, 0.776)		
Aspartic Acid		4.18 ± 0.03	4.19 ± 0.03	4.23 ± 0.03	4.22 ± 0.03	4.22 ± 0.03	4.24 ± 0.03		
		3.85 - 4.45	3.9 - 4.4	4.03 - 4.49	4.02 - 4.59	3.94 - 4.64	3.89 - 4.82	3.58 - 4.94	3.81 - 6.04
(% DW)	0.231		(0.959, 0.977)	(0.093, 0.256)	(0.165, 0.363)	(0.168, 0.365)	(0.055, 0.177)		
Cystine		0.532 ± 0.010	0.556 ± 0.010	0.547 ± 0.010	0.560 ± 0.010	0.549 ± 0.010	0.560 ± 0.010		
		0.458 - 0.647	0.493 - 0.661	0.474 - 0.645	0.489 - 0.672	0.482 - 0.652	0.487 - 0.66	0.429 - 0.71	0.37 - 0.81
(% DW)	**0.002**		(**0.001**, **0.011**)	(**0.032**, 0.120)	(**<0.001**, **0.002**)	(**0.015**, 0.075)	(**<0.001**, **0.002**)		
Glutamic Acid		6.26 ± 0.06	6.22 ± 0.06	6.26 ± 0.06	6.27 ± 0.06	6.26 ± 0.06	6.28 ± 0.06		
		5.75 - 6.74	5.65 - 6.78	5.82 - 6.76	5.8 - 6.95	5.85 - 6.92	5.74 - 7.18	5.85 - 7.77	5.84 - 9.15
(% DW)	0.837		(0.429, 0.646)	(0.960, 0.977)	(0.709, 0.851)	(0.964, 0.978)	(0.602, 0.776)		
Glycine		1.64 ± 0.01	1.63 ± 0.01	1.65 ± 0.01	1.64 ± 0.01	1.65 ± 0.01	1.65 ± 0.01		
		1.53 - 1.75	1.51 - 1.73	1.58 - 1.73	1.55 - 1.75	1.56 - 1.84	1.54 - 1.87	1.55 - 1.89	1.41 - 2.00
(% DW)	0.592		(0.511, 0.720)	(0.378, 0.591)	(0.761, 0.894)	(0.391, 0.607)	(0.434, 0.648)		
Histidine		1.02 ± 0.01	1.02 ± 0.01	1.04 ± 0.01	1.02 ± 0.01	1.03 ± 0.01	1.03 ± 0.01		
		0.943 - 1.1	0.91 - 1.1	0.957 - 1.11	0.964 - 1.13	0.935 - 1.17	0.946 - 1.19	0.197 - 1.18	0.86 - 1.24
(% DW)	0.299		(0.945, 0.976)	(**0.045**, 0.152)	(0.896, 0.960)	(0.551, 0.730)	(0.669, 0.819)		
Isoleucine		1.81 ± 0.02	1.81 ± 0.02	1.83 ± 0.02	1.81 ± 0.02	1.83 ± 0.02	1.82 ± 0.02		
		1.66 - 1.94	1.64 - 1.94	1.67 - 2.02	1.58 - 1.93	1.68 - 2.12	1.64 - 2.06	1.68 - 2.18	1.49 - 2.08
(% DW)	0.458		(0.815, 0.928)	(0.108, 0.281)	(0.608, 0.778)	(0.116, 0.291)	(0.421, 0.639)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Amino Acid									
Leucine		2.84 ± 0.02	2.83 ± 0.02	2.87 ± 0.02	2.85 ± 0.02	2.86 ± 0.02	2.86 ± 0.02		
		2.65 - 3.02	2.62 - 2.99	2.72 - 3.06	2.67 - 3.06	2.69 - 3.2	2.67 - 3.25	2.68 - 3.32	2.2 - 4.0
(% DW)	0.151		(0.522, 0.723)	(0.059, 0.186)	(0.483, 0.695)	(0.209, 0.418)	(0.255, 0.460)		
Lysine		2.46 ± 0.03	2.48 ± 0.03	2.53 ± 0.03	2.48 ± 0.03	2.49 ± 0.03	2.49 ± 0.03		
		2.1 - 2.77	2.11 - 2.76	2.14 - 2.76	2.22 - 2.8	2.06 - 2.97	2.12 - 3.03	2 - 3.04	2.19 - 3.32
(% DW)	0.369		(0.522, 0.723)	(**0.029**, 0.111)	(0.502, 0.713)	(0.344, 0.554)	(0.320, 0.535)		
Methionine		0.504 ± 0.006	0.502 ± 0.006	0.505 ± 0.006	0.501 ± 0.006	0.499 ± 0.006	0.507 ± 0.006		
		0.447 - 0.577	0.435 - 0.595	0.449 - 0.567	0.456 - 0.555	0.445 - 0.564	0.442 - 0.621	0.418 - 0.596	0.39 - 0.68
(% DW)	0.873		(0.821, 0.931)	(0.922, 0.970)	(0.694, 0.838)	(0.450, 0.666)	(0.647, 0.804)		
Phenylalanine		1.9 ± 0.02	1.89 ± 0.02	1.91 ± 0.02	1.9 ± 0.02	1.91 ± 0.02	1.91 ± 0.02		
		1.76 - 2.04	1.72 - 2.02	1.8 - 2.04	1.77 - 2.05	1.77 - 2.13	1.77 - 2.18	1.8 - 2.28	1.6 - 2.44
(% DW)	0.471		(0.633, 0.795)	(0.248, 0.454)	(0.622, 0.786)	(0.356, 0.567)	(0.225, 0.439)		
Proline		1.96 ± 0.02	1.95 ± 0.02	1.95 ± 0.02	1.95 ± 0.02	1.96 ± 0.02	1.95 ± 0.02		
		1.77 - 2.12	1.81 - 2.13	1.84 - 2.09	1.67 - 2.12	1.76 - 2.19	1.77 - 2.34	1.78 - 2.41	1.63 - 2.28
(% DW)	0.986		(0.530, 0.725)	(0.782, 0.901)	(0.537, 0.725)	(0.854, 0.949)	(0.772, 0.899)		
Serine		1.79 ± 0.02	1.80 ± 0.02	1.79 ± 0.02	1.82 ± 0.02	1.82 ± 0.02	1.83 ± 0.02		
		1.62 - 1.98	1.58 - 1.96	1.62 - 1.97	1.62 - 2	1.64 - 1.97	1.6 - 2.1	1.56 - 2.2	1.11 - 2.48
(% DW)	0.264		(0.701, 0.843)	(0.883, 0.956)	(0.114, 0.290)	(0.234, 0.443)	(0.093, 0.256)		
Threonine		1.52 ± 0.01	1.51 ± 0.01	1.52 ± 0.01	1.52 ± 0.01	1.53 ± 0.01	1.53 ± 0.01		
		1.43 - 1.58	1.38 - 1.59	1.42 - 1.59	1.43 - 1.6	1.46 - 1.6	1.42 - 1.75	1.4 - 1.75	1.14 - 1.89
(% DW)	0.171		(0.374, 0.590)	(0.524, 0.723)	(0.487, 0.697)	(0.205, 0.414)	(0.109, 0.281)		
Tryptophan		0.574 ± 0.01	0.588 ± 0.01	0.589 ± 0.01	0.575 ± 0.01	0.583 ± 0.01	0.583 ± 0.01		
		0.512 - 0.667	0.521 - 0.739	0.515 - 0.676	0.512 - 0.641	0.505 - 0.699	0.517 - 0.645	0.495 - 0.704	0.30 - 0.67
(% DW)	0.091		(**0.030**, 0.112)	(**0.021**, 0.092)	(0.860, 0.949)	(0.140, 0.325)	(0.165, 0.363)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Amino Acid									
Tyrosine		1.44 ± 0.01	1.44 ± 0.01	1.46 ± 0.01	1.45 ± 0.01	1.45 ± 0.01	1.45 ± 0.01		
		1.36 - 1.51	1.35 - 1.52	1.39 - 1.54	1.38 - 1.55	1.38 - 1.61	1.35 - 1.62	1.34 - 1.64	0.79 - 1.61
(% DW)	0.148		(0.872, 0.953)	(**0.024**, 0.098)	(0.242, 0.449)	(0.178, 0.377)	(0.219, 0.432)		
Valine		1.85 ± 0.01	1.85 ± 0.01	1.86 ± 0.01	1.85 ± 0.01	1.86 ± 0.01	1.85 ± 0.01		
		1.72 - 1.98	1.71 - 1.96	1.68 - 2.03	1.61 - 1.96	1.71 - 2.14	1.7 - 2.01	1.71 - 2.16	1.5 - 2.44
(% DW)	0.737		(0.921, 0.970)	(0.241, 0.449)	(0.954, 0.976)	(0.459, 0.670)	(1.000, 1.000)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

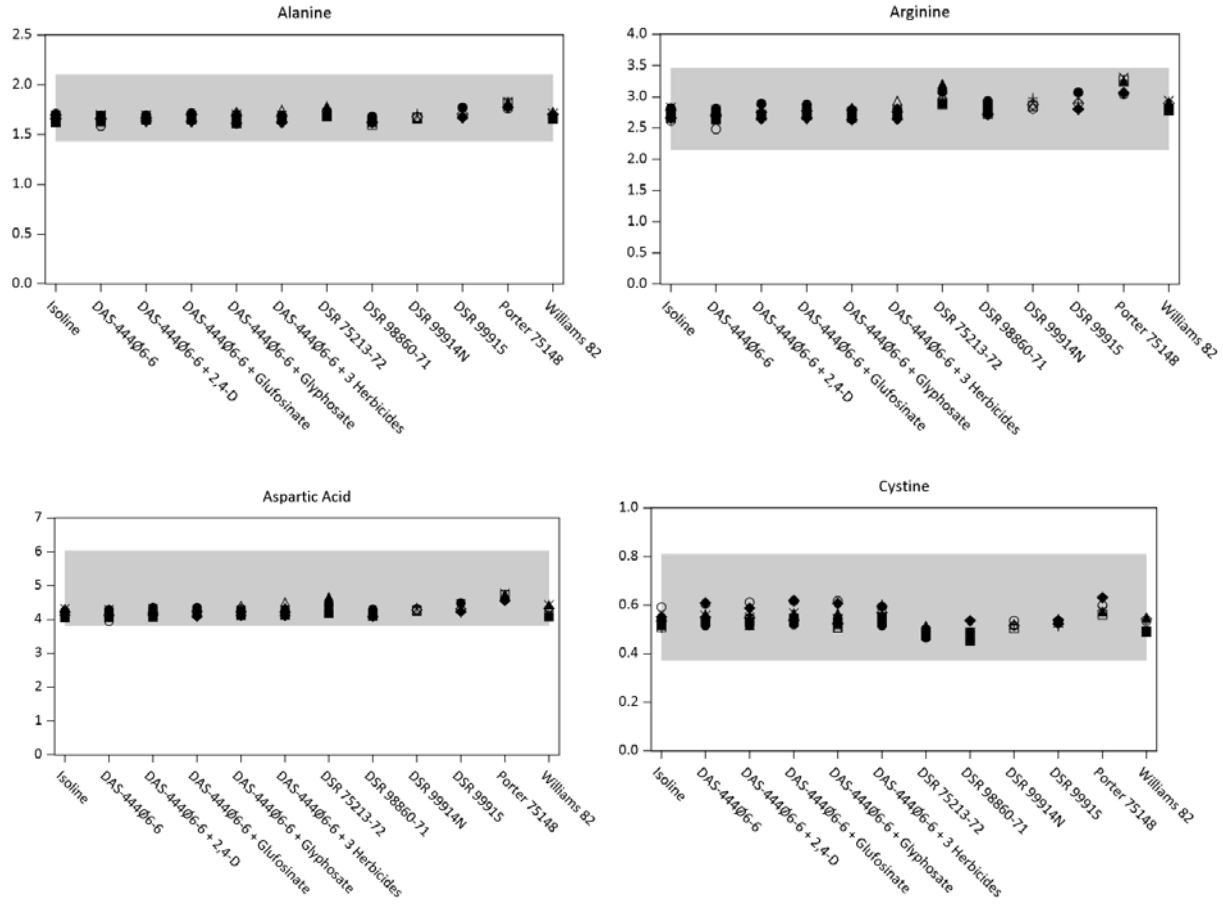


Figure 57. Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

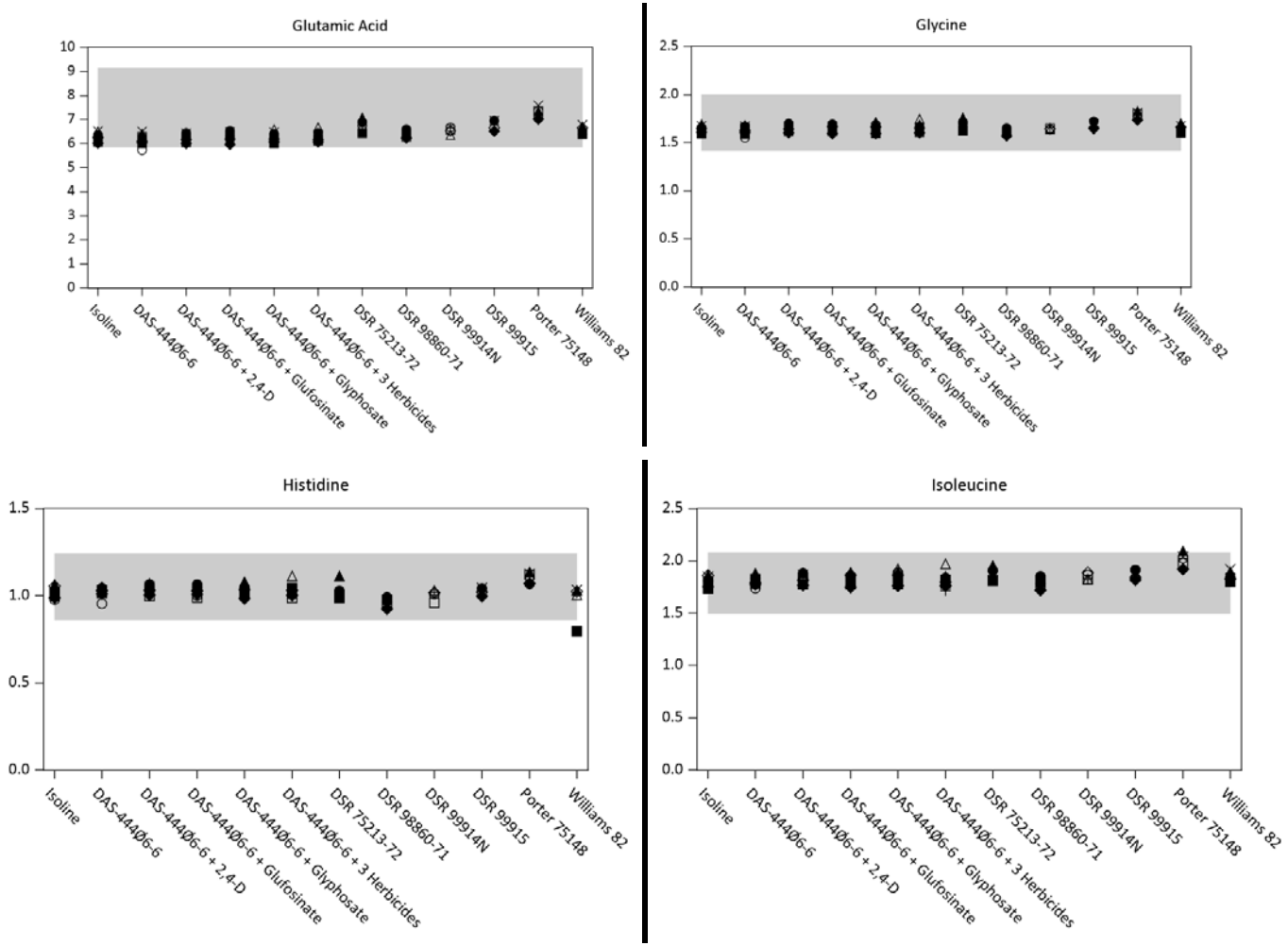


Figure 57 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

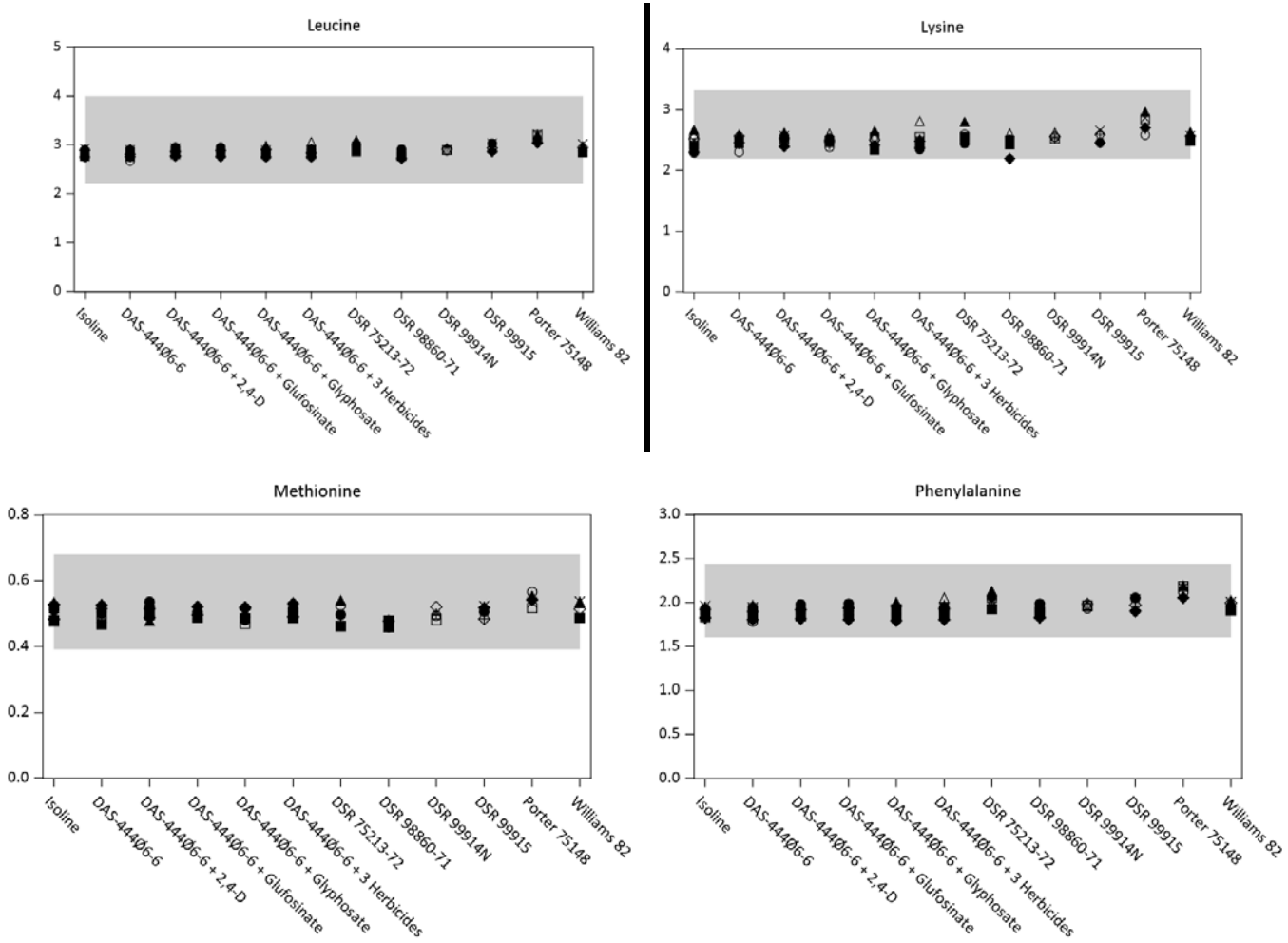


Figure 57 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

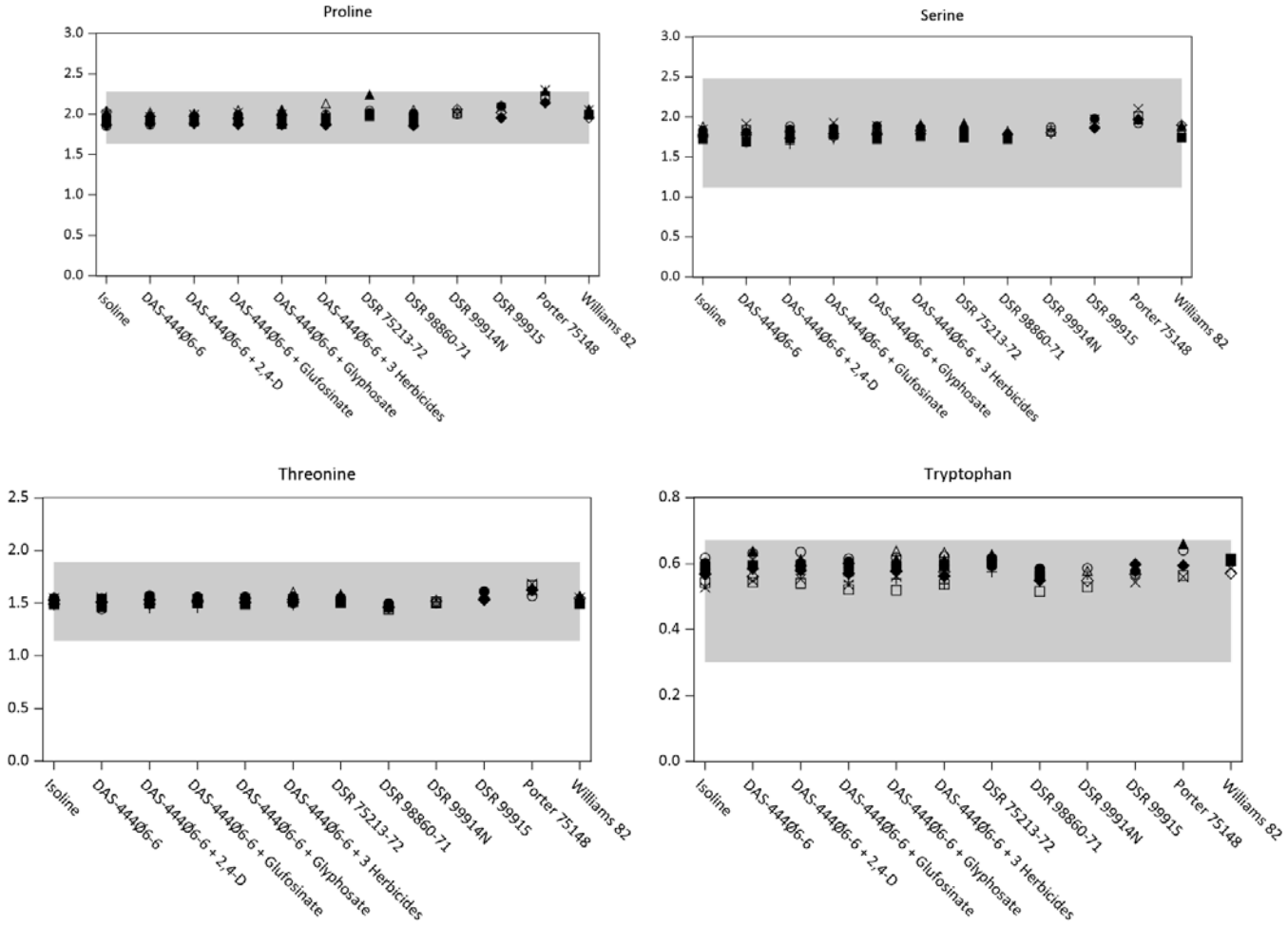


Figure 57 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

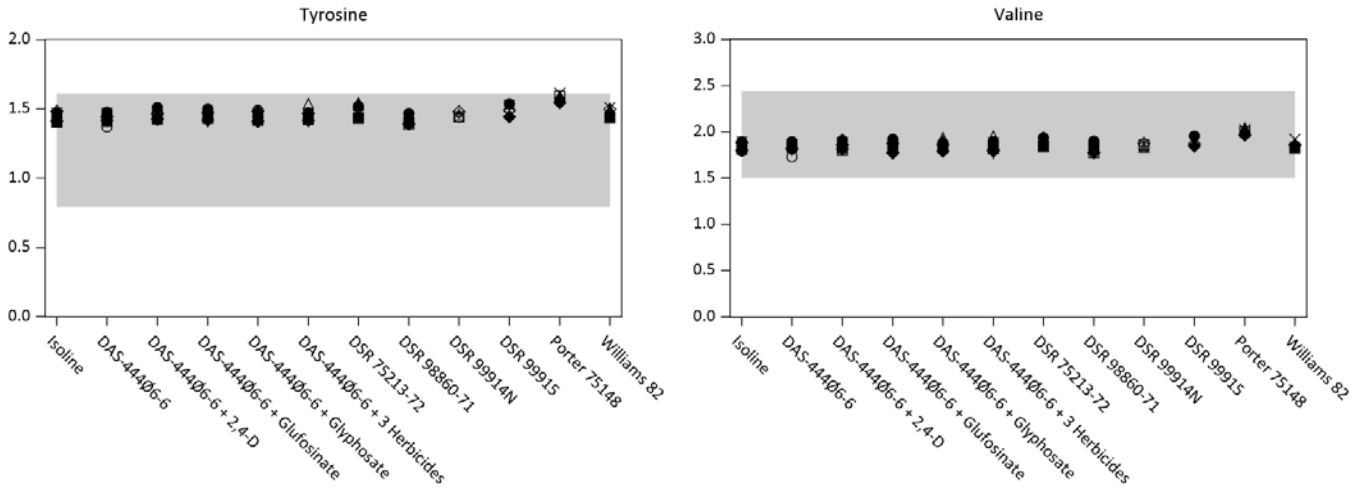


Figure 57 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

Fatty Acid Analysis of Seed

Soybean seed samples from the control, reference, and DAS-444Ø6-6 entries were analysed for fatty acid content. A summary of the results across all locations is presented in Table 212 and Figure 45. All mean results were within literature ranges (when available) and within ranges for reference lines included in the study. Statistical analysis was not performed for the following analytes since greater than 50% of the samples were found to be below the LOQ: caprylic (8:0), capric (10:0), lauric (12:0), myristic (14:0), myristoleic (14:1), pentadecanoic (15:0), pentadecenoic (15:1), palmitoleic (16:1), heptadecanoic (17:0), heptadecenoic (17:1), γ-linolenic (18:3), eicosadienoic (20:2), eicosatrienoic (20:3), and arachidonic (20:4). No statistical differences were observed in the combined-site analysis between the control and DAS-444Ø6-6 entries for stearic (18:0) and eicosenoic (20:1). Statistically significant differences were observed for palmitic (16:0), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidic (20:0), and behenic (22:0) for some DAS-444Ø6-6 entries compared with the control, where mean differences were negligible and not biologically meaningful, as means were within literature ranges and within ranges for reference lines included in the study.

Table 212. Summary of the fatty acid analysis of DAS-444Ø6-6 soybean seed from all sites, and associated literature range.

Analytical Component (Units) [a]	Overall Trt Effect (Pr > F) [b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P) [c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P) [c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P) [c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P) [c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P) [c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Fatty Acid									
8:0 Caprylic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ - 0.148
(% total fatty acid)	NA								
10:0 Capric		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ND - 0.27
(% total fatty acid)	NA								
12:0 Lauric		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ - 0.132
(% total fatty acid)	NA								
14:0 Myristic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ - 0.238
(% total fatty acid)	NA								
14:1 Myristoleic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ - 0.125
(% total fatty acid)	NA								
15:0 Pentadecanoic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ND
(% total fatty acid)	NA								
15:1 Pentadecenoic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ND
(% total fatty acid)	NA								
16:0 Palmitic		10.9 ± 0.1	10.7 ± 0.1	10.7 ± 0.1	10.7 ± 0.1	10.7 ± 0.1	10.6 ± 0.1		
		10.4 - 12.55	10.21 - 11.02	10.24 - 11.11	10.07 - 11.06	10.25 - 11.2	10.05 - 11.03	9.5 - 11.31	9.55 - 15.77
(% total fatty acid)	**<0.001**		(**<0.001** , **<0.001**)	(**<0.001** , **<0.001**)	(**<0.001** , **<0.001**)	(**<0.001** , **<0.001**)	(**<0.001** , **<0.001**)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was converted from % dry wt. to % total fatty acid prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Fatty Acid									
16:1 Palmitoleic		NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	< LOQ	< LOQ - 0.194
(% total fatty acid)	NA								
17:0 Heptadecanoic		NA < LOQ - 0.11	NA < LOQ - 0.136	NA < LOQ - 0.136	NA < LOQ - 0.126	NA < LOQ - 0.135	NA < LOQ - 0.142	< LOQ	< LOQ - 0.146
(% total fatty acid)	NA								
17:1 Heptadecenoic		NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	< LOQ	< LOQ - 0.087
(% total fatty acid)	NA								
18:0 Stearic		4.51 ± 0.07 3.88 - 5	4.47 ± 0.07 3.96 - 4.93 (0.174, 0.372)	4.47 ± 0.07 4.05 - 4.89 (0.159, 0.358)	4.48 ± 0.07 4.07 - 5.04 (0.343, 0.554)	4.48 ± 0.07 4.08 - 4.96 (0.261, 0.468)	4.52 ± 0.07 4.11 - 4.95 (0.785, 0.901)	3.28 - 4.98	2.59 - 5.88
(% total fatty acid)	0.391								
18:1 Oleic		23.5 ± 0.5 20.8 - 28.3	21.4 ± 0.5 18.4 - 23.7 (**<0.001**, **<0.001**)	21.5 ± 0.5 18.8 - 25.9 (**<0.001**, **<0.001**)	21.8 ± 0.5 18.9 - 26 (**<0.001**, **<0.001**)	21.7 ± 0.5 19.1 - 26.3 (**<0.001**, **<0.001**)	21.9 ± 0.5 19.2 - 26.3 (**<0.001**, **<0.001**)	18.1 - 27.9	14.3 - 45.68
(% total fatty acid)	**<0.001**								
18:2 Linoleic		53.0 ± 0.3 50.9 - 54.5	54.8 ± 0.3 53.4 - 56.8 (**<0.001**, **<0.001**)	54.6 ± 0.3 52.6 - 56.5 (**<0.001**, **<0.001**)	54.4 ± 0.3 52.5 - 56.1 (**<0.001**, **<0.001**)	54.5 ± 0.3 52.1 - 56.2 (**<0.001**, **<0.001**)	54.2 ± 0.3 51.7 - 56.2 (**<0.001**, **<0.001**)	50.1 - 56.7	35.36 - 58.8
(% total fatty acid)	**<0.001**								
18:3 Linolenic		7.32 ± 0.33 5.03 - 8.88	7.77 ± 0.33 5.56 - 9.38 (**<0.001**, **<0.001**)	7.79 ± 0.33 5.33 - 9.29 (**<0.001**, **<0.001**)	7.86 ± 0.33 5.48 - 9.47 (**<0.001**, **<0.001**)	7.76 ± 0.33 5.46 - 9.42 (**<0.001**, **<0.001**)	7.92 ± 0.33 5.38 - 9.48 (**<0.001**, **<0.001**)	4.83 - 9.82	3 - 12.52
(% total fatty acid)	**<0.001**								
18:3 γ-Linolenic		NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	< LOQ	ND
(% total fatty acid)	NA								

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was converted from % dry wt. to % total fatty acid prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Fatty Acid									
20:0 Arachidic		0.328 ± 0.005	0.323 ± 0.005	0.323 ± 0.005	0.325 ± 0.005	0.323 ± 0.005	0.327 ± 0.005		
		0.298 - 0.39	0.29 - 0.353	0.293 - 0.358	0.289 - 0.366	0.29 - 0.359	0.296 - 0.357	0.254 - 0.427	0.163 - 0.57
(% total fatty acid)	0.055		(**0.015**, 0.075)	(**0.018**, 0.083)	(0.084, 0.243)	(**0.010**, 0.058)	(0.401, 0.616)		
20:1 Eicosenoic		0.169 ± 0.009	0.171 ± 0.009	0.171 ± 0.009	0.171 ± 0.009	0.172 ± 0.009	0.168 ± 0.009		
		< LOQ - 0.254	< LOQ - 0.239	< LOQ - 0.254	< LOQ - 0.247	< LOQ - 0.24	< LOQ - 0.239	< LOQ - 0.272	< LOQ - 0.350
(% total fatty acid)	0.194		(0.131, 0.307)	(0.181, 0.378)	(0.123, 0.297)	(0.072, 0.215)	(0.865, 0.949)		
20:2 Eicosadienoic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ - 0.245
(% total fatty acid)	NA								
20:3 Eicosatrienoic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ND
(% total fatty acid)	NA								
20:4 Arachidonic		NA	NA	NA	NA	NA	NA		
		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ND
(% total fatty acid)	NA								
22:0 Behenic		0.326 ± 0.004	0.332 ± 0.004	0.331 ± 0.004	0.332 ± 0.004	0.328 ± 0.004	0.335 ± 0.004		
		0.273 - 0.365	0.303 - 0.368	0.298 - 0.371	0.299 - 0.367	0.294 - 0.365	0.309 - 0.371	0.29 - 0.454	0.277 - 0.595
(% total fatty acid)	**0.009**		(**0.018**, 0.083)	(**0.029**, 0.112)	(**0.014**, 0.075)	(0.237, 0.448)	(**<0.001**, **0.003**)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was converted from % dry wt. to % total fatty acid prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

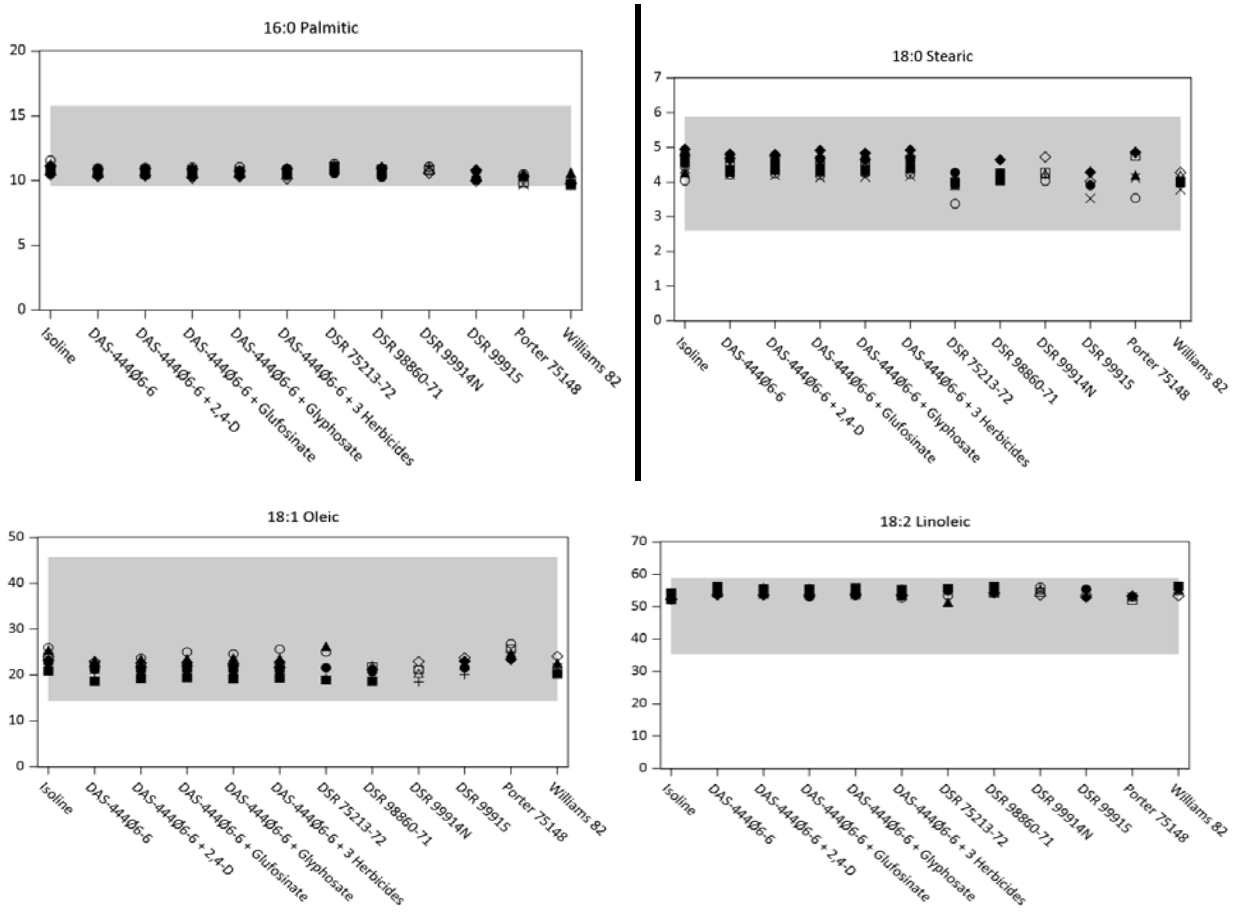


Figure 45. Fatty acids (% total fatty acid) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

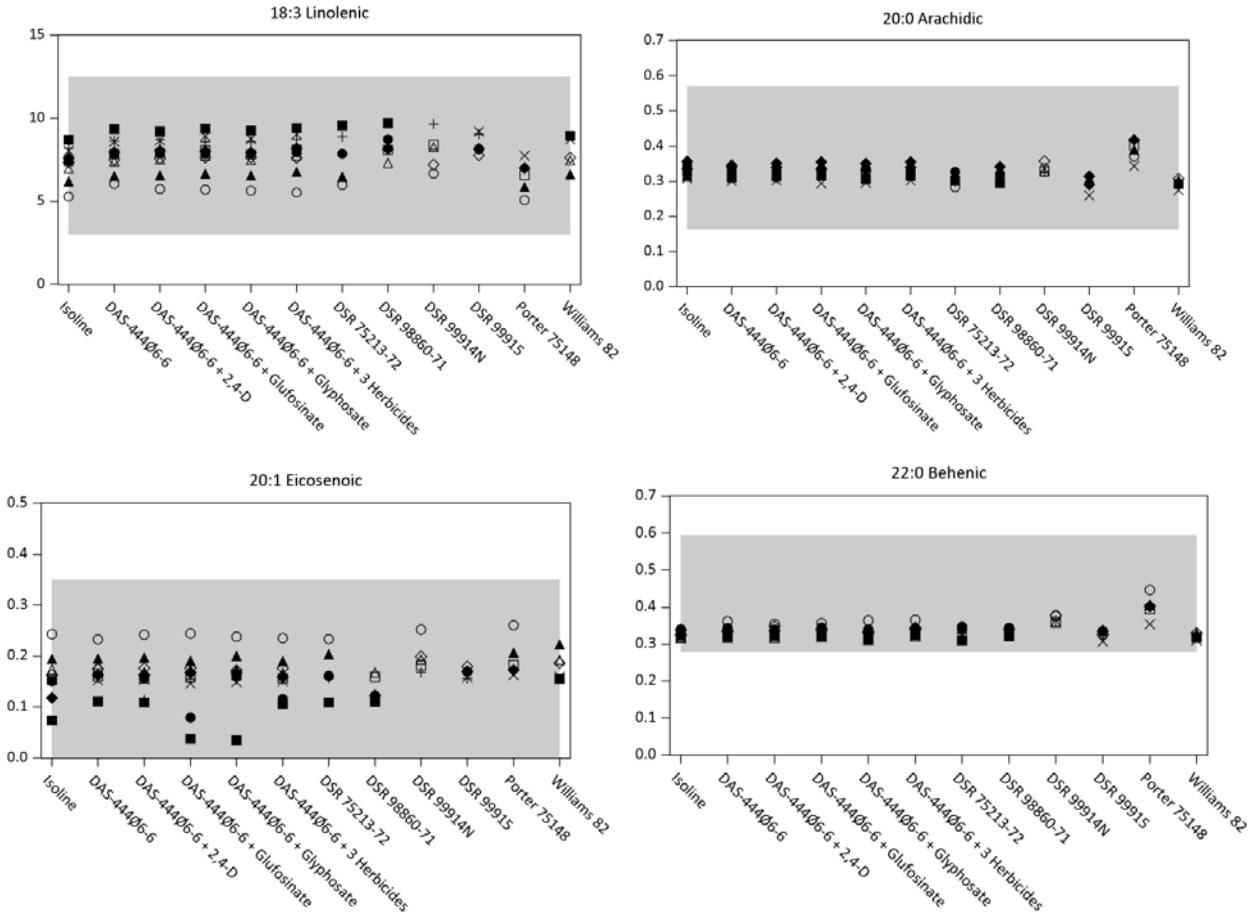


Figure 45 (Cont). Fatty acids (% total fatty acid) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

Vitamin Analysis of Seed

Soybean seed samples from the control, reference, and DAS-444Ø6-6 entries were analysed for vitamin content. A summary of the results across all locations is presented in Table 22 and Figure 46. All mean results were within literature ranges (when available) and/or within ranges for reference lines included in the study. For Vitamin A and β-Tocopherol, statistical analysis was not performed since greater than 50% of the samples were found to be below the LOQ. No statistical differences were observed in the combined-site analysis between the control and DAS-444Ø6-6 entries for Vitamins B_3, B_5, B_6, and δ-Tocopherol. Statistically significant differences were observed for Vitamins B_1, B_2, B_9, C, E, γ-Tocopherol, and total tocopherol for some DAS-444Ø6-6 entries compared with the control, where mean differences were negligible and not biologically meaningful as means were within literature ranges and/or within ranges for reference lines included in the study.

Table 22. Summary of the vitamin analysis of DAS-444Ø6-6 soybean seed from all sites, and associated literature range.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Vitamin									
Vitamin A (β-Carotene) (mg/kg DW)	NA	NA 0 - 0.894	NA 0 - 0.803	NA 0 - 0.928	NA 0 - 0.762	NA 0 - 1.1	NA 0 - 0.78	0 - 0.871	NR
Vitamin B_1 (Thiamine) (mg/kg DW)	0.241	3.64 ± 0.20 2.28 - 5.87	3.42 ± 0.20 2.2 - 4.75 (0.126, 0.303)	3.46 ± 0.20 2.35 - 4.5 (0.215, 0.428)	3.32 ± 0.20 1.14 - 5.12 (**0.026**, 0.105)	3.38 ± 0.20 2.03 - 5.01 (0.067, 0.202)	3.55 ± 0.20 2.13 - 5.54 (0.521, 0.723)	1.65 - 5.48	1.01 - 2.54
Vitamin B_2 (Riboflavin) (mg/kg DW)	0.199	3.99 ± 0.09 3.04 - 4.97	3.90 ± 0.09 2.99 - 4.81 (0.318, 0.535)	3.88 ± 0.09 3.03 - 4.91 (0.244, 0.451)	3.99 ± 0.09 2.98 - 4.71 (0.943, 0.976)	3.88 ± 0.09 2.64 - 5.1 (0.219, 0.432)	3.77 ± 0.09 2.32 - 4.88 (**0.022**, 0.095)	2.72 - 4.76	1.90 - 3.21
Vitamin B_3 (Niacin) (mg/kg DW)	0.765	26.5 ± 1.0 22.5 - 33.8	26.2 ± 1.0 19.2 - 32.8 (0.328, 0.541)	26.5 ± 1.0 22.9 - 34.3 (0.993, 0.996)	26.3 ± 1.0 21.8 - 34.1 (0.557, 0.735)	26.6 ± 1.0 22.9 - 36.8 (0.650, 0.804)	26.4 ± 1.0 22.6 - 35.4 (0.880, 0.956)	20.1 - 33	NR
Vitamin B_5 (Pantothenic Acid) (mg/kg DW)	0.277	15.4 ± 0.6 12.5 - 20.1	15.9 ± 0.6 12.3 - 20.5 (0.123, 0.297)	15.6 ± 0.6 8.29 - 20.4 (0.446, 0.662)	15.3 ± 0.6 13 - 19.8 (0.579, 0.758)	15.6 ± 0.6 12.3 - 21.2 (0.646, 0.804)	15.8 ± 0.6 12.9 - 20.3 (0.178, 0.377)	9.55 - 18.1	NR
Vitamin B_6 (Pyridoxine) (mg/kg DW)	0.268	4.89 ± 0.09 3.95 - 5.81	4.85 ± 0.09 3.68 - 5.7 (0.587, 0.763)	4.86 ± 0.09 3.98 - 5.95 (0.689, 0.834)	4.79 ± 0.09 4.19 - 5.85 (0.166, 0.363)	4.94 ± 0.09 4.15 - 5.88 (0.428, 0.646)	4.93 ± 0.09 4.08 - 6.19 (0.536, 0.725)	2.77 - 6.2	NR
Vitamin B_9 (Folic Acid) (mg/kg DW)	**0.029**	4.29 ± 0.19 2.7 - 5.78	4.09 ± 0.19 2.72 - 5.46 (0.063, 0.195)	4.03 ± 0.19 2.63 - 5.85 (**0.016**, 0.076)	4.02 ± 0.19 2.5 - 5.84 (**0.015**, 0.075)	4.07 ± 0.19 2.88 - 6.02 (**0.039**, 0.137)	3.92 ± 0.19 2.57 - 5.28 (**0.001**, **0.009**)	2.35 - 5.98	2.386 - 4.709

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Vitamin									
Vitamin C (Ascorbic Acid) (mg/kg DW)	0.154	121.4 ± 12.7 0 - 198	107.8 ± 12.7 16.5 - 181 (**0.008**, 0.051)	111.1 ± 12.7 17.6 - 194 (**0.040**, 0.139)	112.1 ± 12.7 16.8 - 173 (0.064, 0.196)	112.8 ± 12.7 23.8 - 193 (0.083, 0.243)	113.6 ± 12.7 25.3 - 171 (0.117, 0.294)	0 - 141	NR
Vitamin E (α-Tocopherol) (mg/kg DW)	0.115	18.6 ± 3.9 10.5 - 46	22.2 ± 3.9 10.9 - 69 (**0.023**, 0.096)	22.4 ± 3.9 11 - 56.8 (**0.016**, 0.076)	22.2 ± 3.9 10.1 - 106 (**0.020**, 0.092)	21.9 ± 3.9 10.9 - 55.7 (**0.035**, 0.128)	22.2 ± 3.9 8.85 - 76.2 (**0.023**, 0.096)	6.43 - 49.9	0.108 - 61.693
β-Tocopherol (mg/kg DW)	NA	NA 0 - 0	NA 0 - 0	NA 0 - 0	NA 0 - 0	NA 0 - 0	NA 0 - 0	0 - 6.42	NR
γ-Tocopherol (mg/kg DW)	**0.005**	174 ± 5 88.4 - 208	185 ± 5 157 - 224 (**0.001**, **0.007**)	184 ± 5 154 - 220 (**0.001**, **0.010**)	179 ± 5 99 - 214 (0.097, 0.261)	183 ± 5 153 - 217 (**0.002**, **0.015**)	181 ± 5 116 - 227 (**0.015**, 0.075)	116 - 215	NR
δ-Tocopherol (mg/kg DW)	0.317	73.3 ± 5.2 22.5 - 96.8	72.0 ± 5.2 40.4 - 94.3 (0.262, 0.468)	71.5 ± 5.2 35.5 - 94.6 (0.109, 0.281)	73.8 ± 5.2 40.5 - 99.6 (0.687, 0.834)	72.1 ± 5.2 40 - 98.2 (0.269, 0.475)	72.2 ± 5.2 31.5 - 96.1 (0.315, 0.535)	40 - 114	NR
Total Tocopherol (mg/kg DW)	**0.035**	266 ± 4 132 - 305	279 ± 4 244 - 316 (**0.003**, **0.023**)	278 ± 4 252 - 306 (**0.005**, **0.034**)	275 ± 4 161 - 375 (**0.040**, 0.139)	277 ± 4 248 - 308 (**0.008**, **0.049**)	276 ± 4 196 - 330 (**0.024**, 0.100)	199 - 321	NR

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

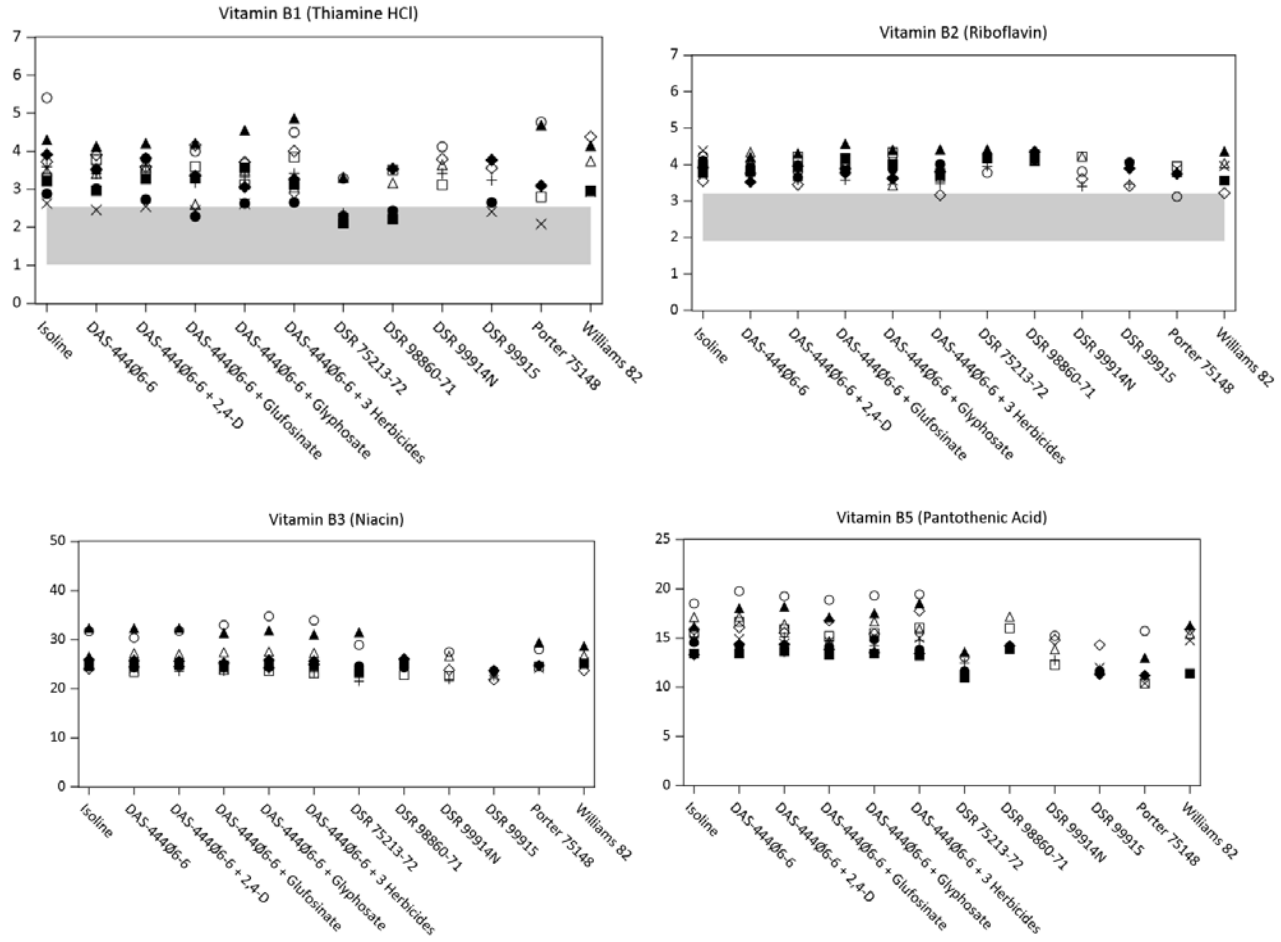


Figure 469. Vitamins (mg/kg dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

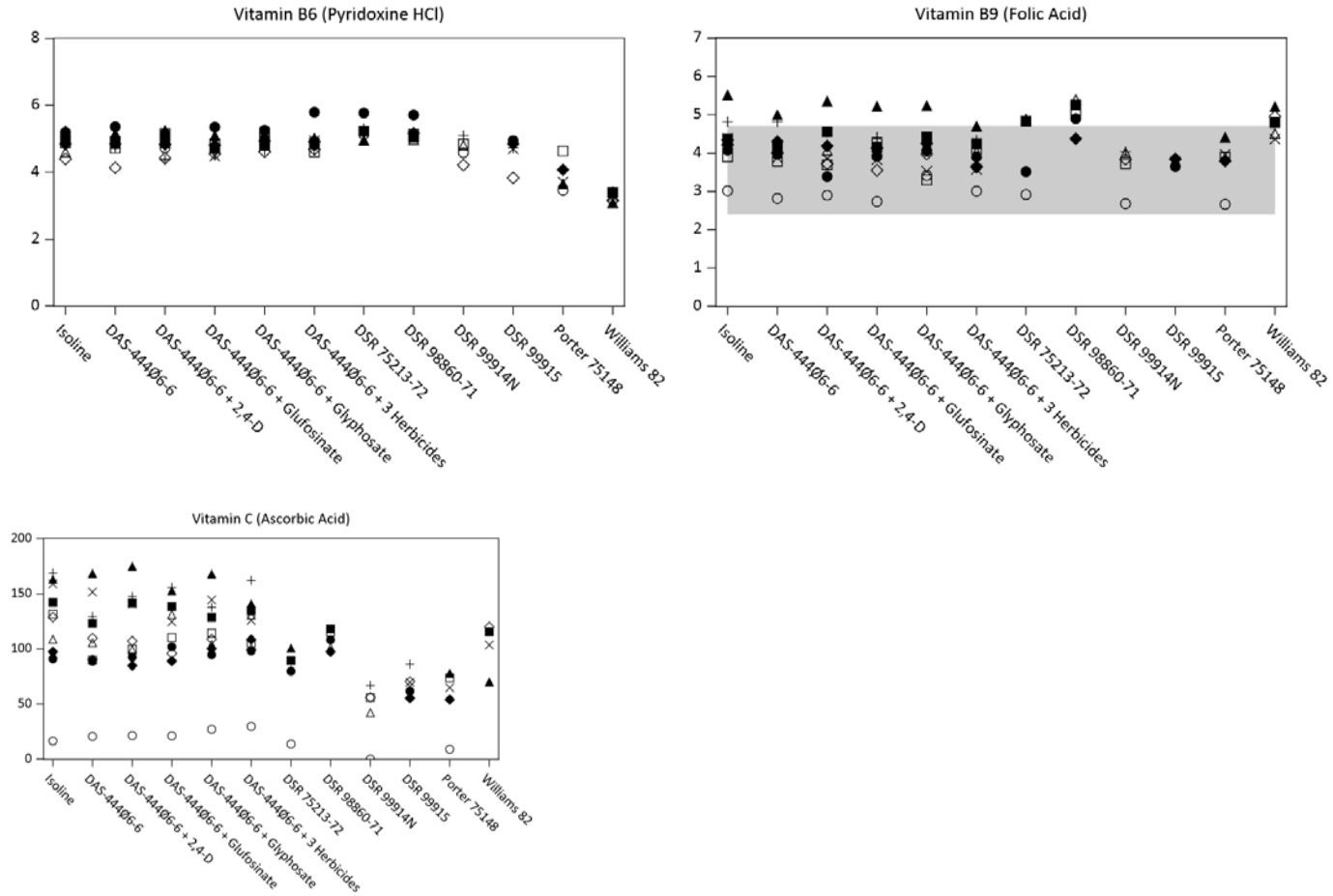


Figure 46 (Cont). Vitamins (mg/kg dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

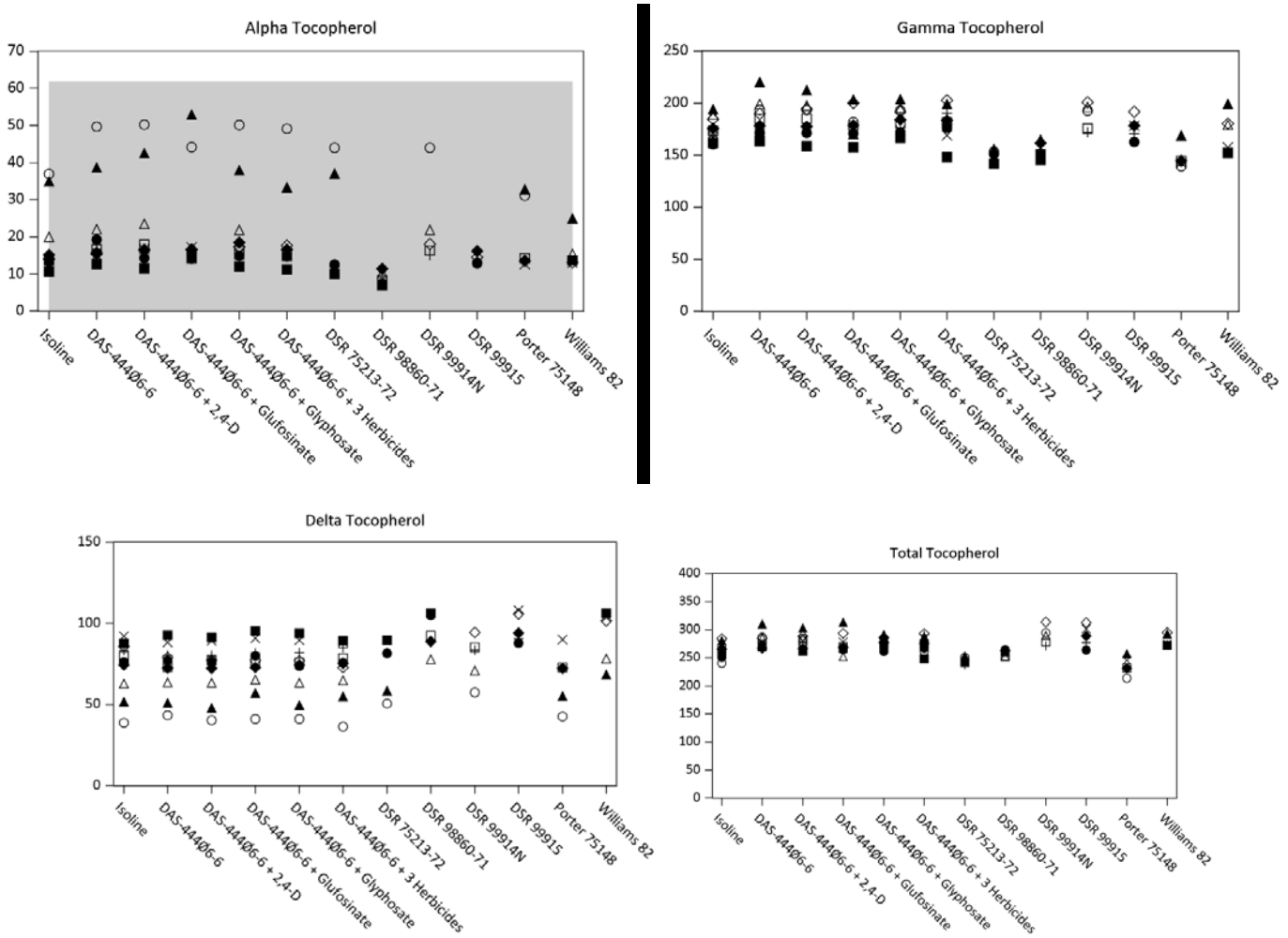


Figure 46 (Cont). Vitamins (mg/kg dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

b. Bioactive and Anti-Nutrient Analysis of Seed

Soybean seed samples from the control, reference, and DAS-444Ø6-6 entries were analysed for bioactive chemical content. A summary of the results across all locations is presented in Table 13, Figure 60 and Figure . All mean results were within literature ranges and/or within ranges for reference lines included in the study. No statistical differences were observed in the combined-site analysis between the control and DAS-444Ø6-6 entries for phytic acid, stachyose, and total glycitein equivalent. Statistically significant differences were observed for lectin, raffinose, trypsin inhibitor, total daidzein equivalent, and total genistein equivalent for some DAS-444Ø6-6 entries compared with the control. The mean differences were negligible and not biologically meaningful as means were within literature ranges and/or within ranges for reference lines included in the study. Additionally, bioactive components including lectin and trypsin inhibitor are inactivated during standard processing of soybean seed prior to consumption (Rackis 1974; Padgette,Taylor et al. 1996; Hammond and Jez 2011).

Table 13. Summary of the bioactive analysis of DAS-444Ø6-6 soybean seed from all sites, and associated literature range.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Bioactive									
Lectin		79 ± 8	107 ± 8	94 ± 8	94 ± 8	92 ± 8	99 ± 8		
(H.U./mg protein		27.9 - 153	60.6 - 228	39.4 - 188	56.3 - 146	44.8 - 151	31 - 196	18.5 - 144	37 - 323
DW)	**0.004**		(**<0.001**, **0.001**)	(**0.021**, 0.092)	(**0.021**, 0.092)	(**0.049**, 0.161)	(**0.004**, **0.026**)		
Phytic Acid		1.19 ± 0.07	1.18 ± 0.07	1.19 ± 0.07	1.18 ± 0.07	1.18 ± 0.07	1.19 ± 0.07		
		0.513 - 1.53	0.679 - 1.53	0.65 - 1.59	0.603 - 1.51	0.707 - 1.46	0.651 - 1.55	0.55 - 1.54	0.41 - 2.74
(% DW)	0.958		(0.789, 0.903)	(0.866, 0.949)	(0.511, 0.720)	(0.632, 0.795)	(0.824, 0.932)		
Raffinose		0.82 ± 0.06	0.80 ± 0.06	0.80 ± 0.06	0.80 ± 0.06	0.80 ± 0.06	0.79 ± 0.06		
		0.497 - 1.29	0.556 - 1.22	0.581 - 1.18	0.569 - 1.22	0.438 - 1.3	0.478 - 1.23	0.569 - 1.4	0.212 - 1.62
(% DW)	0.200		(0.151, 0.345)	(0.092, 0.256)	(0.050, 0.162)	(0.104, 0.275)	(**0.012**, 0.065)		
Stachyose		3.88 ± 0.06	3.88 ± 0.06	3.87 ± 0.06	3.86 ± 0.06	3.83 ± 0.06	3.89 ± 0.06		
		2.98 - 4.22	3.38 - 4.11	3.21 - 4.26	2.95 - 4.29	2.77 - 4.18	3.08 - 4.38	2.92 - 4.48	1.21 - 6.1
(% DW)	0.905		(0.952, 0.976)	(0.816, 0.928)	(0.779, 0.901)	(0.375, 0.590)	(0.827, 0.932)		
Trypsin Inhibitor		30.8 ± 3.0	35.0 ± 3.0	35.5 ± 3.0	36.6 ± 3.0	33.6 ± 3.0	34.2 ± 3.0		
		18.4 - 54.6	19 - 56	17.4 - 71	21.1 - 78.9	21.3 - 51.7	21.8 - 62.6	15.6 - 59.7	18.14 - 118.68
(TIU/mg DW)	**0.025**		(**0.015**, 0.075)	(**0.007**, **0.047**)	(**0.001**, **0.010**)	(0.095, 0.260)	(**0.047**, 0.159)		

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Unit of measure was not converted prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

[e] Combined range for Stachyose includes individual and mean values.

Analytical Component (Units)[a]	Overall Trt Effect (Pr > F)[b]	Isoline Mean ± SE Min - Max	DAS-444Ø6-6 unsprayed Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ 2,4-D Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glufosinate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Glyphosate Mean ± SE Min - Max (P-value, Adj.P)[c]	DAS-444Ø6-6 sprayed w/ Three Herbicides Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Range Min - Max	Combined Literature Range[d] Min - Max
Bioactive									
Total Daidzein Equivalent (mcg/g DW)	0.176	809 ± 114 186 - 1450	777 ± 114 175 - 1420 (0.067, 0.202)	799 ± 114 179 - 1470 (0.540, 0.726)	800 ± 114 182 - 1510 (0.581, 0.758)	771 ± 114 124 - 1490 (**0.029**, 0.111)	781 ± 114 149 - 1430 (0.097, 0.261)	153 - 1710	25 - 2453.5
Total Genistein Equivalent (mcg/g DW)	0.216	890 ± 155 267 - 1670	863 ± 155 300 - 1730 (0.133, 0.311)	870 ± 155 251 - 1690 (0.269, 0.475)	877 ± 155 264 - 1720 (0.458, 0.670)	848 ± 155 215 - 1770 (**0.021**, 0.092)	855 ± 155 186 - 1700 (0.057, 0.181)	205 - 1980	28 - 2837.2
Total Glycitein Equivalent (mcg/g DW)	0.736	453 ± 107 222 - 1300	459 ± 107 237 - 1250 (0.656, 0.806)	465 ± 107 223 - 1290 (0.415, 0.632)	452 ± 107 223 - 1340 (0.948, 0.976)	448 ± 107 197 - 1250 (0.760, 0.894)	443 ± 107 212 - 1270 (0.521, 0.723)	85.2 - 1630	15.3 - 349.19

Abbreviations: NA (Not Available) = analysis not performed, majority of data was < LOQ (Limit of Quantitation); ND (Not Detected) = < LOQ; NR = Not Reported.

[a] Aglycone and glycone forms of each isoflavone were summed to produce a total aglycone equivalent prior to analysis.

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

[c] Comparison to the control using t-tests (P-value); P-values adjusted (Adj. P) using a False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.

[d] Combined range from Appendix 7.

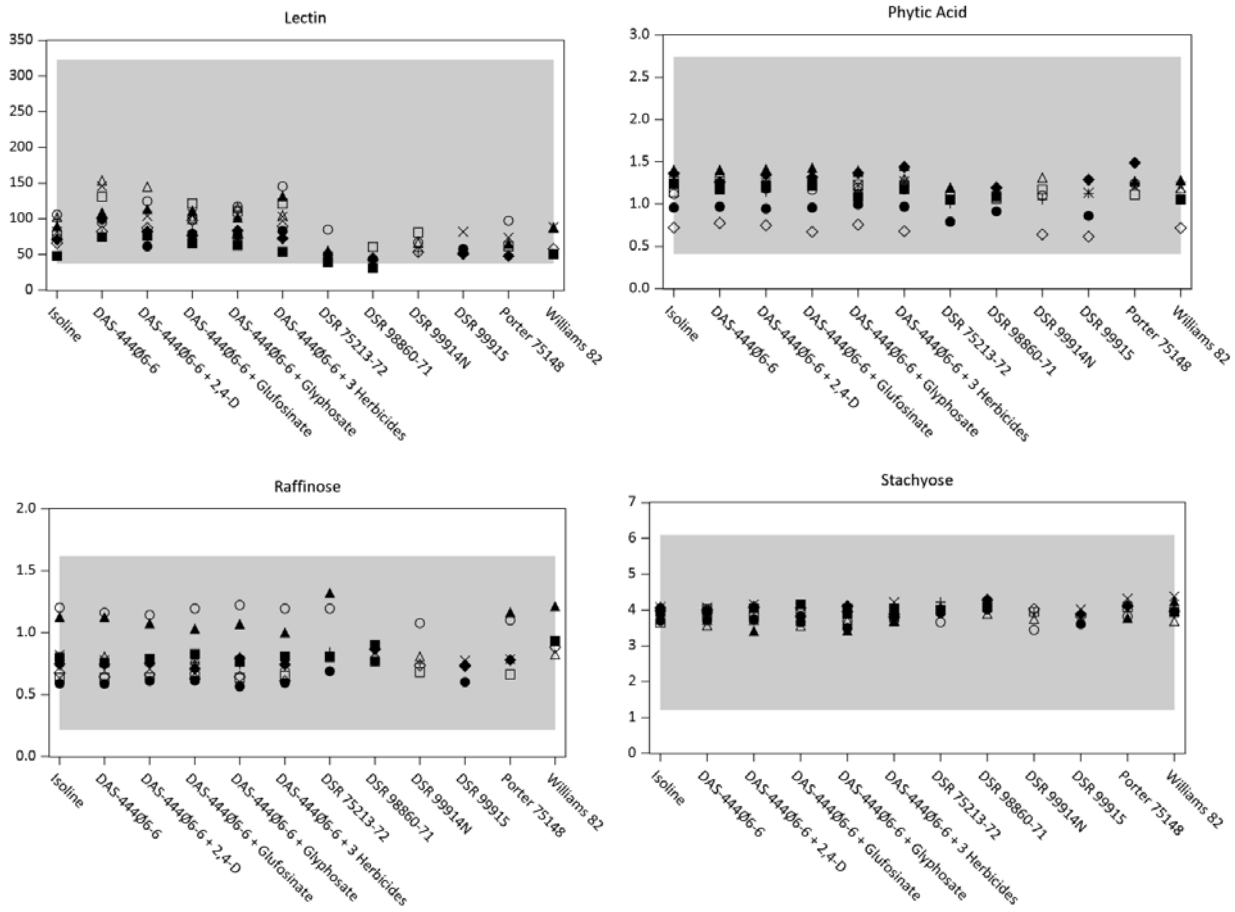


Figure 60. Bioactives (% dry weight (DW) for all bioactives except lectin (H.U./mg protein DW, H.U. = hemagglutination unit) and trypsin inhibitor (TIU/mg DW, TIU = trypsin inhibitor unit)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

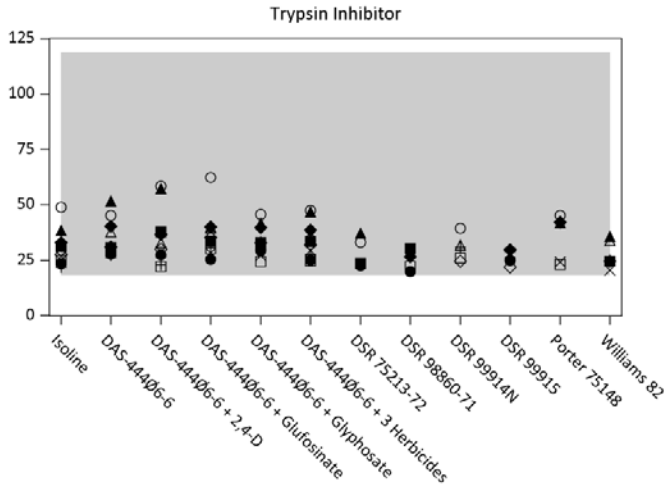


Figure 60 (Cont). Bioactives (% dry weight (DW) for all bioactives except lectin (H.U./mg protein DW, H.U. = hemagglutination unit) and trypsin inhibitor (TIU/mg DW, TIU = trypsin inhibitor unit)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

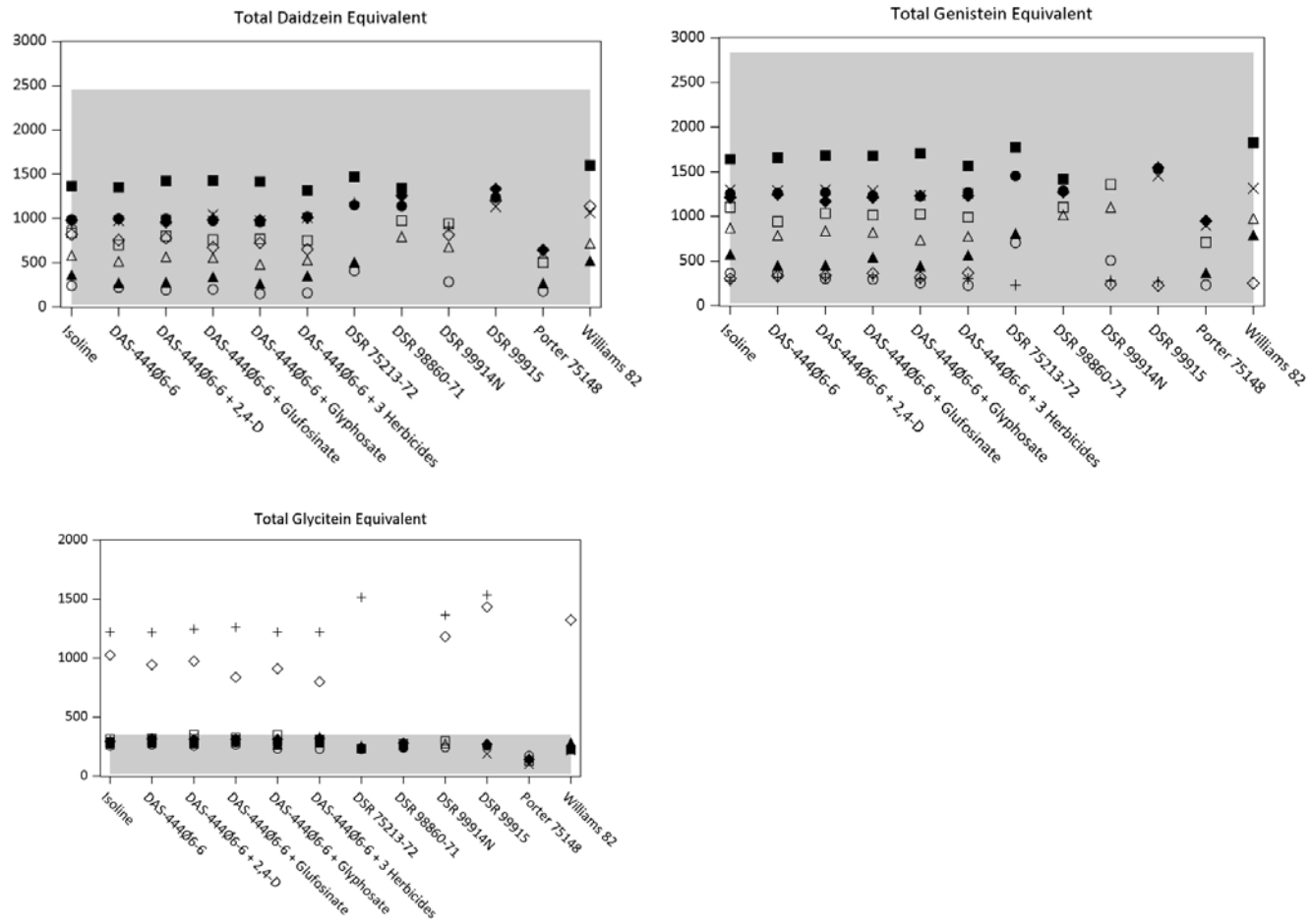


Figure 61. Bioactives: isoflavones (mcg/g dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

Summary of Grain and Forage Composition

All overall mean values for the non-transgenic isogenic control and DAS-444Ø6-6 entries (unsprayed or sprayed with 2,4-D, glyphosate, glufosinate, or all three herbicides) were within literature ranges (when available) for soybean and/or within ranges for non-transgenic reference soybean lines included in the study. A limited number of statistically significant differences between DAS-444Ø6-6 entries (unsprayed and/or sprayed) and the control were observed, but the differences were not biologically meaningful as the results were within ranges found for non-transgenic soybean. In conclusion, the compositional results for DAS-444Ø6-6 soybean, unsprayed or sprayed with 2,4-D, glyphosate, glufosinate, or all three herbicides, confirm equivalence to non-transgenic soybean lines.

Methods for Compositional Analysis

Acid Detergent Fibre (ADFA)
The ANKOM2000 Fibre Analyzer automated the process of removal of proteins, carbohydrates, and ash. Fats and pigments were removed with an acetone wash prior to analysis. The fibrous residue that was primarily cellulose and lignin and insoluble protein complexes remained in the Ankom filter bag, and was determined gravimetrically.

Amino Acid Composition (TALC/TPLC)
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

Total methionine
Total cystine (including cysteine)

The samples were hydrolysed in 6N hydrochloric acid for 24 hours at approximately 110ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine are converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan was hydrolysed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for 20 hours. The samples were analysed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids are derivatized with fluorenylmethyl chloroformate (FMOC) before injection.

Reference Standards:

Component	Manufacturer	Lot Number	Purity (%)
L-Alanine	Fluka	1388605	99.8
L-Arginine Monohydrochloride	Fluka	1361811	100.0
L-Aspartic Acid	Fluka	1337624	99.9
L-Cystine	Fluka	1386158	99.8
L-Glutamic Acid	Fluka	1423805	100.2
Glycine	Fluka	1119375	100.0
L-Histidine Monohydrochloride	Fluka	1388486	99.9
L-Isoleucine	Fluka	1423806	100.0
L-Leucine	Fluka	028K0027	100
L-Lysine Monohydrochloride	Fluka	1362380	100.2
L-Methionine	Fluka	1423807	99.9
L-Phenylalanine	Fluka	048K0662	>99
L-Proline	Fluka	1414414	99.7
L-Serine	Fluka	1336081	99.9
L-Threonine	Fluka	1234249	100.0
L-Tryptophan	Sigma-Aldrich	097K0119	100
L-Tyrosine	Fluka	1419640	100
L-Valine	Fluka	1352709	100.0

Ash (ASHM)

The sample was placed in an electric furnace at 550°C and ignited. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash.

Beta Carotene (BCLC)

The sample was saponified with potassium hydroxide and extracted with hexane. The samples were then injected on a reverse phase high-performance liquid chromatography system with ultraviolet light detection. Quantitation was achieved with a linear regression analysis.

Reference Standard:

Sigma-Aldrich, Beta Carotene, 100%, Lot Number 079K1729

Carbohydrate (CHO)

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

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

Fat by Acid Hydrolysis (FAAH)

The sample was hydrolysed with hydrochloric acid. The fat was extracted using ether and hexane. The extract was dried down and filtered through a sodium sulfate column. The remaining extract was then evaporated, dried, and weighed. The hexane extract was then evaporated again on a steambath under nitrogen, dried, and weighed.

Fat by Soxhlet Extraction (FSOX)

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.

Fatty Acids (FAPM)

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.

Reference Standards:

Component	Lot Number	Component	Weight (%)	Purity (%)
Nu-Chek Prep GLC Reference Standard Hazelton No. 1	JY20-U	Methyl Octanoate	16.66	99.6
		Methyl Decanoate	16.66	99.6
		Methyl Laurate	16.66	99.8
		Methyl Myristate	16.66	99.8
		Methyl Palmitoleate	16.66	99.7
		Methyl Linolenate	16.66	99.5
Nu-Chek Prep GLC Reference Standard Hazelton No. 2	AU16-U	Methyl Arachidate	33.33	99.6
		Methyl 11-Eicosenoate	33.33	99.5
		Methyl Arachidonate	33.33	99.6
Nu-Chek Prep GLC Reference Standard Hazelton No. 3	J28-U	Methyl Myristoleate	12.5	99.5
		Methyl Pentadecanoate	12.5	99.6
		Methyl 10-Pentadecenoate	12.5	99.5
		Methyl Heptadecanoate	12.5	99.6
		Methyl 10-	12.5	99.5
		Methyl 11-14	12.5	99.6
		Methyl Behenate	12.5	99.8
		Methyl 11-14-17	12.5	99.5
Nu-Chek Prep GLC Reference Standard Hazelton No. 4	MA30-U	Methyl Palmitate	27.0	99.6
		Methyl Stearate	19.0	99.5
		Methyl Oleate	27.0	99.8
		Methyl Linoleate	27.0	99.8
Nu-Chek Prep Methyl Gamma	U-63M-M18-U	Not applicable	Not applicable	>99
Nu-Chek Prep Methyl Tridecanoate	N-13M-MA25-T	Not applicable	Not applicable	>99
Nu-Chek Prep Methyl Undecanoate	N-11M-028-T	Not applicable	Not applicable	>99

Folic acid (FOAN)

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.

Reference Standard:

USP, Folic acid, 98.9%, Lot Number Q0G151

ICP Emission Spectrometry (ICPS)

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	Lot Numbers	Concentration (µg/ml)
Calcium	D2-MEB349023MCA, D2-MEB349025	200.00, 1000.00
Copper	D2-MEB349023MCA, D2-MEB349024MCA	2.00, 10.00
Iron	D2-MEB349023MCA, D2-MEB349026	10.00, 50.00
Magnesium	D2-MEB349023MCA, D2-MEB349024MCA	50.00, 250.00
Manganese	D2-MEB349023MCA, D2-MEB349024MCA	2.00, 10.00
Phosphorus	D2-MEB349023MCA, D2-MEB349025	200.00, 1000.00
Potassium	D2-MEB349023MCA, D2-MEB349025	200.00, 1000.00
Sodium	D2-MEB349023MCA, D2-MEB349025	200.00, 1000.00
Zinc	D2-MEB349023MCA, D2-MEB349024MCA	10.00, 50.00

ICP-Mass Spectrometry (MS1)

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

Spex CertiPrep Reference Standards and Limit of Quantitation:

Mineral	Lot Numbers	Purity	Limit of Quantitation (ppb)
Selenium	15-120JB, 24-133JB	100 mg/L	50.0

Isoflavones (ASOF)

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 diluted. The samples were analysed on a high-performance liquid chromatography system with ultraviolet spectrophotometric detection and were compared against an external standard curve.

Reference Standards:

Indofine, Daidzein, 99%, Lot Number 071212146

Chromadex, Daidzein, 97.5%, Lot Number 00004007-121

Indofine, Glycitein, 99%, Lot Number 0704034

Indofine, Glycitein, 97%, Lot Number 0803103

Indofine, Genistein, 99.35%, Lot Number 0604043

Indofine, Daidzin, 96%, Lot Number C1803

Indofine, Glycitin, Minimum 98+%, Lot Number 0310179

Indofine, Genistin, >99%, Lot Number 0701006

Lectin (LCTN)

The determination of lectin was based on the ability of lectin (a hemagglutinin) to bind to specific sugars present on the surface of red blood cells (RBCs) of different animal species resulting in the agglutination of RBCs. Samples were defatted and extracted with a saline solution. Agglutination of trypsinized rabbit RBCs was measured with a spectrophotometer at a wavelength of 620 nm.

Moisture (M100)

The sample was dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture. The results are reported on a fresh weight basis.

Neutral Detergent Fiber (NDFA) using Ankom

The ANKOM2000 Fibre Analyzer automated the process of the removal of protein, carbohydrate, and ash. 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.

Niacin (NIAP)

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.

Reference Standard:

USP, Niacin, 99.8%, Lot Number I0E295

Pantothenic Acid (PANN)

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.

Reference Standard:

USP, Calcium Pantothenate, 99.0%, Lot Number O1H081

Phytic Acid (PHYT)

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.

Reference Standard:

Sigma-Aldrich, Phytic Acid Sodium Salt Hydrate, 96%, Lot Number 089K0159

Protein (PGEN)

The protein and other organic nitrogen in the sample were converted to ammonia by digesting the sample with sulfuric acid containing a catalyst mixture. 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.

Raffinose and Stachyose (SUGT)

Sugars in the sample were extracted with a 50:50 water:methanol solution. Aliquots were taken, dried under inert gas, and then reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucopyranoside as the internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid treatment, and then analysed by gas chromatography using a flame ionization detector.

Reference Standard:

Sigma-Aldrich, D-(+)-Raffinose pentahydrate, 99%, Lot Number 037K1059

Sigma-Aldrich, Stachyose hydrate, 98%, Lot Number 049K3800

Total Tocopherols (TTLC)

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.

Reference Standard:

USP, Alpha Tocopherol, 98.9%, Lot Number N0F068

Matreya, rac-beta-Tocopherol, 98%, Lot Number 22902

Matreya, rac-beta-Tocopherol, 98%, Lot Number 23097

ACROS, D-gamma-Tocopherol, 99.4%, Lot Number A0083534

Sigma-Aldrich, (+)-Delta-Tocopherol, 95%, Lot number 126K1307

Sigma-Aldrich, (+)-Delta-Tocopherol, 92%, Lot number 090M1916V

Total Dietary Fibre (TDF)

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 fibre. The sample was 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 fibre in the sample was calculated using protein and ash values.

Trypsin Inhibitor (TRIP)

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.

Thiamine Hydrochloride (BIDE)

The sample was autoclaved under weak acid conditions to extract the thiamine. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamine. The solution was purified on a cation-exchange column. An aliquot was reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard. The results are reported as thiamine hydrochloride.

Reference Standard:

USP, Thiamine Hydrochloride, 99.8%, Lot Number O1F236

Vitamin B_2 (Riboflavin) (B2FV)

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.

Reference Standard:

USP, Riboflavin, 100.0%, Lot Number N0C021

Pyridoxine Hydrochloride (B6A)

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.

Reference Standard:

USP, Pyridoxine hydrochloride, 99.8%, Lot Number: Q0G409

Vitamin C (VCF)

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.

Reference Standard:

USP, Ascorbic Acid, 99.9%, Lot Number Q1G135

Literature Values for Compositional Analysis

Table 144. Literature ranges reported for soybean seed: proximates, fiber, and minerals.

Analyte	Units	Combined Range		OECD 2001[a]		ILSI 2010[a]		Literature		Literature Citations	
		Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Protein	% Dry weight	32	48.4	32	43.6	33.19	45.48	32.54	48.4	Harrigan et al. 2007	Hartwig and Kilen 1991
Total Fat	% Dry weight	8.104	24.7	15.5	24.7	8.104	23.562	14.10	23.67	Padgette et al. 1996	Berman et al. 2010
Ash	% Dry weight	3.885	6.994	4.5	6.4	3.885	6.994	4.29	6.44	Padgette et al. 1996	Harrigan et al. 2007
Moisture	% Fresh weight	4.7	34.4	NR	NR	4.7	34.4	4.71	14.30	Harrigan et al. 2007	Taylor et al. 1999
Carbohydrates	% Dry weight	29.3	50.2	31.7	31.8	29.6	50.2	29.3	44.35	Padgette et al. 1996	Harrigan et al. 2007
Acid Detergent Fiber (ADF)	% Dry weight	7.81	26.26	9	11.1	7.81	18.61	9.22	26.26	Lundry et al. 2008	Lundry et al. 2008
Neutral Detergent Fiber (NDF)	% Dry weight	8.53	23.90	10	14.9	8.53	21.25	10.79	23.90	Lundry et al. 2008	Lundry et al. 2008
Total Dietary Fiber	% Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Calcium	mg/100g Dry weight	116.55	510	NR	NR	116.55	307.1	258	510	Iskander 1987	Bilyeu et al. 2008
Copper	mg/100g Dry weight	0.632	1.092	NR	NR	NR	NR	0.632	1.092	Bilyeu et al. 2008	Bilyeu et al. 2008
Iron	mg/100g Dry weight	3.734	10.954	NR	NR	5.536	10.954	3.734	6.624	Bilyeu et al. 2008	Bilyeu et al. 2008
Magnesium	mg/100g Dry weight	219.40	312.84	NR	NR	219.40	312.84	261	280	Iskander 1987	Bilyeu et al. 2008
Manganese	mg/100g Dry weight	2.52	3.876	NR	NR	NR	NR	2.52	3.876	Iskander 1987	Bilyeu et al. 2008
Phosphorus	mg/100g Dry weight	506.74	935.24	NR	NR	506.74	935.24	770	790	Bilyeu et al. 2008	Bilyeu et al. 2008
Potassium	mg/100g Dry weight	1868.01	2510	NR	NR	1868.01	2316.14	1910	2510	Iskander 1987	Bilyeu et al. 2008
Selenium	ppb Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Sodium	mg/100g Dry weight	4.05	30	NR	NR	NR	NR	4.05	30	Iskander 1987	Bilyeu et al. 2008
Zinc	mg/100g Dry weight	4.98	7.578	NR	NR	NR	NR	4.98	7.578	Iskander 1987	Bilyeu et al. 2008

NR = Not Reported.

Table 25. Literature ranges reported for soybean seed: amino acids.

		Combined Range		OECD 2001[a]		ILSI 2010[a]		Literature		Literature Citations	
Analyte	Units	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Alanine	% Dry weight	1.43	2.10	NR	NR	1.51	2.10	1.43	1.93	Berman et al. 2009	Berman et al. 2009
Arginine	% Dry weight	2.15	3.46	2.45	3.1	2.29	3.4	2.15	3.46	Berman et al. 2009	Padgette et al. 1996
Aspartic Acid	% Dry weight	3.81	6.04	NR	NR	3.81	5.12	3.90	6.04	Harrigan et al. 2007	Berman et al. 2010
Cystine	% Dry weight	0.37	0.81	0.45	0.67	0.37	0.81	0.41	0.71	Berman et al. 2009	Berman et al. 2009
Glutamic Acid	% Dry weight	5.84	9.15	NR	NR	5.84	8.2	5.97	9.15	Harrigan et al. 2007	Berman et al. 2010
Glycine	% Dry weight	1.41	2.00	NR	NR	1.46	2.00	1.41	1.99	Berman et al. 2009	Berman et al. 2009
Histidine	% Dry weight	0.86	1.24	1	1.22	0.88	1.18	0.86	1.24	Berman et al. 2009	Berman et al. 2009
Isoleucine	% Dry weight	1.49	2.08	1.76	1.98	1.54	2.08	1.49	2.02	Berman et al. 2009	Berman et al. 2009
Leucine	% Dry weight	2.2	4.0	2.2	4.0	2.59	3.62	2.39	3.42	Berman et al. 2009	Lundry et al. 2008
Lysine	% Dry weight	2.19	3.32	2.5	2.66	2.29	2.84	2.19	3.32	Berman et al. 2009	Berman et al. 2010
Methionine	% Dry weight	0.39	0.68	0.5	0.67	0.43	0.68	0.39	0.65	Berman et al. 2009	Berman et al. 2009
Phenylalanine	% Dry weight	1.6	2.44	1.6	2.08	1.63	2.35	1.62	2.44	Berman et al. 2009	Berman et al. 2009
Proline	% Dry weight	1.63	2.28	NR	NR	1.69	2.28	1.63	2.25	Berman et al. 2009	Berman et al. 2009
Serine	% Dry weight	1.11	2.48	NR	NR	1.11	2.48	1.63	2.42	Berman et al. 2009	Lundry et al. 2008
Threonine	% Dry weight	1.14	1.89	1.4	1.89	1.14	1.86	1.28	1.74	Berman et al. 2009	Berman et al. 2009
Tryptophan	% Dry weight	0.30	0.67	0.51	0.67	0.356	0.502	0.30	0.63	Lundry et al. 2008	Padgette et al. 1996
Tyrosine	% Dry weight	0.79	1.61	NR	NR	1.02	1.61	0.79	1.59	Berman et al. 2009	Padgette et al. 1996
Valine	% Dry weight	1.5	2.44	1.5	2.44	1.6	2.2	1.57	2.13	Berman et al. 2009	Berman et al. 2009

NR = Not Reported.

Table 15. Literature ranges for soybean seed: fatty acids.

Analyte	Units	Combined Range		OECD 2001[a]		ILSI 2010[a]		Literature		Literature Citations	
		Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
8:0 Caprylic	% of total fatty acid	<LOQ	0.148	NR	NR	<LOQ	0.148	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
10:0 Capric	% of total fatty acid	ND	0.27	NR	NR	ND	ND	ND	0.27	Harrigan et al. 2007	Berman et al. 2009
12:0 Lauric	% of total fatty acid	<LOQ	0.132	NR	NR	<LOQ	0.132	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
14:0 Myristic	% of total fatty acid	<LOQ	0.238	NR	NR	<LOQ	0.238	ND	0.097	Harrigan et al. 2007	Berman et al. 2009
14:1 Myristoleic	% of total fatty acid	<LOQ	0.125	NR	NR	<LOQ	0.125	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
15:0 Pentadecanoic	% of total fatty acid	ND	ND	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
15:1 Pentadecenoic	% of total fatty acid	ND	ND	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
16:0 Palmitic	% of total fatty acid	9.55	15.77	NR	NR	9.55	15.77	9.80	12.63	Berman et al. 2009	Berman et al. 2009
16:1 Palmitoleic	% of total fatty acid	<LOQ	0.194	NR	NR	<LOQ	0.194	ND	0.14	Harrigan et al. 2007	Berman et al. 2009
17:0 Heptadecanoic	% of total fatty acid	<LOQ	0.146	NR	NR	<LOQ	0.146	ND	0.13	Harrigan et al. 2007	Berman et al. 2009
17:1 Heptadecenoic	% of total fatty acid	<LOQ	0.087	NR	NR	<LOQ	0.087	ND	0.064	Harrigan et al. 2007	Berman et al. 2009
18:0 Stearic	% of total fatty acid	2.59	5.88	NR	NR	2.70	5.88	2.59	5.50	Berman et al. 2010	Berman et al. 2009
18:1 Oleic	% of total fatty acid	14.3	45.68	NR	NR	14.3	32.2	15.80	45.68	Harrigan et al. 2007	Berman et al. 2010
18:2 Linoleic	% of total fatty acid	35.36	58.8	NR	NR	42.3	58.8	35.36	57.72	Berman et al. 2010	Berman et al. 2009
18:3 Linolenic	% of total fatty acid	3	12.52	NR	NR	3	12.52	4.27	9.60	Berman et al. 2009	Berman et al. 2009
18:3 γ-Linolenic	% of total fatty acid	ND	ND	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
20:0 Arachidic	% of total fatty acid	0.163	0.57	NR	NR	0.163	0.482	0.25	0.57	Harrigan et al. 2007	Berman et al. 2009
20:1 Eicosenoic	% of total fatty acid	<LOQ	0.350	NR	NR	<LOQ	0.350	0.13	0.35	Berman et al. 2009	Berman et al. 2010
20:2 Eicosadienoic	% of total fatty acid	<LOQ	0.245	NR	NR	<LOQ	0.245	ND	0.065	Harrigan et al. 2007	Berman et al. 2010
20:3 Eicosatrienoic	% of total fatty acid	ND	ND	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
20:4 Arachidonic	% of total fatty acid	ND	ND	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
22:0 Behenic	% of total fatty acid	0.277	0.595	NR	NR	0.277	0.595	0.28	0.59	Harrigan et al. 2007	Berman et al. 2009

<LOQ = Less than Limit of Quantitation; ND = Not Detected; NR = Not Reported.

Table 16. Literature ranges reported for soybean seed: vitamins.

Analyte	Units	Combined Range		OECD 2001[a]		ILSI 2010[a]		Literature		Literature Citations	
		Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Vitamin A (β-Carotene)	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Vitamin B_1 (Thiamine HCl)	mg/kg Dry weight	1.01	2.54	NR	NR	1.01	2.54	NR	NR	NR	NR
Vitamin B_2 (Riboflavin)	mg/kg Dry weight	1.90	3.21	NR	NR	1.90	3.21	NR	NR	NR	NR
Vitamin B_3 (Niacin)	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Vitamin B_5 (Pantothenic Acid)	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Vitamin B_6 (Pyridoxine HCl)	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Vitamin B_9 (Folic Acid)	mg/kg Dry weight	2.386	4.709	NR	NR	2.386	4.709	NR	NR	NR	NR
Vitamin C (Ascorbic Acid)	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Vitamin E (α-Tocopherol)	mg/kg Dry weight	0.108	61.693	NR	NR	1.934	61.693	0.108	48.0	Berman et al. 2010	Lundry et al. 2008
β-Tocopherol	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
γ-Tocopherol	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
δ-Tocopherol	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Total Tocopherol	mg/kg Dry weight	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

NR = Not Reported.

Table 178. Literature ranges for soybean seed: bioactives.

Analyte	Units	Combined Range		OECD 2001*		ILSI 2010[a]		Literature		Literature Citations	
		Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Lectin	HU/mg Protein Dry weight	37	323	37	323	NR	NR	NR	NR	NR	NR
Phytic Acid	% Dry weight	0.41	2.74	1	2.74	0.634	1.96	0.41	2.68	Lundry et al. 2008	Berman et al. 2010
Raffinose	% Dry weight	0.212	1.62	NR	NR	0.212	0.661	0.22	1.62	Harrigan et al. 2007	Berman et al. 2009
Stachyose	% Dry weight	1.21	6.1[b]	NR	NR	1.21	3.5	1.52	6.1[b]	Harrigan et al. 2007	Harrigan et al. 2010
Trypsin Inhibitor	TIU/mg Dry weight	18.14	118.68	NR	NR	19.59	118.68	18.14	75.5	Berman et al. 2009	McCann et al. 2005
Total Daidzein Equivalent	mcg/g Dry weight	25	2453.5	NR	NR	60	2453.5	25	2099.75	McCann et al. 2005	Berman et al. 2010
Total Genistein Equivalent	mcg/g Dry weight	28	2837.2	NR	NR	144.3	2837.2	28	2600.70	McCann et al. 2005	Harrigan et al. 2007
Total Glycitein Equivalent	mcg/g Dry weight	15.3	349.19	NR	NR	15.3	310.0	45	349.19	McCann et al. 2005	Harrigan et al. 2007

NR = Not Reported.

* Maximum value for stachyose is a mean value reported from the literaure, all other records are individual values.

C. NUTRITIONAL IMPACT

Part C DAS Reports

Cleveland, C.B., 2011a. Global Dietary and Livestock Assessment of 2mEPSPS Protein for DAS Soybean Cultivar Based on Event DAS-444Ø6-6. Study ID 110600. Dow AgroSciences LLC. Indianapolis, IN.

Cleveland, C.B., 2011b. Global Dietary and Livestock Assessment of PAT Protein for DAS Soybean Cultivar Based on Event DAS-444Ø6-6. Study ID 110599. Dow AgroSciences LLC. Indianapolis, IN.

Cleveland, C.B., Stagg, N.J., 2011. Global Dietary and Livestock Assessment of AAD-12 Protein for DAS Soybean Cultivar Based on Event DAS-444Ø6-6. Study ID 110598. Dow AgroSciences LLC. Indianapolis, IN.

Fletcher, D.W., 2011. DAS-68416-4 (AAD-12) Soybean Feeding Study in the Broiler Chicken. Study ID 101088. Dow AgroSciences LLC. Indianapolis, IN.

Lepping, M., 2011. Nutrient Composition of a Transformed Soybean Cultivar Containing Aryloxyalkanoate Dioxygenase-12 (AAD-12), Double Mutant Maize EPSPS Gene (2mEPSPS), and Phosphinothricin Acetyltransferase (PAT) – Event DAS-444Ø6-6. Study ID 101104.03. Dow AgroSciences LLC. Indianapolis, IN.

Moldonado,P.M., 2011. Field Expression of a Transformed Soybean Cultivar Containing Aryloxyalkanoate Dioxygenase (AAD-12), Double Mutant Maize EPSPS Gene (2mEPSPS) and Phosphinothricin Acetyltransferase (PAT) – Event DAS-444Ø6-6. Study ID 101104.02. Dow AgroSciences LLC. Indianapolis, IN.

1. Human Nutritional Impact and Animal Feeding Studies

Estimate of Dietary Exposure of AAD-12 for Humans

Expression levels of AAD-12 protein in plant tissues of DAS-444Ø6-6 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-444Ø6-6 soybean, indicating negligible risk for adverse effects from acute dietary exposure (Cleveland and Stagg 2011).

Potential Human Exposure to AAD-12 Protein via Soybean

The field expression of AAD-12 protein in DAS-444Ø6-6 soybean was measured using an AAD-12 specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (Maldonado 2011). Field expression data is available for trials conducted at multiple test sites located within the major soybean-producing regions of the U.S. Protein expression was analysed in leaf at V5 and V10-12 growth stages, with root, forage, and grain tissues collected throughout the growing season from DAS-444Ø6-6 soybean plants treated with or without the herbicides 2,4-D, glufosinate, and/or glyphosate.

The results showed low level expression of the AAD-12 protein with similar expression values for AAD-12 for all treatments irrespective of the herbicide regime, indicating a low exposure risk to humans. Only the protein expression in the soybean grain is applicable for human dietary consideration.

In soybean grain, the average value of AAD-12 protein was 26.73 ng AAD-12 protein/mg tissue on a dry weight basis. The full range of applicable values was 9.29 to 42.05 ng/mg tissue [based on review of the 50 values for site/test spray], but the use of an average expression value is most appropriate. This is because grain is a blended commodity, making consumption of single-servings of grain at the maximum expression-level highly unlikely. Use of these values are protective estimates for exposure to the AAD-12 protein from soybean; actual dietary exposure and impact of the protein will be lower because: 1) there may be protein degradation during transport and storage, 2) grain containing AAD-12 will be mixed with non-AAD-12 grain, 3) human consumption of soy products is primarily 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 that contain very little protein (e.g. soybean oil).

Margin of Exposure Calculation

A conservative short term intake (STI) consumption of soybean was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint (acute NOAEL of 2000 mg/kg). Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki, Davies et al. 2005). Nevertheless, 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).

MOE = NOEL/Exposure

The larger the MOE value, the less likelihood there is for adverse effects because the exposure is well below the established NOAEL threshold. Regardless of which method is used, all acute MOE are greater than 10000 (Table 29), indicating negligible risk for adverse effects from dietary exposure to AAD-12 protein.

In the assessment, the MOE values were calculated using the DEEM dietary exposure model program (DEEM-FCID version 2.16, Exponent, 2007). The residue value of 26.73 ppm was conservatively applied to all commodities listed in DEEM that are associated with soybean. This is a conservative approach for processed flour and soymilk. No residues were assigned to oil. Using this residue file as input, MOE values were calculated against the acute NOAEL of 2000 mg/kg for several subpopulations (Table 29). The most exposed subpopulation is the non-nursing infants (<1 yr old) with an estimated exposure of 0.092951 mg/kg-bw and an MOE value of 21516 at the 97.5th percentile. MOE values >100 are typically considered acceptable. All relevant MOE values are greater than 10000, indicating there is no concern from acute dietary exposure through soybean.

Table 29. Summary of human dietary margins of exposure for AAD-12 protein in soybean based on US DEEM consumption model for short term exposure.

	Food Intake[b] g/kg-bw	Exposure (mg AAD-12 /kg-bw/day)	NOAEL (mg/kg-bw)	MOE
US DEEM 97.5th				
U.S. population	*0.1302*	0.003479	>2000	>574854
All infants	*3.1816*	0.085045	>2000	>23516
Nursing infants	*1.6401*	0.043840	>2000	>45620
Non-nursing infants	*3.4774*	0.092951	>2000	>21516
Children 1-2 yrs	*0.4405*	0.011775	>2000	>169850
Children 3-5 yrs	*0.2342*	0.006261	>2000	>319437
Children 6-12 yrs	*0.1689*	0.004516	>2000	>442906
Youth 13-19 yrs	*0.0969*	0.002591	>2000	>771995

Estimate of Dietary Exposure of AAD-12 for Livestock

Expression levels of the AAD-12 protein in DAS-444Ø6-6 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-444Ø6-6 soybean (Cleveland and Stagg 2011), indicating no concern for adverse effects from acute dietary exposure.

Animal Feed Exposure

An assessment for livestock exposure was conducted (Cleveland and Stagg 2011) based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (US EPA 2008). The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls, aspirated grain fractions, and optional forage. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average value of 26.73 ng/mg (ppm) for AAD-12

protein in DAS-444Ø6-6 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. Because meal and seed are both protein concentrates, they are not simultaneously used in a diet. When forage was included, a value of 11.48 ng/mg (ppm) was used as the highest residue as the animal feed. This maximum value in forage was used in the conservative calculation of acute dairy animal feed exposure.

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 an impact on feed ration formulation, because nutrient composition analyses have shown that DAS-444Ø6-6 soybean is substantially equivalent to conventional soybean per the general OECD and ILSI guidance (OECD 2001; ILSI 2009). In addition, results from a broiler study indicated that when feed was prepared with AAD-12 protein from DAS-68416-4 soybean meal, it was nutritionally similar to feed prepared with non-transgenic near isogenic soybean meal (non-AAD-12 control) for the production of broiler chickens (Fletcher 2011).

US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (US EPA 2008):

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% complex carbohydrate, CC), forages (15% roughage, R), and protein sources (5% protein concentrate, 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 114.38 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-444Ø6-6 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled in Table 30 (Cleveland and Stagg, 2011).

Table 30. Intake animal dietary burdens for livestock from AAD-12.

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	AAD-12 (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls*	R	90	15	20	Nu	Nu	7.48	1.25	1.66	-	-
Aspirated grain**	CC	85	5	Nu	Nu	Nu	534.6	31.45	-	-	-
Soybean seed	PC	89	*Meal used*	15	*Meal used*	*Meal used*	26.73		4.51	-	-
Soybean meal*	PC	NA	*5*	*Seed used*	25	15	29.4	1.60	-	7.35	4.41
Soybean Forage	R	35	Nu	20	Nu	Nu	114.38	-	65.36	-	-
							Total	34.29	71.53	7.35	4.41

* estimate based on measured value for seed

**based on theoretical estimate of 20X the value in soybean seed

Nu – not used

Because only soybean feeds are considered, the nutritional balance of the diets is 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, which is reported in Table 31.

Table 31. Livestock daily dose estimates of AAD-12 protein from soybean seeds.

	Cattle		Swine	Poultry
	Beef	Dairy	Finishing	Broiler
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum AAD-12 intake (mg/kg feed)	34.29	71.53	4.41	7.35
Maximum intake (mg/kg bw)	0.57	2.81	0.12	0.20
MOE vs. Mammalian NOAEL	3509	713	16667	10000

The highest exposed animal is the dairy cow with 2.81 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; typical MOEs are > 3500 and the worst case is > 700. 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-444Ø6-6 soybean.

Estimate of Dietary Exposure of 2mEPSPS for Humans

Expression levels of 2mEPSPS protein in plant tissues of DAS-444Ø6-6 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 2mEPSPS protein in DAS-444Ø6-6 soybean, indicating negligible risk for adverse effects from acute dietary exposure (Cleveland 2011).

Potential Human Exposure to 2mEPSPS Protein via Soybean

The field expression of 2mEPSPS protein in DAS-444Ø6-6 soybean was measured using a 2mEPSPS specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (Maldonado 2011). Field expression data is available for trials conducted at multiple test sites located within the major soybean-producing regions of the U.S. Protein expression was analysed in leaf at V5 and V10-12 growth stages, with root, forage, and grain tissues collected throughout the growing season from DAS-444Ø6-6 soybean plants treated with or without the herbicides 2,4-D, glufosinate, and/or glyphosate.

The results showed low level expression of the 2mEPSPS protein with similar expression values for 2mEPSPS for all treatments irrespective of the herbicide regime, indicating a low exposure risk to humans. Only the protein expression in the soybean grain is applicable for human dietary consideration. In soybean grain, the average value of 2mEPSPS protein was 22.2 ng 2mEPSPS protein/mg tissue on a dry weight basis. The full range of applicable values was 9.92 to 33.47 ng/mg tissue [based on review of the 50 values for site/test spray], but the use of an average expression value is most appropriate. This is because grain is a blended commodity, making consumption of single-servings of grain at the maximum expression-level highly unlikely. Use of these values are protective estimates for exposure to the 2mEPSPS protein from soybean; actual dietary exposure and impact of the protein will be lower because: 1) there may be protein degradation during transport and storage, 2) grain containing 2mEPSPS will be mixed with non-2mEPSPS grain, 3) human consumption of soy products is primarily 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 that contain very little protein (e.g. soybean oil).

Margin of Exposure Calculation

A conservative short term intake (STI) consumption of soybean was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint (acute NOAEL of 5000 mg/kg). Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki, Davies et al. 2005). Nevertheless, to place the 2mEPSPS 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).
MOE = NOEL/Exposure

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold. Regardless of which method is used, all acute MOE are greater than 10000 (Table 28), indicating negligible risk for adverse effects from dietary exposure to 2mEPSPS protein.
In the assessment, the MOE values were calculated using the DEEM dietary exposure model program (DEEM-FCID version 2.16, Exponent, 2007). The residue value of 22.2 ng 2mEPSPS/mg tissue was conservatively applied to all commodities listed in DEEM that are associated with soybean. This is a conservative approach for processed flour and soymilk. No residues were assigned to oil. Using this residue file as input, MOE values were calculated against the acute NOAEL of 5000 mg/kg for several subpopulations (Table 32). The most exposed subpopulation is the non-nursing infants (<1 yr old) with an estimated exposure of 0.077198 mg/kg-bw and an MOE value of >64768 at the 97.5th percentile. MOE

values >100 are typically considered acceptable. All relevant MOE values are greater than 40000, indicating there is no concern from acute dietary exposure through soybean.

Table 32. Summary of human dietary margins of exposure for 2mEPSPS protein in soybean based on US DEEM consumption model for short term exposure.

	Food Intake[b] g/kg-bw	Exposure (mg 2mEPSPS /kg-bw/day)	NOAEL (mg/kg-bw)	MOE
US DEEM 97.5th				
U.S. Population	*0.1302*	0.002890	>5000	>1000000
All infants	*3.1816*	0.070632	>5000	>70789
Nursing infants	*1.6401*	0.036411	>5000	>137323
Non-nursing infants	*3.4774*	0.077198	>5000	>64768
Children 1-2 yrs	*0.4405*	0.009779	>5000	>511274
Children 3-5 yrs	*0.2342*	0.005200	>5000	>961551
Children 6-12 yrs	*0.1689*	0.003750	>5000	>1000000
Youth 13-19 yrs	*0.0969*	0.002152	>5000	>1000000

Estimate of Dietary Exposure of 2mEPSPS for Livestock

Expression levels of the 2mEPSPS protein in DAS-444Ø6-6 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 2mEPSPS protein in DAS-444Ø6-6 soybean, indicating no concern for adverse effects from acute dietary exposure.

Animal Feed Exposure

An assessment for livestock exposure was conducted (Cleveland 2011) based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures of US EPA (2008)**.** The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls, aspirated grain fractions, and optional forage. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average value of 22.2 ng/mg (ppm) for 2mEPSPS

protein in DAS-444Ø6-6 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. Because meal and seed are both protein concentrates, they are not simultaneously used in a diet. When forage was included, a value of 707.19 ng/mg (ppm) was used as the highest residue in the animal feed. This maximum value in forage was used in the conservative calculation of acute dairy animal feed exposure.

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 2mEPSPS protein in general soybeans is not anticipated to have an impact on feed ration formulation, based on the history of safe use of EPSPS proteins in other commercial offerings.

US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (US EPA 2008):

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% complex carbohydrate, CC), forages (15% roughage, R), and protein sources (5% protein concentrate, 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 2mEPSPS protein. In addition, the higher values of 707.19 ppm of 2mEPSPS 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-444Ø6-6 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled (Table 33).

Table 33. Intake animal dietary burdens for livestock from 2mEPSPS.

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	2mEPSPS (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls*	R	90	15	20	Nu	Nu	6.2	1.04	1.38	-	-
Aspirated grain**	CC	85	5	Nu	Nu	Nu	444.0	26.12	-	-	-
Soybean seed	PC	89	*Meal used*	15	*Meal used*	*Meal used*	22.2		3.74	-	-
Soybean meal*	PC	NA	*5*	*Seed used*	25	15	24.42	1.33	-	6.11	3.66
Soybean Forage	R	35	Nu	20	Nu	Nu	707.19	-	404.11	-	-
							Total	28.48	409.23	6.11	3.66

* estimate based on measured value for seed

**based on theoretical estimate of 20X the value in soybean seed

Nu – not used

Because only soybean feeds are considered, the nutritional balance of the diets is 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, as reported in (Table 34.)

Table 34. Livestock daily dose estimates of 2mEPSPS protein from soybean seeds.

	Cattle		Swine	Poultry
	Beef	**Dairy**	**Finishing**	**Broiler**
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum 2mEPSPS intake (mg/kg feed)	28.48	409.00	3.66	6.11
Maximum intake (mg/kg-bw)	0.47	16.04	0.10	0.17
MOE vs. Mammalian NOEL	10612	312	49797	29901

The highest exposed animal is the dairy cow with 16.04 mg 2mEPSPS/kg bw estimate. When this value is compared to the acute NOEL of >5000 mg/kg bw, there is an adequate margin of safety for livestock; typical MOEs are > 10000 and the worst case is > 300. 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 2mEPSPS protein in DAS-444Ø6-6 soybean.

Estimate of Dietary Exposure of PAT for Humans

Expression levels of PAT protein in plant tissues of DAS-444Ø6-6 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 PAT protein in DAS-444Ø6-6 soybean, indicating negligible risk for adverse effects from acute dietary exposure (Cleveland 2011).

Potential Human Exposure to PAT Protein via Soybean

The field expression of PAT protein in DAS-444Ø6-6 soybean was measured using a PAT specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (Maldonado 2011). Field expression data is available for trials conducted at multiple test sites

located within the major soybean-producing regions of the U.S. Protein expression was analysed in leaf at V5 and V10-12 growth stages, with root, forage, and grain tissues collected throughout the growing season from DAS-444Ø6-6 soybean plants treated with or without the herbicides 2,4-D, glufosinate, and/or glyphosate.
The results showed low level expression of the PAT protein with similar expression values for PAT for all treatments irrespective of the herbicide regime, indicating a low exposure risk to humans. Only the protein expression in the soybean grain is applicable for human dietary consideration.

In soybean grain, the average value of PAT protein was 2.12 ng PAT protein/mg tissue on a dry weight basis. The full range of applicable values was 1.60 to 2.97 ng/mg tissue [based on review of the 50 values for site/test spray], but the use of an average expression value is most appropriate. This is because grain is a blended commodity, making consumption of single-servings of grain at the maximum expression-level highly unlikely. Use of these values are protective estimates for exposure to the PAT protein from soybean; actual dietary exposure and impact of the protein will be lower because: 1) there may be protein degradation during transport and storage, 2) grain containing PAT will be mixed with non-PAT grain, 3) human consumption of soy products is primarily 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 that contain very little protein (e.g. soybean oil).

Margin of Exposure Calculation

A conservative short term intake (STI) consumption of soybean was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint of 5000 mg/kg (OECD 1999). Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki, Davies et al. 2005). Nevertheless, to place the PAT 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).

MOE = NOEL/Exposure

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold. Regardless of which method is used, all acute MOE are greater than 10000 (Table 31), indicating negligible risk for adverse effects from dietary exposure to PAT protein.

In the US assessment, the MOE values were calculated using the DEEM dietary exposure model program (DEEM-FCID version 2.16, Exponent, 2007). The residue value of 2.12 ng PAT/mg tissue was conservatively applied to all commodities listed in DEEM that are associated with soybean. This is a conservative approach for processed flour and soymilk. No residues were assigned to oil. Using this

residue file as input, MOE values were calculated against the acute NOAEL of 5000 mg/kg for several subpopulations (Table 35). The most exposed subpopulation is the non-nursing infants (<1 yr old) with an estimated exposure of 0.00737 mg/kg-bw and an MOE value of >678235 at the 97.5th percentile. MOE values >100 are typically considered acceptable. All relevant MOE values are greater than 400000, indicating there is no concern from acute dietary exposure through soybean.

Table 18. Summary of human dietary margins of exposure for PAT protein in soybean based on US DEEM consumption model for short term exposure.

	Food Intake[b] g/kg-bw	Exposure (mg PAT /kg-bw/day)	NOAEL (mg/kg-bw)	MOE
US DEEM 97.5th				
U.S. Population	*0.1302*	0.000276	>5000	>1000000
All infants	*3.1816*	0.006745	>5000	>741281
Nursing infants	*1.6401*	0.003477	>5000	>1000000
Non-nursing infants	*3.4774*	0.007372	>5000	>678235
Children 1-2 yrs	*0.4405*	0.000934	>5000	>1000000
Children 3-5 yrs	*0.2342*	0.000497	>5000	>1000000
Children 6-12 yrs	*0.1689*	0.000358	>5000	>1000000
Youth 13-19 yrs	*0.0969*	0.000205	>5000	>1000000

Estimate of Dietary Exposure of PAT for Livestock

Expression levels of the PAT protein in DAS-444Ø6-6 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 PAT protein in DAS-444Ø6-6 soybean, indicating no concern for adverse effects from acute dietary exposure.

Animal Feed Exposure

An assessment for livestock exposure was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (US EPA 2008). The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls and aspirated grain fractions, and optional forage. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle

based on the average value of 2.12 ng/mg (or ppm) for PAT protein in DAS-444Ø6-6 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. Because meal and seed are both protein concentrates, they are not simultaneously used in a diet. When forage was included, a value of 9.35 ng/mg was used as the highest residue in the animal feed. This maximum value in forage was used in the conservative calculation of acute dairy animal feed exposure.

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 PAT protein in soybean tissue is not anticipated to have impact for feed ration formulation, because nutrient composition analyses have shown that DAS-444Ø6-6 soybean is substantially equivalent to conventional soybean per the general OECD and ILSI guidance (OECD 2001; ILSI 2011).

US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (US EPA 2008):

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 PAT protein. In addition, the higher values of 114.38 ppm of PAT 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-444Ø6-6 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled in Table 36.

Table 196. Intake animal dietary burdens for livestock from PAT.

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	PAT (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls	R	90	15	20	Nu	Nu	0.59	0.1	0.132	-	-
Aspirated grain**	CC	85	5	Nu	Nu	Nu	42.4	2.49	-	-	-
Soybean seed	PC	89	*Meal used*	15	*Meal used*	*Meal used*	2.33		0.36	-	-
Soybean meal*	PC	NA	*5*	*Seed used*	25	15	29.4	0.13	-	0.58	0.35
Soybean Forage	R	35	Nu	20	Nu	Nu	9.35	-	5.34	-	-
							Total	2.72	5.83	0.58	0.35

* estimate based on measured value for seed

**based on theoretical estimate of 20X the value in soybean seed

Nu – not used

Because only soybean feeds are considered, the 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 is reported (Table 20).

Table 20. Livestock daily dose estimates of PAT protein from soybean seeds.

	Cattle		**Swine**	**Poultry**
	Beef	**Dairy**	**Finishing**	**Broiler**
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum PAT intake (mg/kg feed)	2.72	5.83	0.35	0.58
Maximum intake (mg/kg bw)	0.05	0.23	0.01	0.02
MOE vs. Mammalian NOAEL	111111	21870	520737	314987

The highest exposed animal is the dairy cow with 0.23 mg PAT/kg bw estimate. When this value is compared to the acute NOEL of >5000 mg/kg bw, there is an adequate margin of safety for livestock; typical MOEs are > 100000 and the worst case is > 21000. 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 PAT protein in DAS-444Ø6-6 soybean.

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Attachment 1 – DAS Internal Studies