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Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-444Ø6-6 Soybean

OECD Unique Identifier: DAS-444Ø6-6

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

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Release of Information

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

Certification

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

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Summary

Dow AgroSciences LLC (herein referred to as "DAS") and M.S. Technologies LLC (herein referred to as "MS Tech") are submitting a Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-444Ø6-6 Soybean. DAS and MS Tech request a determination from USDA Animal and Plant Health Inspection Service (APHIS) that soybean transformation event DAS-444Ø6-6 and any soybean lines derived from crosses with DAS-444Ø6-6 soybeans no longer be considered regulated articles under 7 CFR Part 340.

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 analyzed 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 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 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 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 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 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 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.

DAS-444Ø6-6 soybean has been field tested in the major soybean growing regions of the continental United States as well as Puerto Rico. All field tests were conducted under field permits granted by USDA APHIS. Agronomic performance assessments were conducted on DAS-444Ø6-6 soybean in multi-site field studies to measure characteristics such as emergence, seedling vigor, plant height, lodging, and yield. All field trials were also observed for opportunistic disease or insect stressors as well as normal phenotypic characteristics. There were no meaningful differences observed between DAS-444Ø6-6 soybean and the non-transgenic control for plant pest characteristics and no indication of a selective advantage that would result in increased weediness potential of DAS-444Ø6-6 soybean.

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.

Information collected during field trials and laboratory analyses presented herein demonstrate that DAS-444Ø6-6 soybean exhibits no plant pathogenic properties or weediness characteristics. DAS-444Ø6-6 soybean is no more likely to become a plant pest than conventional soybean, and the AAD-12, 2mEPSPS and PAT proteins are unlikely to increase the weediness potential of any other cultivated plant or wild species.

DAS and MS Tech hereby request a determination from APHIS that herbicide-tolerant DAS-444Ø6-6 soybean and all progeny derived from the conventional breeding of this line no longer be considered regulated articles under 7 CFR Part 340.

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Acronyms and Scientific Terms

2,4-D	2,4-Dichlorophenoxyacetic acid
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 fiber
ae	Acid equivalent
ae/A	Acid equivalent per acre
ae/ha	Acid equivalent per hectare
ai	Active ingredient
ai/A	Active ingredient per acre
ai/ha	Active ingredient per hectare
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	Liter
LOD	Limit of Detection
LOQ	Limit of Quantitation
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
Maverick	Publicly available soybean line used in transformation to produce DAS-444Ø6-6 soybean
mEPSPS	Same as 2mEPSPS
MOA	Mode of action
MS Tech	M.S. Technologies LLC
NDF	Neutral detergent fiber
OECD	Organisation for Economic Co-operation and Development
pat	Gene from *Streptomyces viridochromogenes* which encodes the PAT protein
PAT	Phosphinothricin *N*-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
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

1. Rationale for the Development of DAS-444Ø6-6

1.1. Basis for the Request for Nonregulated Status

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and the Plant Quarantine Act (7 U.S.C. 151-167), to prevent the introduction or dissemination of plant pests into or within the United States. Part 340 regulates introduction of organisms altered or produced through genetic engineering which are plant pests or for which there is a reason to believe are plant pests. The APHIS regulations at 7 CFR 430.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Dow AgroSciences LLC (herein referred to as "DAS") and M.S. Technologies LLC (herein referred to as "MS Tech") are submitting data for genetically engineered herbicide-tolerant DAS-444Ø6-6 soybean and request a determination from APHIS that DAS-444Ø6-6 soybean and crosses of DAS-444Ø6-6 with nonregulated soybean lines no longer be considered regulated articles under 7 CFR 340.

1.2. Benefits of DAS-444Ø6-6 Soybean

DAS and MS Tech have developed transgenic soybean plants that are tolerant to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate and glufosinate. The unique identifier for these plants, in accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2004), is DAS-444Ø6-6.

DAS-444Ø6-6 soybean was developed using *Agrobacterium*-mediated transformation to stably incorporate the *aad-12* gene from *Delftia acidovorans*, the *2mepsps* gene from *Zea mays*, and the *pat* gene from *Streptomyces viridochromogenes* into soybean. The *aad-12* gene encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme which, when expressed in plants, degrades 2,4-D to herbicidally-inactive 2,4-dichlorophenol (DCP). The *2mepsps* gene encodes the 2mEPSPS protein, which has a decreased sensitivity to the herbicide glyphosate. This allows the enzyme to function in the presence of the herbicide and thereby makes the plant tolerant to glyphosate. The *pat* gene encodes the enzyme phosphinothricin acetyltransferase that inactivates glufosinate.

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.

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). Additionally, weeds that had previously not been an agronomic problem prior to the wide use of glyphosate-tolerant crops are now becoming more prevalent and difficult to control in the context of glyphosate-tolerant crops, which now comprise >90% of U.S. soybean acres and >60% of U.S. corn and cotton acres (USDA ERS, 2011a). 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 gives 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 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 vigor and yield of the crop, allow an increased herbicide application window for effective weed control, and provide an effective resistance management/prevention solution to the increased incidence of glyphosate- and acetolactate synthase (ALS)-resistant weeds.

1.3. Submission to Other Regulatory Agencies

DAS-444Ø6-6 falls within the scope of the FDA policy statement, published in the Federal Register on May 29, 1992, concerning regulation of products derived from new plant varieties, including those developed via biotechnology. DAS and MS Tech intend to submit a pre-market biotechnology notification (PBN) to FDA in 2011.

The regulation and use of herbicides on DAS-444Ø6-6 soybean is governed by the US under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). The use of herbicides on DAS-444Ø6-6 soybean will be consistent with either currently authorized uses or uses that are currently in review by US EPA, therefore there are no new regulatory submissions to US EPA associated with DAS-444Ø6-6 soybean.

DAS and MS Tech intend to submit dossiers beginning in 2011 to the regulatory authorities of trade partners for import clearance and production approval which may include Canada, Japan, Korea, Taiwan, European Union, Australia/New Zealand, South Africa, Brazil, Argentina and Mexico.

2. The Biology of Soybean

2.1. Overview of Soybean Biology

Refer to the OECD Consensus Document on the Biology of *Glycine max* (L.) Merr. (Soybean) (OECD, 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
- centers 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

2.2. Characterization of the Recipient Soybean Cultivar

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. Development of DAS-444Ø6-6 Soybean

3.1. Description of the Transformation System

DAS-444Ø6-6 soybean was generated through *Agrobacterium*-mediated transformation of soybean (*Glycine max*) cotyledonary node explants. The disarmed *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986), carrying the binary vector with the *pat*, *aad-12* and *2mepsps* 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 analyzed at the molecular level for the presence of the T-DNA insert 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 1 shows a plasmid map of pDAB8264.

Figure 2 shows a diagram of the T-DNA insert in plasmid pDAB8264.

Figure 3 outlines the development of DAS-444Ø6-6 soybean.

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

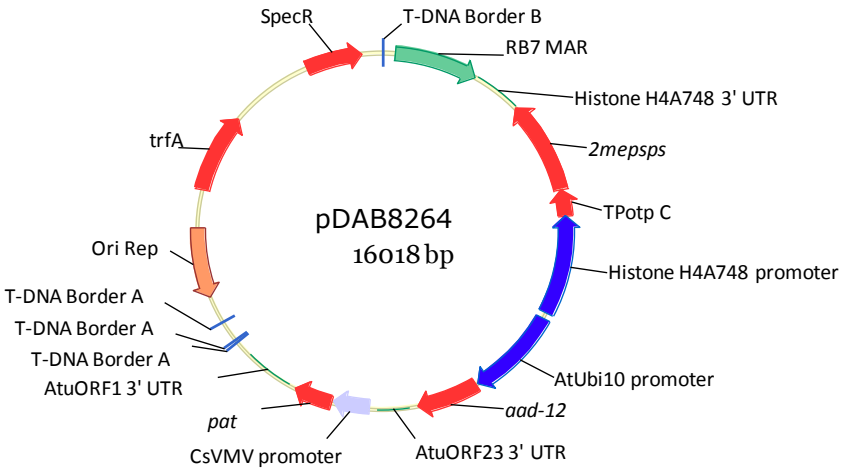


Figure 1. Plasmid map of pDAB8264.

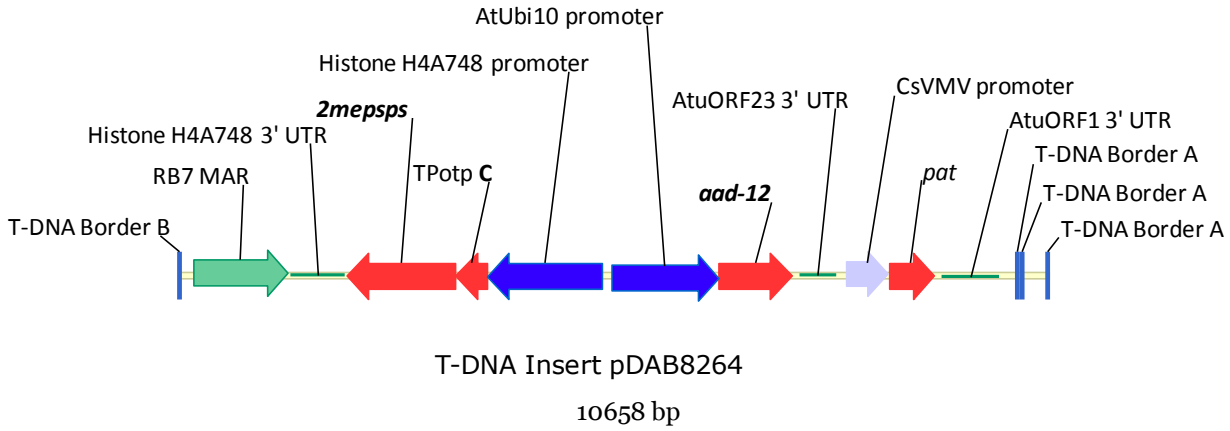


Figure 2. Diagram of intended T-DNA insert in plasmid pDAB8264.

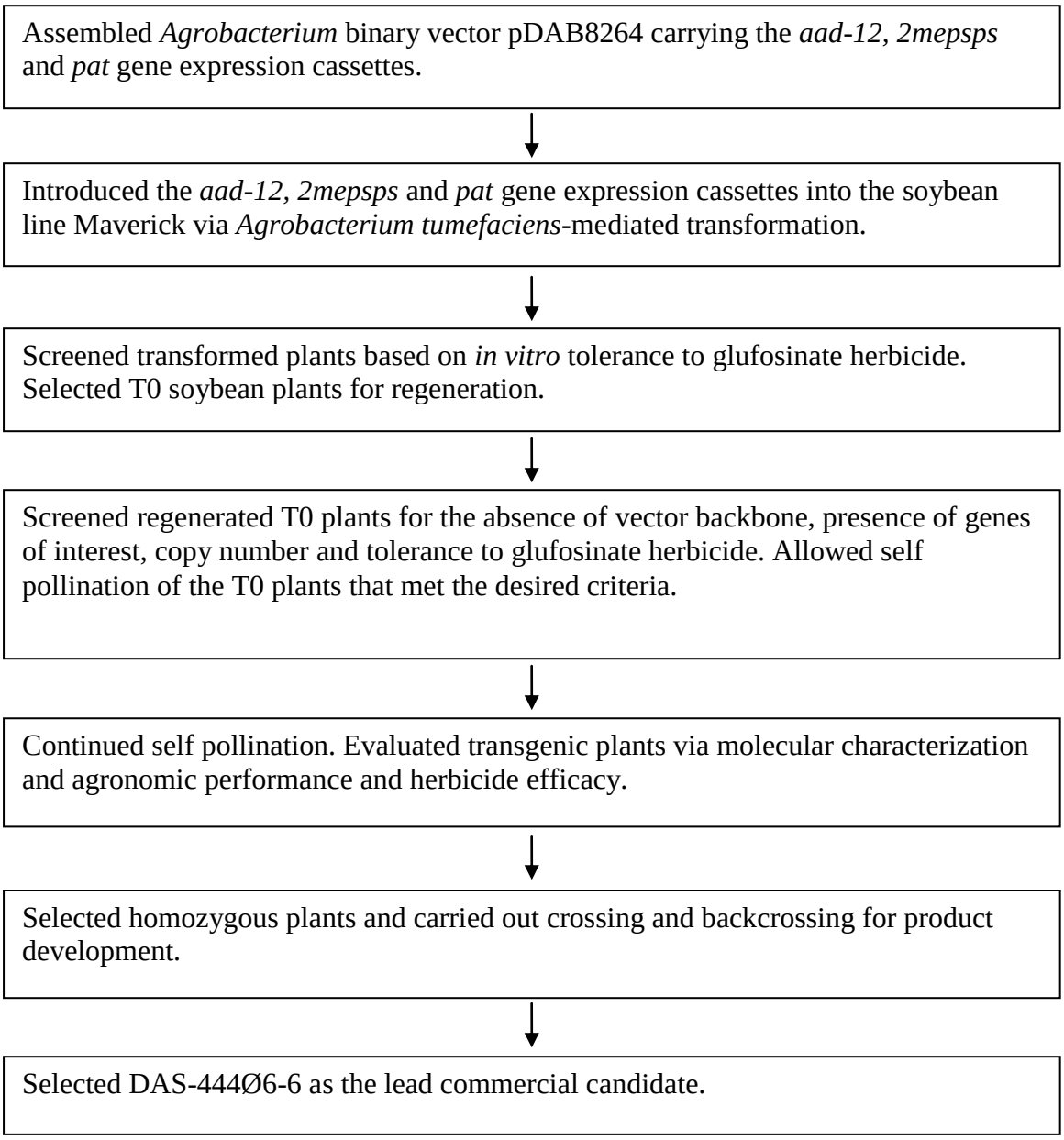
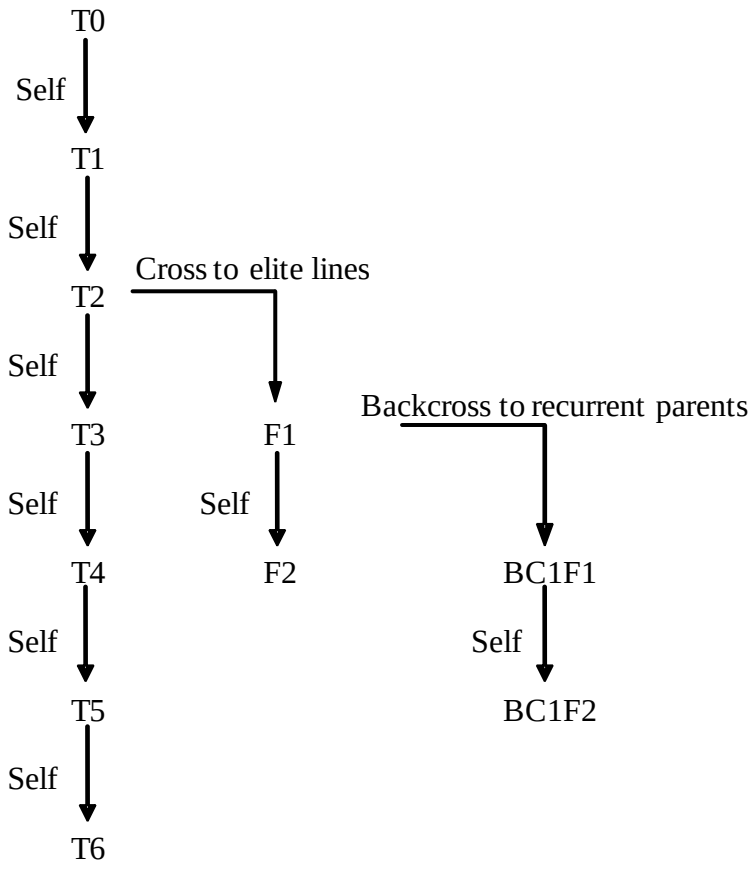


Figure 3. Event sorting and selection process for DAS-444Ø6-6 soybean.



Analysis	Petition Section(s)	DAS-444Ø6-6 Soybean Generations Used	Control
Molecular Analysis	5.1, 5.2, 5.3, 5.4	T2, T3, T4, T6, F2	Maverick
Segregation Analysis	5.5	F2, BC1F2	Maverick
Protein Characterization	6.1.3, 6.2.3, 6.3.3	T4	Maverick
Protein Expression	6.1.3, 6.2.3, 6.3.3	T4	Maverick
Agronomics	7	T4	Maverick
Germination/Dormancy	7.3	T5	Maverick
Composition	8	T4	Maverick
Efficacy	9.1	T4	Maverick

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

3.2. Selection of Comparators for DAS-444Ø6-6 Soybean

To ensure the accurate assessment of the impact of transgene insertion on various characteristics of DAS-444Ø6-6 soybean, a proper selection of comparator plants is important.

The control plants should have a genetic background similar to that of DAS-444Ø6-6 soybean, but lack the transgenic insert. In all cases, the non-transgenic variety Maverick was used as the control. Maverick is the recipient variety that was transformed to generate DAS-444Ø6-6 soybean. The T0 plants and all subsequent self-pollinated generations derived post-transformation were essentially genetically identical to Maverick soybean with the exception of the transgenic insert DNA.

4. Donor Genes and Regulatory Sequences

4.1. Identity and Source of Genetic Material in pDAB8264

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* 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.

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

Feature Name	Feature Start	Feature Stop	Feature Length	Description
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
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)
Intervening sequence	10305	10323	19	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)

Feature Name	Feature Start	Feature Stop	Feature Length	Description
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, also referred to as plant transcription units or PTUs, 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 *et al.*, 1996; Lebrun *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 (USDA, 1997) and in GlyTol™ cotton (OECD unique identifier BCS-GHØØ2-5), which was deregulated by USDA APHIS in 2009 (USDA, 2009). It is also present in soybean event FG-72 (OECD unique identifier MST-FGØ72-3) which is currently in review at USDA APHIS as petition 09-328-01p. 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 *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).

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 *et al.*, 1987; Wen *et al.*, 1999). *Delftia acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Rao and Ravishankar, 2000; Shetty *et al.*, 2006). This utility has led to a history of safe use for *Delftia acidovorans* in the food processing industry. For example, 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).

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 *et al.*, 1993). The function of AtuORF23 (GenBank Accession Number: CAA25184) in pTi15955 (GenBank Accession Number: X00493) was not identified (Barker *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 deregulated products (USDA, 1996, 2001, 2004, 2005).

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 *et al.*, 1996). The function of AtuORF1 (GenBank Accession Number: CAA25163) in pTi15955 (GenBank Accession Number: X00493) was not identified (Barker *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 at the 5′ end of 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 *et al.*, 1997; Abranches *et al.*, 2005; Verma *et al.*, 2005).

5. Genetic Characterization

5.1. Overview of Molecular Analysis

Molecular characterization of DAS-444Ø6-6 soybean was conducted by Southern blot analysis. 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 Maverick served as the positive control for the Southern blot analysis. Materials and methods used for the Southern analyses are further described in Appendix 1.

The expected restriction fragments of the inserted DNA are shown in Table 3 and Figure 7, and the Southern blot results are shown in Figure 8 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 5.2). No vector backbone sequences were detected in DAS-444Ø6-6 soybean (Section 5.3). The hybridization patterns across five generations of DAS-444Ø6-6 soybean (T2, T3, T4, T6, and F2) were identical, indicating that the insertion is stably integrated in the soybean genome (Section 5.4). 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 (Section 5.5). All results confirmed the predicted inheritance pattern of the transgene at a single locus (Section 5.6).

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

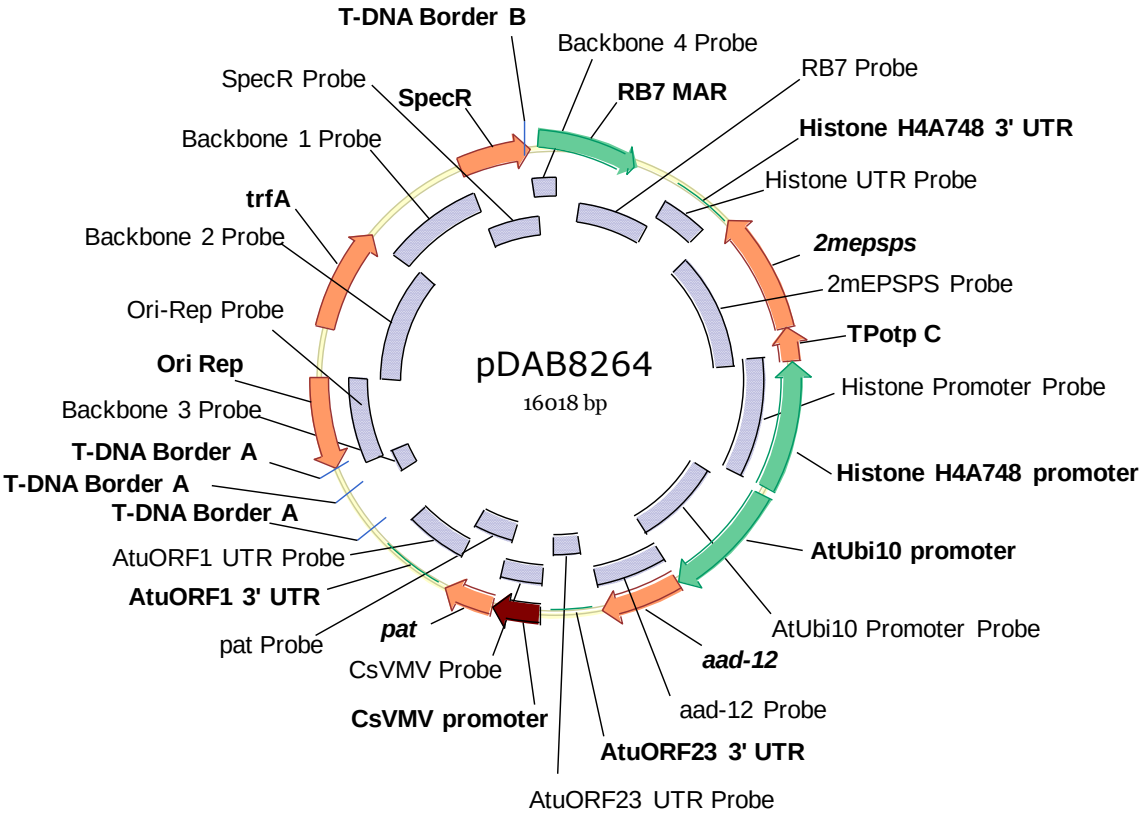


Figure 5. Location of probes on pDAB8264 used in Southern blot analysis of DAS-444Ø6-6 soybean.

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	9A
		Maverick	None	None	
		DAS-444Ø6-6	>4261	~4700	
	*Msc*I	pDAB8264	10089	~10100	9B
		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	

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
CsVMV	*Xho*I	pDAB8264	16018	~16000	13A
		Maverick	None	None	
		DAS-444Ø6-6	>10093	~12000	
	*Hind*III	pDAB8264	4731	~4700	13B
		Maverick	None	None	
		DAS-444Ø6-6	>4432	~7000	
AtuORF1 UTR	*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	

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
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	
		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	

Probe/ Feature	Enzyme	Sample	Expected Result (bp)[1]	Observed Result (bp)[2]	Figure Number
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	
		DAS-444Ø6-6	None	None	
	*Pst*I/*Xho*I	pDAB8264	9288	~9300	22B
		Maverick	None	None	
		DAS-444Ø6-6	None	None	
Backbone 1	*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 pDAB8264 (Figure 6) and the linearized T-DNA map (Figure 7).
2. Observed fragment sizes are considered approximations from these analyses and are based on the indicated sizes of the DIG-labeled 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.

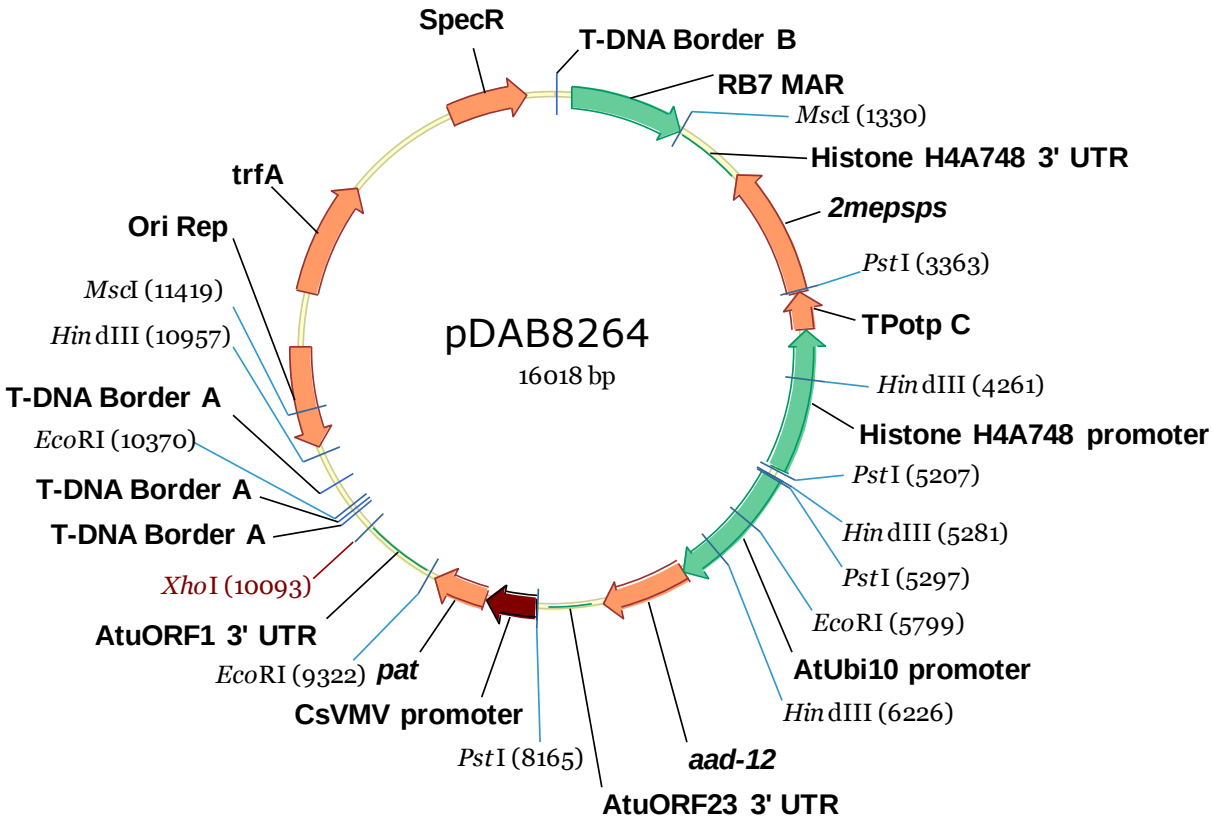


Figure 6. Plasmid map of pDAB8264 with selected restriction enzyme sites used for Southern analysis.

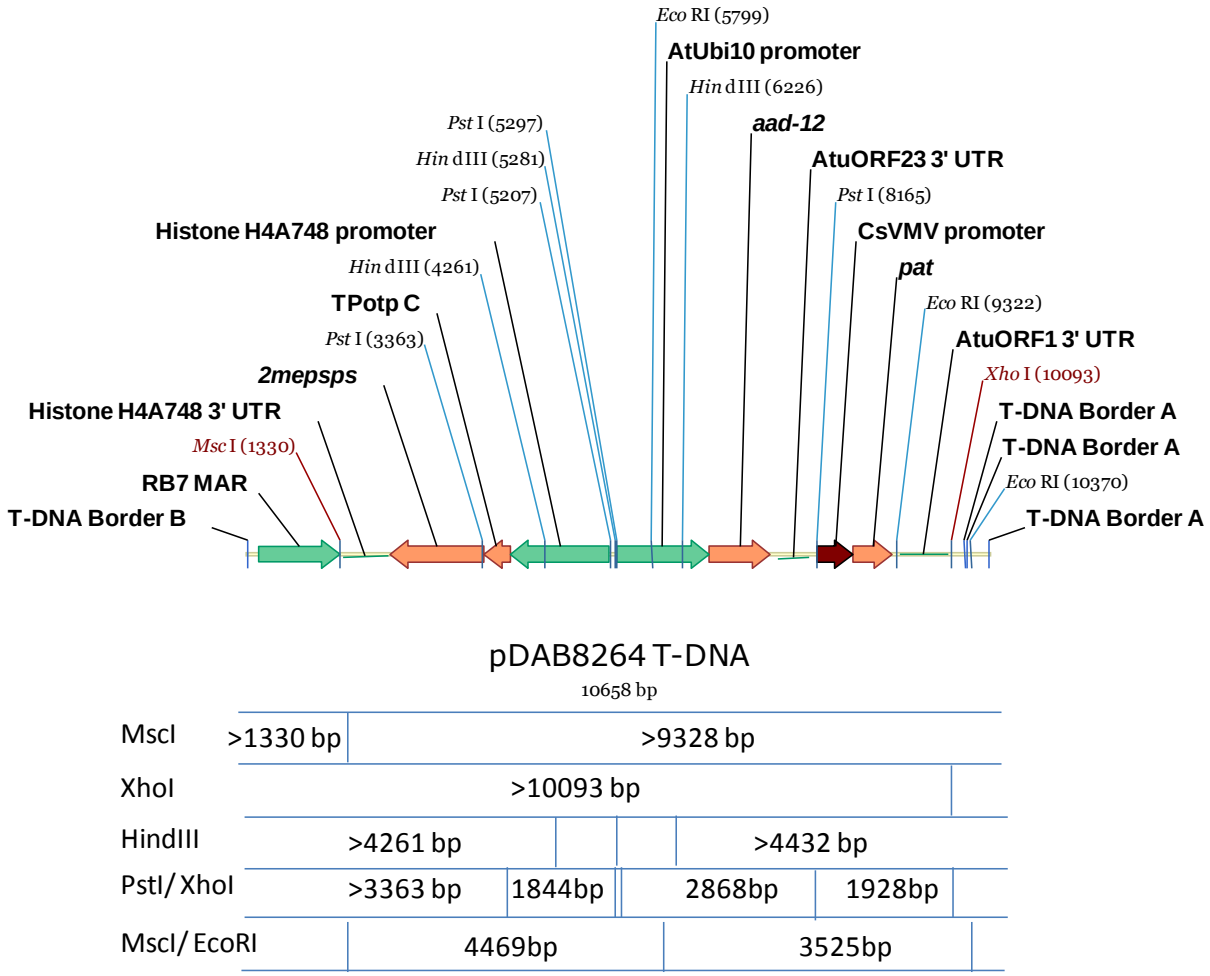


Figure 7. Linearized intended T-DNA insert from pDAB8264 with restriction enzymes used for DNA digestion and the expected hybridization bands.

5.2. Analysis of the Insert and Its Genetic Elements

5.2.1. 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, 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 were 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.

5.2.2. 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 an *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 19B). 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 19A and Figure 19C). 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 *pat* probe, each individual DAS-444Ø6-6 plant across the five generations 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 Figure 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*.

5.3. 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 Southern 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 digestions, 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 digestions. 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 samples 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.

5.4. 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. As described above, 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.

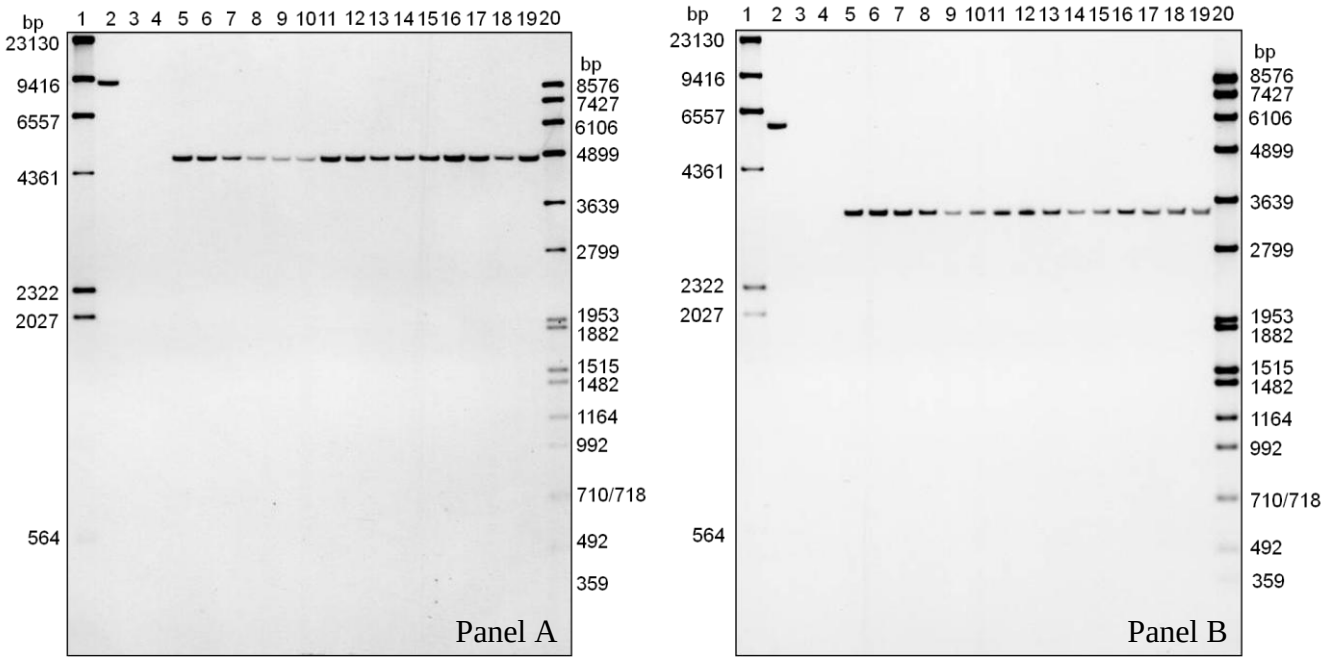


Figure 8. Southern blot analysis of *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: RB7 probe.

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

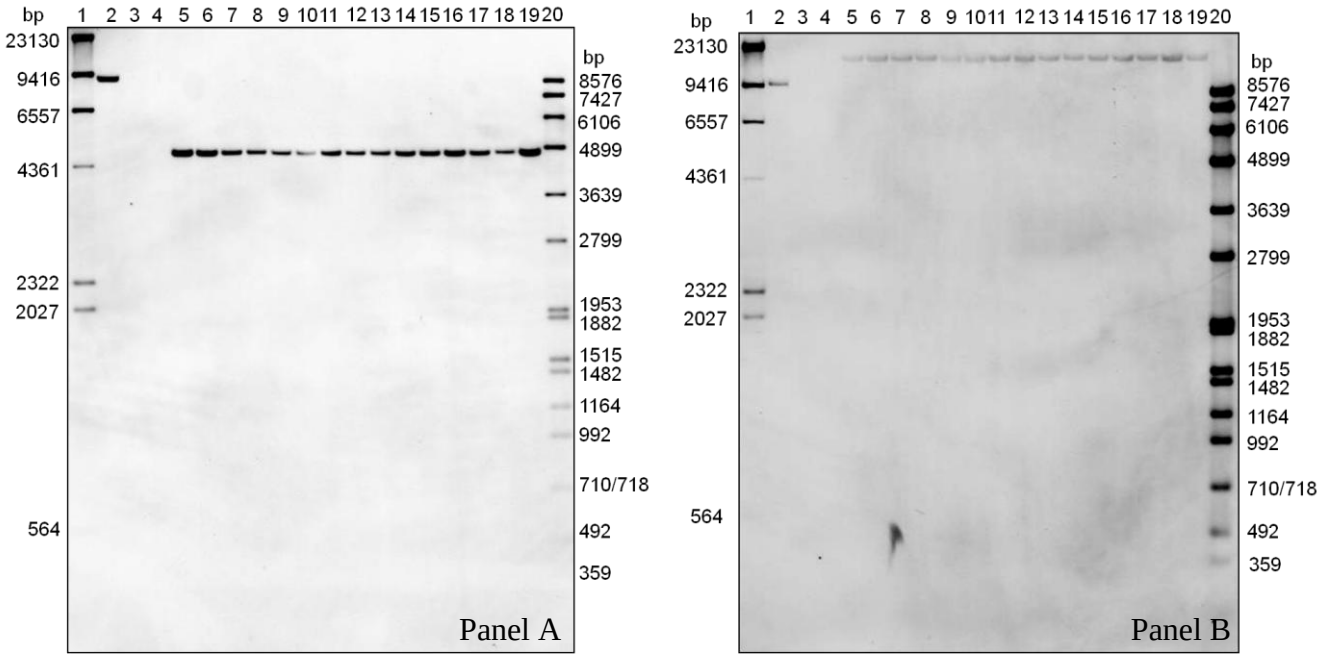


Figure 9. Southern blot analysis of *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: Histone H4A748 UTR probe.

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 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

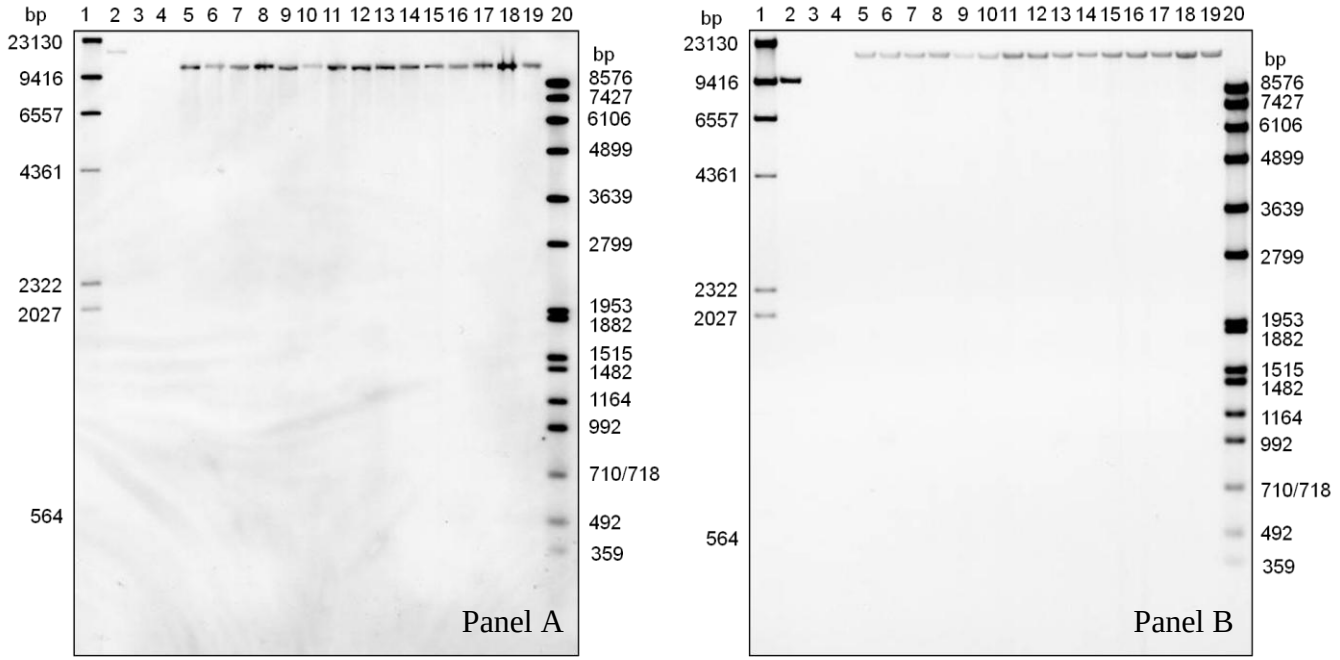


Figure 10. Southern blot analysis of *Xho*I and *Msc*I digested DAS-444Ø6-6 soybean: Histone H4A748 Promoter probe.

Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A) and *Msc*I (Panel B) and hybridized with the Histone 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

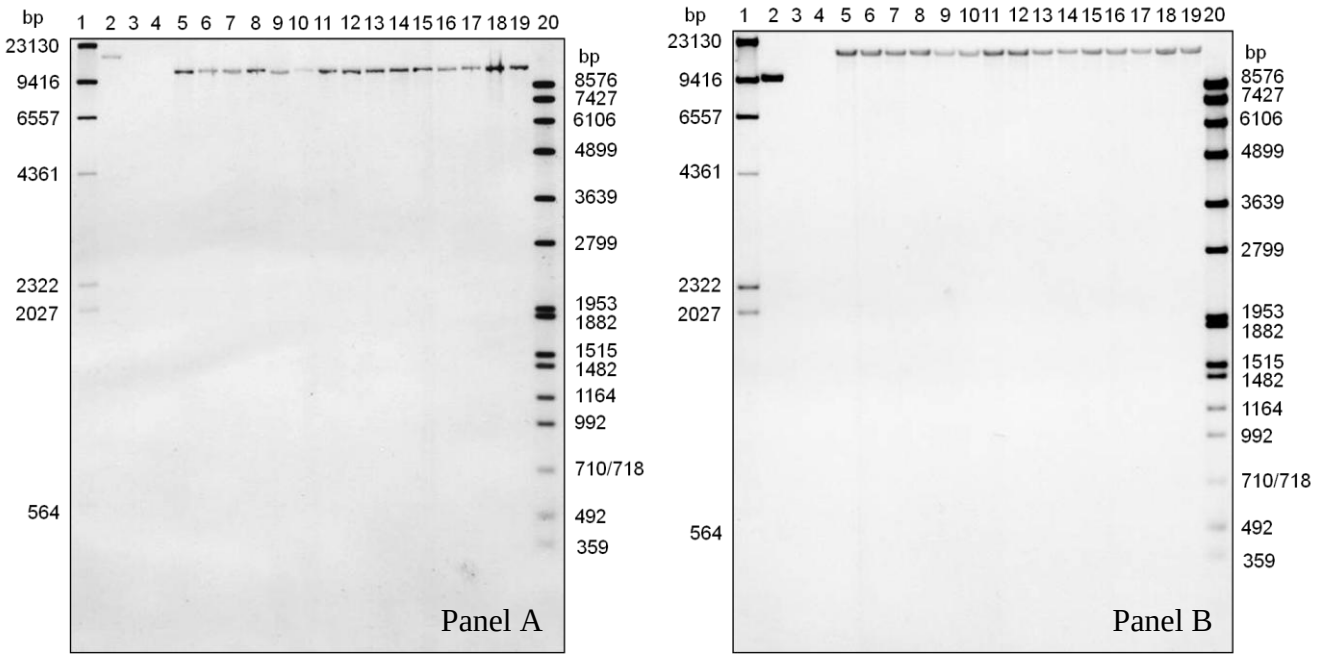


Figure 11. Southern blot analysis of *Xho*I and *Msc*I digested DAS-444Ø6-6 soybean: AtUbi10 Promoter probe.

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

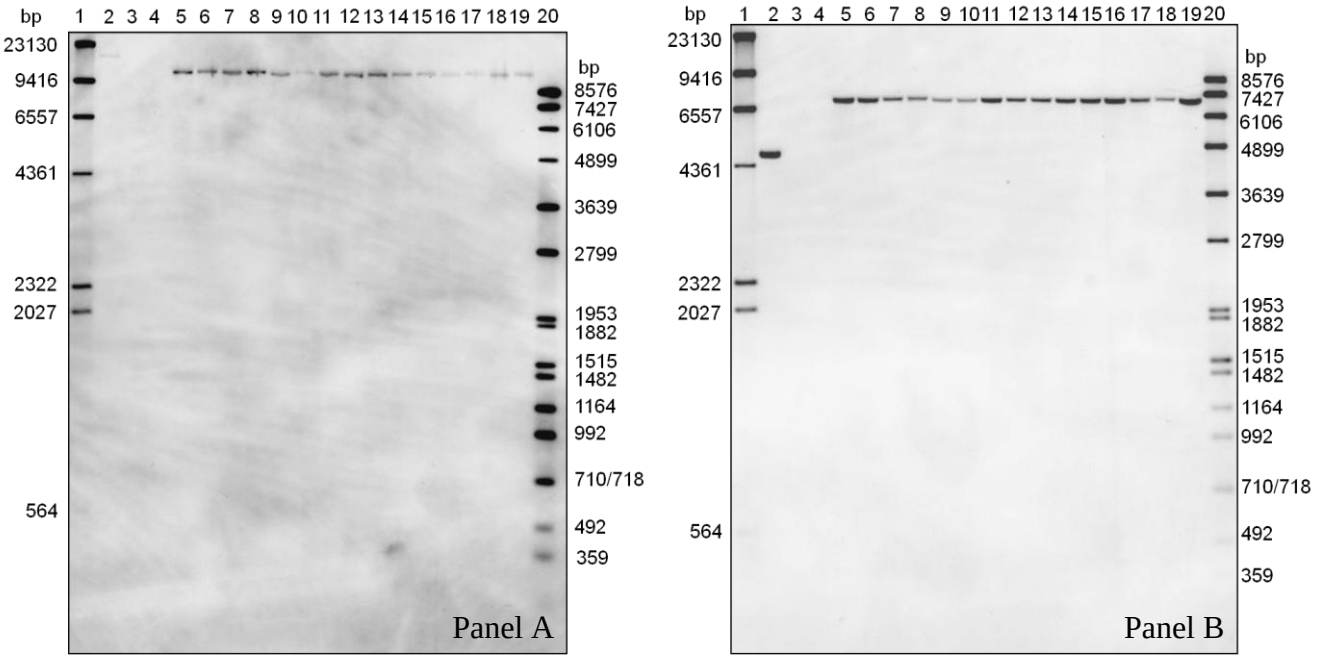


Figure 12. Southern blot analysis of *Xho*I and *Hind*III digested DAS-444Ø6-6 soybean: AtuORF23 UTR probe.
Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A) and *Hin*dIII (Panel B) and hybridized with the AtuORF23 UTR probe. Plasmid pDAB8264 served as the positive control at approximately 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

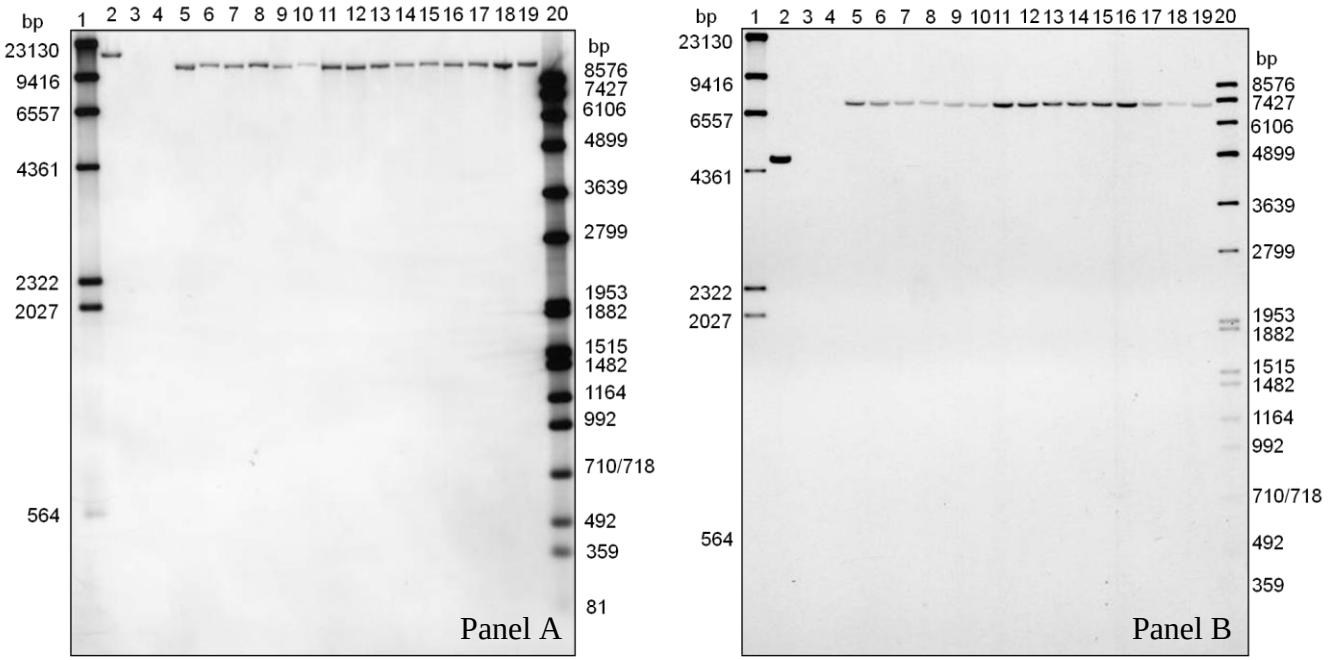


Figure 13. Southern blot analysis of *Xho*I and *Hind*III digested DAS-444Ø6-6 soybean: CsVMV probe.
Approximately 10 µg genomic DNA was digested with *Xho*I (Panel A) and *Hind*III (Panel B) and hybridized with the CsVMV probe. Plasmid pDAB8264 served as the positive control at approximately one copy 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

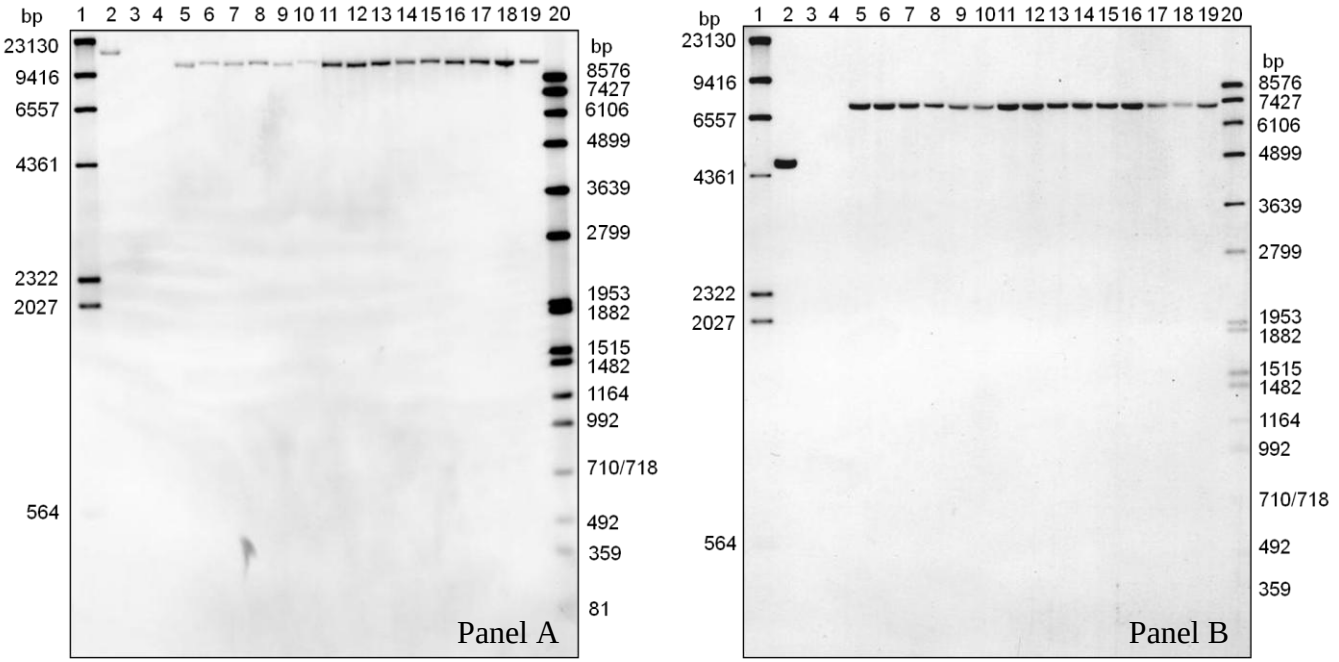


Figure 14. Southern blot analysis of *Xho*I and *Hind*III digested DAS-444Ø6-6 soybean: AtuORF1 UTR probe.
Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A) and *Hind*III (Panel B) and hybridized with the AtuORF1 UTR probe. Plasmid pDAB8264 served as the positive control at approximately 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

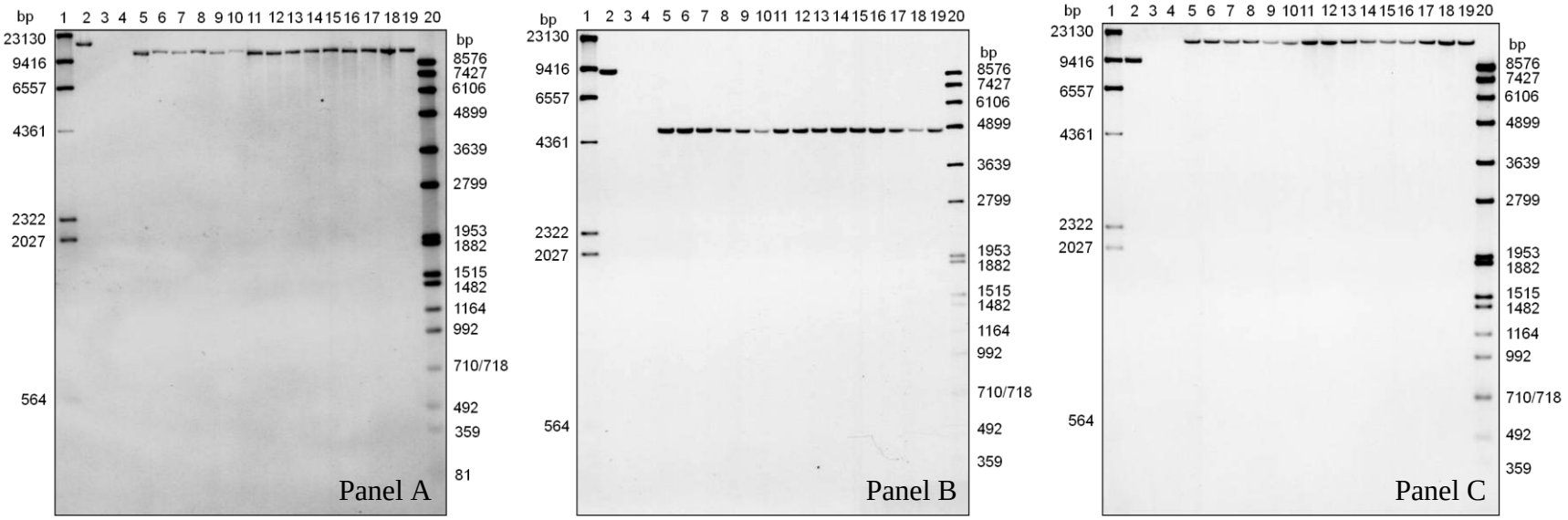


Figure 15. Southern blot analysis of *Xho*I, *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: *2mepsps* probe.
Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A), *Hind*III (Panel B) and *Msc*I (Panel C) followed by hybridization with the *2mepsps* v1 probe. Plasmid pDAB8264 served 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

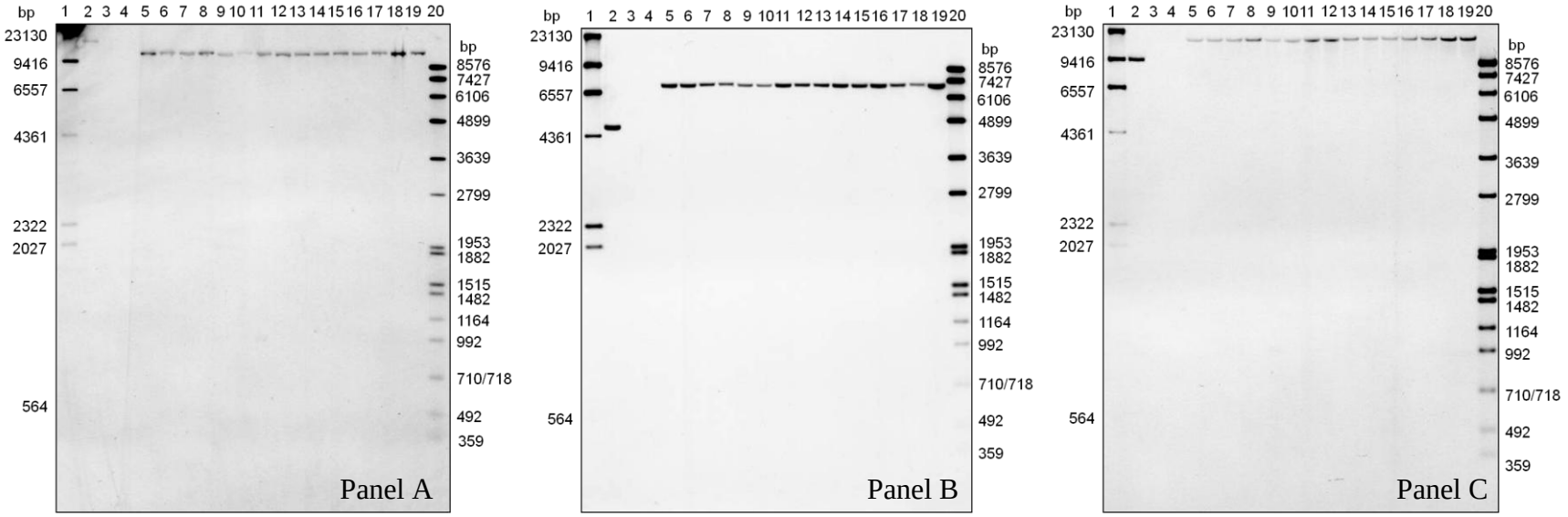


Figure 16. Southern blot analysis of *Xho*I, *Hind*III and *Msc*I digested DAS-444Ø6-6 soybean: *aad-12* probe.
Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A), *Hind*III (Panel B) and *Msc*I (Panel C) followed by hybridization with the *aad-12* probe. Plasmid pDAB8264 served as 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

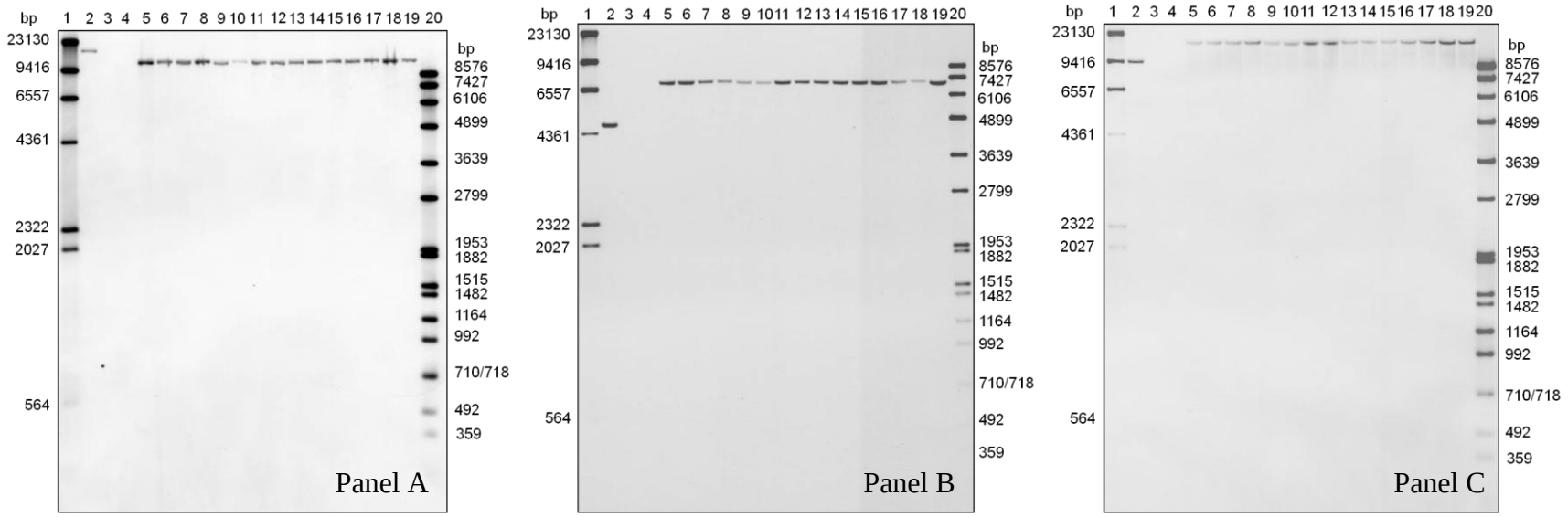


Figure 17. **Southern blot analysis of *Xho*I, *Hind*III, and *Msc*I digested DAS-444Ø6-6 soybean: *pat* probe.**
Approximately 10 µg of genomic DNA was digested with *Xho*I (Panel A), *Hind*III (Panel B) and *Msc*I (Panel C) followed by hybridization with the Pat probe. Plasmid pDAB8264 served as the 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

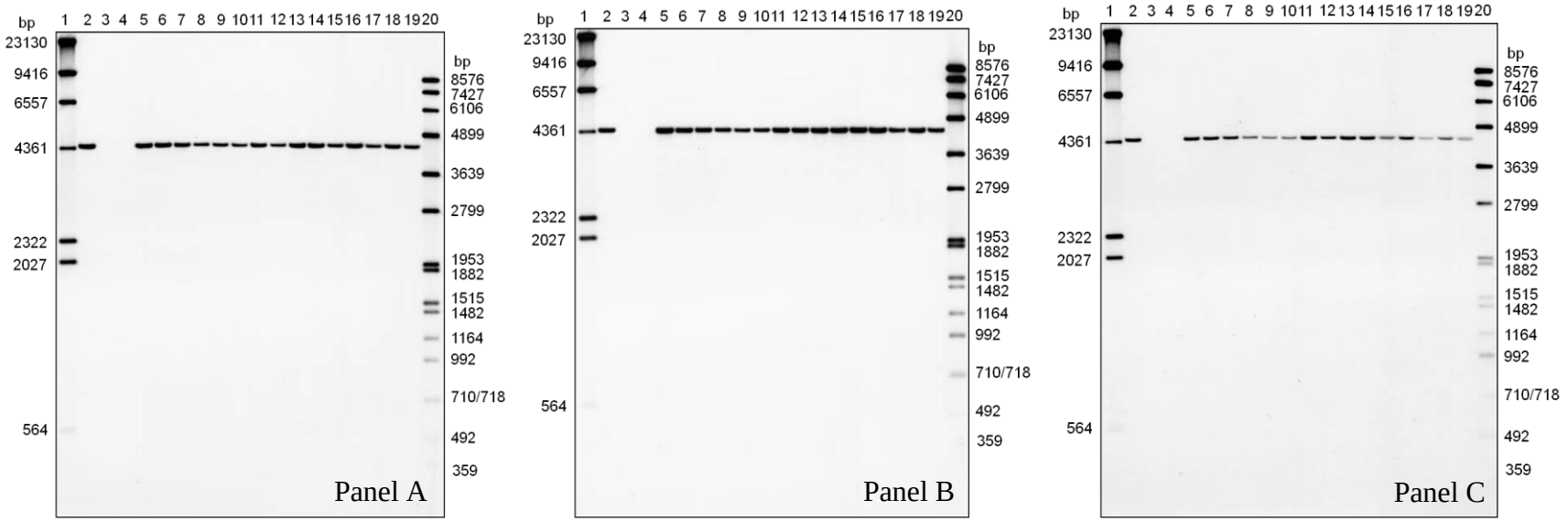


Figure 18. Southern blot analysis of *Msc*I/*Eco*RI digested DAS-444Ø6-6 soybean: Histone H4A748 Promoter, *2mepsps* and Histone H4A748 UTR probes.

Approximately 10 µg of genomic DNA was digested with *Msc*I/*Eco*RI followed by hybridization with the Histone Promoter (Panel A), *2mepsps* (Panel B) and Histone 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

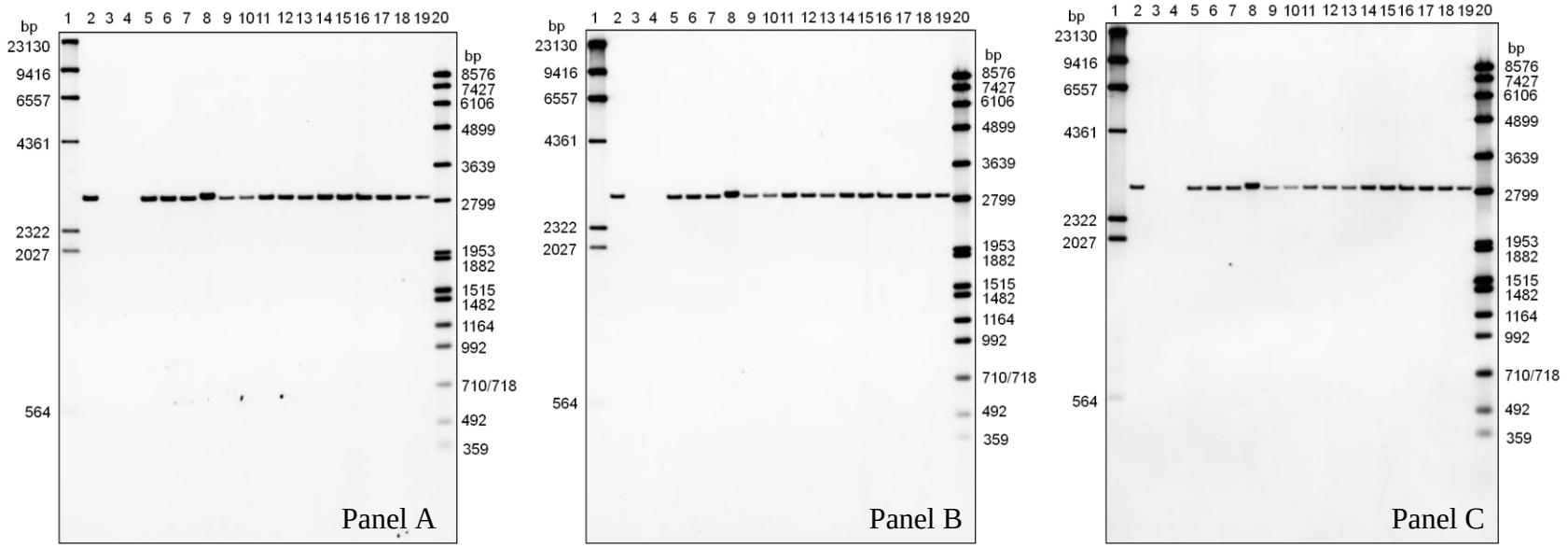


Figure 19. **Southern blot analysis of *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: AtUbi10 Promoter, *aad-12*, and AtuORF23 UTR probes.**

Approximately 10 µg of genomic DNA was with 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
10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII	10	4406-F2-4	20	DIG MWM VII

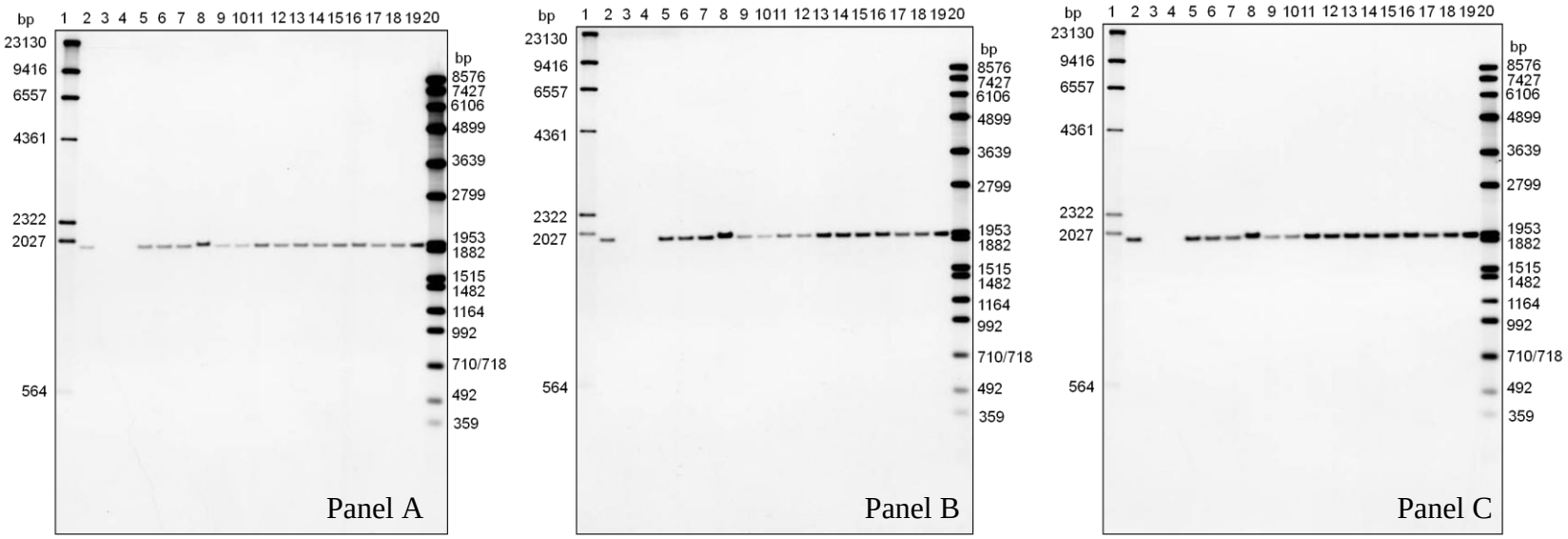


Figure 20. Southern blot analysis of *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: CsVMV, *pat*, and AtuORF1 UTR probes.
Approximately 10 µg of genomic DNA was digested with *Pst*I/*Xho*I followed by hybridization with the CsVMV (Panel A), Pat (Panel B) and AtuORF1 UTR (Panel C) probes. Plasmid pDAB8264 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

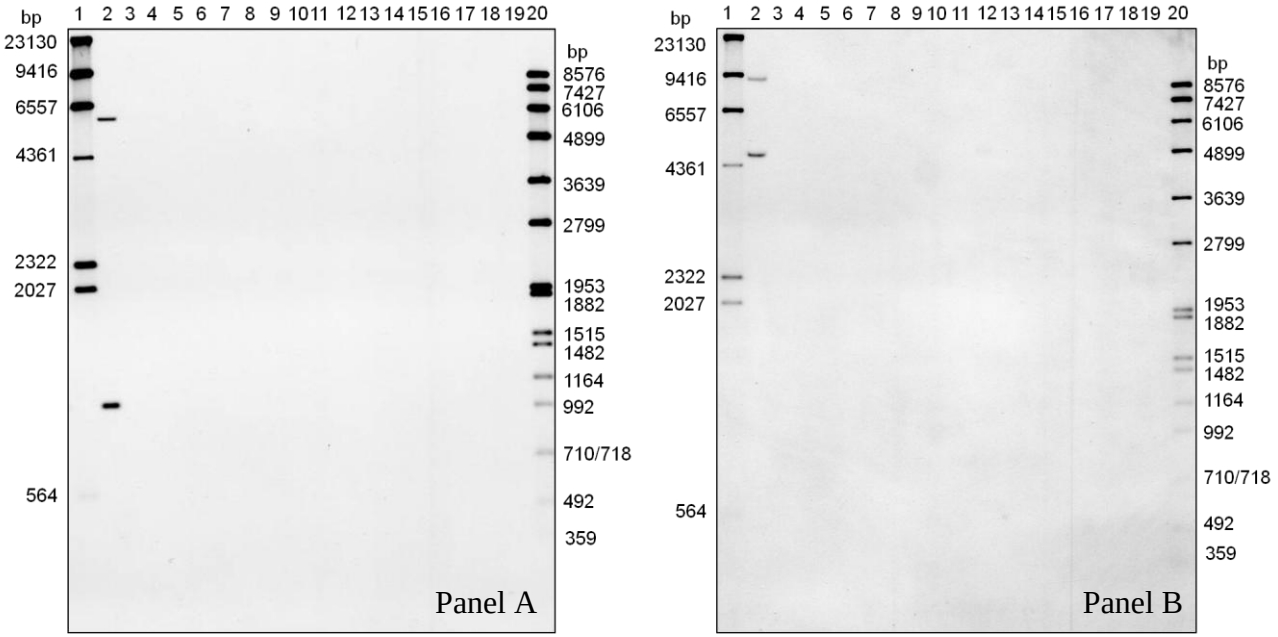


Figure 21. Southern blot analysis of *Msc*I/*Eco*RI and *Hind*III digested DAS-444Ø6-6 soybean: Backbone 3 and Backbone 4 probes.

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

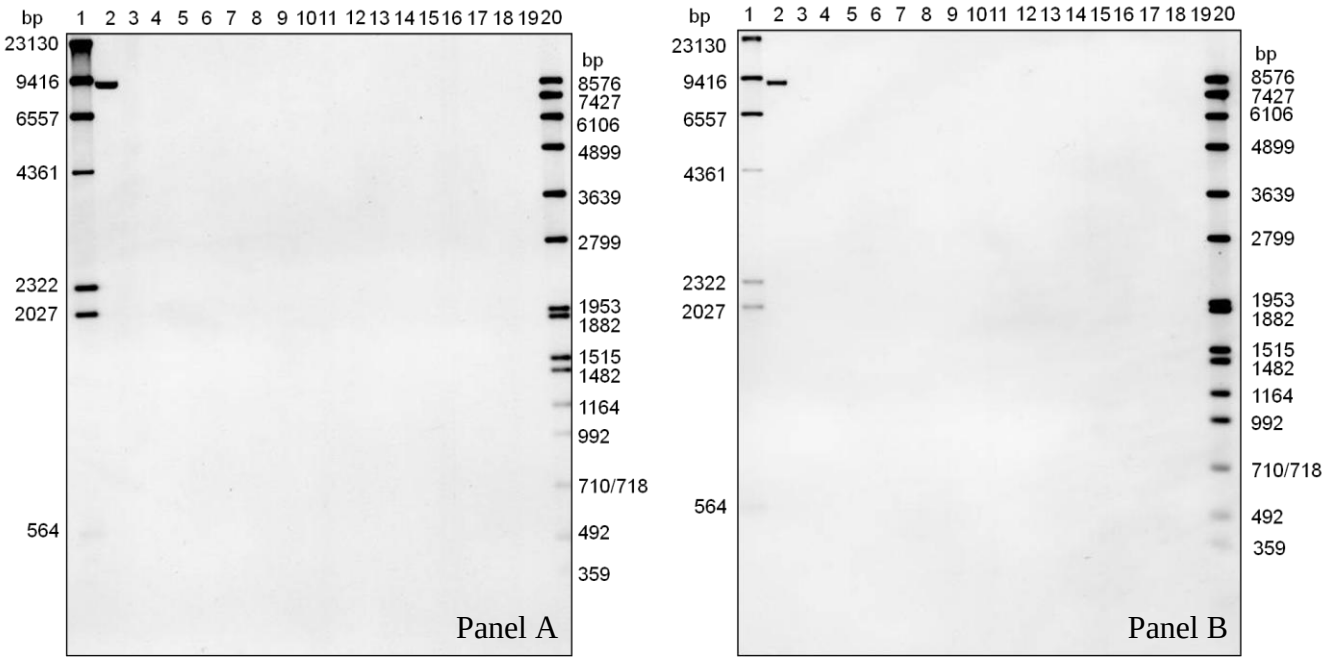


Figure 22. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: Ori-Rep probe.

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

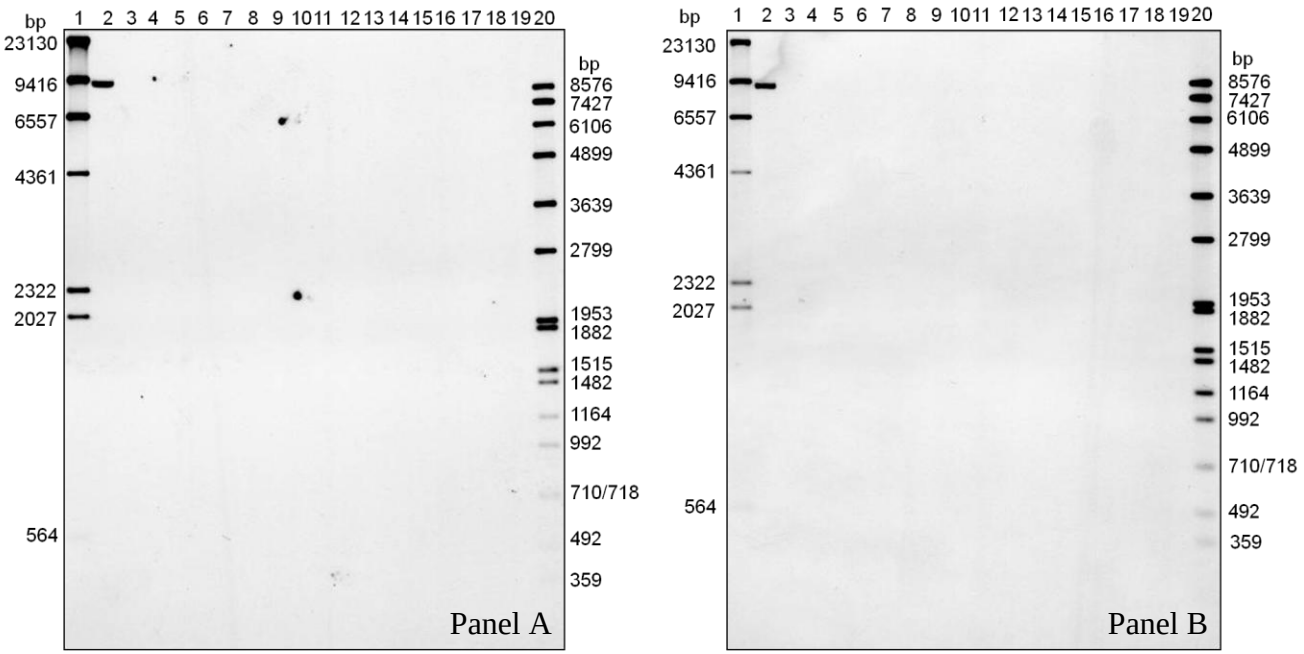


Figure 23. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: Backbone 1 probe.
Approximately 10 µg of genomic DNA was digested with *Hind*III (Panel A) *Pst*I/*Xho*I and (Panel B) and hybridized with the Backbone 1 probe. Plasmid pDAB8264 served as the positive control 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

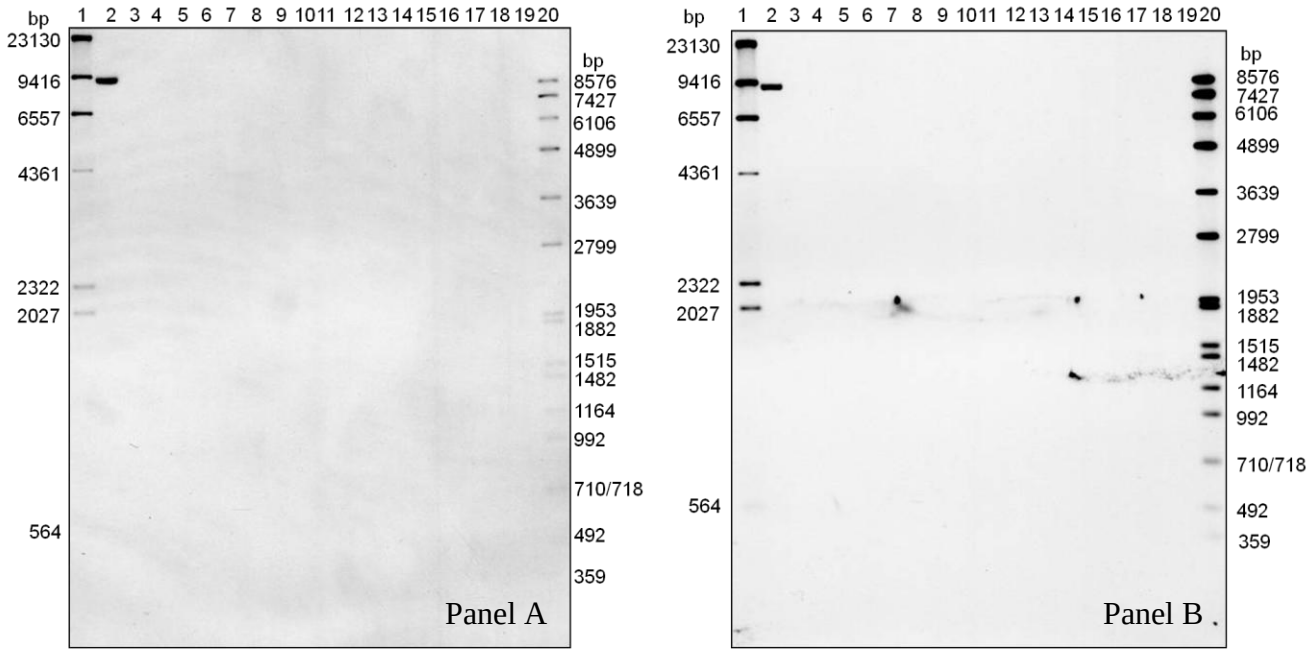


Figure 24. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: Backbone 2 probe.
Approximately 10 µg of genomic DNA was digested with *Hind*III (Panel A) *Pst*I/*Xho*I and (Panel B) and hybridized with the Backbone 2 probe. Plasmid pDAB8264 served as the positive control 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

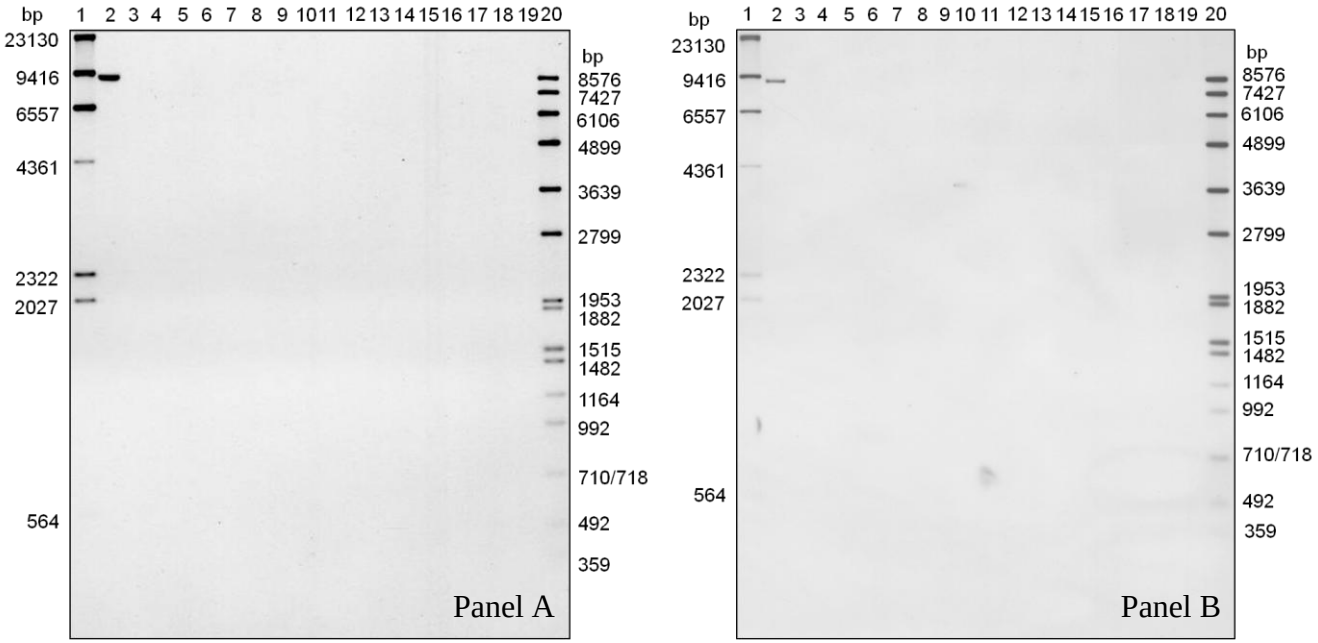


Figure 25. Southern blot analysis of *Hind*III and *Pst*I/*Xho*I digested DAS-444Ø6-6 soybean: SpecR probe.

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

5.5. Segregation Analysis

5.5.1. 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 proprietary 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 Maverick, were analyzed 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.

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

5.5.2. 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 DAS-444Ø6-6 soybean 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

5.6. 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, DAS-444Ø6-6 displayed the expected Mendelian inheritance pattern for a single independent insert/locus in a segregating generation (F2).

6. Characterization of the Introduced Proteins

6.1. AAD-12

6.1.1. Identity of the AAD-12 Protein

The aryloxyalkanoate dioxygenase (AAD-12) protein was derived from *Delftia acidovorans*, a gram-negative soil bacterium. The amino acid sequence is identical to the native enzyme sequence except for the addition of an alanine at position number 2. The additional alanine codon encodes part of an *Nco*I restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. This additional codon serves the dual purpose of facilitating subsequent cloning operations and improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized coding regions are 99.3% identical, differing only at amino acid number 2. The AAD-12 protein is comprised of 293 amino acids and has a molecular weight of ~32 kDa (Figure 26).

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

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

6.1.2. 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 catalyzing 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 catalyze 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.

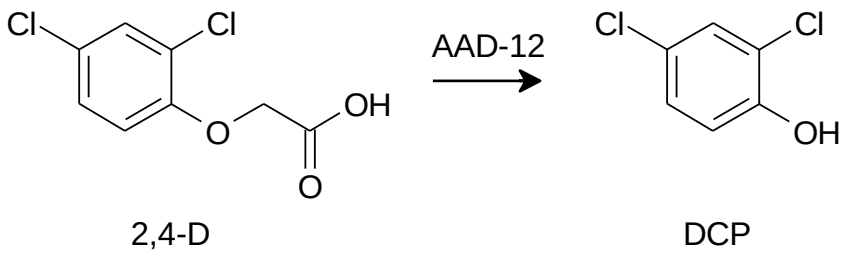


Figure 27. Degradation reaction of 2,4-D catalyzed by AAD-12.

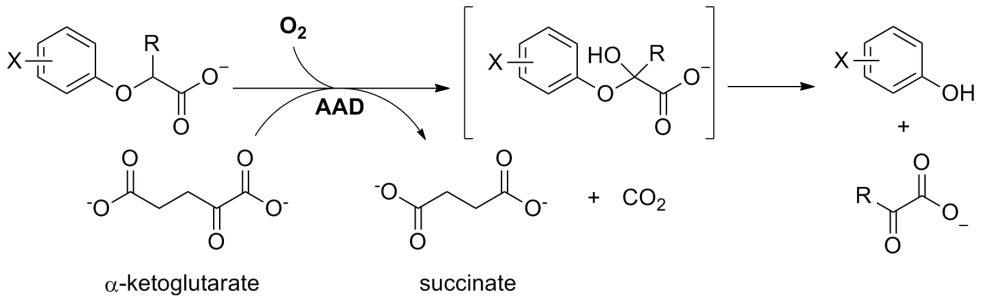


Figure 28. General reaction catalyzed by AAD-12 (R=H or CH_3).

6.1.3. 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. 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 in Appendix 2. 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.

6.1.4. Expression of the 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 (see Appendix 5 for application rates and timings). 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. 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 in Appendix 5.

A summary of the AAD-12 protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 6. 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 6. 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 all 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

6.1.5. Food and Feed Safety Assessment for AAD-12 Protein

Results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause an allergic reaction in humans or be a toxin to humans or animals.

History of Safe Use

- 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 *et al.*, 1987; Wen *et al.*, 1999).
- *Delftia acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Toms and Wood, 1970; Rao and Ravishankar, 2000; Shetty *et al.*, 2006). This utility has led to a history of safe use for *Delftia acidovorans* in the food processing industry. For example, see US Patent 5,128,253 "Bioconversion process for the production of vanillin" issued on July 7, 1992 to Kraft General Foods (Labuda *et al.*, 1992).

Lack of allergenic potential

- 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.
- The AAD-12 protein is rapidly degraded below the level of detection in simulated gastric fluid (SGF). The AAD-12 protein was readily digested, *i.e.*, not detectable after 30 seconds, under *in vitro* SGF conditions (0.32% pepsin, pH 1.2; 37^{o}C) as demonstrated by both SDS-PAGE and western blot analyses.
- The AAD-12 protein is not present in a glycosylated state. No glycosylation of the AAD-12 protein was detected using SDS-PAGE and a glycosylation detection system (Appendix 2).

Lack of toxic potential

- 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 the proteins returned by the BLASTp search are associated with protein toxins that are harmful to humans or animals.

- In acute mouse toxicity testing, there were no mortalities or treatment-related clinical signs in CD-1 mice after oral administration by gavage of AAD-12 protein at 2000 mg protein/kg body weight.

6.1.6. Summary of AAD-12 Protein Characterization

The aryloxyalkanoate dioxygenase (AAD-12) protein was derived from *Delftia acidovorans*, a gram-negative soil bacterium. AAD-12 is comprised of 293 amino acids and has a molecular weight of ~32 kDa. Detailed biochemical characterization of the AAD-12 protein derived from DAS-444Ø6-6 soybean and microbial sources was conducted. Additionally, characterization of AAD-12 protein expression in DAS-444Ø6-6 soybean over the growing season was determined by analyzing leaf, root, whole plant, and grain tissues from DAS-444Ø6-6 soybean sprayed with 2,4-D, glufosinate, glyphosate, all three herbicides in combination, and non-sprayed.

A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the AAD-12 amino acid sequence. The AAD-12 protein 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.

6.2. 2mEPSPS

6.2.1. 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.

6.2.2. 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 *et al.*, 2009).

6.2.3. 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. 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.

The methods and results of the biochemical characterization of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS proteins are described in detail in Appendix 3. 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).

6.2.4. Expression of the 2mEPSPS 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 (see Appendix 5 for application rates and timings). 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. 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 in Appendix 5.

A summary of the 2mEPSPS protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 7. 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 7. 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 all 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

6.2.5. Food and Feed Assessment for 2mEPSPS Protein

Results of the overall safety assessment of the 2mEPSPS protein indicate that it is unlikely to cause allergic reactions in humans or be toxic to humans or animals.

History of Safe 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).
- The 2mEPSPS protein is expressed in other events and crops that have previously been deregulated by the USDA and have received authorizations in numerous countries, for example:
    - GA21 corn (OECD Unique Identifier MON-ØØØ21-9) was deregulated by USDA in 1997 and was approved by the Canadian Food Inspection Agency (CFIA) in 1998.
    - GHB614 cotton (OECD Unique Identifer BCS-GHØØ2-5) was deregulated by USDA in 2008 and was approved by CFIA in 2008.

Lack of allergenic potential

- 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.
- The 2mEPSPS protein is rapidly degraded below the level of detection in simulated gastric fluid (SGF). The 2mEPSPS protein was readily digested (i.e., not detectable at 1 minute) in SGF (0.32% pepsin, pH 1.2; 37ºC) when analyzed by both SDS-PAGE and western blot analyses.
- The 2mEPSPS protein is not present in a glycosylated state. No glycosylation of the 2mEPSPS protein was detected using SDS-PAGE and a glycosylation detection system.

Lack of toxic potential

- 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.

- In acute mouse toxicity testing, there were no mortalities or clinical signs in CD-1 mice after oral administration by gavage of 2mEPSPS at 5000 mg protein/kg body weight.

6.2.6. Summary of 2mEPSPS Protein Characterization

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 microbial sources were conducted. Additionally, characterization of 2mEPSPS protein expression in DAS-444Ø6-6 soybean over the growing season was determined by analyzing leaf, root, whole plant, and grain tissues from DAS-444Ø6-6 soybean sprayed with 2,4-D, glyphosate, and glufosinate, all three herbicides in combination, and non-sprayed.

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.

6.3. PAT

6.3.1. 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).

```
001 MSPERRPVEIRPATAADMAAVCDIVNHYIETSTVNFRTEPQTPQEWIDDL
051 ERLQDRYPWLVAEVEGVVAGIAYAGPWKARNAYDWTVESTVYVSHRHQRL
101 GLGSTLYTHLLKSMEAQGFKSVVAVIGLPNDPSVRLHEALGYTARGTLRA
151 AGYKHGGWHDVGFWQRDFELPAPPRPVRPVTQI
```

Figure 30. Amino acid sequence of the PAT protein.

6.3.2. 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)).

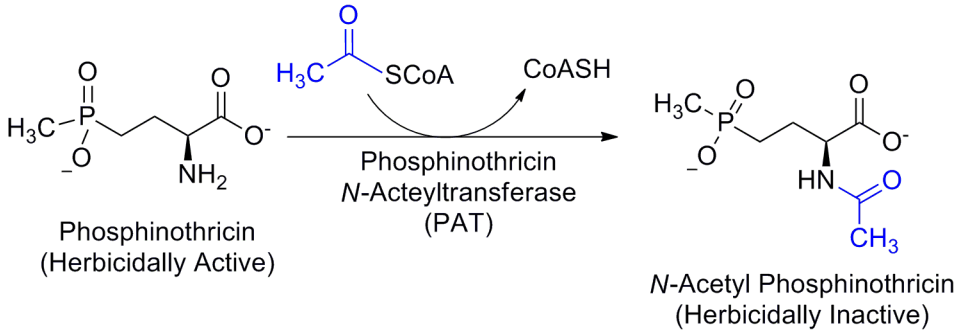


Figure 31. Mode of action of the PAT protein.

6.3.3. 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 in Appendix 4. 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, 2001, 2004, 2005).

6.3.4. 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 (see Appendix 5 for application rates and timings). 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. 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 Appendix 5.

A summary of the PAT protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 8. 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 8. 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 all 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

6.3.5. Food and Feed Safety Assessment for PAT Protein

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 deregulated by USDA (USDA, 1996, 2001, 2004, 2005). 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 has concluded, after reviewing data on the acute toxicity and digestibility of the PAT protein, 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.

6.3.6. Summary of PAT Protein Characterization

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). 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. Characterization of PAT protein expression in DAS-444Ø6-6 soybean over the growing season was determined by analyzing leaf, root, whole plant, and grain tissues from DAS-444Ø6-6 soybean sprayed with 2,4-D, glyphosate, glufosinate, all three herbicides in combination, and non-sprayed. 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.

7. Agronomic Performance

7.1. Phenotypic and Agronomic Characteristics

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. This study used the same plots that were used for protein expression (sections 6.1.4, 6.2.4 and 6.3.4) and nutrient composition (section 8) studies. No agronomically meaningful unintended differences were observed between the non-transgenic near-isogenic control (Maverick) and DAS-444Ø6-6 soybean plots. Results from this study demonstrate agronomic equivalence between DAS-444Ø6-6 soybean (unsprayed and sprayed) and non-transgenic soybean (Maverick).

7.1.1. Agronomic Study

An agronomic study with DAS-444Ø6-6 soybean (unsprayed or sprayed with 2,4-D, glyphosate, glufosinate, or all three herbicides), a near-isogenic non-transgenic control (Maverick) and six non-transgenic reference lines (Dairyland Seed (DSR) 75213-72, 98860-71, 99914N, and 99915; Porter 75148; Williams 82) was conducted in 2010 at ten sites located in Sycamore, Georgia; Richland, Iowa; Bagley, Iowa; Carlyle, Illinois; Wyoming, Illinois; Sheridan, Indiana; Deerfield, Michigan; Fisk, Missouri; Brunswick, Nebraska; and York, Nebraska.

An entry in this study was defined as a soybean line with herbicide treatment, if applicable. For example, DAS-444Ø6-6 with 2,4-D treatment-only was one entry. Each trial site included nine entries, including five entries of DAS-444Ø6-6 (one untreated and four different herbicide treatments), one entry of control (Maverick), and three entries of non-transgenic reference lines. At each of the ten sites, all entries were arranged in a randomized complete block design with four blocks. Across all sites, each control and DAS-444Ø6-6 entry was represented by a total of 40 plots (10 sites, 4 replicate plots per entry at each site). Three of the six reference lines were included at each site by randomizing across sites in a balanced incomplete-block design. Each of the six reference lines was assigned to five sites; therefore, each reference line was represented by a total of 20 plots across sites (5 sites per reference line, 4 replicate plots per entry at each site).

At each site, four replicate plots of each entry were established, with each plot consisting of four 25 ft (7.62 m) rows. Herbicide applications and observations of agronomic characteristics were conducted on the center two rows of each plot; row number one and four were included as additional border rows. Soybean seeds were planted at a seeding rate of approximately 125 seeds per row with seed spacing within each row of approximately 2.4 inches (6 cm). Each soybean plot was bordered by two rows of a non-transgenic soybean cultivar of similar maturity. The entire trial site was surrounded by a minimum of four rows (10 ft or 3.0 m) of a non-transgenic soybean cultivar of similar maturity. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop.

Unsprayed DAS-444Ø6-6 soybean plots and DAS-444Ø6-6 soybean plots treated with one of four 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 following agronomic characteristics were measured and recorded for all test entries at each location on a per plot basis (Table 9).

Table 9. Agronomic characteristics.

Trait	Evaluation Time	Description	Scale
Early Population (Stand Count)	≈V2	Number of plants in a representative 1 meter section of one row per plot	Number of emerged plants in 1 meter
Seedling Vigor	≈V2	Visual estimate of average vigor of plants in each plot	1-9 Rating Scale, 5 = average, 9 = high vigor; e.g. 1 = short plants with small, thin leaves; 9 = tall plants with large, robust leaves; Not based on growth of the control entries; Germination/ emergence (stand count) not considered
Days to 50% Flowering	≈R1/R2	Date at which »50% of plants were flowering	Date recorded when »50% of the plants in each plot were flowering; Days since planting calculated
Disease Incidence	≈R6	Visual estimate of disease incidence	0-100%; Estimated % plant tissue/leaf area diseased over all plants in plot; Did not record % of plants in plot that had detectable disease; 100% = all plant tissues in plot were diseased; Recorded type of disease if incidence was greater than 30%
Insect Damage	≈R6	Visual estimate of insect damage	0-100%; Estimated % plant tissue/leaf area damaged over all plants in plot; Did not record % of plants in plot that had detectable damage; 100% = all plant tissues had feeding damage; Recorded type of damage, e.g. chewing, stippling, distortion if damage was greater than 30%; Recorded type of insect(s) if present
Days to Maturity	≈R8	Date at which »95% of plants had reached physiological maturity/dry down color	Recorded the date when »95% of the plants in each plot reached physiological maturity/dry down color; Days since planting calculated
Lodging	≈R8	Visual estimate of lodging severity	0-100%; Estimated % of plants lodged in plot; 100% = all plants in plot were lodged

Trait	Evaluation Time	Description	Scale
Plant Height	≈R8	Average plant height: from soil surface to growing tip (at senescence / after leaf shed)	Recorded the average height of all plants in plot (stand) in centimeters (cm); One value for each plot; If plot was lodged, a representative group of plants was held up to obtain a measurement
Final Population (Stand Count)	≈R8	Number of plants in a representative 1 meter section of one row per plot	Number of plants in 1 meter; Did not sample a section where plants were removed during previous sampling
Number of Seeds	≈R8 (prior to harvest)	Number of pods and seeds from 5 plants collected from each plot	Recorded the number of pods and seeds present on 5 plants collected from each plot
Shattering	≈R8 (prior to harvest)	Visual estimate of pod shattering	0-100%; Estimated % of shattered pods for each plot; 100% = all pods shattered
Yield	≈R8	Weight of grain harvested from each plot	Recorded the weight in grams of grain harvested from each plot
100 seed weight	≈R8	Weight in grams for 100 representative seeds from bulk yield sample	Recorded the weight in grams for 100 representative seeds taken from the bulk yield sample

Analysis of variance was conducted across field sites (combined-site analysis) for agronomic 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 (Maverick) 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 endpoint (14 analyzed observation types for agronomics), resulting in 70 comparisons made in the combined-site agronomic analysis. Therefore, the probability of declaring one or more false differences based on unadjusted P-values was >97% for agronomics ($1-0.95^{70}$).

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 False Discovery Rate (FDR) control procedures (Benjamini and Hochberg, 1995); FDR methods are commonly applied in studies examining transgenic crops (Herman *et al.*, 2007; Coll *et al.*, 2008; Huls *et al.*, 2008; Jacobs *et al.*, 2008; Stein *et al.*, 2009; Herman *et al.*, 2010). Therefore, the P-values from the agronomics evaluations 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.

7.1.2. Agronomic Results

A statistical analysis of the agronomic data collected from the non-transgenic near-isogenic Maverick, unsprayed DAS-444Ø6-6 and sprayed DAS-444Ø6-6 entries was conducted. For each agronomic character 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) for each agronomic character are reported. Each minimum and maximum value is an individual data point reported for a single plot.

For the combined-site analysis (Table 10), no statistically significant differences were observed following False Discovery Rate (FDR) adjustment of P-values for all of the agronomic characteristics evaluated: early population, seedling vigor, days to flowering, disease incidence, insect damage, days to maturity, lodging, plant height, final population, number of pods per five plants, number of seeds per five plants, shattering, yield, and 100 seed weight. Unadjusted P-values were significant at the 0.05 level for paired t-tests for one comparison each for seedling vigor, lodging, final population, and number of pods per five plants. For each significant unadjusted P-value, mean differences between transgenic and control entries were negligible and transgenic means were within the range observed for reference varieties included in the study. Results from this study demonstrate agronomic equivalence between DAS-444Ø6-6 (unsprayed and sprayed) and non-transgenic Maverick soybean.

Table 10. Combined-site analysis: summary of agronomic characteristics.

Agronomic 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
Early Population - V2 (number of plants in a 1 m section of row)	0.556	14 ± 1 6 - 22	13 ± 1 3 - 19 (0.202, 0.692)	13 ± 1 1 - 19 (0.596, 0.817)	13 ± 1 3 - 20 (0.189, 0.692)	14 ± 1 3 - 24 (1.000, 1.000)	14 ± 1 3 - 23 (0.940, 1.000)	2 - 27
Seedling Vigor - V2 (1-9 scale 1 = low vigor, 9 = high vigor)	0.269	6.2 ± 0.3 5 - 8	6.0 ± 0.3 4 - 8 (0.408, 0.746)	5.9 ± 0.3 3 - 8 (0.102, 0.682)	6.0 ± 0.3 4 - 8 (0.249, 0.692)	6.1 ± 0.3 4 - 8 (0.508, 0.781)	5.8 ± 0.3 3 - 8 (**0.024**, 0.669)	3 - 9
Days to Flowering - R1/R2 (days since planting)	0.664	42 ± 3 22 - 54	43 ± 3 22 - 56 (0.317, 0.692)	42 ± 3 24 - 56 (0.859, 0.986)	43 ± 3 28 - 56 (0.317, 0.692)	42 ± 3 22 - 55 (0.953, 1.000)	42 ± 3 22 - 54 (0.859, 0.986)	22 - 57
Disease Incidence - R6 (0-100% scale, 0% = no disease 100% = all plants diseased)	0.661	5 ± 2 0 - 20	5 ± 2 0 - 25 (0.968, 1.000)	4 ± 2 0 - 15 (0.400, 0.746)	5 ± 2 0 - 15 (0.574, 0.817)	5 ± 2 0 - 20 (0.422, 0.746)	5 ± 2 0 - 15 (1.000, 1.000)	0 - 20
Insect Damage - R6 (0-100% scale, 0% = no damage 100% = all plants damaged)	0.664	12 ± 8 0 - 80	14 ± 8 0 - 90 (0.136, 0.692)	13 ± 8 0 - 80 (0.524, 0.781)	13 ± 8 0 - 90 (0.326, 0.692)	13 ± 8 0 - 80 (0.299, 0.692)	14 ± 8 0 - 90 (0.144, 0.692)	0 - 90
Days to Maturity - R8 (days since planting)	0.323	115 ± 5 97 - 143	114 ± 5 95 - 143 (0.293, 0.692)	114 ± 5 96 - 143 (0.139, 0.692)	115 ± 5 97 - 143 (0.660, 0.839)	114 ± 5 97 - 143 (0.255, 0.692)	115 ± 5 97 - 143 (0.895, 0.999)	96 - 143
Lodging - R8 (0-100% scale, 0% = no lodging 100% = all plants lodged)	0.428	10 ± 4 0 - 30	12 ± 4 0 - 60 (0.437, 0.746)	16 ± 4 0 - 70 (**0.038**, 0.669)	12 ± 4 0 - 70 (0.431, 0.746)	12 ± 4 0 - 50 (0.614, 0.817)	13 ± 4 0 - 50 (0.367, 0.739)	0 - 35
Plant Height - R8 (cm)	0.242	94 ± 4 58 - 122	96 ± 4 70 - 123 (0.218, 0.692)	94 ± 4 48 - 123 (1.000, 1.000)	95 ± 4 68 - 120 (0.478, 0.781)	93 ± 4 61 - 120 (0.641, 0.831)	91 ± 4 64 - 116 (0.266, 0.692)	22 - 112

[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.

Agronomic 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
Final Population - R8 (number of plants in a 1 m section of row)	0.317	13 ± 1 4 - 20	13 ± 1 5 - 20 (0.220, 0.692)	12 ± 1 4 - 18 (0.150, 0.692)	12 ± 1 3 - 18 (**0.027**, 0.669)	12 ± 1 4 - 19 (0.081, 0.682)	12 ± 1 3 - 18 (0.083, 0.682)	2 - 20
Number of Pods - R8 (number of pods on 5 plants)	0.298	361 ± 42 176 - 743	384 ± 42 197 - 698 (0.265, 0.692)	364 ± 42 151 - 683 (0.899, 0.999)	387 ± 42 184 - 786 (0.199, 0.692)	404 ± 42 188 - 1163 (**0.038**, 0.669)	379 ± 42 189 - 765 (0.370, 0.739)	133 - 1008
Number of Seeds - R8 (number of seeds from 5 plants)	0.170	802 ± 81 295 - 1187	826 ± 81 407 - 1183 (0.588, 0.817)	771 ± 81 390 - 1329 (0.499, 0.781)	815 ± 81 380 - 1472 (0.774, 0.939)	881 ± 81 152 - 1824 (0.086, 0.682)	772 ± 82 319 - 1416 (0.514, 0.781)	258 - 1882
Shattering - R8 (0-100% scale, 0% = no shattering 100% = all pods shattered)	0.801	3 ± 3 0 - 30	4 ± 3 0 - 35 (0.318, 0.692)	4 ± 3 0 - 50 (0.318, 0.692)	3 ± 3 0 - 45 (0.948, 1.000)	4 ± 3 0 - 75 (0.500, 0.781)	3 ± 3 0 - 50 (0.760, 0.939)	0 - 60
Yield - R8 (bushels per acre)	0.423	33 ± 3 1.15 - 53.78	30 ± 3 2.05 - 41.87 (0.106, 0.682)	32 ± 3 3.59 - 49.94 (0.433, 0.746)	30 ± 3 2.05 - 52.95 (0.063, 0.682)	31 ± 3 8.45 - 52.5 (0.187, 0.692)	30 ± 3 4.1 - 49.94 (0.107, 0.682)	4.99 - 55.44
100 Seed Weight - R8 (grams)	0.507	13.5 ± 0.4 9.4 - 16.3	13.2 ± 0.4 10.85 - 16.3 (0.241, 0.692)	13.5 ± 0.4 10.17 - 15.2 (0.818, 0.971)	13.6 ± 0.4 11.3 - 16.4 (0.619, 0.817)	13.6 ± 0.4 10.3 - 15.6 (0.575, 0.817)	13.5 ± 0.4 10.76 - 16.2 (0.778, 0.939)	9.4 - 20.7

[a] Unit of measure for yield was converted from grams per plot to bushels per acre prior to analysis; conversion formula:
(X g/125 ft^2 plot) × (43560 ft^2/A) × (bu./27.2155 kg) × (kg/1000 g), where X is the individual sample value.

[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.

7.1.3. Conclusions

Field agronomic characteristics of DAS-444Ø6-6 (unsprayed or sprayed with 2,4-D, glyphosate, glufosinate, or all three herbicides) were evaluated in field trials in 2010. DAS-444Ø6-6 agronomic results were all statistically indistinguishable from the control (Maverick) and/or within reference ranges for non-transgenic soybean, indicating that no unintended agronomic effects were observed for DAS-444Ø6-6 soybean. Results from this study demonstrate agronomic equivalence between DAS-444Ø6-6 soybean and non-transgenic Maverick soybean.

7.2. Ecological Evaluations

The DAS-444Ø6-6 soybean field trials were monitored and observed by personnel familiar with soybean cultivation practices (breeders, field station managers, field agronomists, field associates). The personnel conducting the field tests visually monitored the incidence of plant disease and pests on DAS-444Ø6-6 soybeans compared to the conventional soybean varieties, including Maverick and six reference lines (Section 7.1.1), in the same trials. Disease and insect damage was rated on a numerical scale of 0-100%, with 0% representing no damage due to disease incidence or insect pests (Section 7.1). Table 11 summarizes the results obtained from the field trials conducted in 2010 at ten sites as described in Section 7.1. The results showed that there were no statistically significant differences between the isoline and DAS-444Ø6-6 (with or without herbicide treatment) in susceptibility to and interactions with diseases and insects.

Table 11. Analysis of disease incidence and insect damage.

Agronomic Component (Units)[a]	Overall Treatment 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
Disease Incidence - R6		5 ± 2	5 ± 2	4 ± 2	5 ± 2	5 ± 2	5 ± 2	
(0-100% scale, 0% = no disease		0 - 20	0 - 25	0 - 15	0 - 15	0 - 20	0 - 15	0 - 20
100% = all plants diseased)	0.661		(0.968, 1.000)	(0.400, 0.746)	(0.574, 0.817)	(0.422, 0.746)	(1.000, 1.000)	
Insect Damage - R6		12 ± 8	14 ± 8	13 ± 8	13 ± 8	13 ± 8	14 ± 8	
(0-100% scale, 0% = no damage		0 - 80	0 - 90	0 - 80	0 - 90	0 - 80	0 - 90	0 - 90
100% = all plants damaged)	0.664		(0.136, 0.692)	(0.524, 0.781)	(0.326, 0.692)	(0.299, 0.692)	(0.144, 0.692)	

[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.

Ecological observations were also made from all USDA APHIS notified field trials conducted in 2009-2011 (Appendix 6). Incidence of disease and insect presence in trials of DAS-444Ø6-6 soybeans was recorded and differences in incidence or response of DAS-444Ø6-6 soybeans compared with the conventional control Maverick were examined. Table 12 summarizes the disease and insect stressors observed in trials of DAS-444Ø6-6 and conventional Maverick soybeans. In all cases, no differences between DAS-444Ø6-6 and conventional Maverick soybeans were observed in any of the trials. These observations support the conclusion that the response of DAS-444Ø6-6 soybeans to ecological stressors does not differ from that of conventional soybeans (Maverick).

Table 12. Disease and insect stressors observed in trials of DAS-444Ø6-6 and conventional soybean.

Year	USDA Notification Number	State (County)	Diseases	Insects
2009	08-254-109n	PR (Santa Isabel)	None	None
2009-2010	09-068-103n	IN (Benton), PR (Santa Isabel)	None	None
2009-2010	09-259-108n	PR (Santa Isabel)	Carlavirus, rust	None
2010	10-077-107n	GA (Turner), IL (Champaign, Clinton, Ford, Jackson, Madison, Shelby, Stark), IN (Benton, Boone), IA (Jefferson, Shelby, Story), MD (Queen Anne), MO (Adair, Butler), NE (Polk, York), OH (Fulton), PR (Santa Isabel)	Carlavirus, rust, brown spot, frogeye leaf spot, Sudden Death Syndrome	aphids, bean leaf beetles, grasshoppers, ladybugs, leaf hoppers, green cloverworm, stink bugs
2010	10-083-105n	IN (Benton), IA (Story), MS (Washington), MO (Platte)	None	None
2010	10-085-103n	GA (Turner), IL (Clinton, Stark), IN (Boone), IA (Guthrie, Jefferson), MI (Lenawee), MO (Butler), NE (Antelope, York)	brown spot, brown leaf spot, frogeye leaf spot	aphids, brown leaf beetles, grasshoppers, stink bugs, lady bugs, ground beetles, Lepidoptera spp,

7.3. Germination and Dormancy Evaluations

The germination of DAS-444Ø6-6 seed compared with the non-transgenic control (Maverick) under warm and cold conditions was evaluated to determine any impact on seed dormancy characteristics.

To conduct the germination studies, Metro™ Mix potting soil was placed in plastic flats (10.5 × 21 × 2.5 in.), and flats were sub-irrigated with 3 L of distilled water. Seeds of DAS-444Ø6-6 soybean (T5 generation) and non-transgenic, near-isogenic control (Maverick) soybean were planted in the flats to a depth of 1 in. on the day following irrigation, and each half-flat contained 100 seeds (200 seeds total per flat). Immediately after planting, flats for the cold germination

experiment were placed in a growth chamber where they were incubated at 10°C for seven days followed by an additional seven days at 25°C. Flats for the warm emergence experiment were placed in the growth chamber at 25°C for seven days immediately after planting. For both experiments, the emerged seedlings in each half-flat were counted following the seven day 25°C incubation period.

The experimental design was a completely randomized design with four replicates of 100 seeds per replicate. Data were transformed using the arcsine of the square root of the decimal fraction of seeds emerged per replicate and subjected to analysis of variance (ANOVA) (SAS Institute Inc., 2009).

Emergence data and *P*-values for the significance of the effect of event on emergence are provided in (Table 13). Emergence of DAS-444Ø6-6 soybean did not differ significantly ($\alpha = 0.05$) from that of the non-transgenic, near-isogenic control Maverick under cold ($P = 0.7989$) and warm ($P = 0.8947$) conditions. These results indicate that the seed dormancy characteristics have not been changed in DAS-444Ø6-6 soybeans.

Table 13. Percentage emergence (number of seeds emerged divided by number of seeds planted x 100) for DAS-444Ø6-6 soybean and control soybean.

		Soybean emergence (%)					
Temperature	**Event**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Mean (±SE)**	***P***
Cold	DAS-444Ø6-6	93	94	93	99	94.8 (±1.4)	0.7989
Cold	Control	90	91	95	99	93.8 (±2.1)	
Warm	DAS-444Ø6-6	98	98	92	97	96.3 (±1.4)	0.8947
Warm	Control	99	99	92	95	96.3 (±1.7)	

7.4. Summary of Agronomic, Disease, and Pest Characteristics

Agronomic data evaluating plant growth characteristics throughout the growing season demonstrate the equivalence of DAS-444Ø6-6 soybean with the non-transgenic near-isogenic soybean Maverick. Plant growth and phenotypic characteristics, response to ecological stressors as indicated by susceptibility to disease and insect pressure, and germination and dormancy characteristics were unchanged between DAS-444Ø6-6 soybeans and conventional Maverick soybeans across diverse environments. Therefore, these data support the conclusion that agronomic, disease, and pest characteristics of DAS-444Ø6-6 soybean are not significantly different from that of conventional soybeans, and there is no indication that DAS-444Ø6-6 soybeans will pose an increased plant pest risk.

8. Grain and Forage Composition

Field trials with DAS-444Ø6-6 soybean, the non-transgenic control Maverick, and reference lines were conducted in 2010 at ten sites located in the U.S. This study used the same plots that were used for protein expression (Appendix 5) and agronomic characterization (Section 7) studies. No biologically meaningful unintended compositional differences were observed between the non-transgenic near-isogenic control Maverick 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.

8.1. Field Study Design

A crop composition study with DAS-444Ø6-6 soybean (unsprayed or sprayed with 2,4-D, glyphosate, glufosinate, or all three herbicides), a near-isogenic non-transgenic control (Maverick) and six non-transgenic reference lines (Dairyland Seed (DSR) 75213-72, 98860-71, 99914N, and 99915; Porter 75148; Williams 82) was conducted in 2010 at ten sites located in Sycamore, Georgia; Richland, Iowa; Bagley, Iowa; Carlyle, Illinois; Wyoming, Illinois; Sheridan, Indiana; Deerfield, Michigan; Fisk, Missouri; Brunswick, Nebraska; and York, Nebraska.

An entry in this study was defined as a soybean line with herbicide treatment, if applicable. For example, DAS-444Ø6-6 with 2,4-D treatment-only was one entry. Each trial site included nine entries, including five entries of DAS-444Ø6-6 (one untreated and four different herbicide treatments), one entry of control (Maverick), and three entries of non-transgenic reference lines. At each of the ten sites, all entries were arranged in a randomized complete block design with four blocks. Across all sites, each control and DAS-444Ø6-6 entry was represented by a total of 40 plots (10 sites, 4 replicate plots per entry at each site). Three of the six reference lines were included at each site by randomizing across sites in a balanced incomplete-block design. Each of the six reference lines was assigned to five sites; therefore, each reference line was represented by a total of 20 plots across sites (5 sites per reference line, 4 replicate plots per entry at each site).

At each site, four replicate plots of each entry were established, with each plot consisting of four 25 ft (7.62 m) rows. Herbicide applications and crop composition sampling were conducted on the center two rows of each plot; row number one and four were included as additional border rows. Soybean seeds were planted at a seeding rate of approximately 125 seeds per row with seed spacing within each row of approximately 2.4 inches (6 cm). Each soybean plot was bordered by two rows of a non-transgenic soybean cultivar of similar maturity. The entire trial site was surrounded by a minimum of four rows (10 ft or 3.0 m) of a non-transgenic soybean cultivar of similar maturity. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop.

Unsprayed DAS-444Ø6-6 soybean plots and DAS-444Ø6-6 soybean plots treated with one of four 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.

8.2. Compositional Analysis

Samples of soybean forage and seed were analyzed at Covance Laboratories Inc. for nutrient content. The analytes examined are presented in Table 14.

Table 14. 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)			

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 *et al.*, 1996; Taylor *et al.*, 1999; OECD, 2001; McCann *et al.*, 2005; Harrigan *et al.*, 2007; Bilyeu *et al.*, 2008; Lundry *et al.*, 2008; Berman *et al.*, 2009, 2010; Harrigan *et al.*, 2010; ILSI, 2011). A summary of the compositional data used for comparison can be found in Appendix 7.

8.3. Statistical Analysis

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 (Maverick) 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 analyzed 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 *et al.*, 2007; Coll *et al.*, 2008; Huls *et al.*, 2008; Jacobs *et al.*, 2008; Stein *et al.*, 2009; Herman *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.

8.4. Composition Analysis Results

A statistical analysis of composition data from the non-transgenic near-isogenic Maverick (referred to as isoline in Table 15 to Table 22, Figure 32 to Figure 40), unsprayed DAS-444Ø6-6 and sprayed DAS-444Ø6-6 entries was conducted. A summary of the compositional results across locations is presented in Table 15 to Table 22. 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 the six reference lines across all sites (reference range) and literature range for each analyte are reported. Minimum and maximum values are for individual plot results as are literature ranges (except where noted). Arithmetic means for each analyte from each field site are plotted for the non-transgenic control Maverick, DAS-444Ø6-6 (sprayed and unsprayed), and reference line entries (Figure 32 to Figure 40). Literature ranges are depicted as the shaded area on each plot. Literature ranges reported as not detected (ND) or less than the limit of quantitation (<LOQ) were plotted as zeros.

8.4.1. Proximate, Fiber, and Mineral Analysis of Forage

Soybean forage samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed for proximate content (protein, fat, ash, moisture, and carbohydrates), fiber (acid detergent fiber (ADF), neutral detergent fiber (NDF)), and minerals (calcium and phosphorus). A summary of the results across all locations is presented in Table 15, Table 16, Figure 32 and Figure 33. 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 15. 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 16. Summary of the mineral 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
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
(mg/100 g dry wt.)	0.222		(0.859, 0.949)	(0.187, 0.384)	(0.233, 0.443)	(0.581, 0.758)	(0.345, 0.554)		
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.

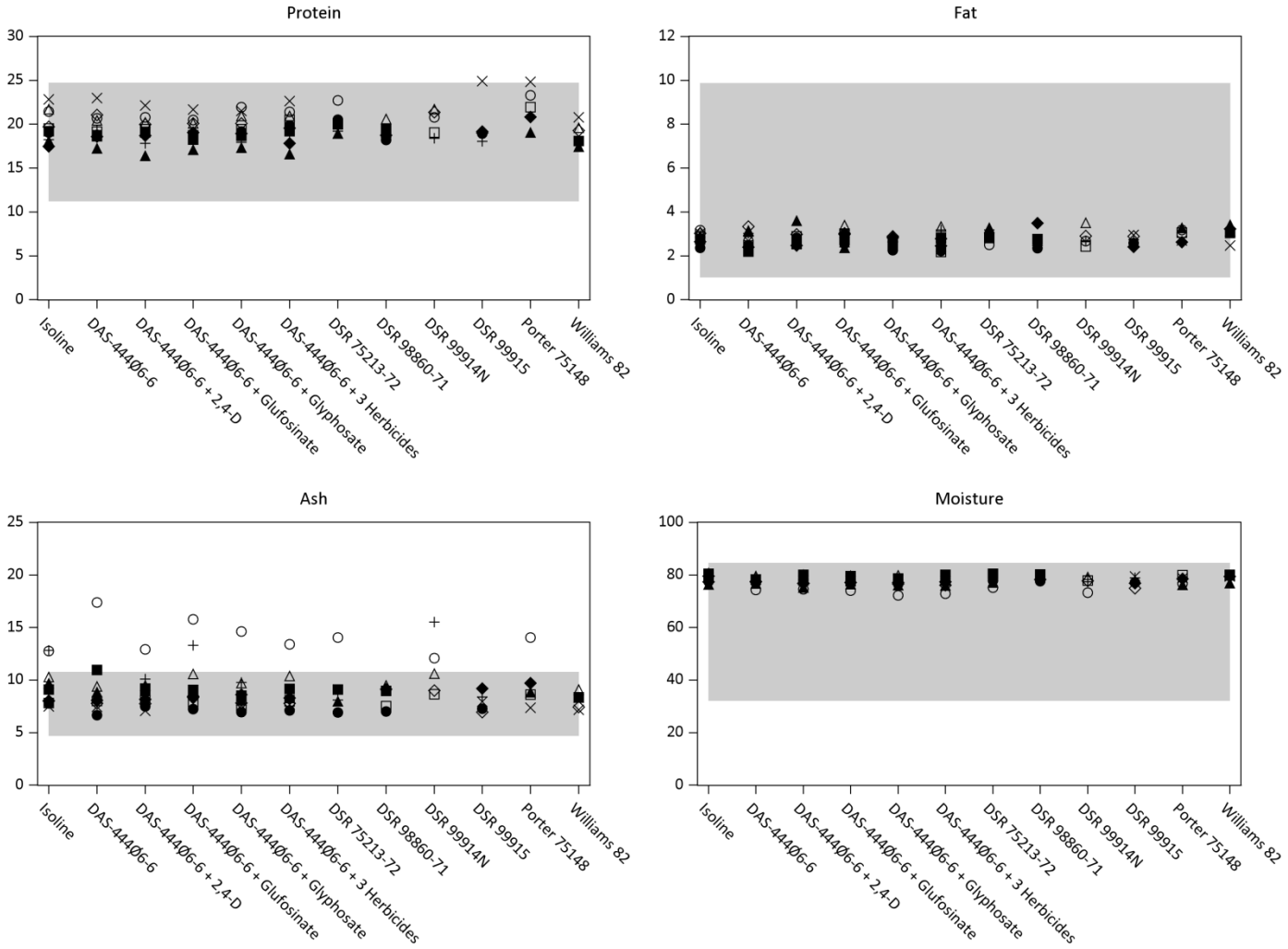


Figure 32. Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean forage.

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.

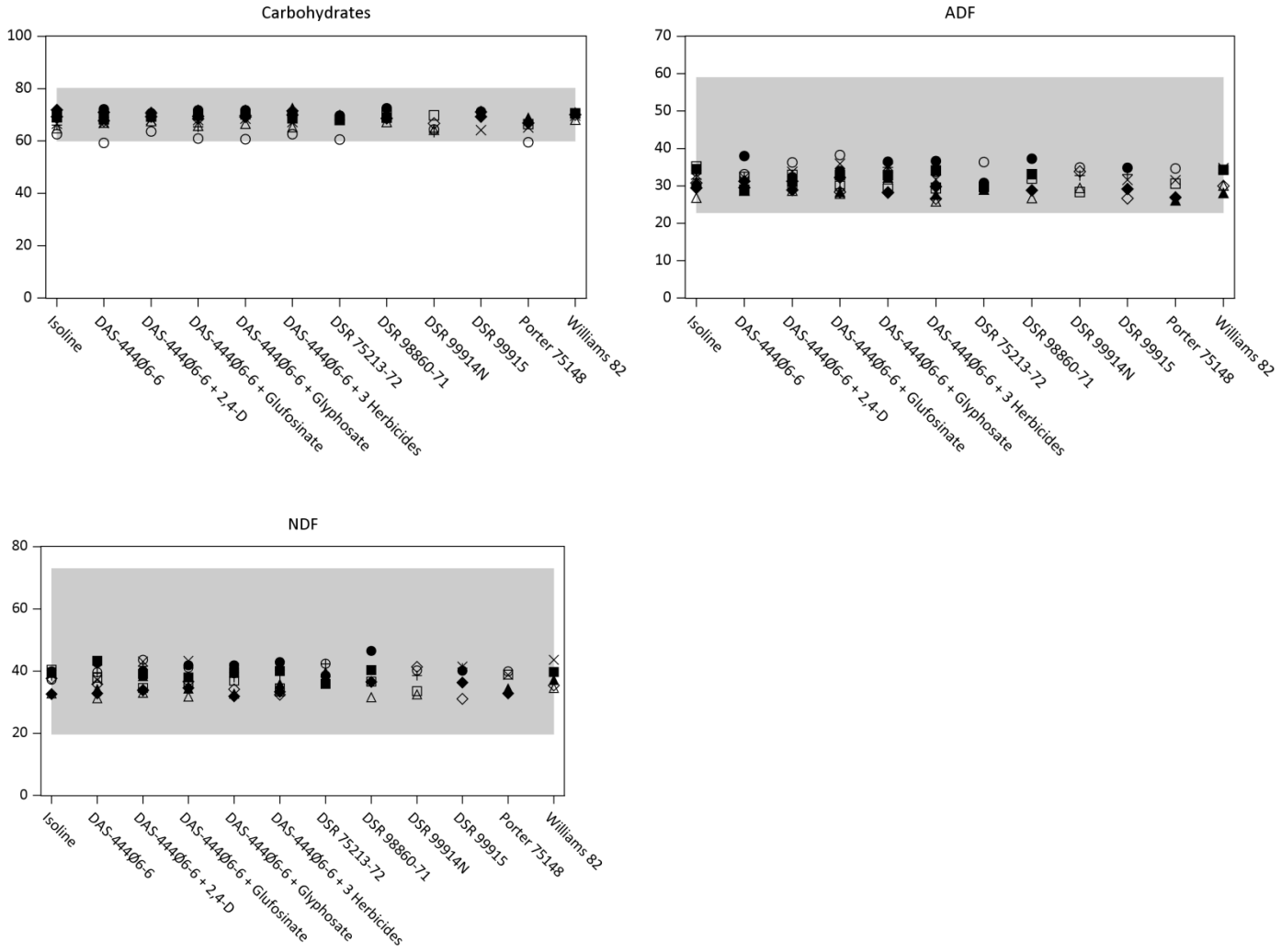


Figure 32. (Cont). Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean forage. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

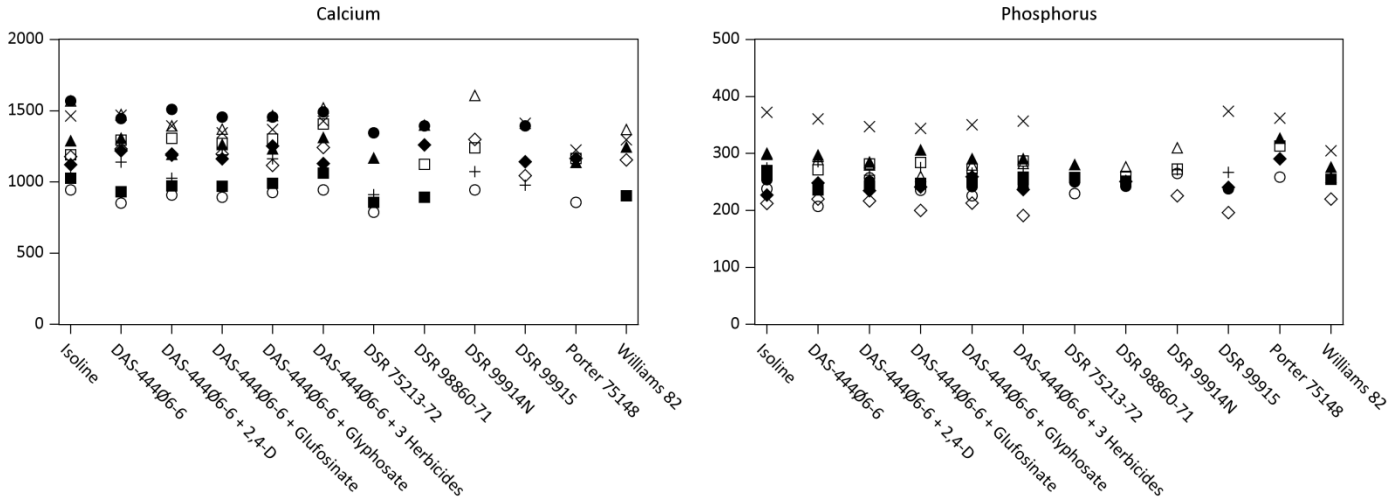


Figure 33. Minerals (mg/100g dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean forage. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

8.4.2. Proximate and Fiber Analysis of Seed

Soybean seed samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed for proximate content (protein, fat, ash, moisture, and carbohydrates) and fiber (acid detergent fiber (ADF), neutral detergent fiber (NDF), and total dietary fiber). A summary of the results across all locations is presented in Table 17 and Figure 34. 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 17). 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 fiber (NDF) and total dietary fiber, 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 fiber were within literature ranges and within ranges for reference lines included in the study.

Table 17. 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)

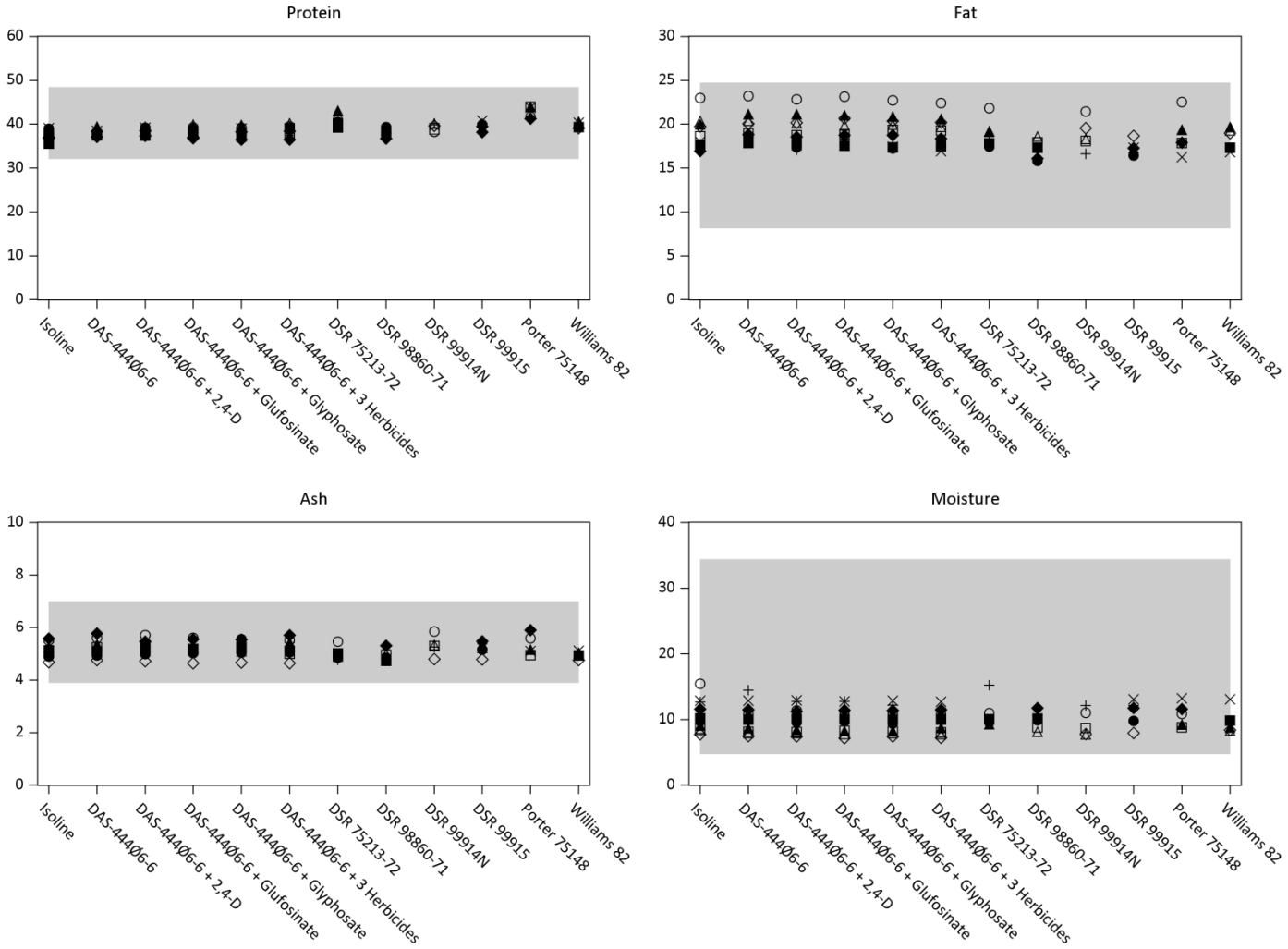


Figure 34. Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

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).

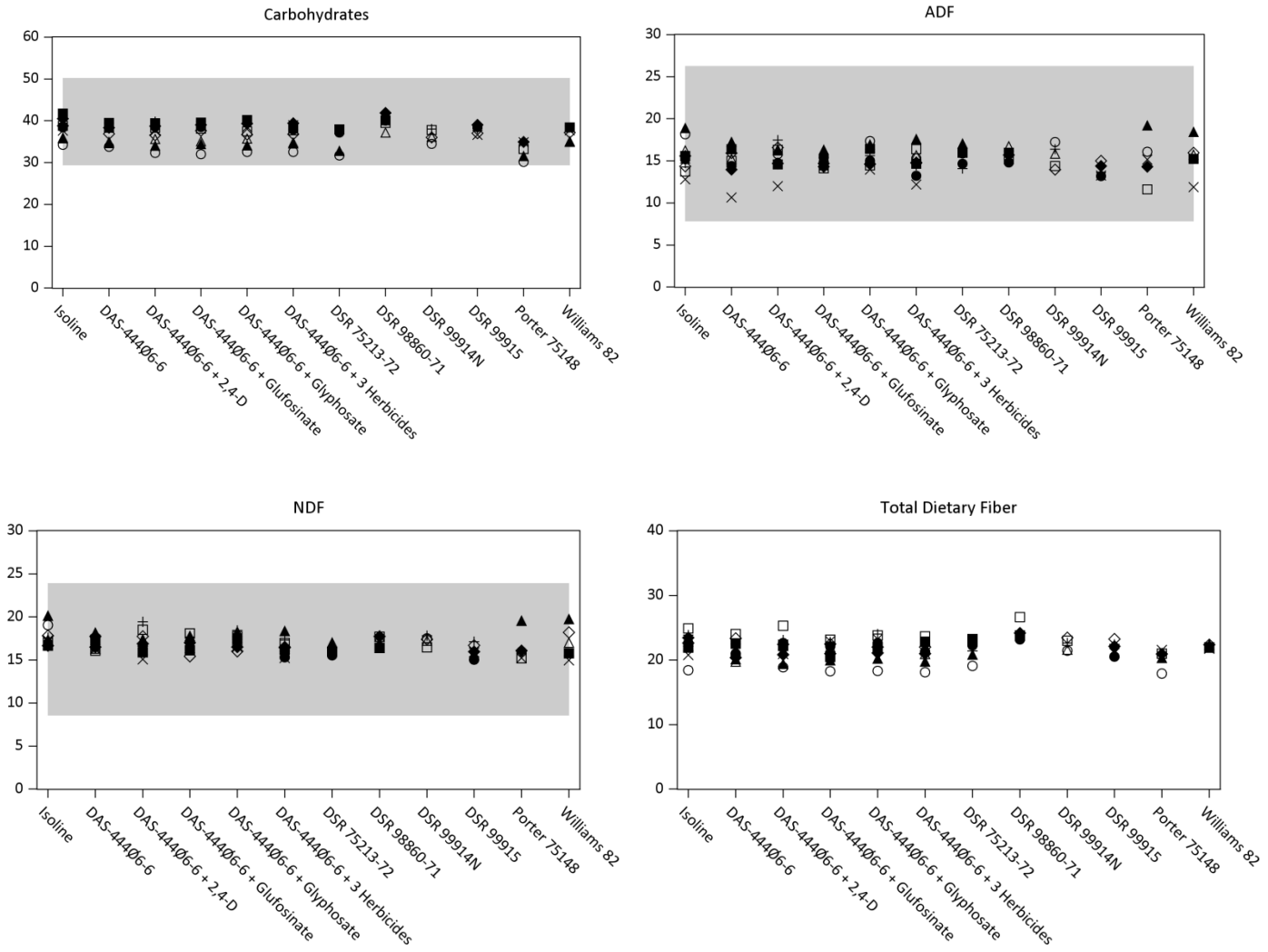


Figure 34 (Cont). Proximate and fiber (% dry weight for all proximate and fiber except moisture (% fresh weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

8.4.3. Mineral Analysis of Seed

Soybean seed samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed 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 18 and Figure 35. 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 18. 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 (**0.010**, 0.058)	4.34 ± 0.12 3.53 - 5.59 (**0.010**, 0.058)	4.25 ± 0.12 3.62 - 5.01 (0.205, 0.414)	4.3 ± 0.12 3.56 - 5.05 (**0.036**, 0.128)	4.3 ± 0.12 3.67 - 6.01 (**0.042**, 0.143)	3.34 - 5.82	4.98 - 7.578
(mg/100 g DW)	0.077								

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.

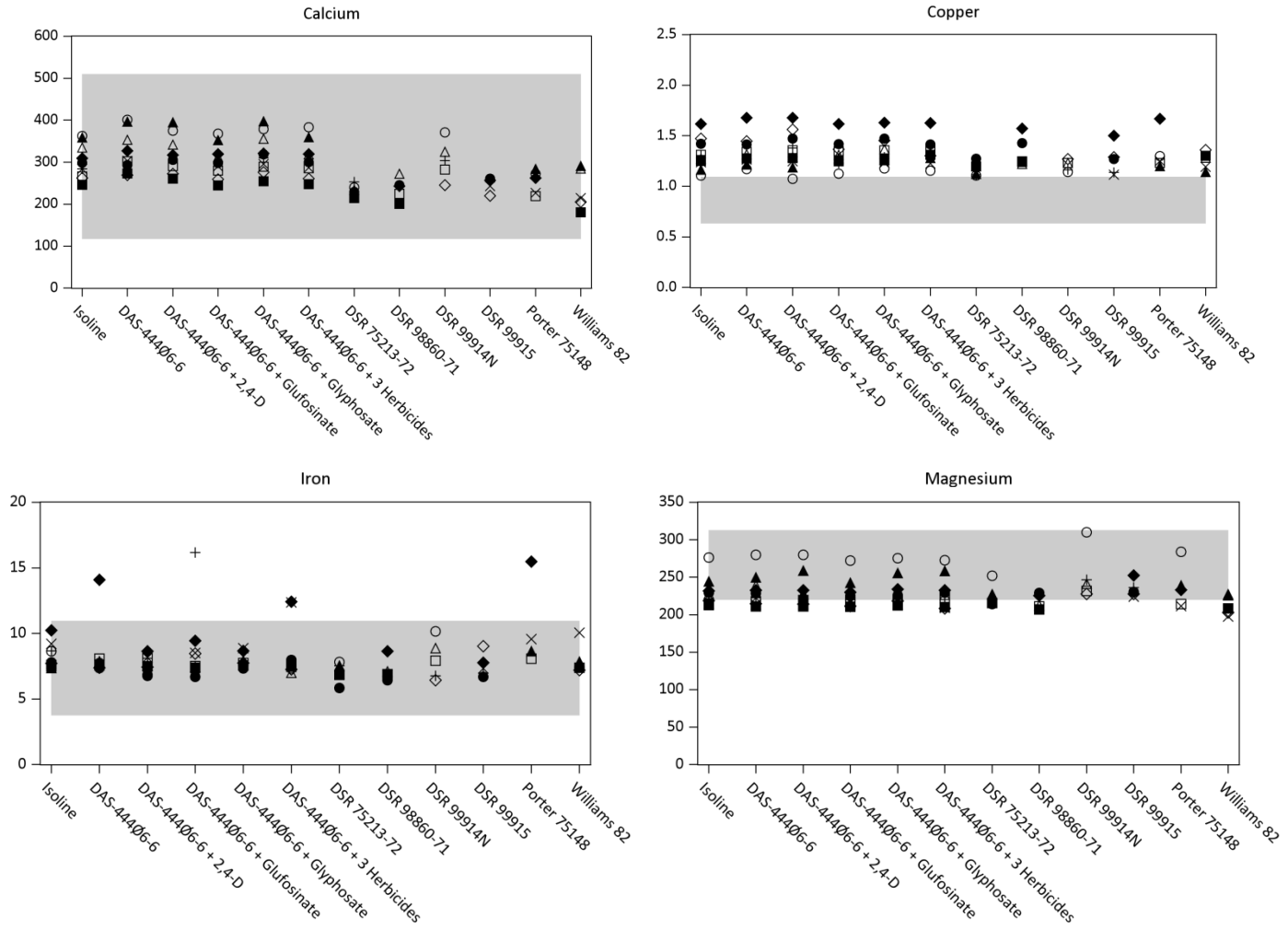


Figure 35. Minerals (mg/100g dry weight for all minerals except selenium (ppb dry weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

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).

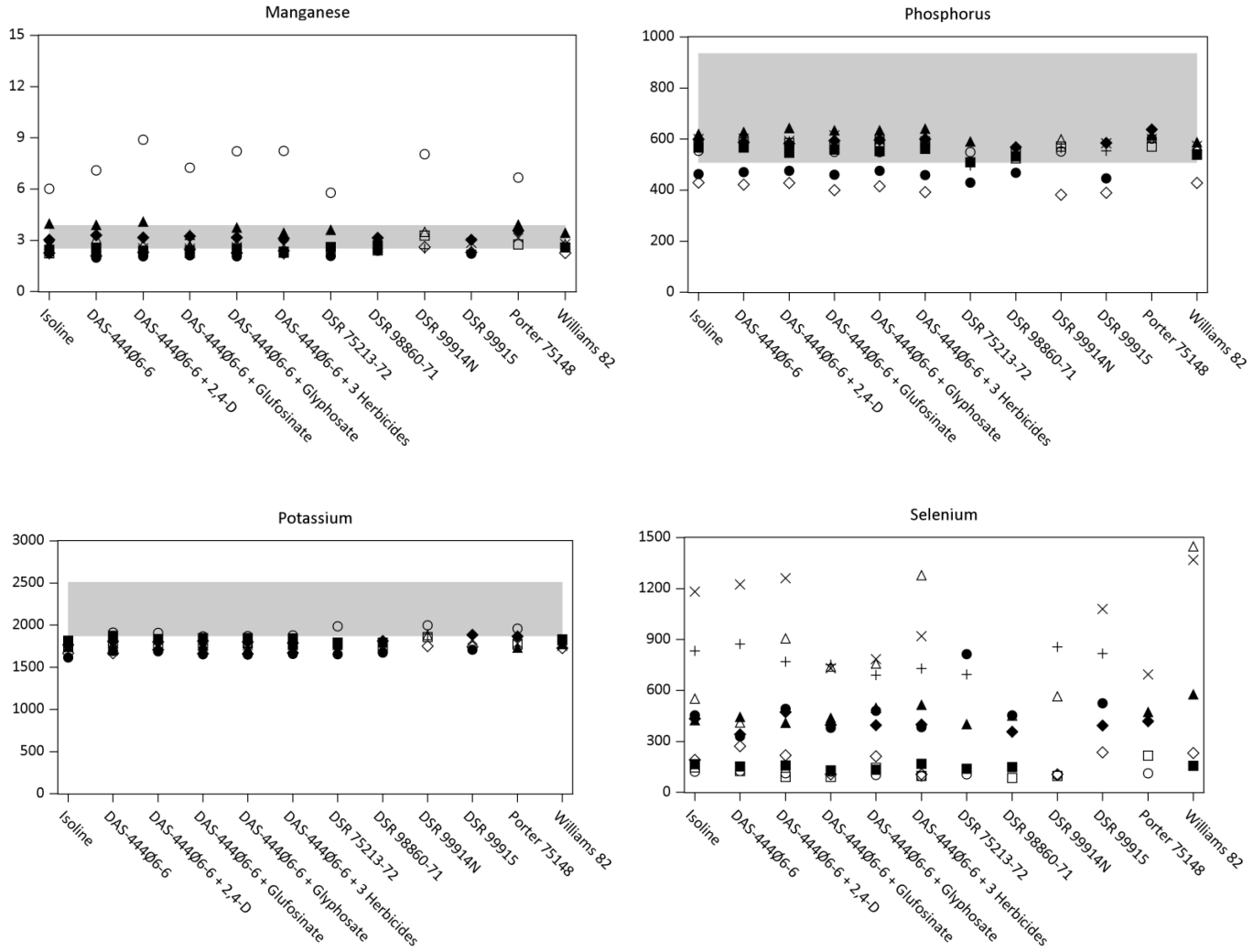


Figure 35 (Cont). Minerals (mg/100g dry weight for all minerals except selenium (ppb dry weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

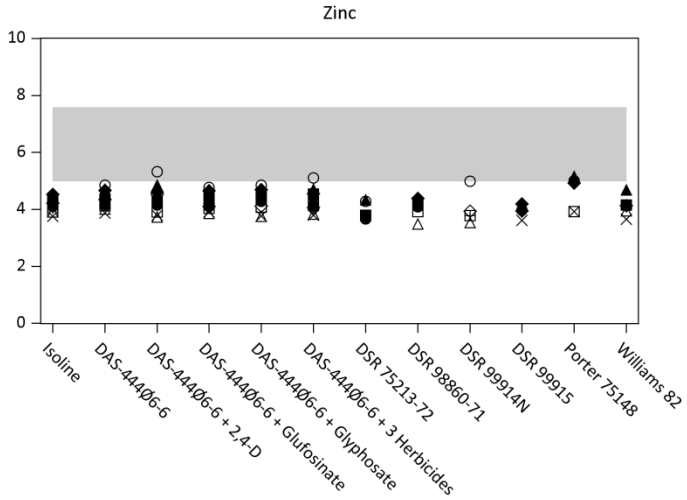


Figure 35 (Cont). Minerals (mg/100g dry weight for all minerals except selenium (ppb dry weight)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

8.4.4. Amino Acid Analysis of Seed

Soybean seed samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed for amino acid content. A summary of the results across all locations is presented in Table 19 and Figure 36. 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 19. 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 1.56 - 1.76	1.67 ± 0.01 1.58 - 1.76	1.67 ± 0.01 1.6 - 1.74	1.68 ± 0.01 1.6 - 1.74	1.67 ± 0.01 1.6 - 1.81	1.68 ± 0.01 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 2.55 - 2.94	2.71 ± 0.02 2.46 - 2.88	2.75 ± 0.02 2.6 - 2.95	2.74 ± 0.02 2.6 - 2.99	2.74 ± 0.02 2.55 - 3	2.75 ± 0.02 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 3.85 - 4.45	4.19 ± 0.03 3.9 - 4.4	4.23 ± 0.03 4.03 - 4.49	4.22 ± 0.03 4.02 - 4.59	4.22 ± 0.03 3.94 - 4.64	4.24 ± 0.03 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 0.458 - 0.647	0.556 ± 0.010 0.493 - 0.661	0.547 ± 0.010 0.474 - 0.645	0.560 ± 0.010 0.489 - 0.672	0.549 ± 0.010 0.482 - 0.652	0.560 ± 0.010 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 5.75 - 6.74	6.22 ± 0.06 5.65 - 6.78	6.26 ± 0.06 5.82 - 6.76	6.27 ± 0.06 5.8 - 6.95	6.26 ± 0.06 5.85 - 6.92	6.28 ± 0.06 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 1.53 - 1.75	1.63 ± 0.01 1.51 - 1.73	1.65 ± 0.01 1.58 - 1.73	1.64 ± 0.01 1.55 - 1.75	1.65 ± 0.01 1.56 - 1.84	1.65 ± 0.01 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 0.943 - 1.1	1.02 ± 0.01 0.91 - 1.1	1.04 ± 0.01 0.957 - 1.11	1.02 ± 0.01 0.964 - 1.13	1.03 ± 0.01 0.935 - 1.17	1.03 ± 0.01 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 1.66 - 1.94	1.81 ± 0.02 1.64 - 1.94	1.83 ± 0.02 1.67 - 2.02	1.81 ± 0.02 1.58 - 1.93	1.83 ± 0.02 1.68 - 2.12	1.82 ± 0.02 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.

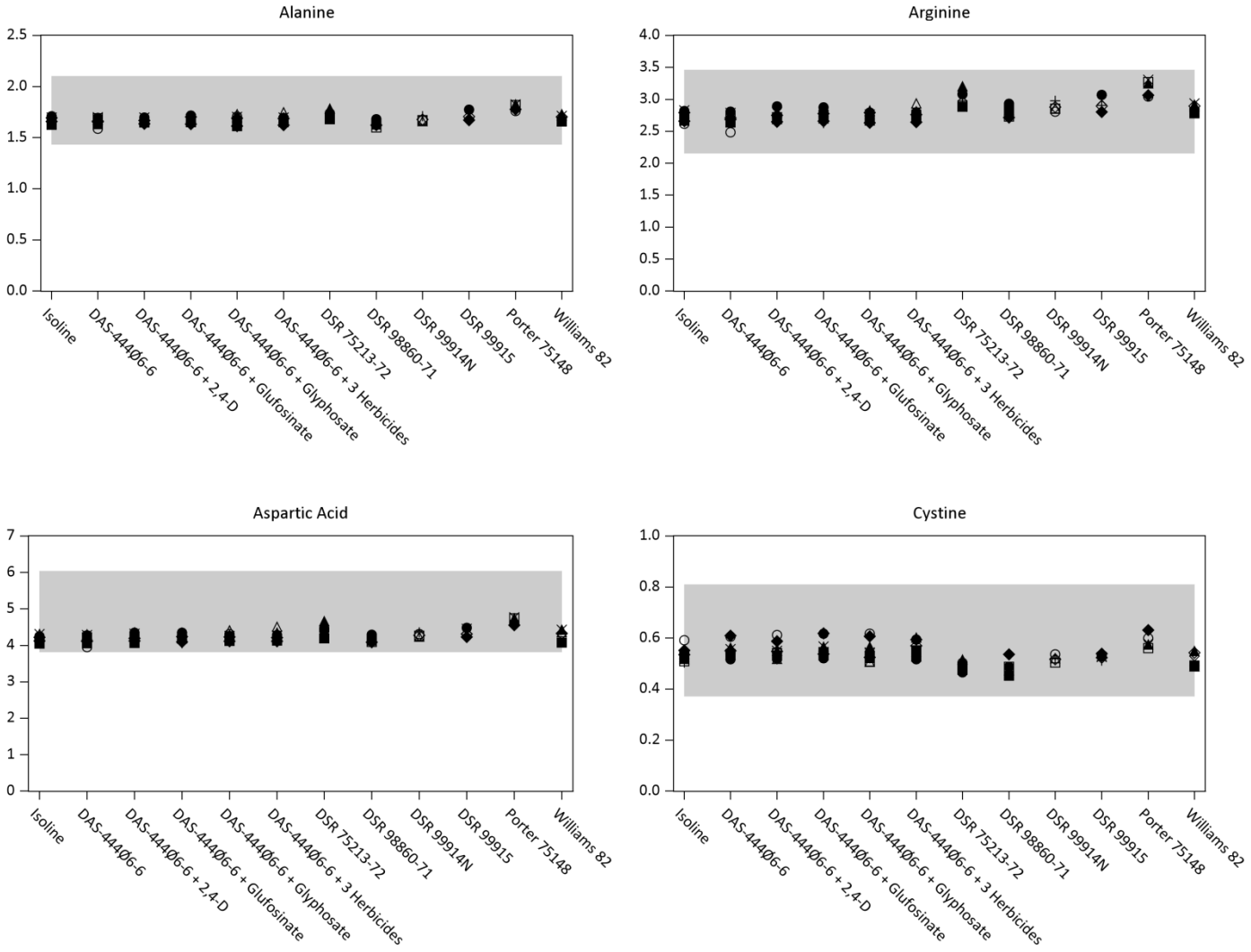


Figure 36. Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.
Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

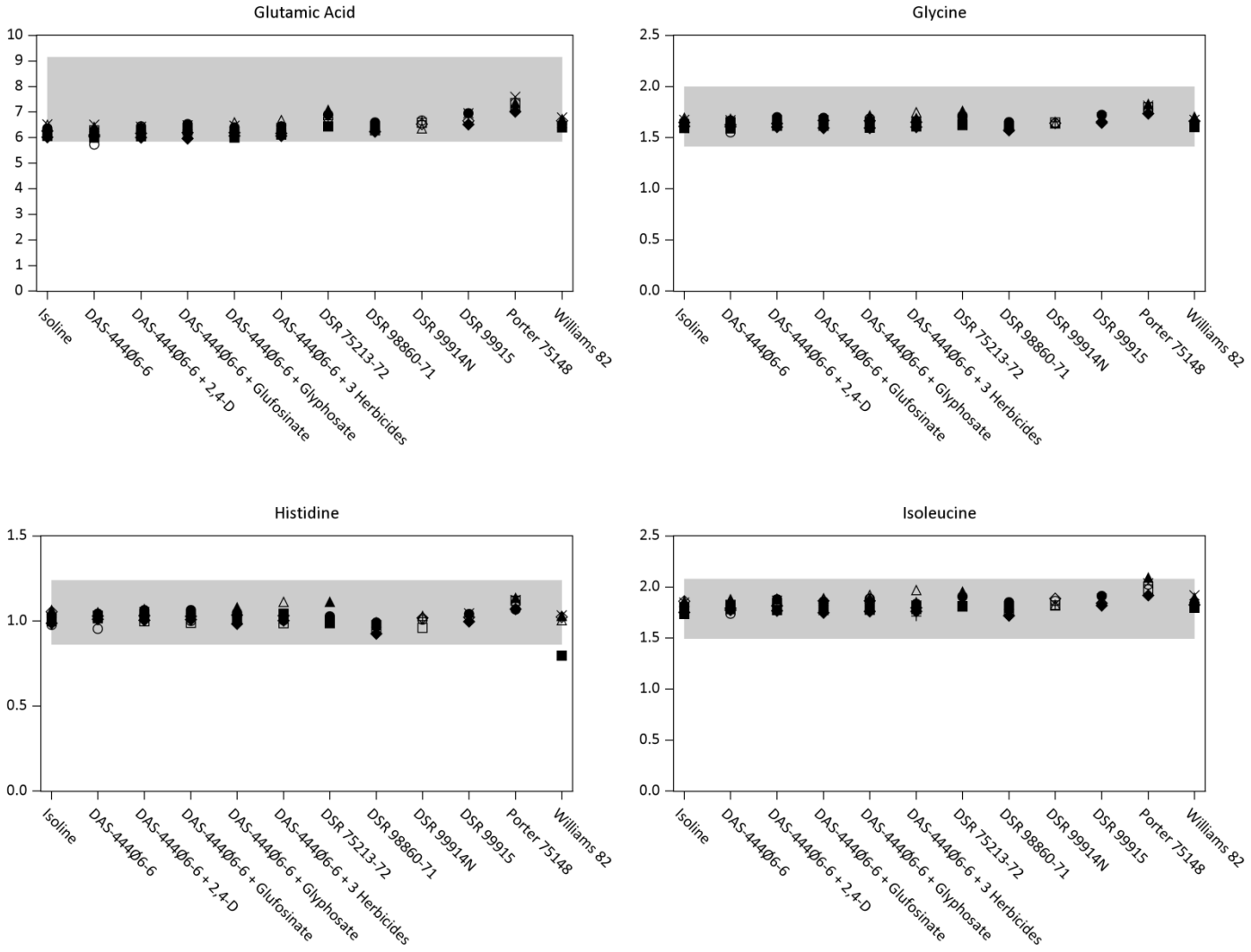


Figure 36 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

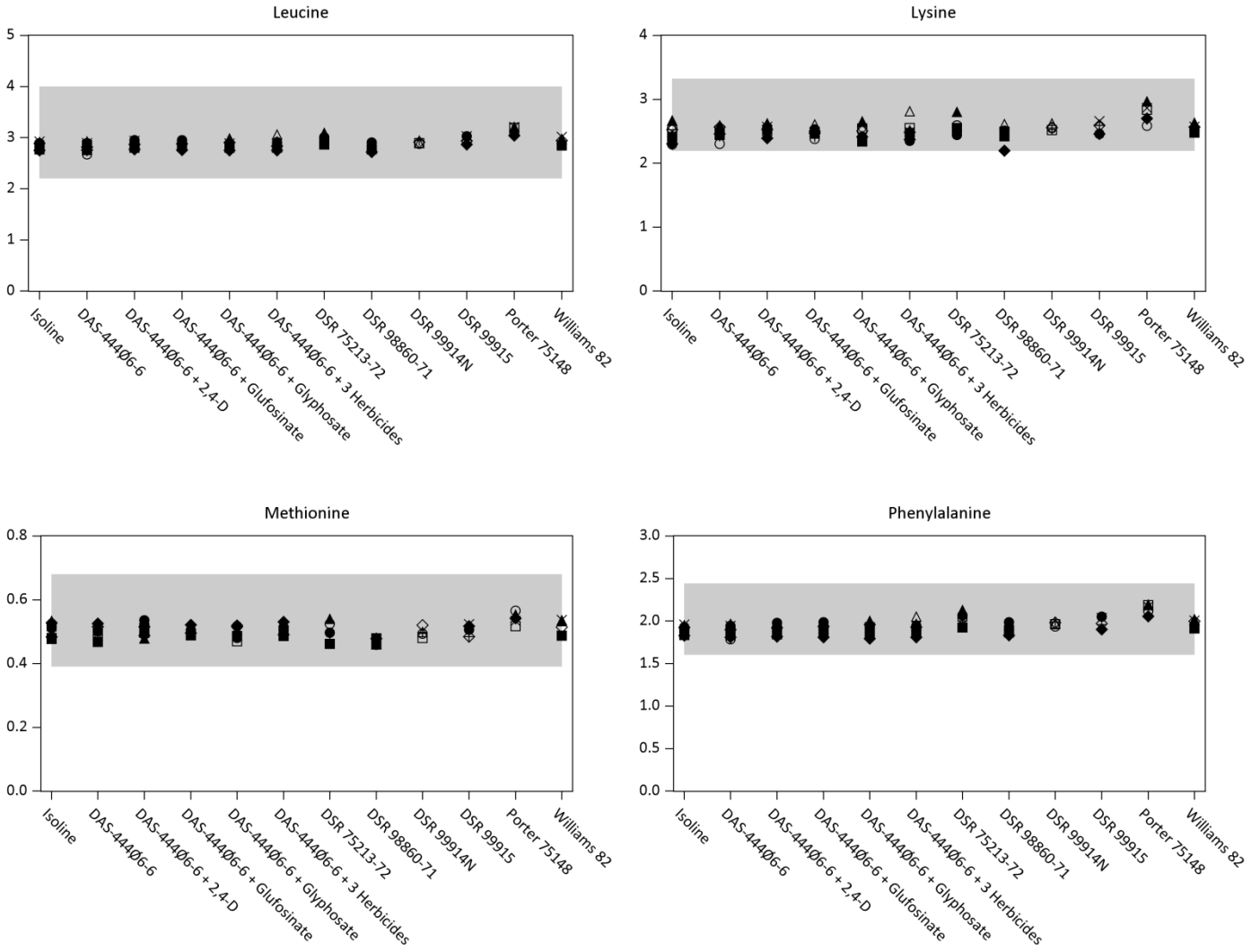


Figure 36 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

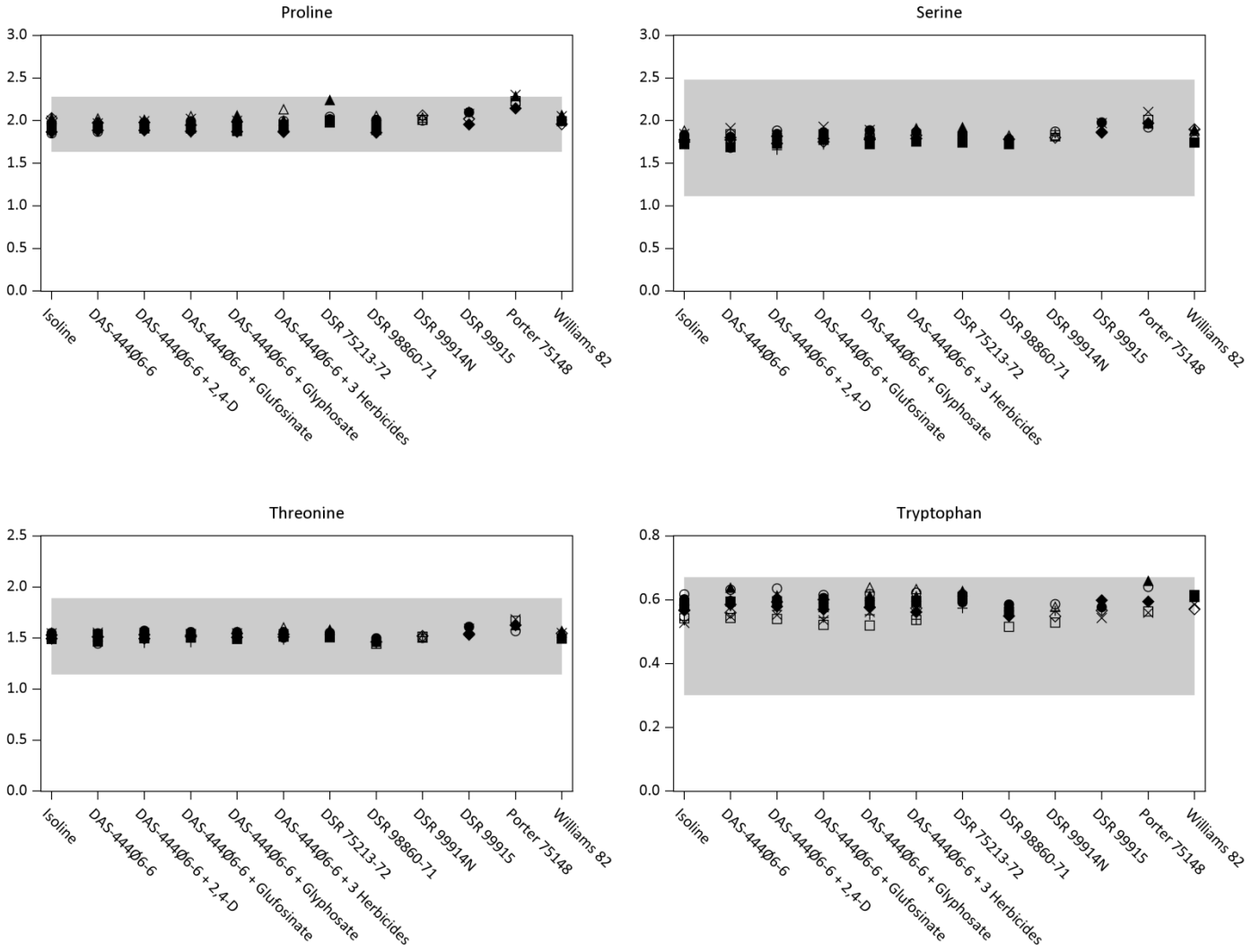


Figure 36 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

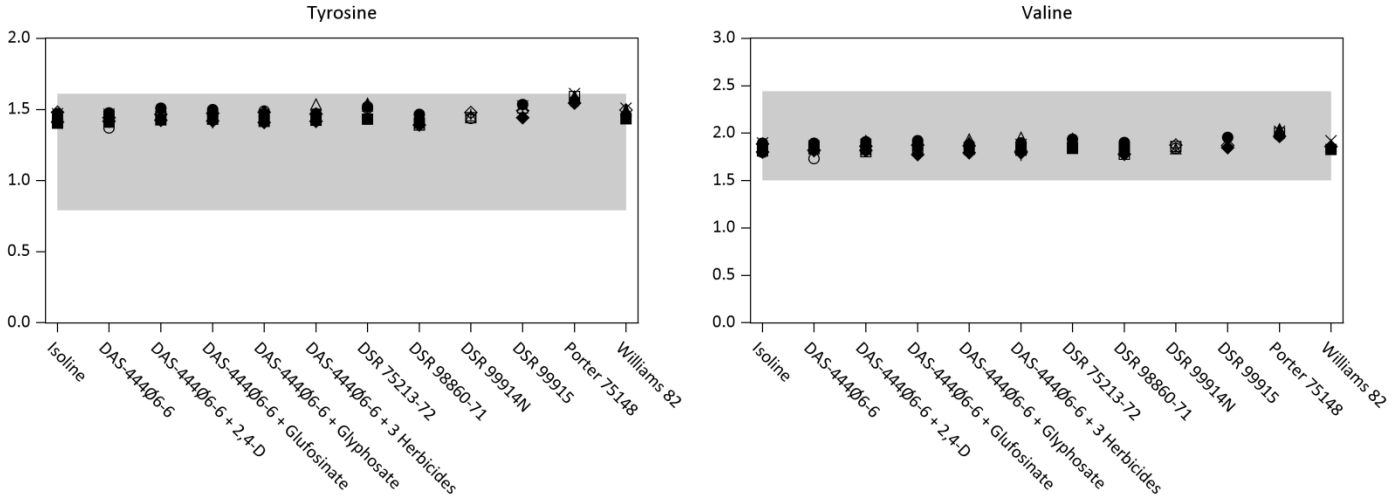


Figure 36 (Cont). Amino acids (% dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

8.4.5. Fatty Acid Analysis of Seed

Soybean seed samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed for fatty acid content. A summary of the results across all locations is presented in Table 20 and Figure 37. 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 20. 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 (% total fatty acid)	NA	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	< LOQ	< LOQ - 0.194
17:0 Heptadecanoic (% total fatty acid)	NA	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
17:1 Heptadecenoic (% total fatty acid)	NA	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	< LOQ	< LOQ - 0.087
18:0 Stearic (% total fatty acid)	0.391	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
18:1 Oleic (% total fatty acid)	**<0.001**	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
18:2 Linoleic (% total fatty acid)	**<0.001**	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
18:3 Linolenic (% total fatty acid)	**<0.001**	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
18:3 γ-Linolenic (% total fatty acid)	NA	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	NA < LOQ	< LOQ	ND

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.

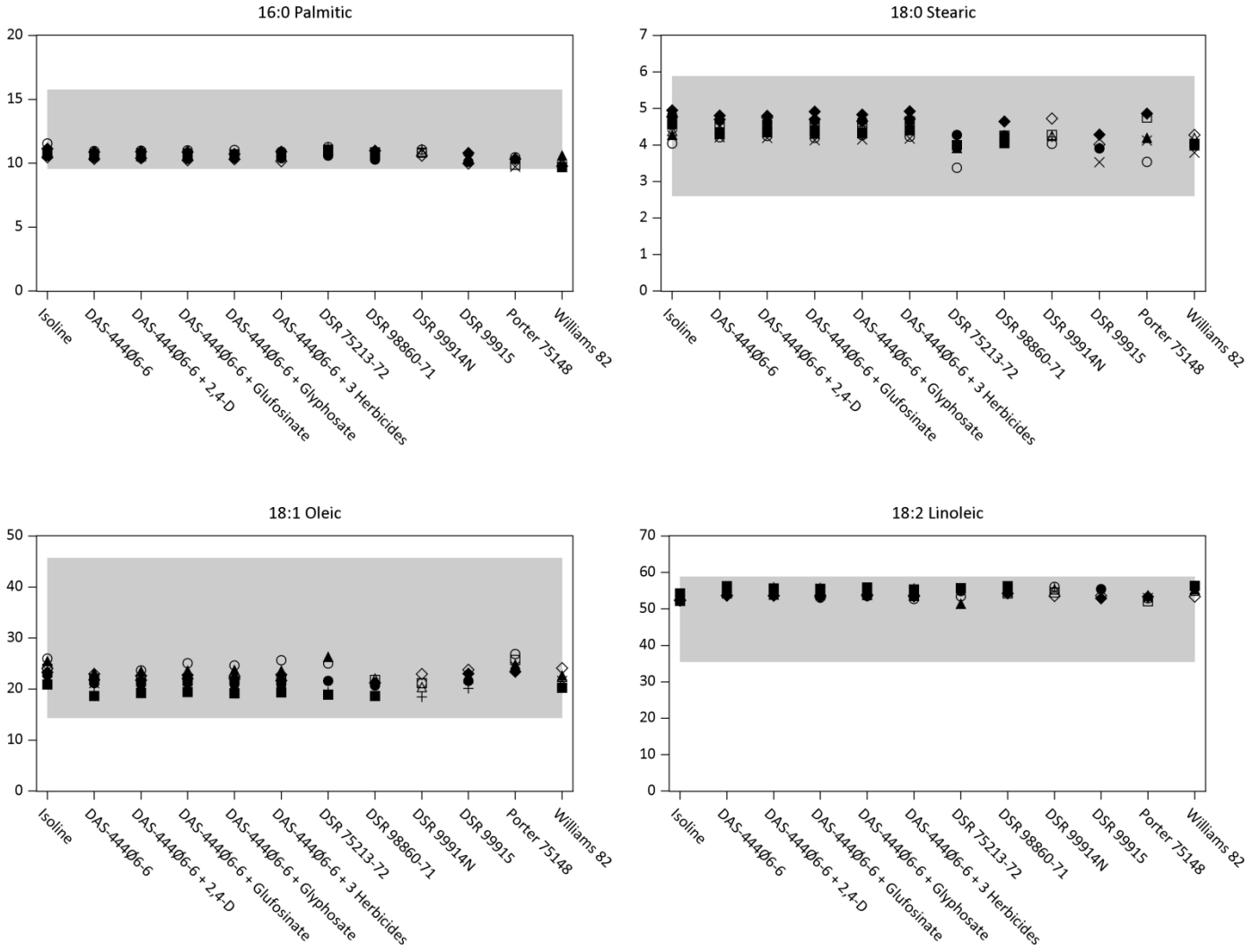


Figure 37. Fatty acids (% total fatty acid) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.
Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

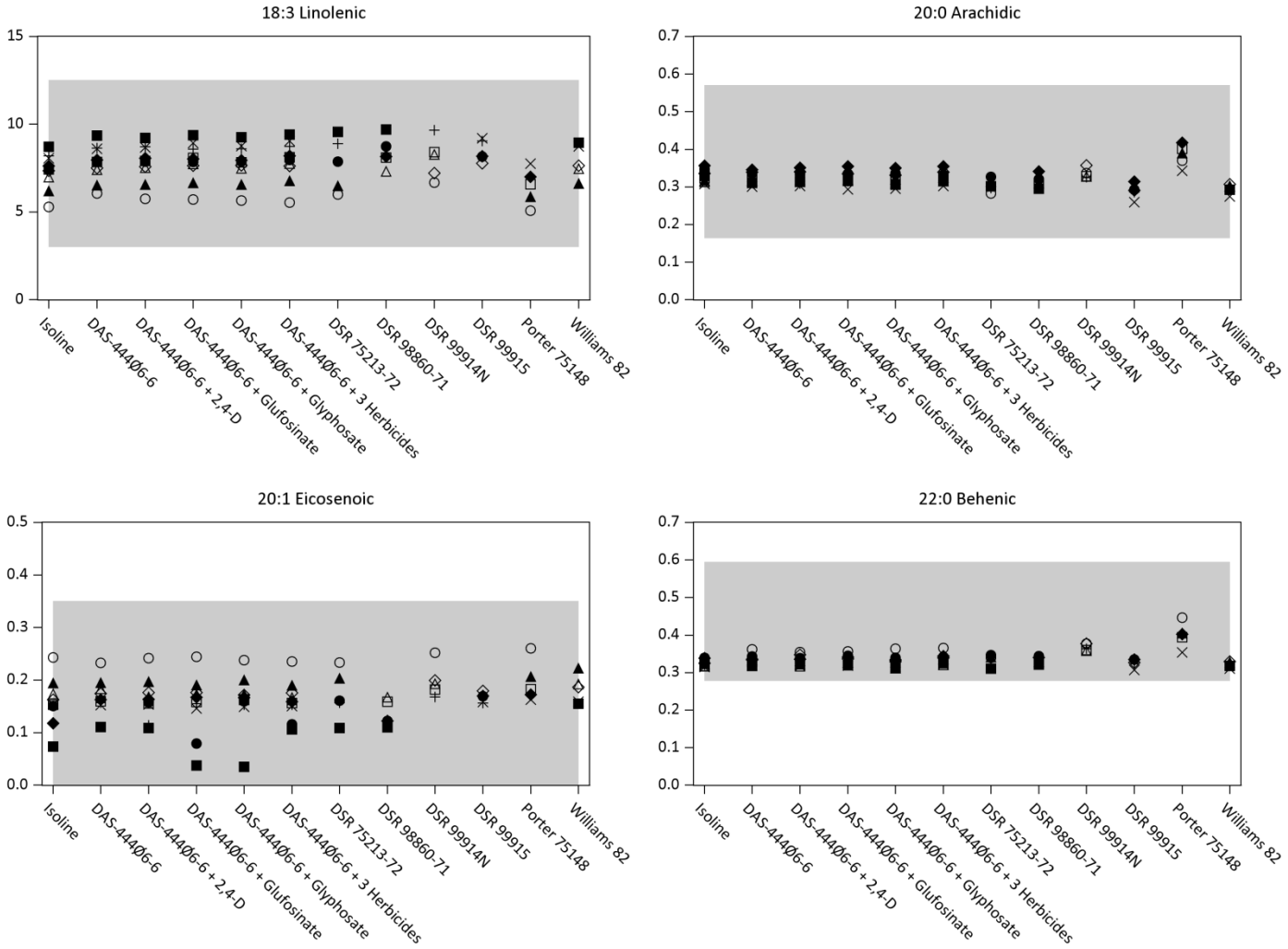


Figure 37 (Cont). Fatty acids (% total fatty acid) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte.

8.4.6. Vitamin Analysis of Seed

Soybean seed samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed for vitamin content. A summary of the results across all locations is presented in Table 21 and Figure 38. 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 21. 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.

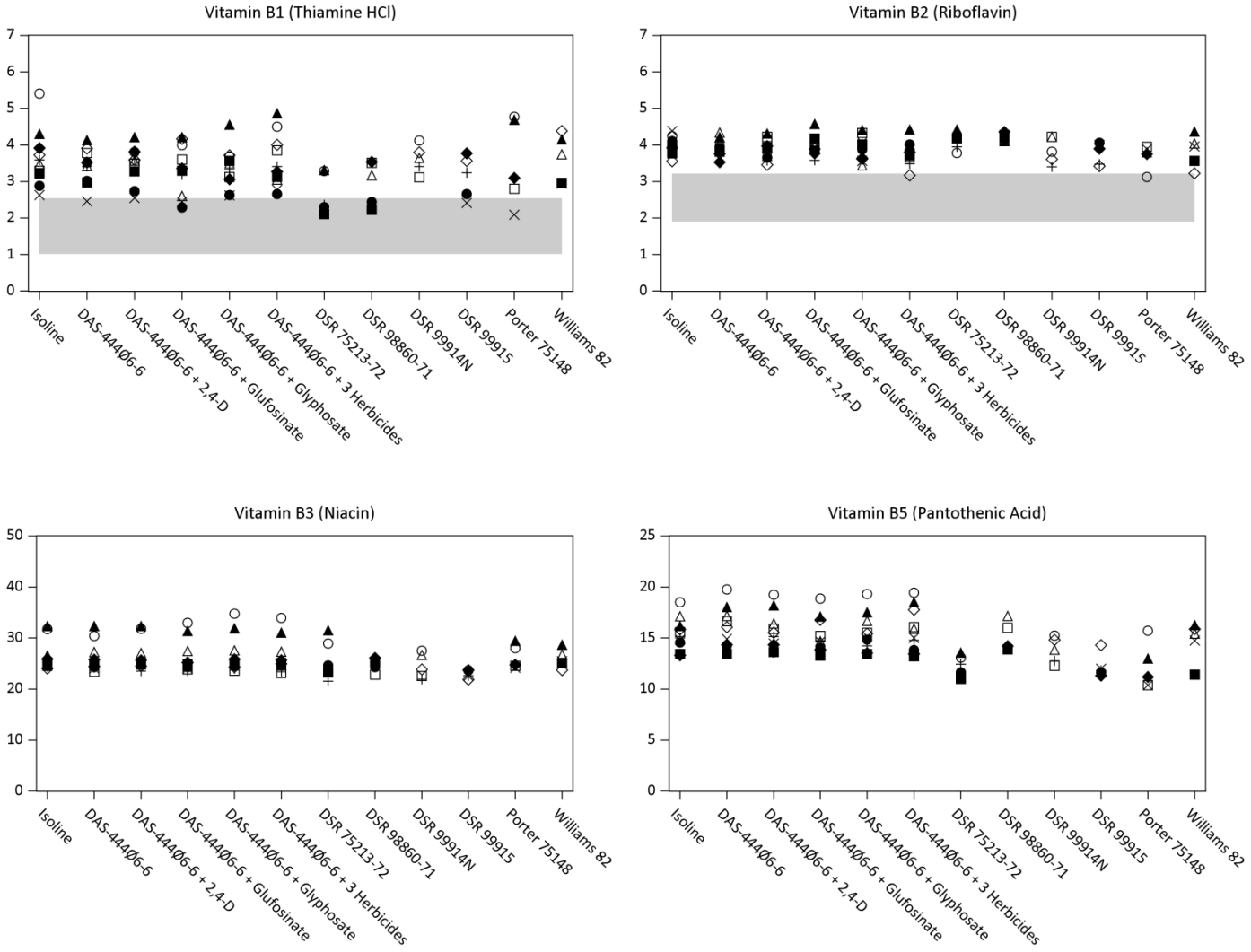


Figure 38. Vitamins (mg/kg dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.
Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

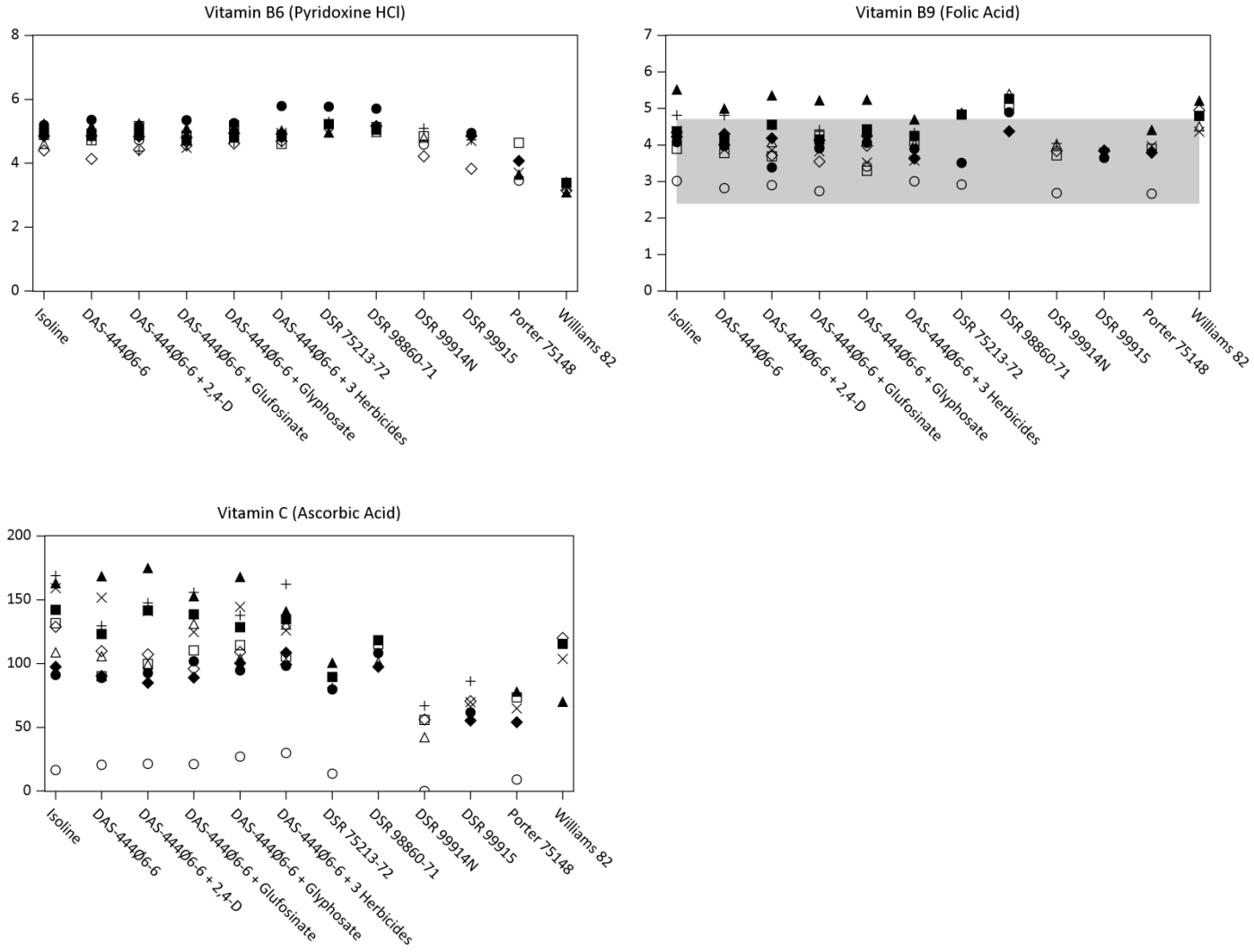


Figure 38 (Cont). Vitamins (mg/kg dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

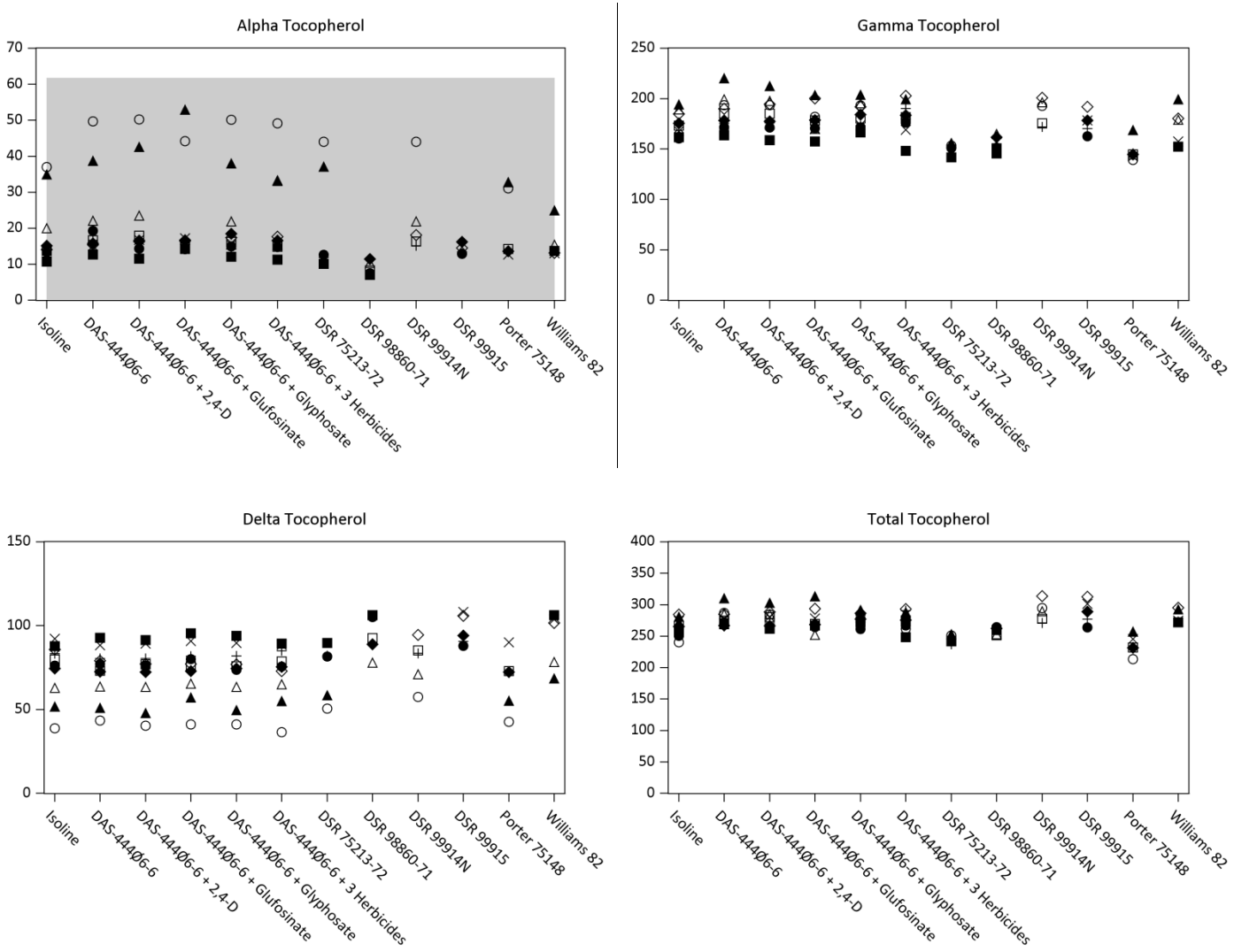


Figure 38 (Cont). Vitamins (mg/kg dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location shown: open circle = GA, × = IA1, + = IA2, open triangle = IL1, open square = IL2, open diamond = IN, filled circle = MI, filled triangle = MO, filled square = NE1, filled diamond = NE2. Literature range is shaded for each analyte (when available).

8.4.7. Bioactive Analysis of Seed

Soybean seed samples from the control (Maverick), reference, and DAS-444Ø6-6 entries were analyzed for bioactive chemical content. A summary of the results across all locations is presented in Table 22, Figure 39 and Figure 40. 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, 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. Additionally, bioactive components including lectin and trypsin inhibitor are inactivated during standard processing of soybean seed prior to consumption (Rackis, 1974; Padgette *et al.*, 1996; Hammond and Jez, 2011).

Table 22. 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 (H.U./mg protein DW)	**0.004**	79 ± 8 27.9 - 153	107 ± 8 60.6 - 228 (<**0.001**, **0.001**)	94 ± 8 39.4 - 188 (**0.021**, 0.092)	94 ± 8 56.3 - 146 (**0.021**, 0.092)	92 ± 8 44.8 - 151 (**0.049**, 0.161)	99 ± 8 31 - 196 (**0.004**, **0.026**)	18.5 - 144	37 - 323
Phytic Acid (% DW)	0.958	1.19 ± 0.07 0.513 - 1.53	1.18 ± 0.07 0.679 - 1.53 (0.789, 0.903)	1.19 ± 0.07 0.65 - 1.59 (0.866, 0.949)	1.18 ± 0.07 0.603 - 1.51 (0.511, 0.720)	1.18 ± 0.07 0.707 - 1.46 (0.632, 0.795)	1.19 ± 0.07 0.651 - 1.55 (0.824, 0.932)	0.55 - 1.54	0.41 - 2.74
Raffinose (% DW)	0.200	0.82 ± 0.06 0.497 - 1.29	0.80 ± 0.06 0.556 - 1.22 (0.151, 0.345)	0.80 ± 0.06 0.581 - 1.18 (0.092, 0.256)	0.80 ± 0.06 0.569 - 1.22 (0.050, 0.162)	0.80 ± 0.06 0.438 - 1.3 (0.104, 0.275)	0.79 ± 0.06 0.478 - 1.23 (**0.012**, 0.065)	0.569 - 1.4	0.212 - 1.62
Stachyose (% DW)	0.905	3.88 ± 0.06 2.98 - 4.22	3.88 ± 0.06 3.38 - 4.11 (0.952, 0.976)	3.87 ± 0.06 3.21 - 4.26 (0.816, 0.928)	3.86 ± 0.06 2.95 - 4.29 (0.779, 0.901)	3.83 ± 0.06 2.77 - 4.18 (0.375, 0.590)	3.89 ± 0.06 3.08 - 4.38 (0.827, 0.932)	2.92 - 4.48	1.21 - 6.1
Trypsin Inhibitor (TIU/mg DW)	**0.025**	30.8 ± 3.0 18.4 - 54.6	35.0 ± 3.0 19 - 56 (**0.015**, 0.075)	35.5 ± 3.0 17.4 - 71 (**0.007**, **0.047**)	36.6 ± 3.0 21.1 - 78.9 (**0.001**, **0.010**)	33.6 ± 3.0 21.3 - 51.7 (0.095, 0.260)	34.2 ± 3.0 21.8 - 62.6 (**0.047**, 0.159)	15.6 - 59.7	18.14 - 118.68

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.

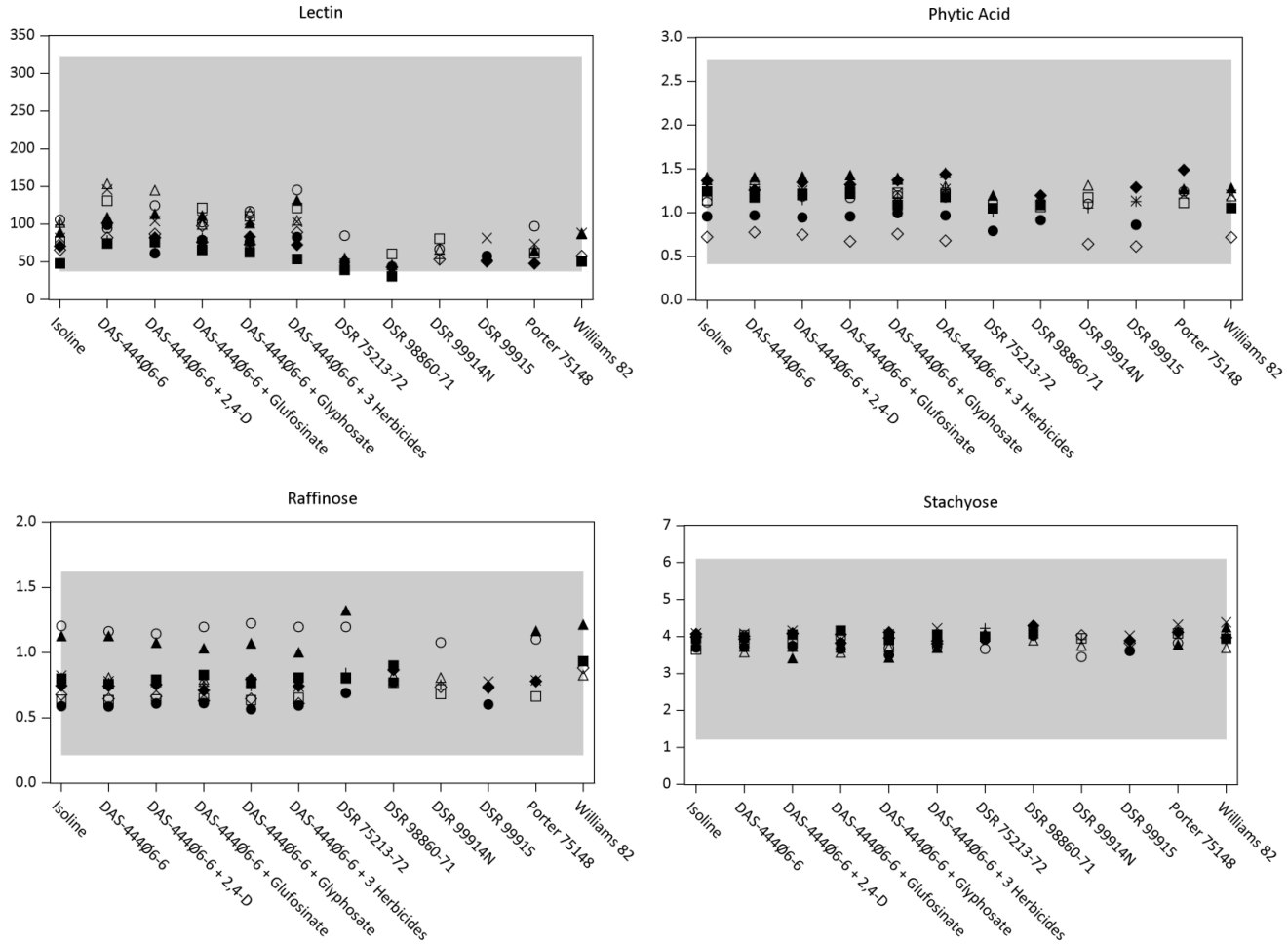


Figure 39. Bioactives (% dry weight (DW) for all bioactives except lectin (H.U./mg protein DW, H.U. = hemagglutination unit) and trypsin inhibitor (TIU/mg DW, TIU = trypsin inhibitor unit)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

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.

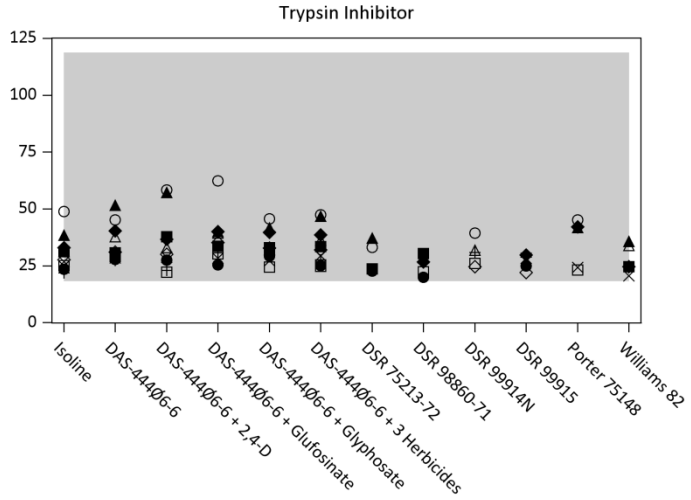


Figure 39 (Cont). Bioactives (% dry weight (DW) for all bioactives except lectin (H.U./mg protein DW, H.U. = hemagglutination unit) and trypsin inhibitor (TIU/mg DW, TIU = trypsin inhibitor unit)) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed. Symbols for each location 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.

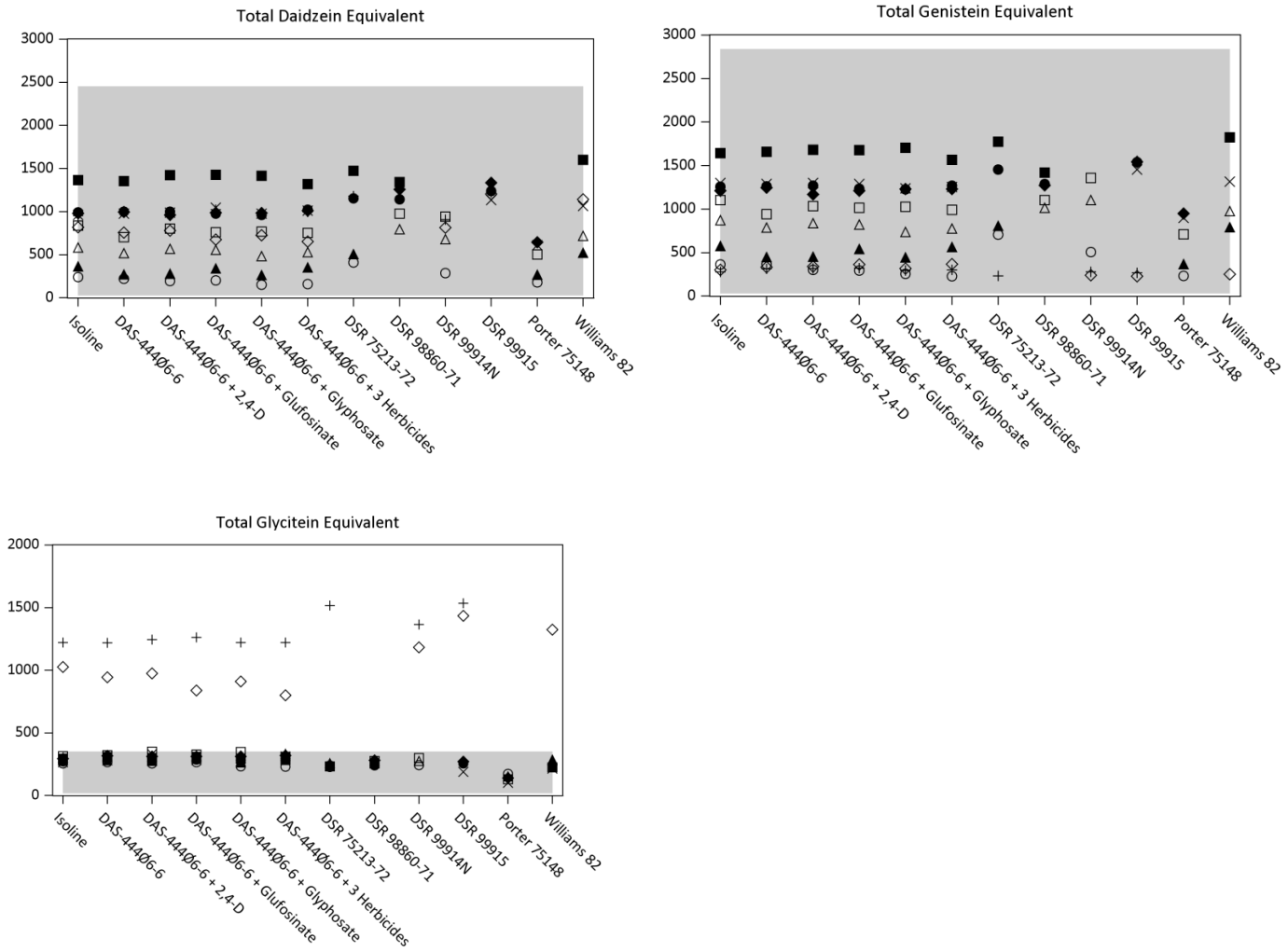


Figure 40. Bioactives: isoflavones (mcg/g dry weight) in non-transgenic (Isoline), Event DAS-444Ø6-6, and reference line soybean seed.

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.

8.5. Composition Summary

All overall mean values for the non-transgenic isogenic control Maverick 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 (Maverick) 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.

8.6. Conclusions

Nutrient composition of DAS-444Ø6-6 soybean (unsprayed or sprayed with 2,4-D, glyphosate, glufosinate, or all three herbicides) was evaluated in field trials in 2010. DAS-444Ø6-6 soybean composition samples were all statistically indistinguishable from the control line and/or within literature or reference ranges for non-transgenic soybean, indicating that no unintended compositional effects were observed for DAS-444Ø6-6 soybean. Results from this study demonstrate compositional equivalence between DAS-444Ø6-6 and non-transgenic soybean.

9. Environmental Consequences and Impact on Agronomic Practices

9.1. Field Efficacy

Field studies were conducted with DAS-444Ø6-6 soybean in 2010 to evaluate herbicide injury in three trials which differed by the timing of application (V2, V6, or R2). All three trials were conducted at Greenville, MS (US) and Weston, MO (US). The trial applied at V2 was also conducted at Huxley, IA (US), and the trials applied at V6 and R2 were also conducted at Fowler, IN (US) and Cambridge, IA (US). Seeds were planted between May 24 and June 6, 2010 in 2-row plots with rows spaced 30 cm apart and seed placed at ~26 seeds per meter in the row. Rows were 5.3 m long with a 76 cm alley between plots. Therefore, each plot included ~275 seeds. Plots were arranged in a randomized complete block design with 3 blocks per trial. Herbicide applications were applied 140 L/ha at between 21 and 32 PSI. Glyphosate (Durango DMA) and 2,4-D (Weedar 64) were applied at 1120 (1X) or 2240 (2X) g ae/ha. Glufosinate (Ignite 280 SL) was applied at 411 (1X) or 822 (2X) g ae/ha. Visual injury ratings were taken on a 0 to 100 scale, which reflects a visual composite of all injury symptoms observed across all plants in a plot. A rating of zero indicates no injury as compared to untreated plots and a rating of 100 indicates death of all plants. Experimental data was analyzed across locations by ANOVA with treatment, replication and experiment being factors. A single replicate of the non-transgenic near-isogenic control Maverick was also planted separately at each location and sprayed with the 1X rate of each herbicide. Injury ratings were collected to verify the negative effects of the herbicide sprays on non-transgenic soybean (Maverick).

Tolerance to 2,4-D

DAS-444Ø6-6 soybean displayed a high level of tolerance to applications of 2,4-D (Table 23). When 2,4-D was sprayed at 1X or 2X, the proposed maximum use rate at the V2 growth stage, plant injury was 1% at both 1X and 2X rates two weeks after application. The same herbicide treatment was applied at the V6 or R2 stage. Plant injury was zero for treatment given at the V6 stage. For treatment at the R2 stage, plant injury after one week of application was zero at 1X, the proposed maximum use rate, and 2% at 2X the proposed maximum use rate. Injury to non-transgenic near-isogenic soybean (Maverick) ranged from 80 to 99% at two weeks after treatment when sprayed at 1X, the proposed use rate of 2,4-D.

Table 23. DAS-444Ø6-6 soybean tolerance to application of 2,4-D (Weedar 64).

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]		
			Unsprayed	DAS-444Ø6-6	Statistical Analysis
2,4-D amine	1120 g ae/ha	V2	0.0	1.0	ns
2,4-D amine	2240 g ae/ha	V2	0.0	1.0	ns
2,4-D amine	1120 g ae/ha	V6	0.0	0.0	ns
2,4-D amine	2240 g ae/ha	V6	0.0	0.0	ns
2,4-D amine	1120 g ae/ha	R2	0.0	0.0	ns
2,4-D amine	2240 g ae/ha	R2	0.0	2.0	ns

[a] ae/ha = acid equivalent/hectare
[b] Application stage in terms of soybean plant growth development.
[c] Ratings were taken two weeks after application for treatment at V2 or V6 stage. Ratings were taken one week after application for treatment at R2 stage. A rating of zero indicates no injury and a rating of 100 indicates death of all plants. ns indicates no statistical differences between sprayed and unsprayed DAS-444Ø6-6 at 95% confidence level.

Tolerance to Glyphosate

DAS-444Ø6-6 soybean displayed a high level of tolerance to applications of glyphosate (Table 24). Plant injury was zero under all of the experimental conditions. Injury to non-transgenic near-isogenic soybean (Maverick) ranged from 80 to 99% at two weeks after treatment when sprayed at 1X the proposed use rate of glyphosate.

Table 24. DAS-444Ø6-6 soybean tolerance to application of glyphosate (Durango DMA).

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]		
			Unsprayed	DAS-444Ø6-6	Statistical Analysis
glyphosate	1120 g ae/ha	V2	0.0	0.0	ns
glyphosate	2240 g ae/ha	V2	0.0	0.0	ns
glyphosate	1120 g ae/ha	V6	0.0	0.0	ns
glyphosate	2240 g ae/ha	V6	0.0	0.0	ns
glyphosate	1120 g ae/ha	R2	0.0	0.0	ns
glyphosate	2240 g ae/ha	R2	0.0	0.0	ns

[a] ae/ha = acid equivalent/hectare
[b] Application stage in terms of soybean plant growth development.
[c] Ratings were taken two weeks after application for treatment at V2 or V6 stage. Ratings were taken one week after application for treatment at R2 stage. A rating of zero indicates no injury and a rating of 100 indicates death of all plants. ns indicates no statistical differences between sprayed and unsprayed DAS-444Ø6-6 at 95% confidence level.

Tolerance to Glufosinate

DAS-444Ø6-6 soybean displayed a high level of tolerance to applications of glufosinate (Table 25). Plant injury was 1% for applications at the V6 and R2 growth stages at 2X, the proposed maximum use rate. Under all other conditions plant injury was zero. Injury to non-transgenic near-isogenic soybean (Maverick) ranged from 65 to 99% at two weeks after treatment when sprayed at 1X, the proposed use rate of glufosinate.

Table 25. DAS-444Ø6-6 soybean tolerance to application of glufosinate (Ignite 280SL).

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]		
			Unsprayed	DAS-444Ø6-6	Statistical Analysis
glufosinate	411 g ae/ha	V2	0.0	0.0	ns
glufosinate	822 g ae/ha	V2	0.0	0.0	ns
glufosinate	411 g ae/ha	V6	0.0	0.0	ns
glufosinate	822 g ae/ha	V6	0.0	1.0	ns
glufosinate	411 g ae/ha	R2	0.0	0.0	ns
glufosinate	822 g ae/ha	R2	0.0	1.0	ns

[a] ae/ha = acid equivalent/hectare
[b] Application stage in terms of soybean plant growth development.
[c] Ratings were taken two weeks after application for treatment at V2 or V6 stage. Ratings were taken one week after application for treatment at R2 stage. A rating of zero indicates no injury and a rating of 100 indicates death of all plants. ns indicates no statistical differences between sprayed and unsprayed DAS-444Ø6-6 at 95% confidence level.

Summary of Herbicide Tolerance

The outcome of the field studies demonstrates that DAS-444Ø6-6 soybean provides excellent tolerance to applications of 2,4-D, glyphosate and glufosinate, at rates of up to two times the proposed maximum use rates.

9.2. Weediness Potential

Commercial soybean varieties in the United States are not considered weeds and are not effective at invading established ecosystems.

Cultivated soybean seed rarely displays any dormancy characteristics and only under certain environmental conditions grows as a volunteer in the year following cultivation. If this should occur, volunteers do not compete well with the succeeding crop, and can easily be controlled mechanically or chemically. The soybean plant is not weedy in character. In North America, *Glycine max* is not found outside of cultivation. In managed ecosystems, soybean does not effectively compete with other cultivated plants or primary colonizers (OECD, 2000).

The introduction of aryloxyalkanoate herbicide, glufosinate, and glyphosate-tolerance into soybean will not alter the weediness characteristics of soybean. Agronomic properties of DAS-444Ø6-6 soybean related to weediness, such as germination (Section 7.3), emergence (Section 7.1.2), seedling vigor (Section 7.1.2), and response to environmental stressors (Section 7.1.2), have been shown to be identical to conventional Maverick soybean. If DAS-444Ø6-6 soybeans were to overwinter, they can still be effectively controlled mechanically or with other herbicide modes of action; such as paraquat in burndown programs, fluometuron in cotton (Hayes, 2000), and atrazine in corn. Therefore, these findings demonstrate that DAS-444Ø6-6 soybean do not have increased weediness potential relative to conventional soybean.

9.3. Gene Flow Assessment

9.3.1. Vertical Gene Flow

Soybean is considered to be a self-pollinated species, although natural crossing can occur at low rates (OECD, 2000). The level of cross pollination can vary as a function of growing conditions, spatial arrangement of plants, and genotype. Cross pollination levels ranging from 0.09% up to 3.6% in adjacent rows have been reported (Beard and Knowles, 1971; Ahrent and Caviness, 1994), but outcrossing frequency rapidly declines with distance. Cross pollination rates have been reported to be <0.1% at distances of >5 m (Caviness, 1966; Ray *et al.*, 2003). Cultivated soybeans can cross only with members of its subgenus *Soja*. Wild soybean species of the subgenus *Soja* are native to Korea, Japan, Taiwan, northeastern China, and areas of the former USSR, but do not exist naturally in the United States (OECD, 2000). Therefore, there is no potential for gene flow from DAS-444Ø6-6 soybeans to wild soybean relatives in the United States.

9.3.2. Horizontal Gene Flow

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Conner *et al.*, 2003). Even if such a transfer were to take place, transfer of the *aad-12, 2mepsps* and *pat* genes from DAS-444Ø6-6 soybean would not present a human health or plant pest risk, based on the safety data presented in this petition.

9.4. Current US Agronomic Practices for Soybeans

9.4.1. Soybean Production

Processed soybeans are the largest source of protein feed and the second largest source of vegetable oil in the world. The United States is the world's leading soybean producer and exporter. Farm value of U.S. soybean production in 2008/09 was $29.6 billion, the second-highest value among U.S.-produced crops, trailing only corn. Soybean and soybean product exports accounted for 54 percent of U.S. soybean production in 2008/09. Soybeans are about 90 percent of U.S. total oilseed production, while other oilseeds—such as cottonseed, sunflower seed, canola, and peanuts—account for the remainder (USDA ERS, 2010).

A total of 77.4 million acres were planted to soybeans in the United States in 2010 (USDA NASS, 2011). Of these planted acres, about 76.6 million acres were harvested, valued at $38.9 billion. The majority of these soybeans were grown within the North Central states. About 80% of the planted soybean acres and 80% of the harvested soybean acres were concentrated in 11 states – Iowa, Illinois, Minnesota, Indiana, Missouri, Nebraska, Ohio, South Dakota, North Dakota, Kansas, and Arkansas.

9.4.2. Weeds in Soybean

Weeds in soybean compete with the crop for light, nutrients and soil moisture. When weeds are uncontrolled for the entire season, yield losses can exceed 68 to 75% (Krausz *et al.*, 2001; Dalley *et al.*, 2002). Hartzler and Pringnitz (2005) define the critical period of competition in soybeans as the point of time when weeds that emerge with the crop begin to impact yields. Most studies have found that soybean yields are protected if weeds are controlled before they reach a height of 6 to 8 inches. The critical period varies widely, depending upon weed species and densities, environmental conditions, and cultural practices.

To maximize soybean yields, weeds must be removed or controlled when they are no more than 6 inches tall (Loux *et al.*, 2006). Field trials conducted in Ohio in 2001 found that applying glyphosate when weeds are already 9 to 12 inches tall resulted in a 6-10% yield loss due to weed competition prior to control of the weeds (Loux *et al.*, 2006). Dalley *et al.* (2002) studied the effect of row width and weed competition on soybean yield in 1998-2001. They found that weed competition could reduce soybean yields by the time weeds reached 6, 6, and >12 inches in height in 7.5, 15, and 30 inch rows, respectively.

The most common weed problems in soybeans are the annual grass and broadleaf weeds (Table 26). Some fields are also infested with perennial weeds (quackgrass, Johnsongrass, field bindweed, Canada thistle, others) which are more difficult to control since they can reproduce from seed or underground root buds or rhizomes.

Table 26. Common troublesome weeds in soybeans in 2006-2008.
(Third Party Proprietary Data).

	Total Soybean Acres Treated[1]		
Weed Species	**2006**	**2007**	**2008**
Annual Broadleaf Weeds			
Lambsquarters, Common	21,859,614	24,459,895	28,242,972
Velvetleaf	23,820,731	23,373,573	26,786,349
Pigweed, Redroot	21,093,224	21,788,121	26,715,150
Cocklebur, Common	23,657,980	22,389,376	23,962,063
Waterhemp, Common	18,399,609	15,970,794	21,364,980
Ragweed, Giant	13,369,296	14,684,000	16,565,209
Marestail	4,044,060	5,382,190	11,257,267
Morningglory Spp.	10,711,087	11,432,904	11,011,185
Ragweed, Common	9,417,252	9,438,871	9,518,051
Sunflower, Wild	5,558,526	5,759,216	5,709,292
Kochia	4,859,759	3,671,795	5,317,528
Smartweed Pennsylvania	2,366,851	1,835,825	3,529,114
Waterhemp, Tall	2,301,380	2,926,358	3,826,647
Horseweed	2,188,359	3,159,712	3,470,274
Mustard, Wild	2,019,346	1,975,291	2,688,590
Sicklepod	2,024,031	1,650,086	2,535,829
Sida, Prickly	1,639,261	1,567,275	2,432,701
Sunflower, Volunteer	1,089,460	1,007,691	1,913,860
Chickweed	1,652,712	1,259,096	1,823,638
Nightshade, Black	1,766,649	1,277,416	1,385,751
Buckwheat, Wild	1,167,746	855,879	1,331,675
Pigweed, Smooth	188,160	801,569	1,322,732
Annual Grass Weeds			
Foxtail Spp.	24,409,043	18,489,746	18,446,420
Foxtail, Giant	11,817,612	17,513,493	17,804,622
Foxtail, Yellow	10,870,761	11,217,512	13,947,018
Foxtail, Green	5,629,880	7,109,316	7,610,855
Crabgrass	5,170,684	5,928,919	7,424,879
Barnyardgrass	4,189,156	3,967,425	3,805,391
Corn, Volunteer	2,292,705	2,088,371	3,704,330
Oat, Wild	1,792,389	1,478,890	2,886,300
Cupgrass, Woolly	1,765,244	2,470,437	2,108,135
Shattercane	2,408,592	2,715,388	1,879,416
Panicum, Fall	2,251,014	2,241,088	1,852,417
Perennial / Biennial Weeds			
Johnsongrass	10,152,393	11,057,825	10,368,155
Thistle, Canada	4,123,437	3,584,676	4,840,383
Quackgrass	2,628,187	2,570,688	2,786,633
Dandelion	1,578,579	1,528,332	2,154,008
Thistle	1,479,038	647,315	1,513,566

[1] Total soybean acres in 2006, 2007, and 2008 was 75.5, 64.7, and 75.7 million acres, respectively (USDA NASS, 2011). However, the total soybean herbicide-treated acreage is much more, due to multiple sprays on each acre.

The economic threshold for weeds is the density of a weed population at which control is economically justified due to the potential for yield reduction, quality loss, harvesting difficulties, or other problems that weeds may cause (Penn State Agronomy Guide, 2011). Broadleaf and grass weeds compete at different levels of intensity depending upon the competitiveness of the crop, tillage system, environmental conditions, and other weeds present. Generally, broadleaf weeds are more damaging to a broadleaf crop, while grass weeds are more competitive in a grass crop. Crop yield loss information is available for certain single weed species growing with soybeans in the U.S. Midwest (Table 27).

Table 27. Yield reduction from specific weed species in soybeans.

	Percent soybean yield reduction[1]					
	1	2	4	6	8	10
Weed Species	**Weeds per 100 feet of row**					
Cocklebur	1	2	4	6	8	10
Pigweed or Lambsquarters	2	4	6	10	15	20
Shattercane (5-8/clump)	2	5	8	11	14	17
Giant foxtail	15	25	80	300	400	600
Velvetleaf	1	3	6	10	13	16
Smartweed	2	4	6	10	15	20
Volunteer corn	1	2	3	4	5	6

[1]Source (Penn State Agronomy Guide, 2011). Interference data are from (Knake and Slife, 1962; Stoller *et al.*, 1987; Werner and Curran, 1995).

9.4.3. Weed Management in Soybean

In 2006, USDA NASS surveyed 19 states (Arkansas, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Michigan, Minnesota, Mississippi, Missouri, Nebraska, North Carolina, North Dakota, Ohio, South Dakota, Tennessee, Virginia, and Wisconsin; about 96% of the total U.S. soybean acreage) and found that 98% of the planted soybean acreage was treated with herbicides, an indication of the intensive weed management that is used in U.S. soybeans (USDA NASS, 2007). In 2006, 97.5% of the planted soybean acres received an average of 2.2 herbicide applications to control weeds (USDA ERS ARMS, 2006). Many herbicides are registered for pre-plant, pre-emergent and/or post-emergent application to selectively control most weed species commonly found in soybeans. In general, soybeans sometimes receive a soil applied or burndown herbicide prior to planting or at plant, but almost always receive a post-emergence herbicide application. In 2006, the most widely used herbicide in soybeans was glyphosate, driven by the high adoption of glyphosate tolerant soybeans (USDA NASS, 2007). Soybeans received an average of 1.7 applications of glyphosate on 96% of the acres planted in 2006. Herbicides such as 2,4-D (10% of acres), chlorimuron (4% of acres), and trifluralin (2% of acres) were applied on significantly fewer acres. All other herbicides applied in soybeans that year comprised 3% or less of the total acres planted.

Integrated weed management (IWM) programs advocate the use of a combination of preventive, cultural, mechanical and chemical tools to keep weed pressure below threshold levels that reduce yield and profits (Knezevic, 2010). Herbicides are only one of several tools available for growers

to consider using in an IWM approach. Herbicide-tolerant soybeans, including DAS-444Ø6-6 soybean, represent another tool that can be used in an IWM program.

As with any technology, some concerns have been raised about using herbicides and herbicide-tolerant crops as part of an IWM program (Knezevic, 2010). Some of these concerns include selection for herbicide-resistant weeds, shifts in weed species, drift to sensitive non-target crops or non-agricultural habitats, herbicide persistence in the soil resulting in carryover to the next crop, herbicide misapplications, and over-reliance on herbicides to control weeds.

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

9.4.4. Crop Rotation and Tillage Practices

In 2006, 71% of U.S. soybean acres were grown in rotation after corn, 13% were grown after soybeans, and 16% were grown after small grains, cotton, fallow or other crops (USDA ERS ARMS). Crop rotation is a widespread management practice that has been recognized and exploited for centuries to increase crop yields (Lauer, 2007). In the Midwestern U.S., a corn-soybean rotation produces at least 10% greater yields in both crops, and sometimes as much as 19% higher corn yields. The exact mechanism for the rotation effect is unknown, but may be influenced by increases in organic matter and soil fertility, as well as management of diseases, insects, and weeds.

The introduction of numerous herbicides in the decades following the launch of 2,4-D in the mid-1940s allowed reduced and conservation tillage systems to become more feasible and popular (Givens *et al.*, 2009). The introduction of glyphosate-resistant (GR) crops in 1996 brought a technology that enabled many producers to adopt reduced-tillage production systems. By 2002, only 17% of U.S. soybeans were produced under conventional tillage (<15% residue) systems (USDA ERS ARMS). Some form of conservation tillage was being used on the other 83% of U.S. soybean acres, as follows: 18% reduced tillage (15-30% residue), 32% mulch till, 1-2% ridge till, and 31% no-till. Increased yields coupled with decreases in erosion and water runoff and lower fuel use and fuel cost strongly support the premise that soybean produced with conservation tillage in any production system is more economically and environmentally sustainable (CAST, 2009). In fact, considering the generally accepted 30% minimum residue cover and the 3 to 5 tons/acre/year tolerable soil loss for a sustainable soybean production system (CAST, 2009), conservation tillage is the only system that seems to support sustainable soybean production from an environmental standpoint.

9.5. Potential Impact on Agronomic Practices

9.5.1. Potential Impact on Cultivation and Management Practices

Soybean lines that contain herbicide-tolerance traits (glyphosate) have been on the market since 1996 and have experienced broad adoption (Figure 41). The adoption track record of biotechnology-derived soybean represents the most rapid case of technology adoption in the

history of agriculture (Sankula and Blumenthal, 2004). Based on USDA survey data, herbicide-tolerant soybeans (glyphosate-tolerant) went from 17 percent of U.S. soybean acreage in 1997 to 68 percent in 2001 and 93 percent in 2010 (USDA ERS, 2011b). Glyphosate has provided simple, inexpensive, and highly effective weed control and has resulted in an increase in no-till soybean production, a practice that is now accepted as improving soil health and agricultural sustainability.

With 93% of soybean acres in the U.S. in 2010 planted to herbicide tolerant (glyphosate-tolerant) soybeans, typical cultivation and management practices used by growers today already take into account the management of herbicide-tolerant traits. DAS-444Ø6-6 soybeans are comparable to conventional soybeans phenotypically and agronomically (Section 7.4), and are not expected to alter the geographic range or seasonality of soybean cultivation. Furthermore, ecological observations during field testing have shown no changes in insect susceptibility of DAS-444Ø6-6 soybeans (Section 7.2) and therefore, no impacts are expected on insect control practices for DAS-444Ø6-6 soybeans. It is anticipated that the same management practices used today for soybeans with the glyphosate-tolerance trait will also be appropriate for DAS-444Ø6-6 soybeans.

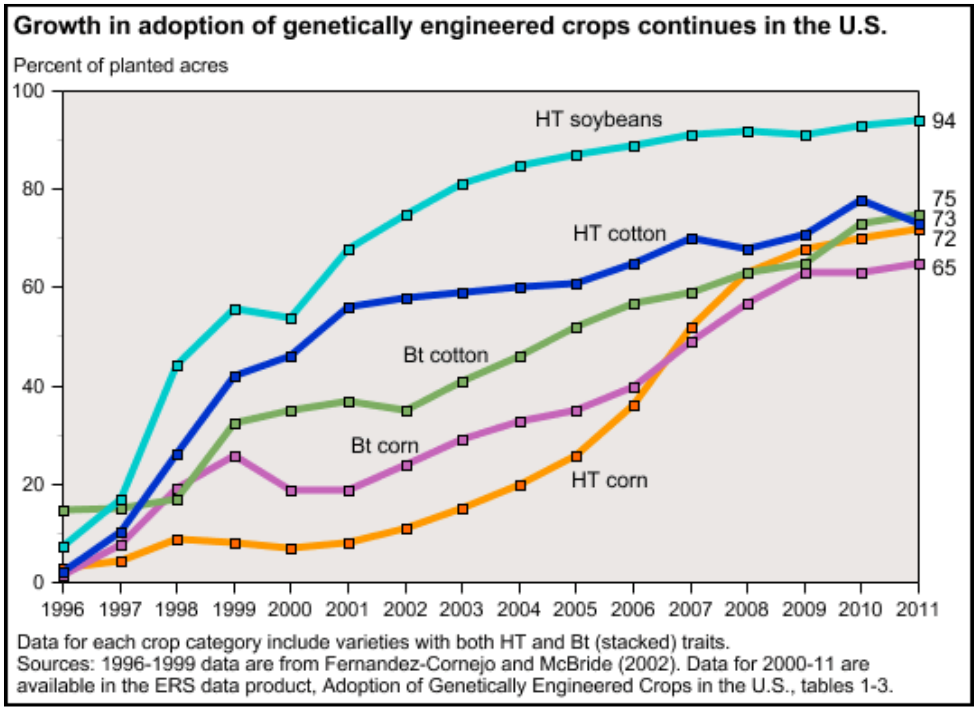


Figure 41. Adoption of genetically engineered crops in the U.S.
(USDA ERS, 2011a)

9.5.2. Potential Impact on Weed Control Practices

DAS-444Ø6-6 soybeans confer tolerance to the herbicides 2,4-D, glyphosate, and glufosinate, all of which will provide expanded weed management options in soybeans.

Post-emergence applications of 2,4-D control a broad spectrum of broadleaf weeds. 2,4-D also has some short-lived soil residual activity (4-10 day soil half life) which provides limited residual control of later germinating broadleaf weeds. Post-emergence applications of glyphosate

will control a broad spectrum of grass and broadleaf weeds. Thus, DAS-444Ø6-6 soybeans will provide flexibility to farmers over glyphosate alone in weed management systems. Inclusion of 2,4-D (and possibly glufosinate) with glyphosate would control the already glyphosate-resistant and hard to control broadleaf weeds, plus slow down the selection for more glyphosate-resistant broadleaf weeds (Powles, 2008a).

Currently, for soybeans without the *aad-12* gene, 2,4-D can be applied only as a burndown or pre-emergence application at up to 1.0 lb ae/A (1120 g ae/ha). 2,4-D currently cannot be applied at burndown or pre-emergence to conventional soybeans any later than 7-15 days (0.5 - 1.0 lbs ae/A, or 560 - 1120 g ae/ha of ester formulations) or 15-30 days (0.5 – 1.0 lbs ae/A, or 560 - 1120 g ae/ha of amine formulations) prior to planting, due to potential for crop injury.

In DAS-444Ø6-6 soybeans, the proposed use pattern will be to allow application of 2,4-D at burndown or pre-emergence (1 lb ae/A or 1120 g ae/ha) without plant back restrictions, followed by one or two post-emergence (0.5 – 1.0 lb ae/A or 560-1120 g ae/ha) applications at least 12 days apart over-the-top of the soybeans up to the R2 stage (full flower) of development (Figure 42). Thus, the proposed maximum seasonal rate of 2,4-D on soybeans will increase from 1.0 lb ae/A to 3.0 lbs ae/ha. DAS-444Ø6-6 soybeans will allow growers to apply 2,4-D from burndown or pre-emergence up through R2 stage soybeans without risk of crop injury. This will provide new options for improved weed control during the soybean development period when weeds have the greatest potential yield impact. The proposed use pattern for 2,4-D on DAS-444Ø6-6 soybeans is identical to that for DAS-68416-4 soybeans.

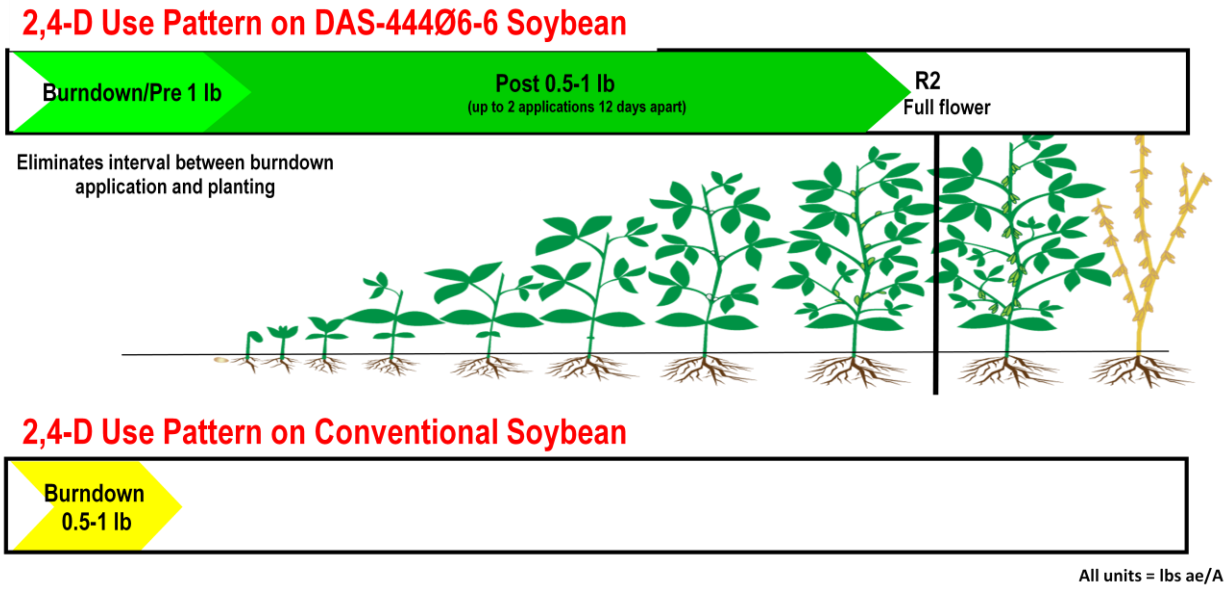


Figure 42. 2,4-D herbicide application timing and rates for conventional and DAS-444Ø6-6 soybeans.

The use pattern for glyphosate and glufosinate on DAS-444Ø6-6 soybeans will be consistent with the current use pattern of glyphosate on other soybean products that are tolerant to glyphosate or glufosinate.

While 2,4-D is currently registered as a pesticide, supporting information on proposed label changes for its use with DAS-444Ø6-6 soybeans is being provided by Dow AgroSciences to U.S. EPA for review, and will be provided to the PMRA shortly. Dow AgroSciences is also developing an extensive stewardship program that will include technological advancements in application to reduce potential for off-target movement, as well as utilizing several media venues to educate and facilitate adoption of the technology and decision management tools to ensure the proper use and stewardship of both the trait and chemical technologies.

Products such as DAS-444Ø6-6 that provide tolerance to multiple modes of action will improve weed control by allowing use of herbicide combinations or mixtures which can provide more consistent performance in post-emergence weed control programs. They counteract glyphosate "rate-creep" (steady increase in rates needed over time to obtain effective weed control; Figure 43) on hard-to-control weeds (Jaehnig, 2005). DAS-444Ø6-6 soybeans, which will enable the use of 2,4-D, will allow use of a low cost, high performance solution to reduce the escalation of glyphosate- and ALS-resistance in weed populations.

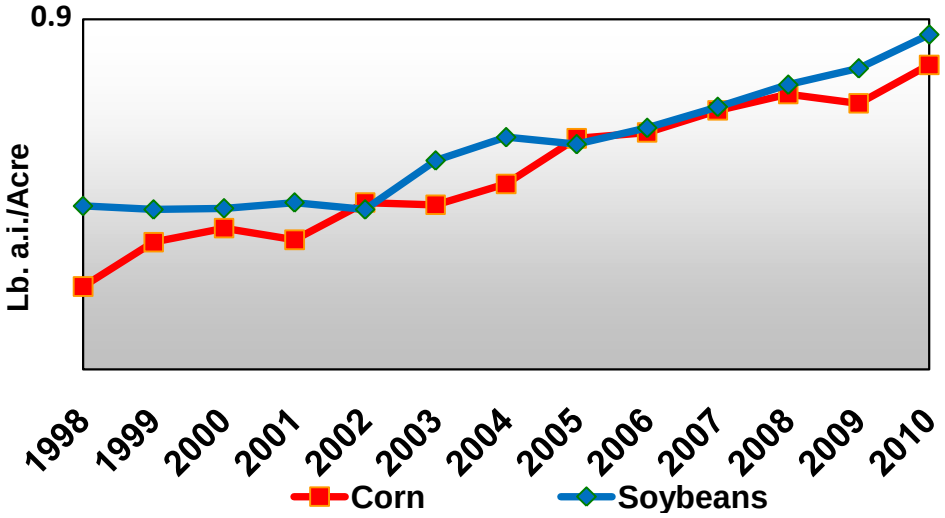


Figure 43. Glyphosate application rates in U.S. corn and soybeans from 1998 -2010. (Third Party Proprietary Data)

9.5.3. Potential Impact on Volunteer Management

Cultivated soybean seed rarely displays any dormancy characteristics, and only under certain environmental conditions grows as a volunteer in the year following cultivation (OECD 2000). If this should occur, volunteers do not compete well with the succeeding crop, and can easily be controlled mechanically or chemically. The soybean plant is not weedy in character. In North America, *Glycine max* is not found outside of cultivation. In managed ecosystems, soybean does not effectively compete with other cultivated plants or primary colonizers.

DAS-444Ø6-6 soybeans are tolerant to 2,4-D, glyphosate and glufosinate. In the unlikely event that they would grow as volunteers in the year following cultivation, they can still be effectively controlled with other herbicides (York *et al.*, 2005).

Soybean is considered a self-pollinated species (OECD, 2000). Crossing does not generally occur. Soybean can only cross with other members of *Glycine* subgenus *Soja*. Approximately 93% of all soybeans planted in the U.S. in 2010 were glyphosate-tolerant soybeans (USDA NASS, 2010). If DAS-444Ø6-6 soybeans were to cross with soybean varieties expressing tolerance to herbicides with different modes of action to produce soybean volunteers with multiple herbicide-tolerance, they can still be effectively controlled mechanically or with other herbicide modes of action; such as paraquat in burndown programs, fluometuron in cotton (Hayes, 2000), and atrazine in corn. Additionally, agronomic practices such as appropriate variety selections, crop rotation, and rotation of herbicides with different modes of action can be used to avoid or manage volunteer soybeans tolerant to one or a few herbicides.

9.5.4. Potential Impact on Non-Target Organisms and Endangered Species

Based on substrate specificity of the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme activity and the biological activity of the 2mEPSPS enzyme, no effect on non-target organisms or endangered species is anticipated for DAS-444Ø6-6 soybean. The *aad-12* gene and expressed protein are present in nature in the soil bacterium *Delftia acidovorans*, while the *2mepsps* and *pat* genes, as well as the expressed proteins are expressed in other crops grown in the United States with no effect on non-target organisms or endangered species. AAD-12, 2mEPSPS, and PAT are not potential food allergens or toxins (Sections 6.1.5, 6.2.5 and 6.3.5) and DAS-444Ø6-6 soybean has been shown to be substantially equivalent to conventional soybean based on the compositional analysis of grain and forage (Section 8). Observations made during field testing of DAS-444Ø6-6 soybean revealed no effects on invertebrate populations (Section 7.2) and agronomic characteristics were found to be equivalent to conventional soybean (Section 7.1).

The Endangered Species Act (ESA) (16 USC 1531) is administered by the U.S. Fish & Wildlife Service (FWS). Under ESA, Section 6 requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact. It is not anticipated that DAS-444Ø6-6 soybean will impact any currently listed species of concern since it is not anticipated that DAS-444Ø6-6 soybean will cause new soybean acres to be planted in areas that are not already in agricultural use. Commercial cropping systems routinely disturb the ground in which crops are grown. Habitat disruption within DAS-444Ø6-6 soybean fields would be comparable to other no-till or herbicide-tolerant cropping systems.

Soybean is not an invasive or weedy species, there are no invasive or weedy sexually compatible relatives of soybean in the US, and these properties are not anticipated to be altered by the insertion of the genes conferring tolerance to herbicides. It is therefore reasonable to conclude that DAS-444Ø6-6 soybean will not affect threatened or endangered species or adversely affect or change designated critical habitats as compared to current commercial agricultural practices.

2,4-D is currently registered for use in soybean production for pre-plant and burndown herbicide treatment. The environmental fate and ecological effects on non-target organisms for the use of 2,4-D in soybeans will be addressed by the EPA as part of their review process.

Glyphosate and glufosinate are currently registered for use in soybean production as a post emergent herbicide treatment in glyphosate- and glufosinate-tolerant soybean varieties. The

environmental fate and ecological effects of these herbicides on non-target organisms and endangered species has been addressed by the EPA as part of its review process.

Corn and soybean are typically planted in rotation in the US. In corn, 2,4-D is used both as a pre-plant burn down prior to planting and post-emergence. The post-emergent and seasonal use patterns for 2,4-D in DAS-444Ø6-6 soybeans are consistent with those currently approved for use in conventional corn, and are consistent with those proposed for use in DAS-40278-9 corn and DAS-68416-4 soybean that also provide tolerance to 2,4-D. Therefore, no significant new geography will be treated with 2,4-D beyond what is already available through pre-emergent applications in soybeans and pre- and post-emergent applications in corn. This consistency in the application rates, timings, and the maximum seasonal rate with currently registered use patterns indicates that there should be no change in the ecological risk assessments or endangered species assessments for 2,4-D with DAS-444Ø6-6 soybean.

9.6. Herbicide Resistance Management

9.6.1. Herbicide Resistance

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

The Weed Science Society of America defines herbicide resistance as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type"(WASS, 1998). Target plants with genes conferring resistance to a given herbicide can occur naturally within a population, but in extremely small numbers. Such plants remain reproductively compatible with the wild-type and can pass resistance genes on to their progeny (Mallory-Smith, 2010). Repeated use of the herbicide may allow these resistant plants to survive and reproduce. The number of resistant plants then increases in the population until the herbicide no longer effectively controls the weed. Thus, this is an evolutionary process (Moss, 2002), whereby a population changes from being susceptible to being resistant. Individual plants do not change from being susceptible to being resistant; rather, the proportion of resistant individuals within the population increases over time.

Herbicide-resistant weeds have been a problem for growers for decades (Heap, 1997). The earliest documented reports of herbicide-resistant weeds were resistance to 2,4-D in wild carrot (*Daucus carota*) in 1952 and spreading dayflower (*Commelina diffusa*) in 1957 (Heap, 2011). During the 1970s, up to 30 different weed species were reported to be resistant to the triazine herbicides (Bandeen *et al.*, 1982). Today, more than 355 weed biotypes around the world have been reported to have some populations that are resistant to one or more herbicides (Heap, 2011). A weed biotype is a sub-type or sub-population of a weed species, in this case one that has developed resistance to one or more herbicides. Report of a resistant biotype for a given weed species does not mean that weed resistance is common, widespread, or persistent in that species. There are generally many other options available to control these resistant biotypes. Table 28 shows a tabular summary of the total number of resistant species for each herbicide mode of action as of May 2011. Figure 44 shows the number of resistant weed biotypes that have been

reported over time for each herbicide mode of action. Additional information on glyphosate, 2,4-D, glufosinate, and the evolution of herbicide resistant weeds can be found in Appendix 8.

Table 28. Number of herbicide resistant weeds reported globally by herbicide group and mode of action.
(Heap, 2011)

Herbicide Group	Mode of Action	HRAC Group[a]	WSSA Group[a]	Example Herbicide	Total Count
ALS Inhibitors	Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	B	2	Chlorsulfuron	109
Photosystem II Inhibitors	Inhibition of photosynthesis at photosystem II	C1	5	Atrazine	69
ACCase Inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	A	1	Diclofop-methyl	40
Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	O	4	2,4-D	28
Bipyridiliums	Photosystem-I-electron diversion	D	24	Paraquat	25
Ureas and Amides	Inhibition of photosynthesis at photosystem II	C2	7	Chlorotoluron	21
Glycines	Inhibition of EPSP synthase	G	9	Glyphosate	21
Dinitroanilines and others	Microtubule assembly inhibition	K1	10	Trifluralin	10
Thiocarbamates and others	Inhibition of lipid synthesis – not ACCase inhibition	N	8	Triallate	8
Chloroacetamides and others	Inhibition of cell division (inhibition of very long chain fatty acids)	K3	15	Butachlor	5
Triazoles, Ureas, Isoxazolidiones	Bleaching: Inhibition of carotenoids biosynthesis (unknown target)	F3	11	Amitrole	4
PPO Inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	E	14	Oxyfluorfen	4
Nitriles and others	Inhibition of photosynthesis at photosystem II	C3	6	Bromoxynil	3
Carotenoid Biosynthesis Inhibitors	Bleaching: Inhibition of carotenoids biosynthesis at the phytoene desaturase	F1	12	Flurtamone	2
Arylaminopropionic Acids	Unknown	Z	25	Flamprop-methyl	2
Mitosis Inhibitors	Inhibition of mitosis / microtubule polymerization inhibitor	K2	23	Propham	1
Cellulose Inhibitors	Inhibition of cell wall (cellulose) synthesis	L	27	Dichlobenil	1
Unknown	Unknown	N	8	(Butylate	1
Unknown	Unknown	Z	227	(chloro)-flurenol	1
Total Number of Unique Herbicide Resistant Biotypes					**355**

[a] Two different classification systems have been developed independently by HRAC (alphabetical) and the WSSA (numerical) to communicate the mode of action of herbicides. Weeds which have developed resistance to one herbicide may also be resistant to other herbicides which have the same mode of action.

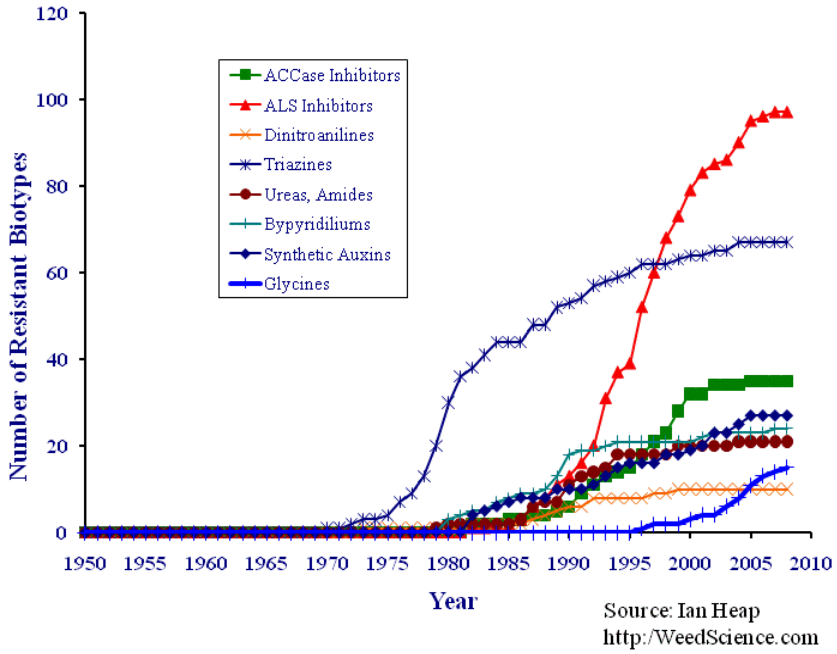


Figure 44. Resistant weed biotypes per herbicide mode of action.

9.6.2. Factors Impacting Development of Resistance

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

A matrix that can be used to evaluate the risk of selection for herbicide-resistant weeds based on cropping system practices in shown in Table 29. This table assesses the risk of herbicide-resistance development for each management practice as either "low", "medium", or "high". The greatest chance for resistance development occurs when several of these management practices fall into the "high" category.

Table 29. Assessment of resistance risk by evaluation of cropping systems.
(Nevill *et al.*, 1998)

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

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

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

9.6.3. Herbicide Resistance Management

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

As the number of glyphosate-resistant weed species increases, it becomes increasingly important for growers to introduce greater diversity into their weed management programs (Powles, 2008a). This diversity could be achieved with herbicide rotations/sequences, mixtures of robust herbicides with different modes of action, and use of non-herbicide weed control tools. Glyphosate is increasingly being mixed with effective doses of other herbicides to manage these hard-to-control and resistant weed species. New herbicide-tolerant traits that allow selective use of additional herbicides with a wider weed control spectrum compared to conventional herbicides can be used to control glyphosate-resistant weed populations and reduce selection pressure for additional glyphosate-resistant weed species. Table 30 shows that several common weeds in U.S. corn and soybeans which are resistant to or difficult to control with glyphosate or ALS herbicides can be effectively controlled with 2,4-D.

Introduction of DAS-444Ø6-6 soybeans will give farmers one more tool for use in their weed management programs which will help insure the long term sustainability of weed management programs, including the use of glyphosate. Importantly, because DAS-444Ø6-6 will provide tolerance to 2,4-D and glyphosate, these herbicides can be tank mixed to provide an effective, flexible and cost effective approach to weed management. 2,4-D controls several key broadleaf weed species that are resistant to, or difficult to control with, glyphosate and ALS herbicides (Table 30). Mixtures of 2,4-D with glyphosate provide control of glyphosate-resistant biotypes that is superior to glyphosate alone.

DAS-444Ø6-6 soybeans will allow use of 2,4-D to control glyphosate-resistant weeds and significantly delay the selection for glyphosate-resistance in other weed species. The same benefits of DAS-444Ø6-6 soybeans can be extended to other herbicide-tolerant cropping systems, such as those with tolerance to glufosinate or ALS-inhibiting herbicides.

Table 30. Glyphosate- and ALS-resistant weeds controlled by 2,4-D.
(Heap, 2011)

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

DAS-444Ø6-6 soybeans will expand the range of herbicides that can be used in herbicide-tolerant soybean production systems, improving the ease and effectiveness of managing resistant and hard-to-control weeds and delaying the evolution of resistance to glyphosate and other herbicides.

9.7. Summary of Environmental Consequences and Impact on Agronomic Practices

Field testing results confirm that AAD-12, 2mEPSPS, and PAT proteins expressed in DAS-444Ø6-6 soybean provides robust tolerance to 2,4-D, glyphosate, and glufosinate respectively. There are no new phenotypic characteristics in DAS-444Ø6-6 soybean to indicate it is any different from conventional soybean in weediness potential, and like conventional soybean, the risk of gene flow from DAS-444Ø6-6 soybean to wild relatives in the U.S. is negligible.

No significant impact is expected on current crop management practices, non-target or endangered species, crop rotation, or volunteer management from the introduction of DAS-444Ø6-6 soybean. The availability of DAS-444Ø6-6 soybean will have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The availability of DAS-444Ø6-6 soybean will allow growers to proactively manage

weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance.

10. Adverse Consequences of Introduction

Field and laboratory testing of DAS-444Ø6-6 soybean has demonstrated that it has no significant differences from the non-transgenic near-isogenic control soybean Maverick apart from the intended change of herbicide-tolerance. DAS and MS Tech know of no study results or other observations associated with DAS-444Ø6-6 soybean that would be anticipated to result in adverse consequences from introduction.

11. Appendices

Appendix 1. Methods for Molecular Characterization of DAS-444Ø6-6 Soybean

Appendix 2. Methods and Results for Characterization of AAD-12 Protein

Appendix 3. Methods and Results for Characterization of 2mEPSPS Protein

Appendix 4. Methods and Results for Characterization of PAT Protein

Appendix 5. Methods for AAD-12, 2mEPSPS and PAT Protein Expression Analysis

Appendix 6. USDA Notifications for DAS-444Ø6-6 Soybean

Appendix 7. Literature Ranges for Compositional Analysis

Appendix 8. Glyphosate, 2,4-D, Glufosinate and Herbicide Resistant Weeds

Appendix 9. Stewardship of Herbicide Tolerant DAS-444Ø6-6 Soybean

Appendix 10. References

Appendix 1. 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°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) labeled 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.

Labeled probes were hybridized to the target DNA on the nylon membranes using the DIG Easy Hyb Solution according to manufacturer's instructions (Roche Diagnostics). DIG-labeled DNA molecular weight marker II and VII were used to determine the size of the hybridizing fragments on the Southern blots.

Detection

DIG-labeled probes bound to the nylon membranes after stringent washing were incubated with AP (Alkaline Phosphatase)-conjugated anti-Digoxigenin antibody for ~1 hr 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 labeled probes from the membranes, allowing them to be re-hybridized with a different DNA probe.

Appendix 2. 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. 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 of AAD-12 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 sequence analysis 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.

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 45). 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 46). 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.

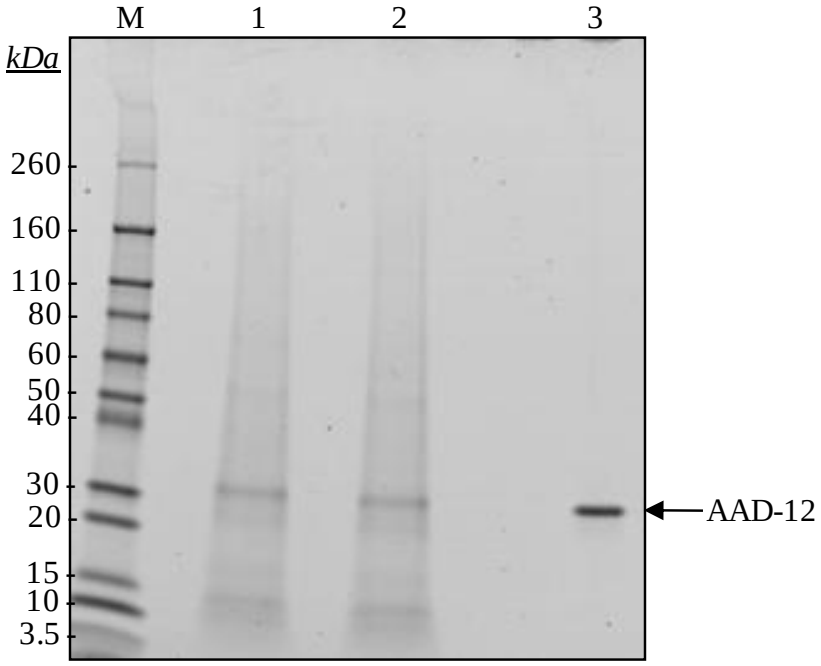
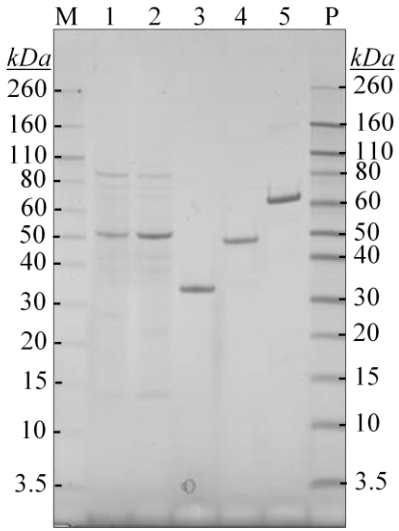


Figure 45. SDS-PAGE of DAS-444Ø6-6 soybean- and microbe-derived AAD-12.
Note: Affinity-purified soybean-derived AAD-12 and microbe-derived AAD-12 were separated by SDS-PAGE. Following electrophoresis, the gel was stained with Thermo-Pierce GelCode Blue stain for total protein according to the manufacturer's 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

Panel A. SDS-PAGE



Panel B. Western blot.

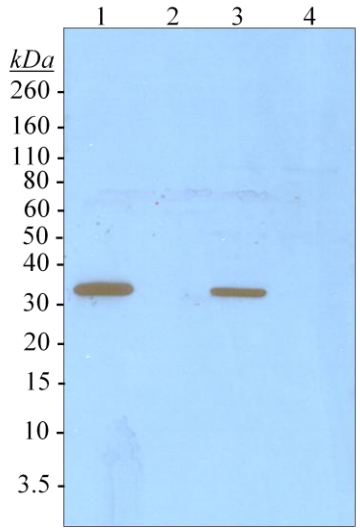
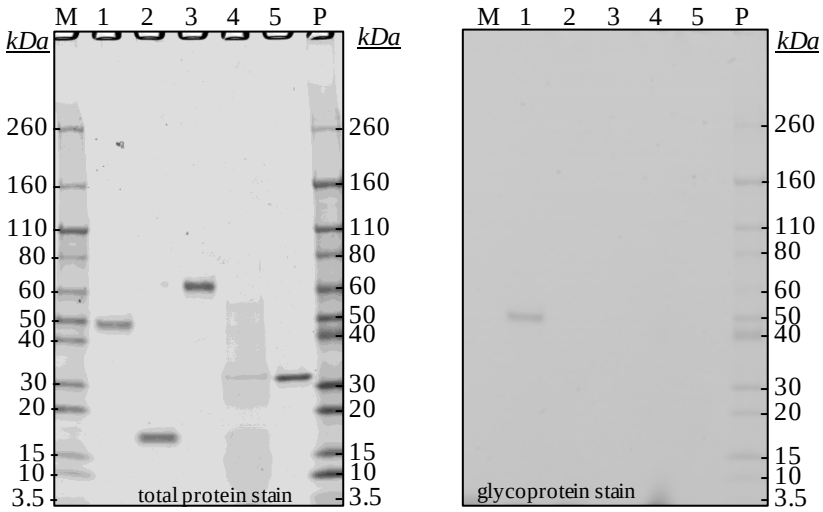


Figure 46. SDS-PAGE and western blot analysis of DAS-444Ø6-6 soybean- and microbe-derived AAD-12 protein.
Note: Crude extracts from fresh leaf tissue of event DAS-444Ø6-6 and Maverick were separated by SDS-PAGE along with microbe-derived AAD-12 and bovine serum albumin. Following electrophoresis the 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-labeled 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 47). 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 47. 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 48 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 49). The detected peptide fragments covered nearly the entire protein sequence lacking only four peptide fragments (Figure 49), 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)

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

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

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

Figure 48. 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 49. 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 31).

Table 31. Summary of N-terminal sequence data of DAS-444Ø6-6 soybean- and *P. fluorescens*-derived AAD-12 protein.

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 (Appendix 2).

[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 31 and Figure 49). In addition, a portion of the protein extracts was missing the N-terminal methionine and the second amino acid, alanine, was acetylated (Table 31). 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 *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 32, Figure 49, and Figure 50).

Table 32. Summary of C-terminal sequence data of DAS-444Ø6-6 soybean- and *P. fluorescens*-derived AAD-12 protein.

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 50. 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.

Conclusions

The biochemical characteristics of microbe-derived AAD-12 protein were equivalent to the protein purified from root tissue of event DAS-444Ø6-6. The plant- and microbe-derived AAD-12 proteins showed the expected molecular weight of ~32 kDa by SDS-PAGE and were immunoreactive to AAD-12 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, MALDI-TOF MS/MS, and ESI-LC/MS. In addition, the lack of glycosylation of the plant-derived AAD-12 protein provided additional evidence that the AAD-12 protein produced by *P. fluorescens* and DAS-444Ø6-6 soybean are biochemically equivalent.

Appendix 3. 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. 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. 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 33 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 33. 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 event DAS-444Ø6-6 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 34) using a Geno/Grinder (Certiprep, Metuchen, NJ). The supernatants collected were mixed with Laemmli sample buffer (containing β-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 34. 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 β -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. 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.

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 (Figure 51). 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).

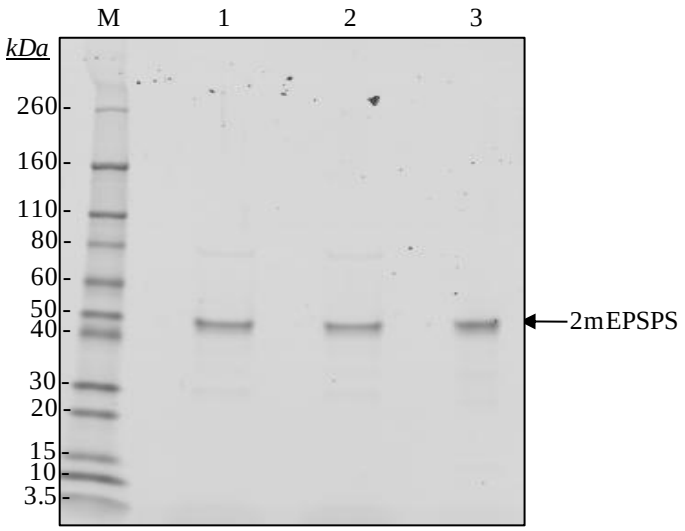


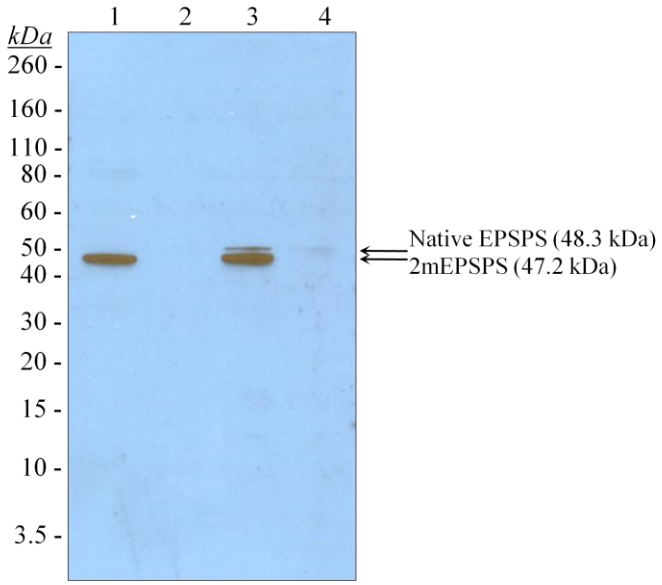
Figure 51. SDS-PAGE of DAS-444Ø6-6- and microbe-derived 2mEPSPS.
Note: The purified soybean-derived 2mEPSPS and microbe-derived 2mEPSPS were separated by SDS-PAGE. Following electrophoresis, the gel was stained with Thermo-Pierce GelCode Blue stain for total protein according to the manufacturer's protocol.

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 52, 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 preparation and DAS-444Ø6-6 tissue, with no immunoreactive proteins observed in the Maverick control extract (Figure 52, 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

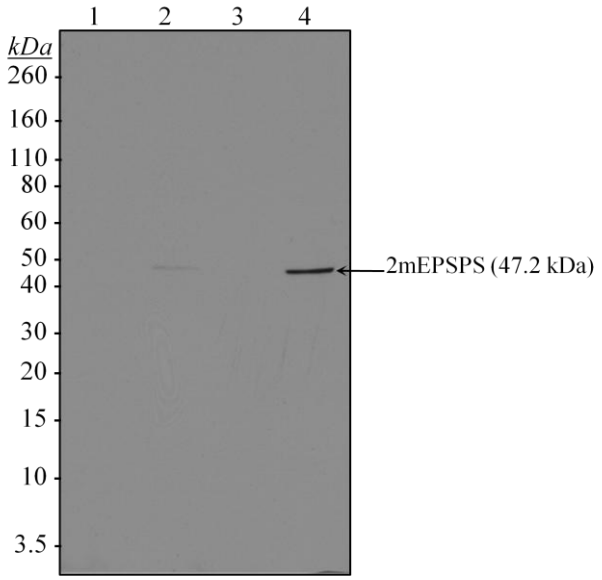
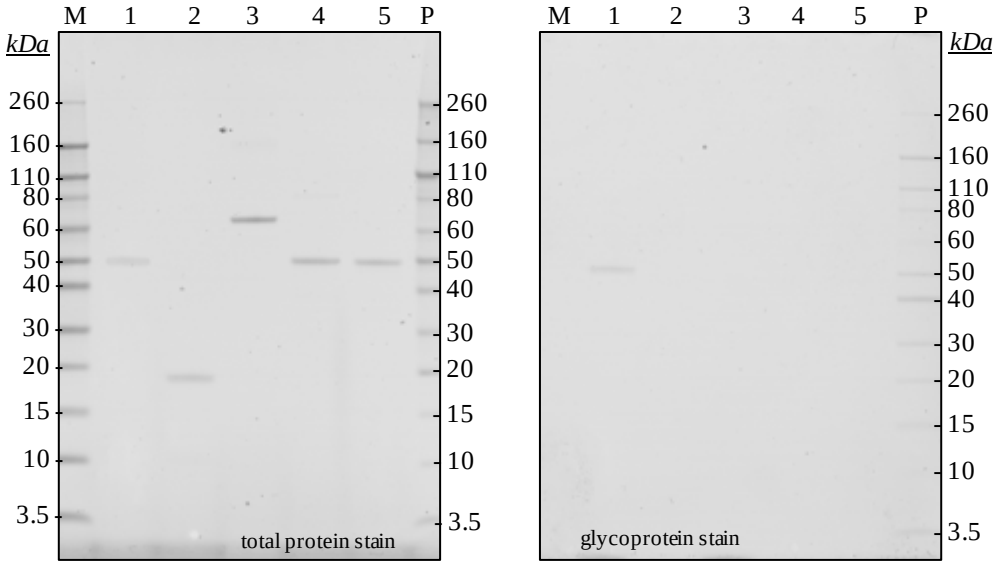


Figure 52. Western blot analysis of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS protein.
Note: Crude extracts from leaf tissue of event DAS-444Ø6-6 and Maverick were separated by SDS-PAGE along with microbe-derived 2mEPSPS and bovine serum albumin. The nitrocellulose membranes were then probed with 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 (TSN033171)	~1 ng	1	Bovine Serum Albumin (BSA)	~1 ng
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 53).



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

Figure 53. Glycosylation analysis of DAS-444Ø6-6 soybean- and microbe-derived 2mEPSPS protein.

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 51). 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 mixtures were 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 54 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 shown in Figure 54 and Figure 55. 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 55). The detected peptide fragments covered nearly the entire protein sequence lacking only six peptide fragments (Figure 55), 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 56) at both the N- and C-terminus as well as a major portion of the internal sequence. 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.

Digestion at K (lysine) and R (arginine)

```
  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
 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
 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
 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
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
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
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
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
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
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
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
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
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
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
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
```

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

```
  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
 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
 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
 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
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
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
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
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
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
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
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
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
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
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
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
```

Figure 54. Theoretical trypsin (top panel) and chymotrypsin (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 55. 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 56. 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. 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 35 and Figure 54).

Table 35. 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 36 and Figure 54).

Table 36. Summary of C-terminal sequence data of DAS-444Ø6-6 soybean- and *P. fluorescens*-derived 2mEPSPS.

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 soybean event DAS-444Ø6-6 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.

Appendix 4. 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.

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.

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 -80ºC to maintain activity.

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 57). The soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal

antibodies and showed the expected band at approximately 21 kDa (Figure 58). 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.

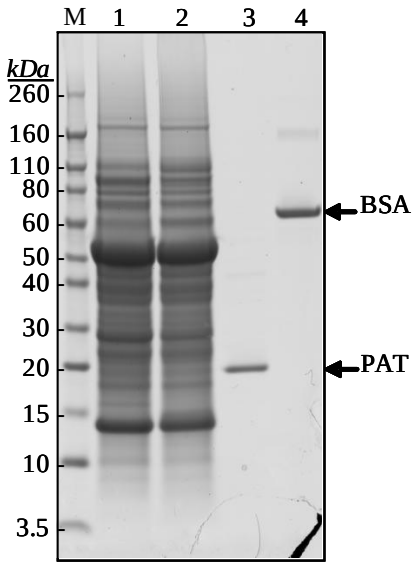


Figure 57. SDS-PAGE of DAS-444Ø6-6 and non-transgenic Maverick soybean extracts, microbe-derived PAT protein.

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

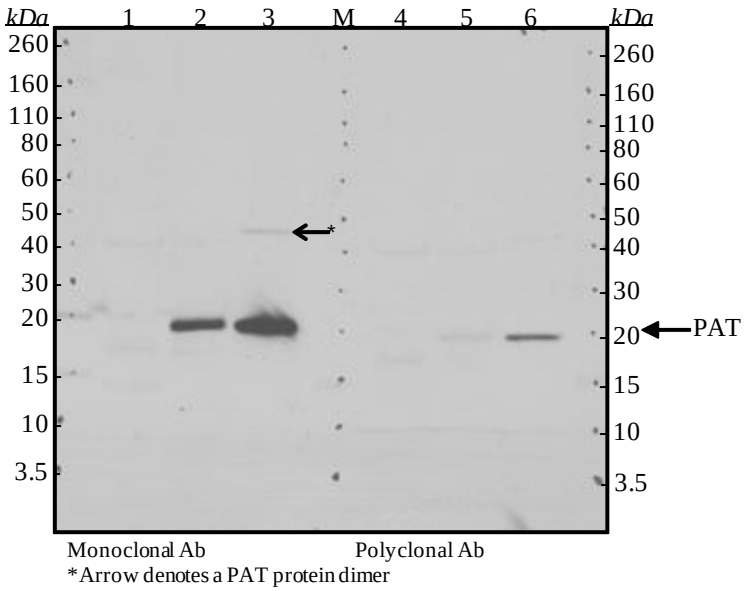


Figure 58. Western blot analysis of DAS-444Ø6-6 and Maverick soybean extracts.
Note: Crude extracts from lyophilized leaf tissue of event DAS-444Ø6-6 and Maverick were separated by SDS-PAGE along with microbe-derived PAT and bovine serum albumin. Following electrophoresis the gel was cut in half, one half was stained with Thermo Scientific GelCode Blue stain for total protein (Figure 57) and the remaining half was electro-blotted onto a nitrocellulose membrane (Figure 58). 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 Pre-stained 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).

Appendix 5. 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. 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 in Soybean Tissue Samples

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

Appendix 6. USDA Notifications for DAS-444Ø6-6 Soybean

USDA Notification Number	Notification Authorization Date	Notification Expiration Date	State(s)	Total Number of Trials Planted	Status of Trial[1]
11-095-105n	4/29/2011	4/29/2012	MS	2	Pending
11-087-114n	4/20/2011	4/20/2012	AL, AR, GA, IL, IN, MD, NE	34	Pending
11-067-105n	3/30/2011	3/30/2012	AR, CA, IA, IN, IL, LA, MN, MO, MS, OH, WI	28	Pending
10-243-104n	9/30/2010	9/30/2011	PR	9	Pending
10-085-103n	4/19/2010	4/19/2011	GA, IA, IN,IL, MI, MO, NE	18	Submitted
10-083-105n	4/22/2010	4/22/2011	IA, IN, MO,MS	8	Submitted
10-077-107n	4/14/2010	4/14/2011	GA, IA, IN,IL, MD, MO,NE, OH, PR	27	Submitted
09-259-108n	10/5/2009	10/5/2010	PR	3	Submitted
09-068-103n	4/1/2009	4/1/2010	IN, PR	4	Submitted
08-254-109n	9/30/2008	9/30/2009	PR	1	Submitted

[1]Pending reports as of June 21, 2011 to be submitted within six months of the notification expiration date.

Appendix 7. Literature Ranges for Compositional Analysis

Table 37. 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 38. 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 39. 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 40. 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 41. 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.

Appendix 8. Glyphosate, 2,4-D, Glufosinate and Herbicide Resistant Weeds

Herbicide Tolerant Crops

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

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

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

Herbicide-tolerant crops which were made available to farmers up to 2005 are listed in Table 42 (Duke, 2005). Transgenes were only used to confer tolerance to bromoxynil, glufosinate, and glyphosate. The bromoxynil-tolerant crops are no longer sold. This leaves only glyphosate- and glufosinate-tolerant transgenic crops, and of those, glyphosate has had the strongest impact on weed management (Duke, 2005).

Table 42. Herbicide-tolerant crops available to farmers in North America in 2005.

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

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

Growers choose glyphosate-tolerant crops because they make weed control easier and more effective, increase profit, require less tillage, and do not restrict crop rotations (Green, 2009). Thus, glyphosate-tolerant corn and soybeans have experienced an unprecedented rapid adoption rate by U.S. farmers (Figure 59). The planting of glyphosate-tolerant crops has increased steadily since their introduction in 1996 (glyphosate-tolerant soybean plantings are currently >90% of all soybean planting in the U.S.).

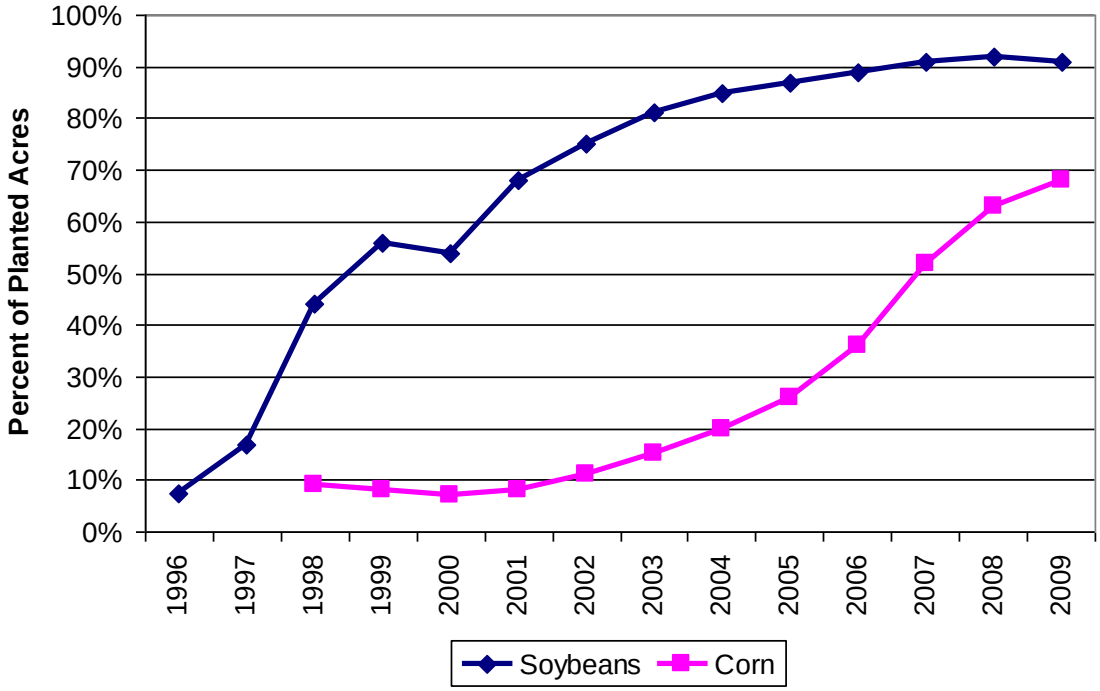


Figure 59. U.S. adoption rates of glyphosate-tolerant soybean and corn.
Sources: (Duke and Powles, 2008b; USDA NASS, 2010; USDA ERS, 2011a)

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

Characteristics of Glyphosate, 2,4-D, and Glufosinate Herbicides

Glyphosate

Glyphosate is a nonselective, foliar applied herbicide which is registered for use on over one hundred food and feed crops, several non-food field crops (fallow, fencerows, woody ornamentals, golf courses, etc.), forestry uses (conifer release and reforestation), and non-crop areas where total vegetation control is desired (aquatics, rights of way, industrial sites, etc.) (US EPA, 1993). When applied at lower rates, glyphosate also is a plant growth regulator. Pre-plant or pre-emergence uses of glyphosate in food and feed crops include most of the major agricultural crops around the globe, including alfalfa, barley, buckwheat, corn, dry beans, grass

forage/fodder/hay, lentils, millet, oats, pastures, rye, sorghum, soybeans, and wheat. In addition, it can be foliar applied over-the-top of recent glyphosate-tolerant transgenic crops, including corn, soybeans, cotton, canola, sugarbeets and alfalfa.

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

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

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

2,4-Dichlorophenoxyacetic Acid (2,4-D)

2,4-Dichlorophenoxyacetic acid (2,4-D) was introduced in 1946 as the first selective herbicide, and rapidly became the most widely used herbicide in the world (Industry Task Force II, 2005). Today, it is still the third most widely used herbicide in the U.S. and Canada, as well as the most widely used herbicide globally. Approximately 46 million pounds of 2,4-D is used in the U.S. annually, with 30 million pounds (66%) used by agriculture and 16 million pounds (34%) used in non-agriculture settings such as pasture/rangeland and lawn/garden (US EPA, 2006). 2,4-D is an ingredient in approximately 660 agricultural and home use products as a sole active ingredient and in conjunction with other active ingredients. Agriculturally, it is used on a variety of crops including corn, rice, sorghum, sugar cane, wheat, rangeland and pasture as well as being used on rights-of-way, roadsides, non-crop areas, forestry, lawn and turf care and on aquatic weeds (Industry Task Force II, 2005). A major use of 2,4-D is in combination with other herbicides because it economically enhances the weed control spectrum of many other herbicides such as glyphosate, dicamba, mecoprop, ALS herbicides, etc (US EPA, 2006). 2,4-D controls many broadleaf weeds including carpetweed, dandelion, cocklebur, horseweed, morning glory,

pigweed sp., lambsquarters, ragweed spp., shepherd's-purse and velvetleaf. It has little to no activity on grasses (Industry Task Force II, 2005).

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

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

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

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

Glufosinate

Glufosinate was first reported as a herbicide in 1981(Vencill, 2002). Glufosinate (phosphinothricin; DL-homoalanin-4-yl-(methyl)-phosphinic acid) is a racemic phosphinic amino acid (Vencill, 2002). Its ammonium salt (glufosinate-ammonium) is widely used as a non-

selective herbicide and is the active ingredient of the commercial herbicide formulations, Liberty and Ignite. The L-isomer of glufosinate is a structural analogue of glutamate and, therefore, is a competitive inhibitor of the enzyme glutamine synthetase (GS) of bacteria and plants. The D-isomer is not a GS inhibitor and is not herbicidally active.

Due to the inhibition of GS, non-tolerant plant cells accumulate large amounts of toxic ammonia produced by nitrate assimilation and photorespiration and the level of available glutamine drops (OECD, 2002). Damage to cell membranes and inhibition of photosynthesis are followed by plant cell death.

In genetically modified glufosinate-tolerant plants (OECD, 2002), the L-isomer of glufosinate is rapidly metabolized by the action of the enzyme phosphinothricin acetyltransferase (PAT) into the non-phytotoxic stable metabolite N-acetyl-L-glufosinate (2-acetamido-4-methylphosphinico-butanoic acid). N-acetyl-L-glufosinate does not inhibit glutamine synthetase. Therefore, no phytotoxic physiological effects are observed in genetically modified glufosinate-tolerant plants.

Glufosinate is a contact herbicide which is taken up by the plant primarily through the leaves. There is no uptake from the soil through the roots, presumably because of the rapid degradation of glufosinate by soil microorganisms. There is limited translocation of glufosinate within the plant.

Glufosinate is a nonselective herbicide (Vencill, 2002). It controls a broad spectrum of annual and perennial grasses and broadleaf weeds. Due to its limited systemic action, there is no enduring effect on perennial weeds. Weeds which emerge after herbicide application are not affected.

Glufosinate is rapidly broken down in soil due to microbial degradation (Vencill, 2002). At 20°C, the soil half life is less than 7 days. The end products of microbial degradation are CO_2 and natural phosphorus compounds.

Evolution of Resistance to Glyphosate, 2,4-D, and Glufosinate

Glyphosate Resistance and Weed Shifts

Glyphosate Resistance

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

Table 43 shows a summary of the twenty-one glyphosate-resistant weed species that have been reported from 1996 to November 2010 (Heap, 2011). The data clearly shows that glyphosate-resistance in weeds is expanding around the globe. Most notably, there have been reports of ten new weed species with some biotypes resistant to glyphosate in the U.S. since 2000 (Figure 60). Two of these glyphosate-resistant weed species have already become a significant problem for farmers across a large geographic area. *Conyza canadensis* infests at least two million hectares of glyphosate-tolerant crops in the U.S. (Main *et al.*, 2004) and glyphosate-resistance in Palmer amaranth has serious ramifications for future weed management in the Southeast U.S. due to its rapid growth rate, extremely competitive nature, and resistance to other herbicide modes of action (Culpepper *et al.*, 2008). Researchers have also reported that individual biotypes of ten of the 21 glyphosate-resistant species are also resistant to herbicides with other modes of action (Table 44). The Palmer amaranth, common waterhemp, and horseweed/marestail biotypes were reported in the corn and soybean growing states. Although herbicide options to control these biotypes with multiple herbicide resistance will be more limited, 2,4-D is still a viable control option for the broadleaf weeds.

Table 43. Weed species with reported glyphosate-resistant biotypes.

		First Confirmed Report		Later Confirmed Reports
Common Name	**Species Name**	**Year**	**Country****	
Rigid ryegrass	*Lolium rigidum*	1996	Australia	USA, S.Africa, France, Spain
Goosegrass	*Eleusine indica*	1997	Malaysia	Colombia
Horseweed/Marestail*	*Conyza Canadensis*	2000	USA	Brazil, China, Spain, Czech Republic
Italian ryegrass	*Lolium multiflorum*	2001	Chile	Brazil, USA, Spain, Argentina
Hairy fleabane	*Conyza bonariensis*	2003	S.Africa	Spain, Brazil, Colombia, USA
Buckhorn plantain	*Plantago lanceolata*	2003	S.Africa	
Common ragweed*	*Ambrosia artemisiifolia*	2004	USA	
Giant ragweed*	*Ambrosia trifida*	2004	USA	
Ragweed parthenium	*Parthenium hysterophorus*	2004	Colombia	
Palmer amaranth*	*Amaranthus palmeri*	2005	USA	
Common waterhemp*	*Amaranthus rudis*	2005	USA	
Johnsongrass*	*Sorghum halepenses*	2005	Argentina	USA
Sourgrass	*Digitaria insularis*	2006	Paraguay	Brazil
Wild poinsettia*	*Euphorbia heterophylla*	2006	Brazil	
Junglerice	*Echinochloa colona*	2007	Australia	
Kochia	*Kochia scoparia*	2007	USA	
Liverseedgrass	*Urochloa panicoides*	2008	Australia	
Perennial ryegrass	*Lolium perenne*	2008	Argentina	
Sumatran fleabane	*Coryza sumatrensis*	2009	Spain	
Australian fingergrass	*Chloris truncate*	2010	Australia	
Annual bluegrass	*Poa annua*	2010	USA	

* Important weeds in US corn, soybean, and cotton production.
** Ten new species confirmed resistant in US since 2000.

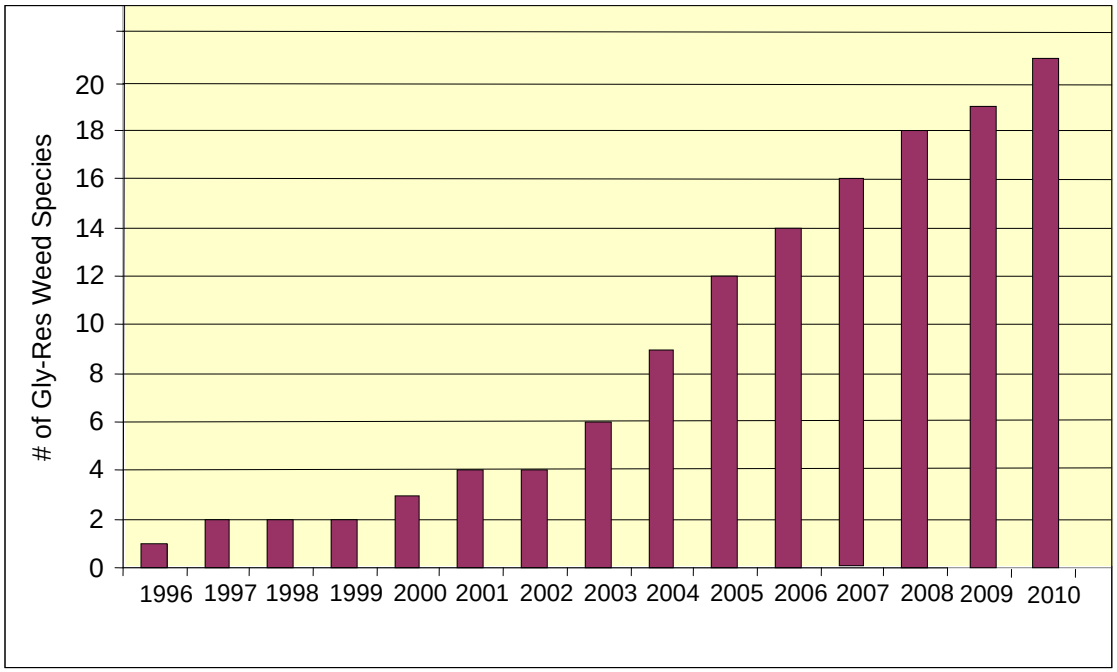


Figure 60. Number of glyphosate-resistant weeds reported globally by year from 1996 to 2010.
(Compiled from (Heap, 2011))

Table 44. Global reports of glyphosate-resistant weed biotypes with resistance to other herbicide modes of action.
(Heap, 2011)

Common Name	Species Name	Year – Country (State)	Multiple Resistance to Other Herbicide MOAs
Palmer amaranth	*Amaranthus palmeri*	2008 – US (MS)	ALS
Common waterhemp	*Amaranthus rudis*	2005 – US (MO)	ALS, PPO
		2006 – US (IL)	ALS
Common ragweed	*Ambrosia artemisiifolia*	2006 – US (OH)	ALS
Hairy fleabane	*Conyza bonariensis*	2009 – US (CA)	Bipyridiliums
Horseweed	*Conyza canadensis*	2003 – US (OH)	ALS
		2007 – US (MS)	Bipyridiliums
Giant ragweed	*Ambrosia trifida*	2006 – US (OH)	ALS
		2008 – US (MN)	ALS
Goosegrass	*Eleusine indica*	1997 – Malaysia	ACCase
Wild poinsettia	*Euphorbia heterophylla*	2006 – Brazil	ALS
Italian ryegrass	*Lolium multiflorum*	2002 – Chile	ALS
		2006 – Chile	ACCase
		2008 – Chile	ACCase, ALS
Rigid ryegrass	*Lolium rigidum*	1999 - Australia	ACCase, ALS, Dinitroanilines
		2003 – S. Africa	ACCase, Bipyridiliums
		2008 – Australia	Triazoles, Ureas, Isoxazolidiones
		2010 – Australia	Bipyridiliums

In addition, researchers in Virginia have been testing a biotype of common lambsquarters that survived 1.0 lb ae/acre (1120 g ae/ha) glyphosate, and thus appears to have low level resistance to glyphosate (Hite *et al.*, 2008). Weed scientists in Ohio and Indiana have also identified a biotype of common lambsquarters in at least a dozen fields that appears to have low-level glyphosate-resistance (Curran *et al.*, 2007). The increased reports of glyphosate-resistant species, plus the geographic spread of their infestations, have caused some to raise concerns about the long term sustainability for glyphosate. Some researchers have stated that applying glyphosate alone over wide areas on highly variable and prolific weeds made the evolution of resistant weeds inevitable (Owen, 2001; Thill and Lemerle, 2001).

Weed Shifts

When glyphosate-tolerant crops are grown intensively with high reliance on glyphosate for weed control, species which possess some level of natural tolerance to glyphosate will become more prevalent. These "weed shifts" can occur more rapidly than selection for glyphosate-resistance (Shaner, 2000). Coble and Warren (1997) demonstrated that continuous use of glyphosate caused an increase in the infestation of morningglory (*Ipomoea spp.*) species over a three year period compared with other herbicide programs. Some common hard to control weed species that could become "weed shifts" in U.S. corn and soybeans are listed below in Table 45.

Table 45. Potential weed shifts with use of glyphosate in U.S. corn and soybeans.
(Duke and Powles, 2008b; Owen, 2008)

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

2,4-D Resistance

The earliest documented reports of herbicide-resistant weeds were for resistance to 2,4-D in wild carrot (*Daucus carota*) (observed in 1952 but not reported until 1957) and spreading dayflower (*Commelina diffusa*) in 1957 (Heap, 2011). Today, a total of sixteen weed species have documented reports of 2,4-D resistant biotypes someplace around the globe (Table 46). Wild carrot in soybeans and roadsides, field bindweed in cropland, and prickly lettuce in cereals are the only ones reported on the U.S. mainland (Heap, 2011). Wild carrot, yellow bur-head, wild radish, musk thistle, and corn poppy are the only 2,4-D resistant weeds that have reported infestations in more than 1,000 acres. Some of these 2,4-D resistant biotypes have documented cross resistance to other auxin herbicides or multiple resistance to some ALS-inhibiting herbicides. It is notable that most of these resistant species do not appear to be spreading, as indicated by few reports of additional sites after the initial report.

Table 46. Weed species with reported 2,4-D-resistant biotypes.
(Heap, 2011)

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

Few of these auxin resistant weeds have had a significant economic impact due to the wide array of alternatives that successfully control these resistant weeds (Heap, 1997). The overall incidence of auxinic herbicide-resistance after more than 60 years of use is low compared with other herbicide families such as the ALS inhibitors (imidazolinones, sulfonylureas, and sulfonamides), triazines, and ACCase herbicides in a much shorter period of use (Section 9.6). Furthermore, there is no widespread resistance to auxinic herbicides. It has been suggested by various researchers that the rarity of auxinic herbicide-resistant biotypes in the field is due to: a) a commonly held belief that these herbicides have multiple sites of action in the plant (Jasieniuk *et al.*, 1996), b) redundancy in auxin receptors (AFBs) and other components of the auxin signal response (Walsh *et al.*, 2006), c) moderate selection pressure and their use in mixtures with other herbicides (Kern *et al.*, 2005), d) fitness penalties (Bourdot *et al.*, 1996), and e) quantitative inheritance of the resistance trait (Cranston *et al.*, 2001)

The mechanism of resistance to auxinic herbicides has been investigated, in varying degrees for only a few of these resistant biotypes. Resistance mechanisms in these biotypes have proven to

be difficult to elucidate. A lack of differences between biotypes in auxinic herbicide absorption, translocation, and metabolism has led to the hypothesis that auxinic herbicide-resistance is most often likely due to differences at the target site or differences along the signal transduction pathway (Van Eerd *et al.*, 2005).

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

To summarize, selection for auxin resistant weed bioytpes after more than 60 years of use has been slow; none show significant spread from initial sites, none are of significant economic importance, and none have been found in corn fields to date. Use of 2,4-D in DAS-444Ø6-6 soybeans should not result in 2,4-D resistant weeds becoming a significant issue in soybeans. 2,4-D will likely be used in a mixture with one or more other herbicides. Other alternative herbicides which are effective on the same weeds can be used to control any 2,4-D resistant weeds that might occur.

Glufosinate Resistance

There is currently only one report of a weed biotype that has developed resistance to glufosinate, Goosegrass (*Eleusine indica*) in Malaysia (Heap, 2011). Thus, glufosinate is an excellent tool to include in a weed management program.

Appendix 9. Stewardship of Herbicide Tolerant DAS-444Ø6-6 Soybean

1. Introduction

DAS-444Ø6-6 soybean is a transgenic soybean product that provides tolerance to 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 resistance management prevention solution to the increased incidence of hard-to-control and glyphosate resistant weeds.[1]

Dow AgroSciences is committed to promoting the responsible use and stewardship of this new herbicide tolerant trait technology and will implement a comprehensive stewardship program for DAS-444Ø6-6 soybean and its associated herbicide technology containing a new 2,4-D choline salt. The stewardship program will focus on educating and training retailers, growers and applicators on the appropriate use of this new technology. This will be accomplished by using a multi-faceted approach, including use of a variety of tools and delivery methods, and working with customers, stakeholders and industry organizations to promote responsible use of the technology (Dietz, 2010).

The stewardship program for DAS-444Ø6-6 soybean will be identical to that associated with DAS-68416-4 soybean.

2. Stewardship Goals

Specific goals of Dow AgroSciences' comprehensive stewardship program for DAS-444Ø6-6 soybean and 2,4-D choline salt include:

- Promoting the responsible use of this new technology to sustain its viability,
- Promoting compatibility with other crops and cropping systems,
- Providing distributors, retailers, growers, and applicators comprehensive guidance on responsible technology use, and
- Supporting compliance with state and federal regulatory requirements.

3. Stewardship Program Components

[1] *The following definitions are applied throughout this appendix: 1). Herbicide Tolerant Crop: A crop plant that has been developed (using techniques such as genetic engineering or selection of variants produced by tissue culture or mutagenesis) to survive and reproduce after treatment with a specific herbicide or herbicides that would normally damage an unmodified crop; 2). Herbicide Resistant Weed: A herbicide-resistant weed is a member of a population within a species that has an inherited ability to survive and reproduce following exposure to a dose of a herbicide normally lethal to susceptible populations of the species. Through repeated herbicide selection, the resistant population becomes dominant in a given area, and 3). Hard-to-Control Weed: A weed species that is inherently able to withstand treatment with a herbicide as a result of natural factors not involving herbicide selection or genetic mutation.*

To meet the above-described stewardship goals, Dow AgroSciences is developing a comprehensive stewardship program based on its experience and input from stakeholders. Key components of the stewardship program include:

- Authorized use through grower agreements,
- New 2,4-D choline based herbicide technologies,
- Comprehensive product use information,
- Education and training, and
- Compliance monitoring.

3.1 Authorized Use Through Grower Agreements

Growers who purchase and plant DAS-444Ø6-6 soybeans will be required to sign a grower agreement that provides the terms and conditions for the authorized use of the technology. The grower agreement will include a provision requiring them to follow the product use guide and all pesticide label requirements.

3.2 2,4-D Choline Herbicide Technology

Dow AgroSciences has discovered and developed a novel (non-ester or non-amine) 2,4-D choline salt herbicide product for use with DAS-44406-6 soybean. New herbicide formulations containing the active ingredient, 2,4-D choline salt,[2] are focused on improving non-target crop and environmental safety by offering growers and applicators herbicide products with ultra low volatility and minimized potential for physical drift.

An exceptionally low volatility of 2,4-D choline salt has been demonstrated in both laboratory studies and preliminary field studies. For example, in a laboratory study, wheat plants treated with rates ten times higher than specified on the label of 2,4-D choline were placed in an enclosed chamber with grape and tomato plants (Ouse et al., 2010). After 24 hours of exposure in the chamber at 104°F, neither sensitive crop showed any symptoms of injury. The lack of any symptoms on the grape and tomato plants is a clear indication that there was no detectable volatility following application of 2,4-D choline salt. Preliminary field results are consistent with these laboratory findings (Hillger et al., 2010).

Dow AgroSciences is also developing an innovative premix formulation that combines 2,4-D choline with glyphosate for use with DAS-444Ø6-6 soybean.[3] As compared to tank mixes of traditional 2,4-D products and commercial glyphosate, this premix uses formulation technologies that will result in a significant reduction in herbicide off-target movement from particle drift

[2] *The United States Environmental Protection Agency (EPA) has accepted and is currently reviewing DAS's submission for a wide range of crop and non-crop herbicide uses for 2,4-D choline salt.*
[3] *An application for 2,4-D choline and glyphosate premix use with DAS-44406-6 soybean will be submitted to EPA in 2012.*

(Wilson et al., 2010). This premix product will reduce the potential for off-target movement from both particle drift and volatility.

3.3 Comprehensive Product Use Information

3.3.1 Product Use Guides

Dow AgroSciences will establish product use guidelines and educate the technology users on responsible use via channel partner standards and agreements, grower agreements and product use guides. The product use guide will require that growers only use authorized 2,4-D choline products for use with DAS-444Ø6-6 soybeans. It will include information on herbicide resistance management, application and management of off-target herbicide movement, as well as seed planting directions and grain stewardship.

3.3.1.1 Herbicide Resistance Management

Based on the mode-of-action classification system of the Weed Science Society of America, 2,4-D is a Group 4 growth regulator herbicide (synthetic auxin). Some naturally occurring weed biotypes that are tolerant (resistant) to 2,4-D may exist due to genetic variability in a weed population. Where resistant biotypes exist, the repeated use of herbicides with the same mode of action can lead to the selection for resistant weeds. Certain agronomic practices reduce the likelihood that resistant weed populations will develop and can be utilized to manage weed resistance once it occurs.

Proactively implementing diversified weed control strategies to minimize selection for weed populations resistant to one herbicide or more is recommended. A diversified weed management program may include the use of multiple herbicides with different modes of action and overlapping weed spectrum with or without tillage operations and/or other cultural practices. Research has demonstrated that using the labeled rate and directions for use is important to delay selection for resistance.

To aid in the prevention of developing 2,4-D resistant weeds, Dow AgroSciences recommends the following practices for herbicide selection, crop selection and cultural practices:

Herbicide Selection

- Rotate the use of 2,4-D choline salt with non-auxin (non-Group 4) herbicides.
- Utilize a broad spectrum soil-applied herbicide as a foundation treatment.
- Utilize premixes, tank mixes or sequential applications of herbicides with alternative modes of action.
- Avoid using more than two applications of a Group 4 herbicide, such as 2,4-D choline salt, within a single growing season unless mixed with another mode of action herbicide with overlapping spectrum.
- Apply full rates of 2,4-D choline salt at the specified time (correct weed size) to minimize escapes of tolerant weeds.

Crop Selection and Cultural Practices

- Incorporate additional weed control practices whenever possible, such as delayed planting, crop rotation, mechanical cultivation and weed-free crop seeds, as part of an integrated weed control program.
- Do not allow weed escapes to produce seeds, roots or tubers.
- Thoroughly clean plant residues from equipment before leaving fields suspected to contain resistant weeds.
- Scout fields after application to detect weed escapes or shifts in weed species.
- If resistance is suspected, treat weed escapes with an alternate mode of action or cultivation.
- Report any incidence of repeated non-performance of this product against a particular weed species to the local retailer, county extension agent, or Dow AgroSciences representative.

3.3.1.2 Herbicide Application and Management of Off-Target Movement

The new 2,4-D choline containing herbicide products will minimize off target movement by reducing volatility and, in the case of the glyphosate + 2,4-D choline premix, off target movement will be minimized by reducing volatility and reducing the number of driftable fines.

To further minimize the potential for off-target movement with the new 2,4-D choline containing herbicide products, the product use guide will include recommendations on application equipment and methods such as following proper equipment maintenance, calibration and use.

A variety of factors including weather conditions (e.g., wind direction, wind speed, temperature, and relative humidity) and method of application can influence pesticide drift. Product use guides will require applicators to evaluate factors and make appropriate adjustments when applying 2,4-D choline salt for use with DAS-444Ø6-6 soybean consistent with the final product

label approved by EPA. Dow AgroSciences is conducting significant research on nozzles for spray application. The product use guide will include instructions on using specific nozzles that minimize driftable spray droplets, specified environmental conditions such as wind speed and field temperature that minimize the potential for off-target movement due to particle or vapor drift.

3.3.2 **Product Labels, Seed Bags and Tags**

3.3.2.1 Product Labels

Dow AgroSciences has submitted a product label to EPA that includes comprehensive information and requirements for responsible use of 2,4-D choline with DAS-444Ø6-6 soybean. Such label directions are designed to minimize the potential for weed resistance development and off-target movement of 2,4-D choline. In addition, the submitted label does not allow herbicide application through any type of irrigation equipment and prohibits aerial application.

3.3.2.2 Seed Bags and Tags

Seed bags and tags of soybean seed containing this new herbicide tolerant trait technology will have tags that provide stewardship information such as where to find the product use guide and grower agreement, as well as a customer information telephone number. Herbicide icons have been developed and will be included in marketing materials to clearly communicate applicable herbicide requirements to growers. When growers order seed, the icons and information will be included in the product use guide and on product fact sheets. Icons will appear on seed bag tags to remind growers of herbicide requirements at the time of planting.

3.3.3 **Technical Bulletins**

Dow AgroSciences will create comprehensive technical bulletins on these products including elements that address weed resistance management strategies, herbicide application technologies, responsible application practices to reduce the potential for off-target herbicide movement, and education on other elements of the herbicide label. Dow AgroSciences will make these available to channel and grower customers. They will also be made available to university cooperators, agronomists, crop consultants, other technical professionals and the general public through publication on internet websites.

3.3.4 Product Literature and Direct Mailings

Dow AgroSciences will also position the herbicide tolerant trait technology and promote its responsible use in its product literature by including crop agronomy, weed management, herbicide use and application information. Dow AgroSciences commonly uses direct mailings to customers, and will promote responsible stewardship of the herbicide tolerant trait technology through newsletters and correspondence. These direct mailings will emphasize responsible use

practices, label compliance, weed resistance management, and application guidance to minimize the potential for off-target movement.

3.3.5 Internet Resources

Information on the technology including agronomic and proper product placement, specific guidelines for planting transgenic traits, and herbicide use and application will be made available on technology provider websites. Relevant industry and university links may also be included to provide the user with additional technical information. Dow AgroSciences provides product stewardship information on its corporate and product websites.

3.4 Education and Training for Retailers, Growers and Applicators

An extensive network of Dow AgroSciences sales representatives, field scientists, and agronomists will play an important role in educating and training retailers, growers and applicators on the proper use of the technology and application guidelines. Dow AgroSciences field personnel, in addition to product suppliers, will work with growers to select the seed and herbicide products appropriate to their needs, growing conditions, and proper application equipment for field conditions.

Dow AgroSciences will communicate to customers the importance of stewardship and responsible product use. Dow AgroSciences will utilize a variety of approaches to educate and train retailers, growers, and applicators. These will include written communications, face-to-face meetings, field visits, television, radio, and/or computer-based training such as webinars and self-paced learning modules. Product stewardship plans for the herbicide tolerant trait technology will be based on applicator, grower and grain channel education, with reinforcement through written and verbal communications, including grower agreements, product use guides, product profiles, technical bulletins, sales literature, direct mailings, and websites.

Education topics for retailers, growers and applicators will include: DAS-444Ø6-6 soybean trait technology (including herbicide tolerance and agronomic practices), 2,4-D choline herbicide solutions (including label education, weed resistance management and herbicide application practices to minimize off-target movement). As an example, weed resistance management training will include information on:

- The value of rotating herbicides with different modes of action,
- How to use herbicides with different modes of action to achieve effective weed control,
- Recommendations to follow all labeled application rates and recommendations,
- How to apply herbicides at the proper weed size or growth stage, and
- Appropriate cultural and mechanical practices for weed control and weed resistance management.

As part of the various delivery methods and tools to educate retailers, growers, and applicators on the responsible use of this technology, Dow AgroSciences will use an online, interactive Learning Management System that will include training modules and information targeted to the users of the technology.

3.5 Compliance Monitoring

Dow AgroSciences is committed to implementing a compliance monitoring program for DAS-444Ø6-6 soybean and its associated new 2,4-D choline herbicide technology. The monitoring program will be developed for retailers, growers, and applicators to promote responsible use of the technology.

Compliance with the grower agreement and product use guides will be tracked and monitored through surveys, communications, and on-farm visits. Failure to follow the requirements set forth in the grower agreement and product use guide may result in the loss of a grower's access to the technology. The grower agreement to be signed by the grower will require that upon request by Dow AgroSciences, the grower must provide information indicating the location of all fields planted with DAS-444Ø6-6 soybean and the herbicide applied to these fields. In addition, the grower will be required to cooperate with any on-farm visits and field inspections.

4. Stakeholder Outreach and Input

Dow AgroSciences is using a variety of methods to obtain stakeholder input in the development of this product stewardship program. Dow AgroSciences is consulting with retailers, growers and applicators to understand and address their needs in order to develop an effective and comprehensive stewardship program. In addition, Dow AgroSciences is proactively seeking input from recognized experts with a diverse range of experience in U.S. agriculture as well as producers and processors of non-target crops. These efforts will support implementation of stewardship practices for this technology as broadly as possible throughout the value chain.

Dow AgroSciences will evaluate additional feedback from retailers, growers and applicators for the continuous improvement of this stewardship program following the launch of DAS-444Ø6-6 soybean.

5. Industry Commitment and Involvement

Dow AgroSciences participates in several organizations and associations globally to promote the safe research and development, production, distribution, and responsible use of agricultural chemical and biotechnology products.

Dow AgroSciences is a participant in the Herbicide Resistance Action Committee (HRAC) [http://www.hracglobal.com], an industry-based group supported by CropLife International [http://www.croplife.org/public/resistance_management]. HRAC focuses on encouraging responsible herbicide usage, communicating herbicide resistance management strategies and supporting their implementation through practical guidelines. HRAC engages in active collaboration with public and private researchers, especially in the areas of problem identification and devising and implementing herbicide management strategies.

Dow AgroSciences personnel interact with academic weed scientists in addressing weed resistance management [http://www.wssa.net/Weeds/Resistance/] issues. Dow AgroSciences conducts joint trials at university sites as well as seeking input from university researchers regarding weed management. Dow AgroSciences also participates in a wide range of professional organizations including agronomy societies, seed trade groups, weed science societies, and crop commodity groups.

In addition to managing the technology in accordance with the applicable requirements of federal and state government agencies, Dow AgroSciences is a Founding Member of the biotechnology industry's Excellence Through Stewardship® organization which encourages effective and comprehensive stewardship programs and quality management systems throughout a trait product's life cycle.

6. Conclusion

Dow AgroSciences is committed to promoting the responsible use and stewardship of this new herbicide tolerant trait technology and will implement a comprehensive stewardship program for DAS-444Ø6-6 soybean and its associated new herbicide technology, 2,4-D choline salt. The stewardship program will focus on educating and training retailers, growers and applicators on the appropriate use of this new technology. This will be accomplished by using a multi-faceted approach, including use of a variety of tools and delivery methods, and working with customers, stakeholders and industry organizations to promote responsible use of the technology.

Appendix 10. References

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