RECEIVED
By caeck at 2:52 pm, Dec 15, 2009

Dow AgroSciences

09-349-01p

Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-68416-4 Soybean

OECD Unique Identifier: DAS-68416-4

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

Submitting Company:

Dow AgroSciences LLC

Submitted by:

b6

Mark S. Krieger, Ph.D.
Regulatory Leader

Regulatory Sciences & Government Affairs
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
Telephone: (317) 337-3458
FAX: (317) 337-4649
mkrieger2@dow.com

December 8, 2009

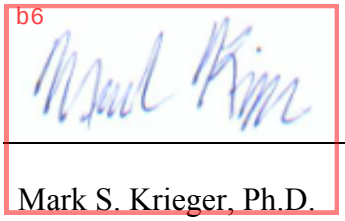
Release of Information

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

Certification

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

b6



Mark S. Krieger, Ph.D.
Regulatory Leader

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268
Telephone: (317) 337-3458
Fax: (317) 337-4649
mkrieger2@dow.com

Summary

Dow AgroSciences LLC (herein referred to as "DAS"), is submitting a Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-68416-4 Soybean. DAS requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that soybean transformation event DAS-68416-4 and any soybean lines derived from crosses with DAS-68416-4 soybeans no longer be considered regulated articles under 7 CFR Part 340.

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

DAS-68416-4 soybean plants have been genetically modified to express the aryloxyalkanoate dioxygenase-12 (AAD-12) and phosphinothricin acetyltransferase (PAT) proteins. The AAD-12 protein is an enzyme with an alpha ketoglutarate-dependent dioxygenase activity which results in metabolic inactivation of the herbicides of the aryloxyalkanoate family. The *aad-12* gene, which expresses the AAD-12 protein, was derived from *Delftia acidovorans,* a gram-negative soil bacterium. The PAT enzyme acetylates the primary amino group of phosphinothricin rendering it inactive. The *pat* gene expressing the PAT protein was derived from *Streptomyces viridochromogenes*.

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

The AAD-12 and PAT proteins in DAS-68416-4 soybean was characterized biochemically and measured using AAD-12 and PAT 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-68416-4 plants treated with 2,4-D, glufosinate, both 2,4-D and glufosinate, or not treated with either herbicide. The results showed a low level of expression of the AAD-12 and PAT proteins across herbicide treatments and environments, indicating a low exposure to humans and animals.

The AAD-12 protein was assessed for any potential adverse effects to humans or animals resulting from the environmental release of crops containing the AAD-12 protein. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 protein. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-12 amino acid sequence. The AAD-12 protein

hydrolyzes rapidly in simulated gastric fluid and there was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-12 protein. Glycosylation analysis of the plant- and microbe-derived AAD-12 proteins revealed no detectable covalently linked carbohydrates. Results of the overall safety assessment of the AAD-12 protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals. The safety of the PAT protein has been assessed previously and it has been approved for use in canola, corn, cotton, rice, soybeans, and sugar beets.

DAS-68416-4 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-68416-4 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-68416-4 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-68416-4 soybean.

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

Since DAS-68416-4 soybean is agronomically and nutritionally similar to conventional soybean, and the safety of the AAD-12 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-68416-4 soybean is expected to have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The use of DAS-68416-4 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-68416-4 soybean exhibits no plant pathogenic properties or weediness characteristics. DAS-68416-4 soybean is no more likely to become a plant pest than conventional soybean, and the AAD-12 and PAT proteins are unlikely to increase the weediness potential of any other cultivated plant or wild species.

DAS hereby requests a determination from APHIS that herbicide-tolerant DAS-68416-4 soybean and all progeny derived from the conventional breeding of this line no longer be considered regulated articles under 7 CFR Part 340.

Table of Contents

Release of Information **2**
Certification **3**
Summary **4**
Table of Contents **6**
List of Figures **9**
List of Tables **11**
Acronyms and Scientific Terms **13**
I. Rationale for the Development of DAS-68416-4 Soybean **15**
I-A. Basis for the Request for Nonregulated Status 15
I-B. Benefits of DAS-68416-4 Soybean 15
I-C. Submissions to Other Regulatory Agencies 17
I-D. References 17
II. The Biology of Soybean **18**
II-A. Overview of Soybean Biology 18
II-B. Characterization of the Recipient Soybean Cultivar 18
II-C. References 18
III. Development of DAS-68416-4 Soybean **19**
III-A. Description of the Transformation System 19
III-B. Selection of Comparators for DAS-68416-4 Soybean 23
III-C. References 23
IV. Donor Genes and Regulatory Sequences **24**
IV-A. Identity and Source of Genetic Material in pDAB4468 24
IV-B. References 26
V. Genetic Characterization **29**
V-A. Overview of Molecular Analysis 29
V-B. Analysis of the Insert and Its Genetic Elements 35
V-B.1. Analysis of the *aad-12* Gene 35
V-B.2. Analysis of the AtUbi10 Promoter 35
V-B.3. Analysis of the AtuORF23 3'UTR 35
V-B.4. Analysis of the *pat* Gene 36
V-B.5. Analysis of the CsVMV Promoter 36
V-B.6. Analysis of the AtuORF1 3'UTR 36
V-B.7. Analysis of the RB7 MAR 36
V-C. Absence of Vector Backbone DNA 37
V-D. Stability of the Insert across Generations 37
V-E. Segregation Analysis 63
V-E.1. Genetic and Molecular Analysis of a Segregating Generation 63
V-E.2. Segregation Analysis of Breeding Generations 67
V-F. Summary of the Genetic Characterization 67
VI. Characterization of the Introduced Proteins **68**
VI-A. AAD-12 68
VI-A.1. Identity of the AAD-12 Protein 68
VI-A.2. Biochemical Characterization of the AAD-12 Protein 68
VI-A.3. Expression of the AAD-12 Protein in Plant Tissues 69
VI-A.4. Food and Feed Safety Assessment for AAD-12 Protein 71

VI-A.5. Summary of AAD-12 Protein Characterization ... 72
VI-B. PAT ... 72
VI-B.1. Identity of the PAT Protein ... 72
VI-B.2. Biochemical Characterization of the PAT Protein ... 72
VI-B.3. Expression of the PAT Protein in Plant Tissues ... 73
VI-B.4. Summary of PAT Protein Characterization ... 74
VI-C. References ... 75
VII. Agronomic Performance ... 77
VII-A. Phenotypic and Agronomic Characteristics ... 77
VII-A.1. Experiment 1 ... 77
VII-A.2. Experiment 2 ... 81
VII-A.3. Conclusions ... 83
VII-B. Ecological Evaluations ... 84
VII-C. Germination and Dormancy Evaluations ... 85
VII-D. Summary of Agronomic, Disease, and Pest Characteristics ... 86
VII-E. References ... 86
VIII. Grain and Forage Composition ... 87
VIII-A. Compositional Analysis of Soybean Forage ... 88
VIII-B. Compositional Analysis of Soybean Grain ... 91
VIII-B.1. Proximates and Fiber ... 91
VIII-B.2. Minerals ... 94
VIII-B.3. Amino Acids ... 97
VIII-B.4. Fatty Acids ... 101
VIII-B.5. Vitamins ... 105
VIII-B.6. Isoflavones ... 108
VIII-B.7. Antinutrients ... 110
VIII-C. Summary of Grain and Forage Composition ... 112
VIII-D. References ... 112
IX. Environmental Consequences and Impact on Agronomic Practices ... 113
IX-A. Mode of Action of the AAD-12 Protein ... 113
IX-A.1. Field Efficacy ... 113
IX-B. Weediness Potential ... 116
IX-C. Gene Flow Assessment ... 117
IX-C.1. Vertical Gene Flow Assessment ... 117
IX-C.2. Horizontal Gene Flow Assessment ... 117
IX-D. Current US Agronomic Practices for Soybeans ... 117
IX-D.1. Soybean Production ... 117
IX-D.2. Weeds in Soybean ... 118
IX-D.3. Weed Management in Soybean ... 120
IX-D.4. Crop Rotation and Tillage Practices ... 121
IX-E. Potential Impact on Agronomic Practices ... 121
IX-E.1. Potential Impact on Cultivation and Management Practices ... 121
IX-E.2. Potential Impact on Weed Control Practices ... 122
IX-E.3. Potential Impact on Volunteer Management ... 124
IX-E.4. Potential Impact on Non-Target Organisms and Endangered Species ... 125
IX-F. Herbicide Resistance Management ... 126

IX-F.1. Herbicide Resistance 126
IX-F.2. Factors Impacting Development of Resistance 128
IX-F.3. Herbicide Resistance Management 129
IX-G. Summary of Environmental Assessment 130
IX-H. References 131
X. Adverse Consequences of Introduction 135
XI. Appendices 136
Appendix 1. Methods for Molecular Characterization of DAS-68416-4 Soybean 137
Appendix 2. Methods and Results for Characterization of AAD-12 Protein 140
Appendix 3. Methods and Results for Characterization of PAT Protein 152
Appendix 4. Methods for AAD-12 and PAT Protein Expression Analysis 155
Appendix 5. USDA Notifications for DAS-68416-4 Soybean 157
Appendix 6. Literature Ranges for Compositional Analysis 158
Appendix 7. Glyphosate, 2,4-D, Glufosinate and Herbicide Resistant Weeds 162
Appendix 8. Stewardship of Herbicide Tolerant DAS-68416-4 Soybeans 175

List of Figures

Figure 1. Plasmid map of pDAB4468. 20
Figure 2. Diagram of T-DNA insert in plasmid pDAB4468. 20
Figure 3. Development of DAS-68416-4 soybean. 21
Figure 4. Breeding diagram of DAS-68416-4 soybean. 22
Figure 5. Location of probes on pDAB4468 used in Southern blot analysis of DAS-68416-4 soybean. 30
Figure 6. Plasmid map of pDAB4468 with restriction enzyme sites used for Southern blot analysis. 33
Figure 7. pDAB4468 T-DNA insert, restriction enzymes used in DNA digestion and expected hybridization bands. 34
Figure 8. Southern blot analysis of *Nco* I digest with *aad-12* probe. 38
Figure 9. Southern blot analysis of *Sph* I/*Xho* I digest with *aad-12* probe. 39
Figure 10. Southern blot analysis of *Nhe* I/*Xho* I digest with *aad-12* probe. 40
Figure 11. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with *aad-12* probe. 41
Figure 12. Southern blot analysis of *Pst* I digest with *aad-12* probe. 42
Figure 13. Southern blot analysis of *Pst* I digest of T5 generation with *aad-12*, AtUbi10, and AtuORF23 probes. 43
Figure 14. Southern blot analysis of *Pst* I digest with AtUbi10 probe. 44
Figure 15. Southern blot analysis of *Pst* I digest with AtuORF23 probe. 45
Figure 16. Southern blot analysis of *Nco* I digest with *pat* probe. 46
Figure 17. Southern blot analysis of *Sph* I/*Xho* I digest with *pat* probe. 47
Figure 18. Southern blot analysis of *Nhe* I/*Xho* I digest with *pat* probe. 48
Figure 19. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with *pat* probe. 49
Figure 20. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe. 50
Figure 21. Southern blot analysis of *Pst* I/*Xho* I digest of T5 generation with *pat,* CsVMV, and AtuORF1 probes. 51
Figure 22. Southern blot analysis of *Pst* I/*Xho* I digest with CsVMV probe. 52
Figure 23. Southern blot analysis of *Pst* I/*Xho* I digest with AtuORF1 probe. 53
Figure 24. Southern blot analysis of *Bam*H I/*Nco* I digest with RB7 probe. 54
Figure 25. Southern blot analysis of *Bam*H I/*Nco* I digest with AtUbi10 probe. 55
Figure 26. Southern blot analysis of *Bam*H I/*Nco* I digest of T5 generation with RB7 and AtUbi10 probes. 56
Figure 27. Southern blot analysis of *Nco* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone. 57
Figure 28. Southern blot analysis of *Nco* I digest with backbone probe set 2 from plasmid pDAB4468 vector backbone. 58
Figure 29. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone. 59
Figure 30. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 2 plasmid pDAB4468 vector backbone. 60
Figure 31. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generations with the backbone probe set 1 from pDAB4468 vector backbone. 61

Figure 32. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with the backbone probe set 2 from pDAB4468 vector backbone. 62
Figure 33. Southern blot analysis of *Nco* I digest with *aad-12* probe. 65
Figure 34. Southern blot analysis of *Nco* I digest with *pat* probe..... 66
Figure 35. Amino acid sequence of the AAD-12 protein. 68
Figure 36. Amino acid sequence of the PAT protein..... 72
Figure 37. Summary of the proximate, fiber and mineral analysis of soybean forage (% dry weight). 90
Figure 38. Summary of the proximate and fiber analysis of soybean grain. 93
Figure 39. Summary of the mineral analysis of soybean grain (mg/100 g dry weight). 96
Figure 40. Summary of the amino acid analysis of soybean grain (% dry weight). 99
Figure 41. Summary of the fatty acid analysis of soybean grain (% total fatty acids). 104
Figure 42. Summary of vitamin analysis of soybean grain (mg/kg dry weight). 107
Figure 43. Summary of isoflavone analysis of soybean grain (µg/g). 109
Figure 44. Summary of anti-nutrient analysis of soybean grain (% dry weight)..... 111
Figure 45. Degradation reaction of 2,4-D catalyzed by AAD-12. 113
Figure 46. Adoption of genetically engineered crops in the U.S. 122
Figure 47. 2,4-D herbicide application timing and rates for conventional and DAS-68416-4 soybeans. 123
Figure 48. Glyphosate application rates in U.S. corn and soybeans from 1990 thru 2007..... 124
Figure 49. Resistant weed biotypes per herbicide mode of action. 128
Figure 50. SDS-PAGE of soybean- and microbe-derived AAD-12. 142
Figure 51. SDS-PAGE and western blot of soybean- and microbe-derived AAD-12 protein extracts. 143
Figure 52. Glycosylation analysis of soybean- and microbe-derived AAD-12 proteins. 144
Figure 53. Theoretical trypsin (top panel) and Asp-N (bottom panel) cleavage of the AAD-12 protein. 146
Figure 54. Sequence coverage in the tryptic and Asp-N peptide mapping analysis of plant-derived AAD-12 protein with MALDI-TOF and ESI/LC MS. 147
Figure 55. Sequence coverage in the peptide mapping analysis of microbe-derived AAD-12 protein with MALDI-TOF and ESI/LC MS. 148
Figure 56. SDS-PAGE and western blots of DAS-68416-4 and non-transgenic Maverick soybean. 153
Figure 57. U.S. adoption rates of glyphosate-tolerant soybean, cotton and corn. 163
Figure 58. Number of glyphosate-resistant weeds reported globally by year from 1996 to 2008..... 168
Figure 59. Example weed resistance management statement on DAS glyphosate brand labels. 176

List of Tables

Table 1. Genetic elements of the T-DNA insert from plasmid pDAB4468. 24
Table 2. List of probes and their positions in plasmid pDAB4468. 30
Table 3. Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-68416-4 soybean. 31
Table 4. Results of F2 individual plants tested for of AAD-12 expression within a single segregating generation. 64
Table 5. Predicted and observed hybridizing fragments in Southern blot analysis of F2 population. 64
Table 6. Results of F2 individual plants analyzed by Southern blot with *aad-12* and *pat* probes within a single segregating generation. 64
Table 7. Results of F2 individual plants tested for *aad-12* within a single segregating generation. 67
Table 8. Summary of AAD-12 protein levels in tissues collected from DAS-68416-4 produced in the U.S. and Canada during 2008. 70
Table 9. Summary of PAT protein levels in tissues collected from DAS-68416-4 produced in the U.S. and Canada during 2008. 74
Table 10. Agronomic parameters evaluated in Experiment 1. 78
Table 11. Analysis of agronomic characteristics from Experiment 1. 80
Table 12. Locations for Experiment 2 agronomic and yield trials, 2009. 81
Table 13. Data collected in Experiment 2 agronomic and yield trials, 2009. 82
Table 14. Agronomic parameters for sprayed (2,4-D) and unsprayed DAS-68416-4 soybean. 82
Table 15. Agronomic parameters for DAS-68416-4 and Maverick soybean. 83
Table 16. Analysis of disease incidence and insect damage from Experiment 1. 84
Table 17. Disease and insect stressors observed in trials of DAS-68416-4 and conventional soybean. 85
Table 18. Germination of DAS-68416-4 seeds under warm and cold conditions. 86
Table 19. Summary of the proximate, fiber and mineral analysis of soybean forage (% dry weight). 89
Table 20. Summary of the proximate and fiber analysis of soybean grain (% dry weight). 92
Table 21. Summary of the mineral analysis of soybean grain (mg/100g dry weight). 95
Table 22. Summary of the amino acid analysis of soybean grain (% dry weight). 98
Table 23. Summary of the fatty acid analysis of soybean grain (% total fatty acids). 103
Table 24. Summary of vitamin analysis of soybean grain (mg/kg dry weight). 106
Table 25. Summary of isoflavone analysis of soybean grain (µg/g). 109
Table 26. Summary of anti-nutrient analysis of soybean grain (% dry weight). 111
Table 27. DAS-68416-4 soybean tolerance to preemergence applications of 2,4-D, 2008. 114
Table 28. DAS-68416-4 soybean tolerance to post-emergence applications of 2,4-D, 2008. 115
Table 29. DAS-68416-4 soybean tolerance to postemergence applications of glufosinate, 2008. 116
Table 30. Common troublesome weeds in soybeans in 2006-2008. 119
Table 31. Yield reduction from specific weed species in soybeans. 120
Table 32. Herbicide-resistant weeds. 127
Table 33. Assessment of resistance risk by evaluation of cropping systems. 129

Table 34. Glyphosate- and ALS-resistant weeds controlled by 2,4-D. 130
Table 35. Summary of N-terminal sequence data of AAD-12 soybean- and microbe-derived proteins..... 149
Table 36. Summary of C-terminal sequence data of AAD-12 soybean- and microbe-derived proteins..... 150
Table 37. Summary of literature values for proximates in soybean forage. 158
Table 38. Summary of literature values for proximates in soybean grain. 158
Table 39. Summary of literature values for amino acids in soybean grain..... 159
Table 40. Summary of literature values for isoflavones in soybean grain..... 159
Table 41. Summary of literature values for fatty acids in soybean grain. 160
Table 42. Summary of literature values for vitamins in soybean grain. 160
Table 43. Summary of literature values for minerals in soybean grain. 161
Table 44. Summary of literature values for anti-nutrients in soybean grain. 161
Table 45. Summary of literature values for tocopherols in soybean grain. 161
Table 46. Herbicide-tolerant crops available to farmers in North America in 2005. 162
Table 47. Weed species with reported glyphosate-resistant biotypes..... 167
Table 48. Global reports of glyphosate-resistant weed biotypes with resistance to other herbicide modes of action. 168
Table 49. Potential weed shifts with use of glyphosate in U.S. corn and soybeans. 169
Table 50. Weed species with reported 2,4-D-resistant biotypes..... 170

Acronyms and Scientific Terms

2,4-D	2,4-Dichlorophenoxyacetic acid
A	Acre
aad-12	Gene from *Delftia acidovorans* which encodes the AAD-12 protein
AAD-12	Aryloxyalkanoate Dioxygenase-12 protein
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-68416-4	Soybean line containing event DAS-68416-4
DCP	2,4-Dichlorophenol
DMA	Dimethylamine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (US)
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ESA	Endangered Species Act
ESI-LC/MS	Electrospray ionization-liquid chromatography mass spectrometry
Event DAS-68416-4	OECD identifier for the soybean event expressing the AAD-12 protein
FDA	Food and Drug Administration (US)
FDR	False Discovery Rate
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
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 event DAS-68416-4
MCPA	4-chloro-2-methylphenoxyacetic acid
MOA	Mode of action
NDF	Neutral detergent fiber
OECD	Organisation for Economic Co-operation and Development
pat	Gene from *Streptomyces viridochromogenes* which encodes the PAT protein
PAT	Phosphinothricin N-acetyl transferase protein
PBN	US FDA Pre-market Biotechnology Notice
pDAB4468	DNA vector carrying the transgenes (*aad-12* and *pat*) for insertion into the plant genome
Pf	*Pseudomonas fluorescens*
PPO	Protoporphyrinogen oxidase
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
WSSA	Weed Science Society of America

I. Rationale for the Development of DAS-68416-4 Soybean

I-A. Basis for the Request for Nonregulated Status

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. 7701-7772) and 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 is submitting data for genetically engineered herbicide-tolerant DAS-68416-4 soybean and requests a determination from APHIS that event DAS-68416-4 and crosses of this event with nonregulated soybean lines no longer be considered regulated articles under 7 CFR 340.

I-B. Benefits of DAS-68416-4 Soybean

Dow AgroSciences LLC (herein referred to as "DAS") has developed transgenic soybean plants that are tolerant to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate. DAS-68416-4 is the unique identifier of these plants, in accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2004).

DAS-68416-4 soybean was developed using *Agrobacterium*-mediated transformation to stably incorporate the *aad-12* gene from *Delftia acidovorans* and *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 into herbicidally-inactive 2,4-dichlorophenol (DCP). The *pat* gene encodes the enzyme phosphinothricin acetyl transferase that inactivates glufosinate. The availability of DAS-68416-4 soybean is expected to have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The availability of DAS-68416-4 soybean will allow growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance, particularly glyphosate-resistance in weeds.

With the introduction of genetically engineered, glyphosate-tolerant crops in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds that was unparalleled in agriculture. Consequently, producers were quick to adopt glyphosate-tolerant crops, and in many instances, abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, and incorporation of mechanical with chemical and cultural weed control. Currently glyphosate-tolerant soybean, cotton, corn, sugar beets, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More glyphosate-tolerant crops (*e.g.,* wheat, rice, turf, *etc.*) are poised for introduction pending global market

acceptance. Many other glyphosate-tolerant species are in experimental or development stages (*e.g.*, alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias) (USDA APHIS, 2009). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate-tolerant crops systems.

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts) (Heap, 2009). Although glyphosate has been widely used globally for more than 30 years, only a handful of weeds have been reported to have developed resistance to glyphosate; however, most of these have been identified in the past 5-8 years. Resistant weeds in the U.S. include both grass and broadleaf species—*Lolium rigidum* (Rigid ryegrass), *Lolium multiflorum* (Italian ryegrass), *Sorghum 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 2009). These weed shifts are occurring predominantly, but not exclusively, with difficult-to-control broadleaf weeds. Some examples include *Ipomoea, Amaranthus, Chenopodium, Taraxacum,* and *Commelina* species.

In areas where growers are faced with glyphosate-resistant weeds or a shift to more difficult-to-control weed species, growers can compensate by tank mixing or alternating with other herbicides that will control the surviving weeds. One popular and efficacious tank mix active ingredient for controlling broadleaf escapes has been 2,4-diclorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant weed species have been reported, but 2,4-D remains one of the most widely used herbicides globally. The development of 2,4-D-tolerant soybeans provides an excellent option for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications, allowing the grower to focus applications at the critical weed control stages and extending the application window without the need for specialized sprayer equipment. Combining the 2,4-D-tolerance trait and a glyphosate-tolerance trait through conventional breeding ("stacking" traits) would give growers the ability to use tank mixes of glyphosate/2,4-D over-the-top of the tolerant plants to control the glyphosate-resistant broadleaf species.

DAS-68416-4 soybeans also provide tolerance to glufosinate herbicides. Glufosinate is a non-selective, contact herbicide that controls a broad spectrum of annual and perennial grasses and broadleaf weeds. 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 and glufosinate will bring new weed control alternatives for growers. This new weed management tool will allow for the improved control of key broadleaf weeds which can affect the vigor and yield of the crop, allow an increased herbicide application window for effective weed control, and provide an effective resistance management/prevention solution to the increased incidence of glyphosate- and acetolactate synthase (ALS)-resistant weeds.

I-C. Submissions to Other Regulatory Agencies

AAD-12 soybean event DAS-68416-4 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 will submit a pre-market biotechnology notification (PBN) to FDA.

As per EPA's authority over the use of pesticidal substances under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), plant metabolism and residue data for 2,4-D on DAS-68416-4 soybeans as well as proposed labeling for the use of 2,4-D on DAS-68416-4 soybeans will be submitted to EPA.

DAS intends to submit dossiers in 2010 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.

I-D. References

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

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

USDA APHIS (2009) Field Test Release Applications in the U.S. Accessed November 1, 2009. http://www.isb.vt.edu/cfdocs/fieldtests1.cfm

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

II. The Biology of Soybean

II-A. Overview of Soybean Biology

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

- general description, including taxonomy, morphology, and the uses of soybean as a crop plant
- agronomic practices
- 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*

II-B. Characterization of the Recipient Soybean Cultivar

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

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

II-C. References

OECD (2000) Consensus Document on the Biology of *Glycine max* (L.) Merr. (Soybean). Organisation for Economic Co-operation and Development, Paris, France. Series on Harmonization of Regulatory Oversight in Biotechnology No. 15. ENV/JM/MONO(2000)9. (http:/www.olis.oecd.org/olis/2000doc.nsf/LinkTo/env-jm-mono(2000)9)

Sleper, D.A., Nickell, C.D.; Noel, G.R., Cary, T.R., Thomas, D.J., Clark, K.M., Rao Arelli, A.P. (1998) Registration of 'Maverick' Soybean. Crop Science 38:549.

III. Development of DAS-68416-4 Soybean

III-A. Description of the Transformation System

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

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

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

Figure 1 shows a plasmid map of pDAB4468 with all genetic elements identified.

Figure 2 shows a diagram of the T-DNA insert in plasmid pDAB4468.

Figure 3 shows the steps used to develop DAS-68416-4 soybean.

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

Figure 1. Plasmid map of pDAB4468.

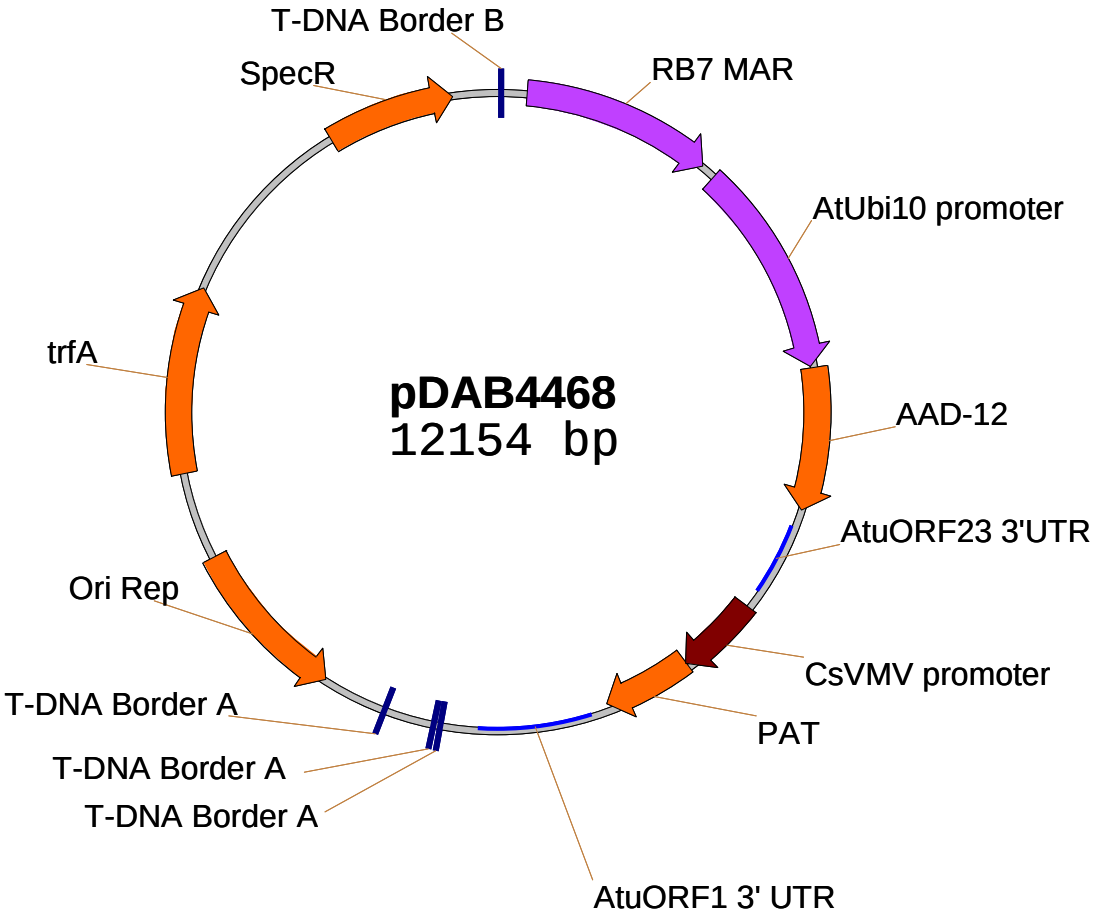


Figure 2. Diagram of T-DNA insert in plasmid pDAB4468.

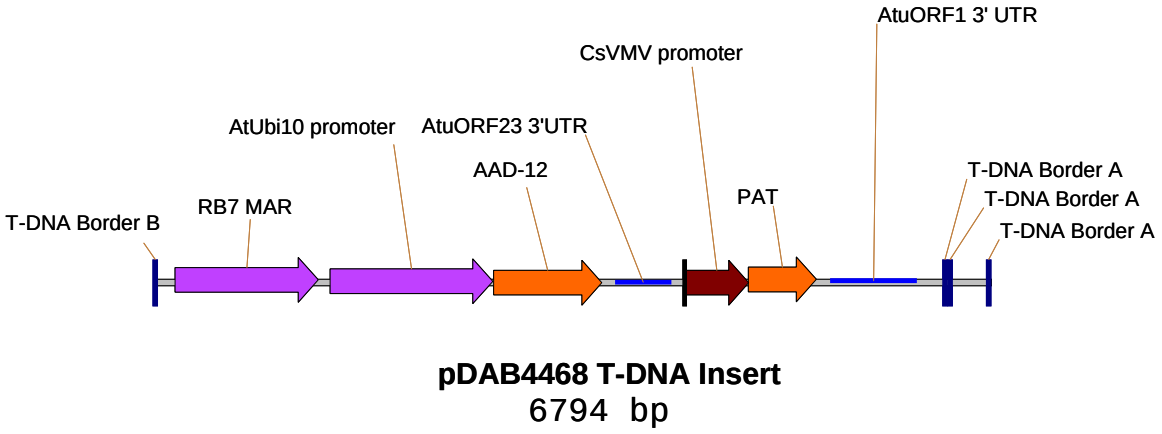


Figure 3. Development of DAS-68416-4 soybean.

The *aad-12* gene sequence that encodes the AAD-12 protein from *Delftia acidovorans* was synthesized to produce an *aad-12* gene sequence for expression in soybean.

↓

A plasmid containing *aad-12* gene and *pat* gene cassettes was assembled and inserted into a plant expression cassette to make transformation plasmid pDAB4468.

↓

The *aad-12* gene was introduced into the soybean line Maverick via *Agrobacterium tumefaciens*-mediated transformation.

↓

Transformed plants were selected based on *in vitro* tolerance to glufosinate herbicide and regeneration of T0 soybean plants.

↓

T0 plants were screened for absence of vector backbone, presence of gene of interest, copy number, and tolerance to 2,4-D and glufosinate herbicides. T0 plants that passed the screen were self-pollinated.

↓

Continued self pollination, molecular characterization, and evaluation of agronomic performance and herbicide efficacy were performed.

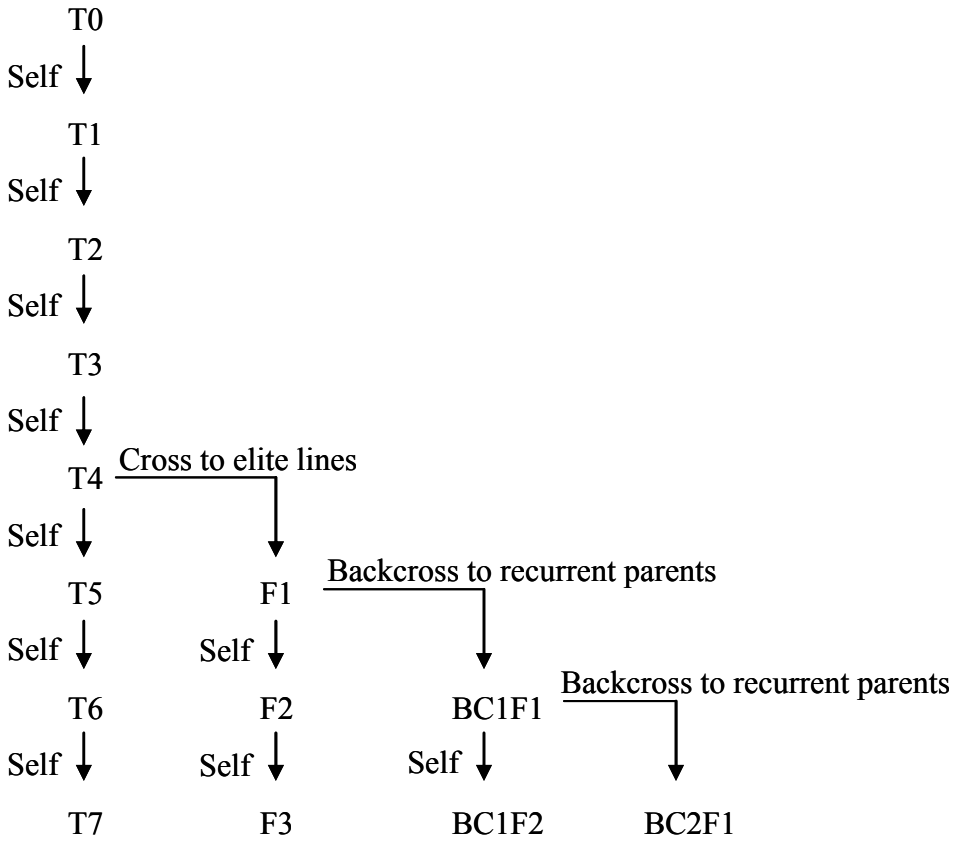
↓

Selection of homozygous plants, backcrossing and crossing for product development.

↓

Selection of DAS-68416-4 event as the lead commercial candidate.

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



Analysis	Petition Section(s)	DAS-68416-4 Soybean Generation Used	Control
Molecular Analysis	V-A, V-B, V-C, V-D	T2, T3, T4, T5	Maverick
Segregation Analysis	V-E	F2	Maverick
Protein Characterization	VI-A.2, VI-B.2	T4	Maverick
Protein Expression	VI-A.3, VI-B.3	T4	Maverick
Agronomics	VII	T4, T6	Maverick
Germination/Dormancy	VII-C	T6	Maverick
Composition	VIII	T4	Maverick
Efficacy	IX-A	T4	Maverick

III-B. Selection of Comparators for DAS-68416-4 Soybean

To ensure the accurate assessment of the impact of transgene insertion on various characteristics of DAS-68416-4 soybean, a proper selection of comparator plants is important.

The control plants should have a genetic background similar to that of DAS-68416-4 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-68416-4 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.

III-C. References

Hood, E.E.; Helmer, G.L.; Fraley, R.T.; Chilton, M.-D. The Hypervirulence of *Agrobacterium tumefaciens* A281 Is Encoded in a Region of pTiBo542 Outside of T-DNA, *Journal of Bacteriology*, **1986** *168*(3):1291-1301.

Zeng, P.; Vadnais, D.A.; Zhang, Z.; Polacco, J.C. Refined glufosinate selection in *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill], *Plant Cell Reports* **2004** *22*:478–482

IV. Donor Genes and Regulatory Sequences

IV-A. Identity and Source of Genetic Material in pDAB4468

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

Table 1. Genetic elements of the T-DNA insert from plasmid pDAB4468.

Location on T-DNA insert of pDAB4468[1]	Genetic Element	Size (base pairs)	Description
1−24	T-DNA Border B	24	Transferring DNA sequences
25−160	Intervening sequence	136	Sequence from Ti plasmid pTi15955 (Barker *et al.*, 1983)
161−1326	RB7-MAR	1166	Matrix attachment region (MAR) from *Nicotiana tabacum* (Hall *et al.*, 1991)
1327−1421	Intervening sequence	95	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
1422−2743	AtUbi10	1322	*Arabidopsis thaliana* polyubiquitin UBQ10 comprising the promoter, 5' untranslated region and intron (Norris *et al.,* 1993)
2744−2751	Intervening sequence	8	Sequence used for DNA cloning
2752−3633	*aad-12*	882	Synthetic, plant-optimized version of an aryloxyalkanoate dioxygenase gene from *Delftia acidovorans* (Wright *et al*., 2007)
3634−3735	Intervening sequence	102	Sequence used for DNA cloning
3736−4192	AtuORF23	457	3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 23 (ORF23) of *Agrobacterium tumefaciens* pTi15955 (Barker *et al*., 1983)
4193−4306	Intervening sequence	114	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
4307−4819	CsVMV	513	Promoter and 5' untranslated region derived from the cassava vein mosaic virus (Verdaguer *et al.*, 1996)
4820−5371	*pat*	552	Synthetic, plant-optimized version of phosphinothricin N-acetyl transferase (PAT) gene, isolated from *Streptomyces viridochromogenes* (Wohlleben *et al.* 1988)
5372−5484	Intervening sequence	113	Sequence from plasmid pCRI2.1(Invitrogen Cat. No. K205001) and multiple cloning sites

Table 1 (cont.). Genetic elements of the T-DNA insert from plasmid pDAB4468.

Location on T-DNA insert of pDAB4468[1]	Genetic Element	Size (base pairs)	Description
5485−6188	AtuORF1	704	3' untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of *Agrobacterium tumefaciens* pTi15955 (Barker *et al.*, 1983)
6189−6416	Intervening sequence	228	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
6417−6440	T-DNA border A	24	Transferring DNA sequence
6441−6459	Intervening sequence	19	Sequence from Ti plasmid C58 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
6460−6483	T-DNA border A	24	Transferring DNA sequence
6484−6770	Intervening sequence	287	Sequence from Ti plasmid pTi15955 (Baker *et al.*, 1983)
6771−6794	T-DNA border A	24	Transferring DNA sequence

[1] Base pair position.

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

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 Gravenitz 1985, Tamaoka *et al.* 1987, Wen *et al.*, 1999). *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Toms and Wood, 1970; Ramachandra Rao and Ravishankar, 2000; Shetty *et al.*, 2006). This utility has led to a history of safe use for *D. acidovorans* in the food processing industry. For example, US Patent 5,128,253 "Bioconversion process for the production of vanillin" was issued on July 7, 1992 to Kraft General Foods (Labuda *et al.*, 1992).

Expression of *aad-12* in the T-DNA insert of pDAB4468 is controlled by the AtUbi10 promoter from *Arabidopsis thaliana* and AtuORF23 3' UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The AtUbi10 promoter is known to drive constitutive expression of the genes it controls (Norris *et al.,* 1993). AtuORF23 UTR has been used in previously deregulated products (USDA 2004).

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

attachments regions are natural and abundant regions found in genomic DNA that are thought to attach to the matrix or scaffold of the nucleus. When positioned on the flanking ends of gene cassettes, some MARs have been shown to increase expression of transgenes and to reduce the incidence of gene silencing (Abranches *et al*., 2005; Han *et al.*, 1997; Verma *et al.*, 2005). It is hypothesized that MARs may act to buffer effects from neighboring chromosomal sequences that could destabilize the expression of genes (Allen *et al*., 2000). A MAR was included at the 5' end of *aad-12* PTU to potentially increase the consistency of *aad-12* expression in transgenic plants.

The *pat* expression cassette contained in the T-DNA insert of pDAB4468 is designed to express the plant-optimized phosphinothricin *N*-acetyl transferase (*pat*) gene that encodes the PAT protein. The *pat* gene was isolated from *Streptomyces viridochromogenes* and the synthetic version of the gene was optimized to modify the G+C codon bias to a level more typical for plant expression. The insertion of the *pat* gene into soybean genome confers tolerance to glufosinate and was used as a selectable marker during the soybean transformation. The *pat* gene encodes a protein of 183 amino acids that has a molecular weight of approximately 21 kDa. The *pat* gene has been widely used both as a selectable marker and herbicide tolerance trait in previously deregulated products (e.g., USDA 1996, USDA 2001, USDA 2004, USDA 2005)

Expression of the *pat* gene in the T-DNA insert of pDAB4468 is controlled by the CsVMV promoter from cassava vein mosaic virus and AtuORF1 3' UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The cassava vein mosaic virus is a double stranded DNA virus which infects cassava plants (*Manihot esculenta* Crantz) and has been characterized as a plant pararetrovirus belonging to the caulimovirus subgroup. The CsVMV promoter is known to drive constitutive expression of the genes it controls (Verdaguer *et al.*, 1996). AtuORF1 UTR has been used in previously deregulated products (USDA 2004).

IV-B. References

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

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

Barker, R.F., Idler, K.B., Thompson, D.V. and Kemp, J.D. (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

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

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

Labuda, I.M., Goers, S.K., Keon, K.A. (1992) Bioconversion Process for the Production of Vanillin. US Patent #5,128,253.

Norris, S.R., Meyer, S.E., Callis, J. (1993) The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Molecular Biology 21(5):895-906.

Ramachachandra Rao, S. and Ravishankar, G.A. (2000) Vanilla Flavor: Production by Conventional and Biotechnological Routes. Journal of the Science of Food and Agriculture 80:289-304.

Shetty, K., Paliyath, G., Pometto, A., Levin, R.E. (2006) Food Biotechnology, CRC Press, ISBN 0824753291, pp. 1672-1673.

Tamaoka, J., Ha, D.M., Komagata, K. (1987) Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas.* International Journal of Systematic Bacteriology 37:52-59.

Toms, A., Wood, J.M. (1970) The Degradation of trans-Ferulic Acid by *Pseudomonas acidovorans*. Biochemistry 9:337-343.

USDA (1996) Availability of Determination of Nonregulatated status for Soybeans Genetically Engineered for Glufosinate Herbicide Tolerance. Federal Register Volume 61, Number 160:42581-42582.

USDA (2001) Availability of Determination of Non-regulatated status for Genetically Engineered Corn for Insect Resistance and Glufosinate Herbicide Tolerance (Corn line 1507). Federal Register Volume 66, Number 157:42624-42625.

USDA (2004) Availability of Determination of Nonregulated Statusfor Cotton Lines Genetically Engineered for Insect Resistance. Federal Register Volume 69, Number 156:50154-50155.

USDA (2005) Availability of Determination of Non-regulated Status for Genetically Engineered Corn (Corn line DAS-59122-7). Federal Register Volume 70, Number 194:58663-58664.

Verdaguer, B., Kochko de, A., Beachy, R. N. and Fauquet, C. (1996) Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter. Plant Molec. Biol. 31:1129-1139.

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

Von Gravenitz, A, (1985) Ecology, clinical significance, and antimicrobial susceptibility encountered glucose-non-fermenting gram-negative rods, p. 181-232. In G. L. Gilardi (ed.), Nonfermentative gram-negative rods, laboratory identification and clinical aspects. Marcel Dekker, Inc., New York.

Wen, A., Fegan, M., Hayward, C., Chakraborty, S., Sly, L.I. (1999) Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren

de Jong 1926 and Tamaoka *et al.* 1987) gen. nov., comb. nov. International Journal of Systematic Bacteriology 49:567-576.

Wohlleben, W,, Arnold, W., Broer, I., Hillemann, D., Strauch, E. and Pühler, A. (1988) Nucleotide sequences of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene 70: 25-37.

Wood, D.W.; Setubal, J.C.; Kaul, R.; Monks, D.E.; Kitajima, J.P.; Okura, V.K.; Zhou, Y.; Chen, L.; Wood, G.E.; Almeida, N.F. Jr.; Woo, L.; Chen, Y.; Paulsen, I.T.; Eisen, J.A.; Karp, P.D.; Bovee, D. Sr.; Chapman, P.; Clendenning, J.; Deatherage, G.; Gillet, W.; Grant, C.; Kutyavin, T.; Levy, R.; Li, M.J.; McClelland, E.; Palmieri, A.; Raymond, C.; Rouse, G.; Saenphimmachak, C.; Wu, Z.; Romero, P.; Gordon, D.; Zhang, S.; Yoo, H.; Tao, Y.; Biddle, P.; Jung, M.; Krespan, W.; Perry, M.; Gordon-Kamm, B.; Liao, L.; Kim, S.; Hendrick, C.; Zhao, Z.Y.; Dolan, M.; Chumley, F.; Tingey, S.V.; Tomb, J.F.; Gordon, M.P.; Olson, M.V.; Nester, E.W. (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science 294(5550):2317-2323.

Wright, T.R.; Lira, J.M.; Walsh, T.; Merlo, D.J., Jayakumar, P., Lin, G. (2007) Novel Herbicide Resistance Genes. World Intellectual Property Organization International Publication Number WO 2007/053482 A2

Zambryski, P.; Depicker, A.; Kruger,K.; Goodman,H.M. (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. Journal of Molecular and Applied Genetics 1(4):361-370.

V. Genetic Characterization

V-A. Overview of Molecular Analysis

Molecular characterization of event DAS-68416-4 was conducted by Southern blot analysis. The results demonstrate that the transgene insert in soybean event DAS-68416-4 occurred as a simple integration of the T-DNA insert from plasmid pDAB4468, including a single, intact copy of the *aad-12* and *pat* expression cassettes. The event is stably integrated and inherited across and within breeding generations, and no plasmid backbone sequences are present in DAS-68416-4 soybean.

Detailed Southern blot analysis was conducted using probes specific to the gene coding sequences, promoters, terminators, and other regulatory elements contained in the pDAB4468 transformation plasmid. The locations of each probe on the pDAB4468 plasmid are described in Table 2 and shown in Figure 5. The expected and observed fragment sizes with specific digest and probe combinations, based on the known restriction enzyme sites of the pDAB4468 plasmid are shown in Table 3, Figure 6 and Figure 7, respectively. The Southern blot analyses described here made use of two types of restriction fragments: a) internal fragments in which known enzyme restriction sites are completely contained within the T-DNA insert of pDAB4468 and b) border fragments in which a known enzyme site is located within the T-DNA insert and a second site is located in the soybean genome flanking the insert. Border fragment sizes vary by event because they rely on the DNA sequence of flanking genomic region. Since integration sites are unique for each event, border fragments provide a means to determine the number of DNA insertions and to specifically identify the event.

Genomic DNA for Southern blot analysis was prepared from leaf material of individual DAS-68416-4 soybean plants from four distinct breeding generations. Genomic DNA from leaves of non-transgenic variety Maverick was used as the control material. Plasmid DNA of pDAB4468 added to genomic DNA from the conventional control served as the positive control for the Southern blot analysis. Materials and methods used for the Southern analyses are further described in Appendix 1.

The expected restriction fragments of the inserted DNA are shown in Figure 7. Southern blot analysis showed that event DAS-68416-4 contains a single intact copy of the *aad-12* and *pat* expression cassettes integrated at a single locus (Section V-B). No vector backbone sequences were detected in event DAS-68416-4 (Section V-C). The hybridization patterns across four generations of DAS-68416-4 soybean (T2, T3, T4, and T5) were identical, indicating that the insertion is stably integrated in the soybean genome (Section V-D). The inheritance of DAS-68416-4 soybean in segregating generations was investigated using Southern blot analysis, detection of the AAD-12 protein, and detection of the *aad-12* gene; all results confirmed the predicted inheritance of the transgene in a single locus (Section V-E).

Table 2. List of probes and their positions in plasmid pDAB4468.

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

Figure 5. Location of probes on pDAB4468 used in Southern blot analysis of DAS-68416-4 soybean.

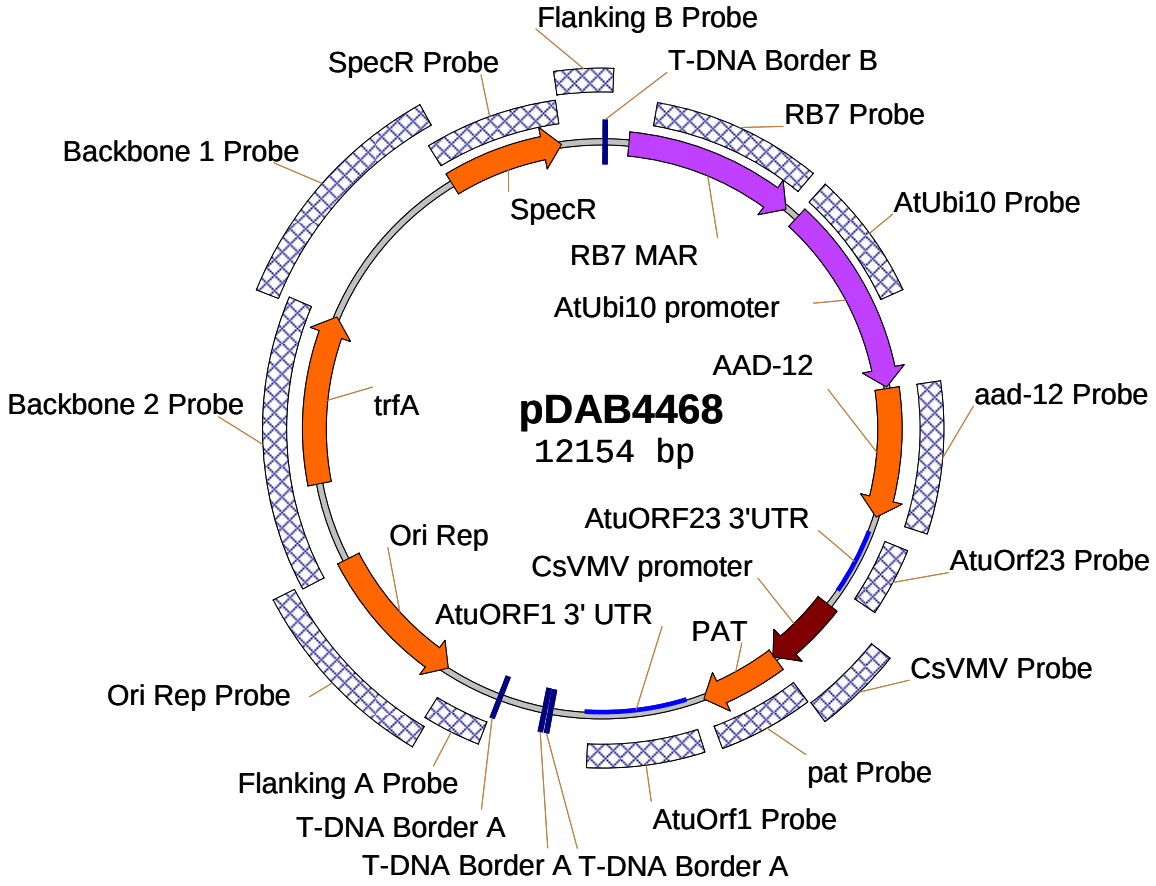


Table 3. Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-68416-4 soybean.

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
aad-12	*Nco* I	Plasmid pDAB4468	Figure 8, Figure 11	7957	7957
		DAS-68416-4	Figure 8, Figure 11	> 4043*	~5500*
		Control (Maverick)	Figure 8, Figure 11	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 9, Figure 11	12146	12146
		DAS-68416-4	Figure 9, Figure 11	> 6229*	~8500*
		Control (Maverick)	Figure 9, Figure 11	none	none
	Nhe I/*Xho* I	Plasmid pDAB4468	Figure 10, Figure 11	12148	12148
		DAS-68416-4	Figure 10, Figure 11	> 6229*	~7200*
		Control (Maverick)	Figure 10, Figure 11	none	none
pat	*Nco* I	Plasmid pDAB4468	Figure 16, Figure 19	7957	7957
		DAS-68416-4	Figure 16, Figure 19	> 4043*	~5500*
		Control (Maverick)	Figure 16, Figure 19	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 17, Figure 19	12146	12146
		DAS-68416-4	Figure 17, Figure 19	> 6229*	~8500*
		Control (Maverick)	Figure 17, Figure 19	none	none
	Nhe I/*Xho* I	Plasmid pDAB4468	Figure 18, Figure 19	12148	12148
		DAS-68416-4	Figure 18, Figure 19	> 6229*	~7200*
		Control (Maverick)	Figure 18, Figure 19	none	none
aad-12	*Pst* I (Release PTU)	Plasmid pDAB4468	Figure 12, Figure 13	2868	2868
		DAS-68416-4	Figure 12, Figure 13	2868	2868
		Control (Maverick)	Figure 12, Figure 13	none	none
AtUbi10		Plasmid pDAB4468	Figure 14, Figure 13	2868	2868
		DAS-68416-4	Figure 14, Figure 13	2868	2868
		Control (Maverick)	Figure 14, Figure 13	none	none
AtuORF23		Plasmid pDAB4468	Figure 15, Figure 13	2868	2868
		DAS-68416-4	Figure 15, Figure 13	2868	2868
		Control (Maverick)	Figure 15, Figure 13	none	none
pat	*Pst* I/*Xho* I (Release PTU)	Plasmid pDAB4468	Figure 20, Figure 21	1928	1928
		DAS-68416-4	Figure 20, Figure 21	1928	1928
		Control (Maverick)	Figure 20, Figure 21	none	none
CsVMV		Plasmid pDAB4468	Figure 22, Figure 21	1928	1928
		DAS-68416-4	Figure 22, Figure 21	1928	1928
		Control (Maverick)	Figure 22, Figure 21	none	none
AtuORF1		Plasmid pDAB4468	Figure 23, Figure 21	1928	1928
		DAS-68416-4	Figure 23, Figure 21	1928	1928
		Control (Maverick)	Figure 23, Figure 21	none	none
RB7	*Bam*H I/*Nco* I	Plasmid pDAB4468	Figure 24, Figure 26	2617	2617
		DAS-68416-4	Figure 24, Figure 26	2617	2617
		Control (Maverick)	Figure 24, Figure 26	none	none
AtUbi10		Plasmid pDAB4468	Figure 25, Figure 26	2617	2617
		DAS-68416-4	Figure 25, Figure 26	2617	2617
		Control (Maverick)	Figure 25, Figure 26	none	none

Table 3 (cont.). Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-68416-4 soybean.

Probe	Restriction Enzyme	Sample	Southern Blot Figure	Fragment Size (bp)	
				Expected	Observed
Flanking A	*Nco* I	Plasmid pDAB4468	Figure 27, Figure 31	7957	7957
		DAS-68416-4	Figure 27, Figure 31	none	none
		Control (Maverick)	Figure 27, Figure 31	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 29, Figure 31	12146	12146
		DAS-68416-4	Figure 29, Figure 31	none	none
		Control (Maverick)	Figure 29, Figure 31	none	none
Backbone1	*Nco* I	Plasmid pDAB4468	Figure 27, Figure 31	4197, 7957	4197, 7957
		DAS-68416-4	Figure 27, Figure 31	none	none
		Control (Maverick)	Figure 27, Figure 31	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 29, Figure 31	12146	12146
		DAS-68416-4	Figure 29, Figure 31	none	none
		Control (Maverick)	Figure 29, Figure 31	none	none
SpecR	*Nco* I	Plasmid pDAB4468	Figure 27, Figure 31	4197	4197
		DAS-68416-4	Figure 27, Figure 31	none	none
		Control (Maverick)	Figure 27, Figure 31	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 29, Figure 31	12146	12146
		DAS-68416-4	Figure 29, Figure 31	none	none
		Control (Maverick)	Figure 29, Figure 31	none	none
Flanking B	*Nco* I	Plasmid pDAB4468	Figure 28, Figure 32	4197	4197
		DAS-68416-4	Figure 28, Figure 32	none	none
		Control (Maverick)	Figure 28, Figure 32	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 30, Figure 32	12146	12146
		DAS-68416-4	Figure 30, Figure 32	none	none
		Control (Maverick)	Figure 30, Figure 32	none	none
Backbone2	*Nco* I	Plasmid pDAB4468	Figure 28, Figure 32	7957	7957
		DAS-68416-4	Figure 28, Figure 32	none	none
		Control (Maverick)	Figure 28, Figure 32	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 30, Figure 32	12146	12146
		DAS-68416-4	Figure 30, Figure 32	none	none
		Control (Maverick)	Figure 30, Figure 32	none	none
Ori-Rep	*Nco* I	Plasmid pDAB4468	Figure 28, Figure 32	7957	7957
		DAS-68416-4	Figure 28, Figure 32	none	none
		Control (Maverick)	Figure 28, Figure 32	none	none
	Sph I/*Xho* I	Plasmid pDAB4468	Figure 30, Figure 32	12146	12146
		DAS-68416-4	Figure 30, Figure 32	none	none
		Control (Maverick)	Figure 30, Figure 32	none	none

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

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

Figure 6. Plasmid map of pDAB4468 with restriction enzyme sites used for Southern blot analysis.

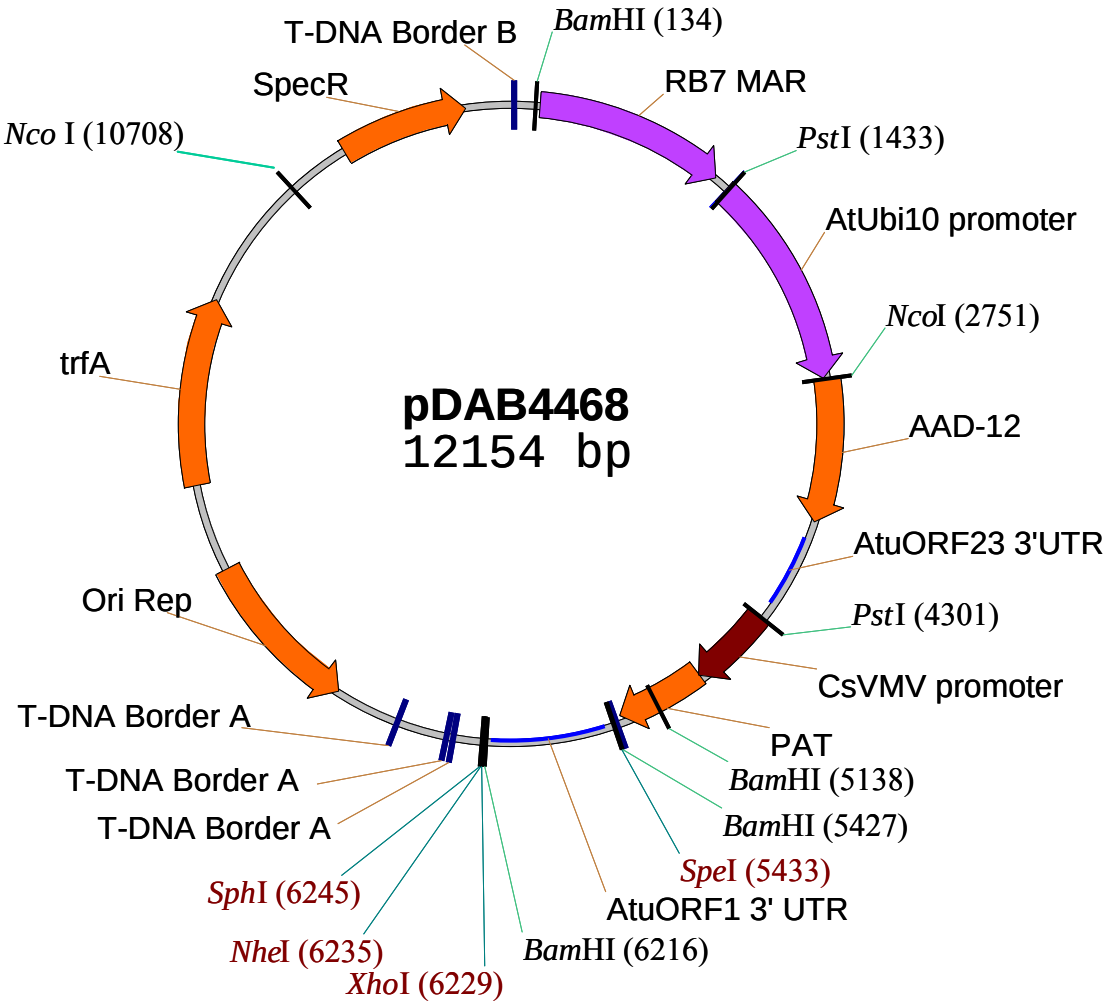
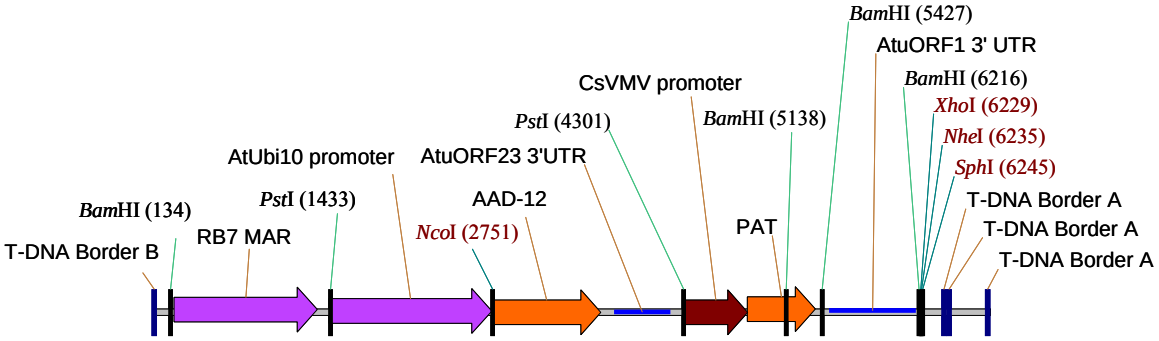


Figure 7. pDAB4468 T-DNA insert, restriction enzymes used in DNA digestion and expected hybridization bands.



pDAB4468 T-DNA Insert
6794 bp

Nco I	> 2751 bp	> 4043 bp				
Nhe I/*Xho* I	> 6229 bp					> 559 bp
Sph I/*Xho* I	> 6229 bp					> 549bp
Pst I	> 1433 bp	2868 bp	> 2493 bp			
Pst I/*Xho* I	> 1433 bp	2868 bp	1928 bp			> 565 bp
*Bam*H I/*Nco* I	2617 bp	2387 bp	289 bp	789 bp		> 578 bp

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

V-B.1. Analysis of the *aad-12* Gene

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

V-B.2. Analysis of the AtUbi10 Promoter

Restriction enzyme *Pst* I was used to characterize the AtUbi10 promoter region for *aad-12* in event DAS-68416-4. *Pst* I digestion was expected to release a PTU (plant transcription unit) fragment of 2868 bp which contains the AtUbi10 promoter, *aad-12* gene, and AtuORF23 terminator sequences. The predicted 2868 bp fragment was observed following the *Pst* I digestion and hybridization with AtUbi10 promoter probe (Figure 13B, Figure 14). The AtUbi10 promoter was further characterized with a double digestion of *Bam*H I and *Nco* I which releases a fragment of 2617bp containing AtUbi10 promoter and RB7 MAR element. The predicted 2617 bp fragment was detected following the enzyme digestion and hybridization with AtUbi10 promoter probe (Figure 25, Figure 26B). Results obtained with *Pst* I or *Bam*H I/*Nco* I digestion of the DAS-68416-4 sample followed by AtUbi10 promoter probe hybridization further confirmed that a single copy of an intact *aad-12* PTU from plasmid pDAB4468, along with a RB7 MAR element at its 5' end, was inserted into the soybean genome of event DAS-68416-4.

V-B.3. Analysis of the AtuORF23 3'UTR

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

V-B.4. Analysis of the *pat* Gene

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

V-B.5. Analysis of the CsVMV Promoter

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

V-B.6. Analysis of the AtuORF1 3'UTR

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

V-B.7. Analysis of the RB7 MAR

Restriction enzyme combination of *BamH* I and *Nco* I was selected to characterize the RB7 MAR elements from the T-DNA insert in pDAB4468 (Table 3). A double digestion with *BamH* I and *Nco* I was expected to release a fragment of 2617 bp containing the RB7 MAR and AtUbi10 promoter. The predicted 2617 bp fragment was observed following the double enzyme digestion and hybridization with RB7 MAR and Atubi10 probe, respectively (Figure 24, Figure

26A, Figure 26B). Results obtained with *BamH* I/*Nco* I double digestion of the DAS-68416-4 sample followed by hybridization with RB7 MAR (Figure 25, Figure 26B) further confirmed that a single copy of an intact RB7 MAR, along with an intact *aad-12* PTU from plasmid pDAB4468, was inserted into the soybean genome of event DAS-68416-4.

V-C. Absence of Vector Backbone DNA

To verify that no plasmid vector backbone sequences exist in event DAS-68416-4, six probes covering the whole backbone region of pDAB4468 were used to hybridize the blots from digestions with *Nco* I and *Sph* I/*Xho* I (Table 2, Figure 5). For the T5 generation, a blot from digestion with *Nhe* I/*Xho* I was also hybridized with backbone probes. The probes were grouped into 2 sets by mixing them with equal ratio for hybridization purposes. Probe Set 1 included backbone1, flanking A, and *Spec*R, and Probe Set 2 included backbone 2, flanking B, and *Ori-Rep* (Figure 5, Table 2). The blots were hybridized with Probe Set 1, and then followed by Probe Set 2 after complete removal of previously deployed probes. No hybridization signals were detected in any sample across the T2 to T5 generations (Table 3, Figure 27, Figure 28, Figure 29, Figure 30, Figure 31, Figure 32) except for the positive controls, indicating no backbone sequences from pDAB4468 were incorporated into event DAS-68416-4.

V-D. Stability of the Insert across Generations

Southern blot hybridizations were conducted with four distinct generations, T2, T3, T4, and T5, of event DAS-468416-4. Prior to initiation of Southern blot analysis, all plants were tested for AAD-12 protein expression using a lateral flow strip test kit to allow confirmation of AAD-12 expression positive plants. All of the genetic element probes: *aad-12* gene, AtUbi10 promoter, AtuORF23 terminator, CsVMV promoter, *pat* gene, AtuORF1 terminator, and RB7 MAR, and the backbone of plasmid pDAB4468, were hybridized with the four generations of DAS-68416-4 soybean. Results across all DAS-68416-4 samples in four generations were as expected (Table 3, Figure 8 − Figure 32), indicating stable integration and inheritance of the intact, single copy insert across multiple generations of DAS-68416-4 soybean.

Figure 8. Southern blot analysis of *Nco* I digest with *aad-12* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with *aad-12* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 15 was due to a larger amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

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

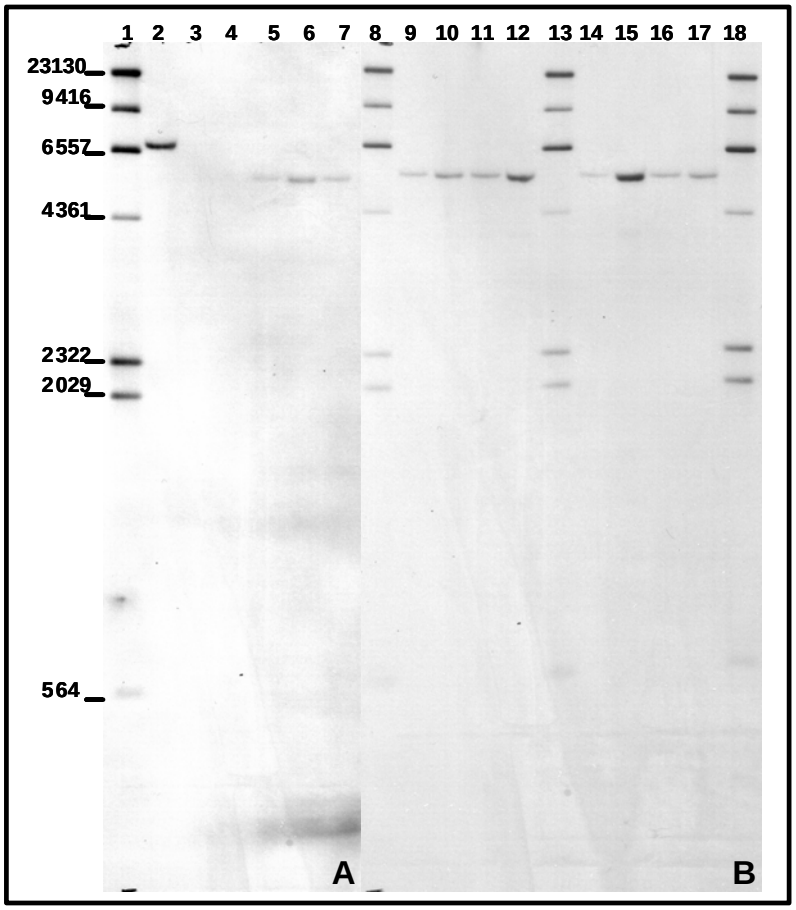


Figure 9. Southern blot analysis of *Sph* I/*Xho* I digest with *aad-12* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with *aad-12* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 10 and 15 were due to a larger amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

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

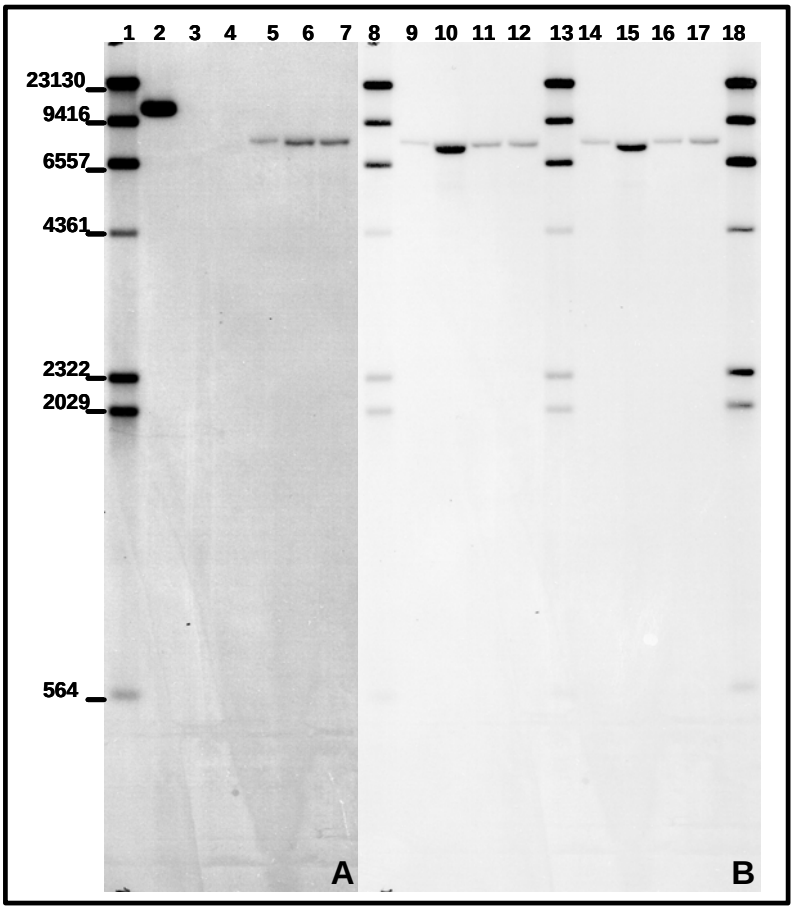


Figure 10. Southern blot analysis of *Nhe* I/*Xho* I digest with *aad-12* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Nhe* I/*Xho* I and hybridized with *aad-12* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 9 was due to a larger amount of DNA recovered after digestion).

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

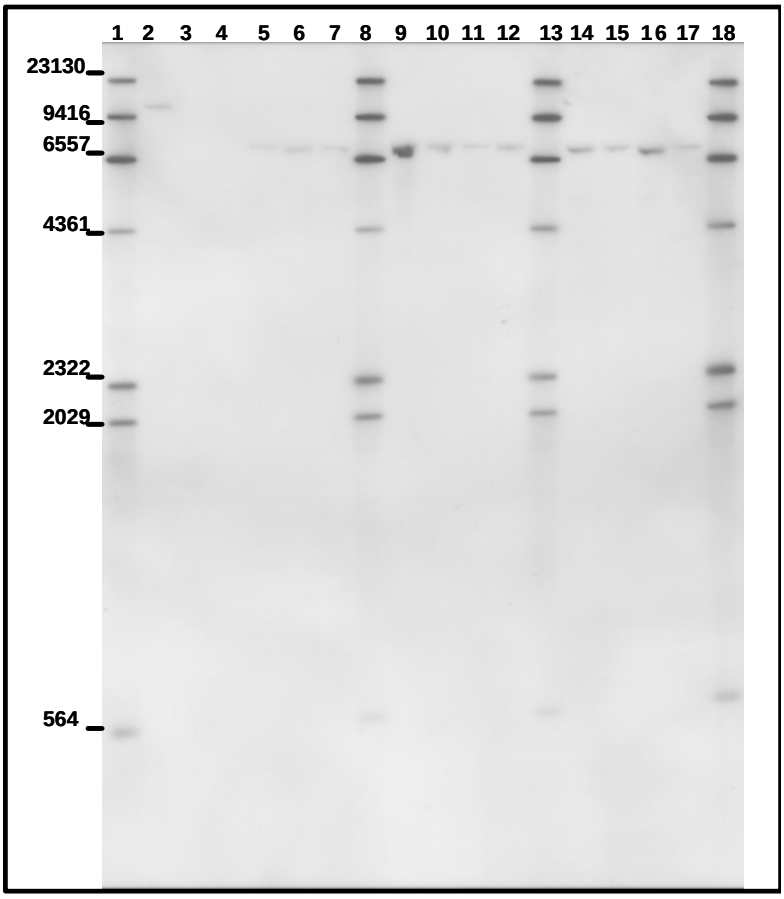


Figure 11. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with *aad-12* probe.

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

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

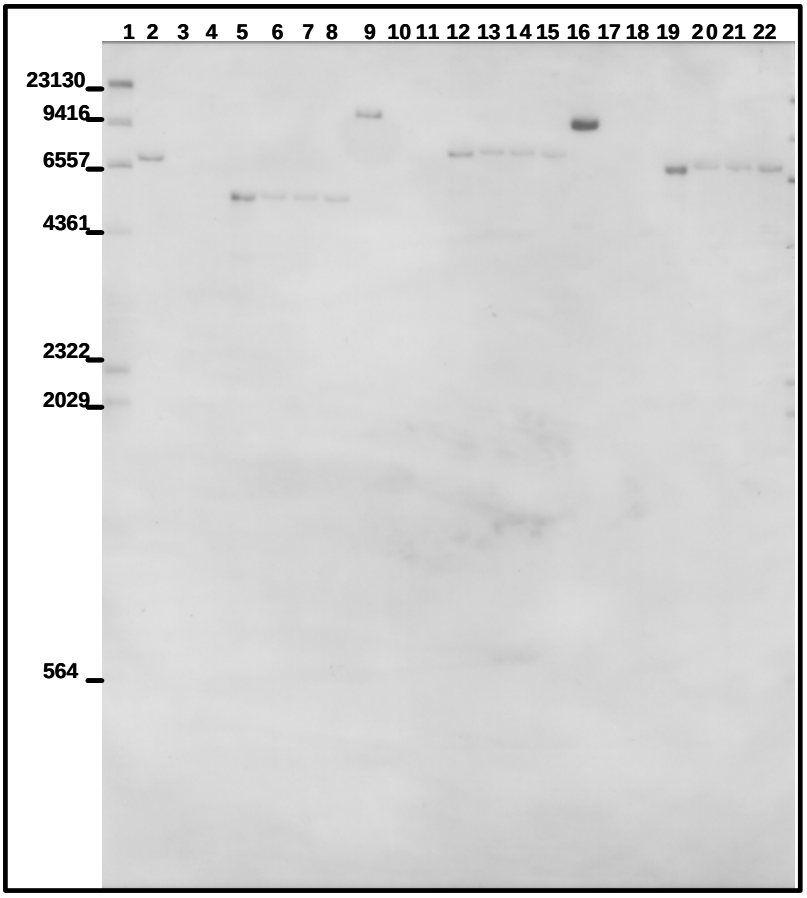


Figure 12. Southern blot analysis of *Pst* I digest with *aad-12* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Pst* I and hybridized with *aad-12* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).

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

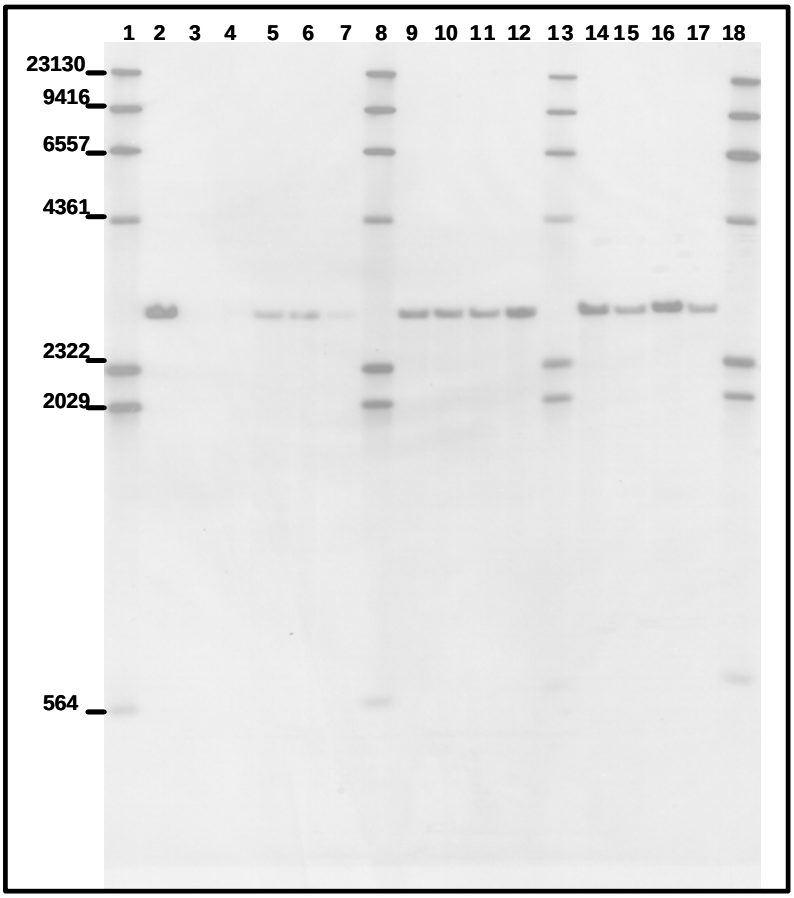


Figure 13. Southern blot analysis of *Pst* I digest of T5 generation with *aad-12*, AtUbi10, and AtuORF23 probes.

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

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

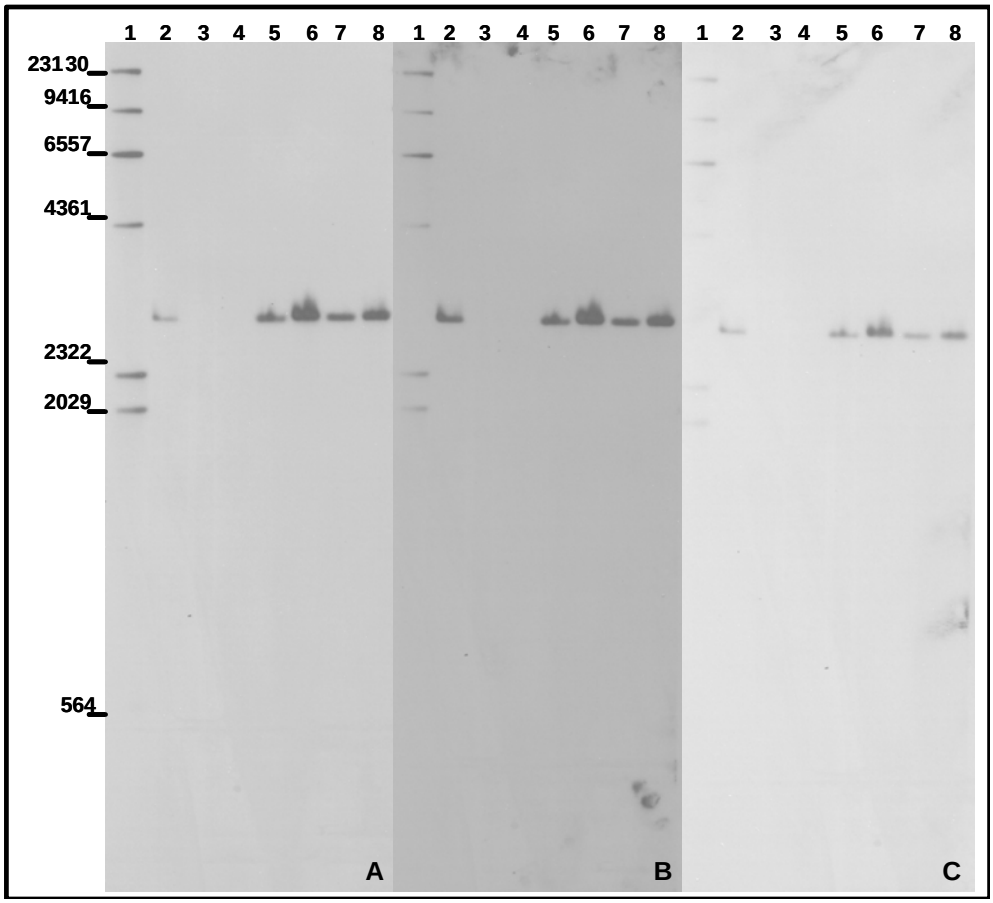


Figure 14. Southern blot analysis of *Pst* I digest with AtUbi10 probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Pst* I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).

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

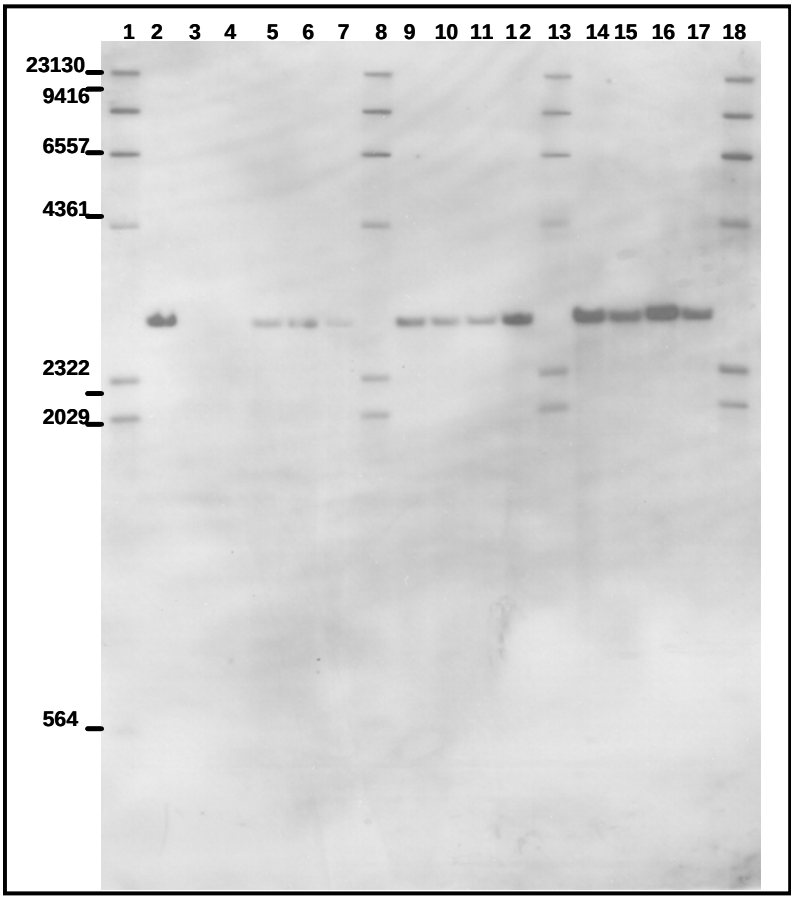


Figure 15. Southern blot analysis of *Pst* I digest with AtuORF23 probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Pst* I and hybridized with AtuORF23 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signal in Lane 7 was due to a lesser amount of DNA recovered after digestion).

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

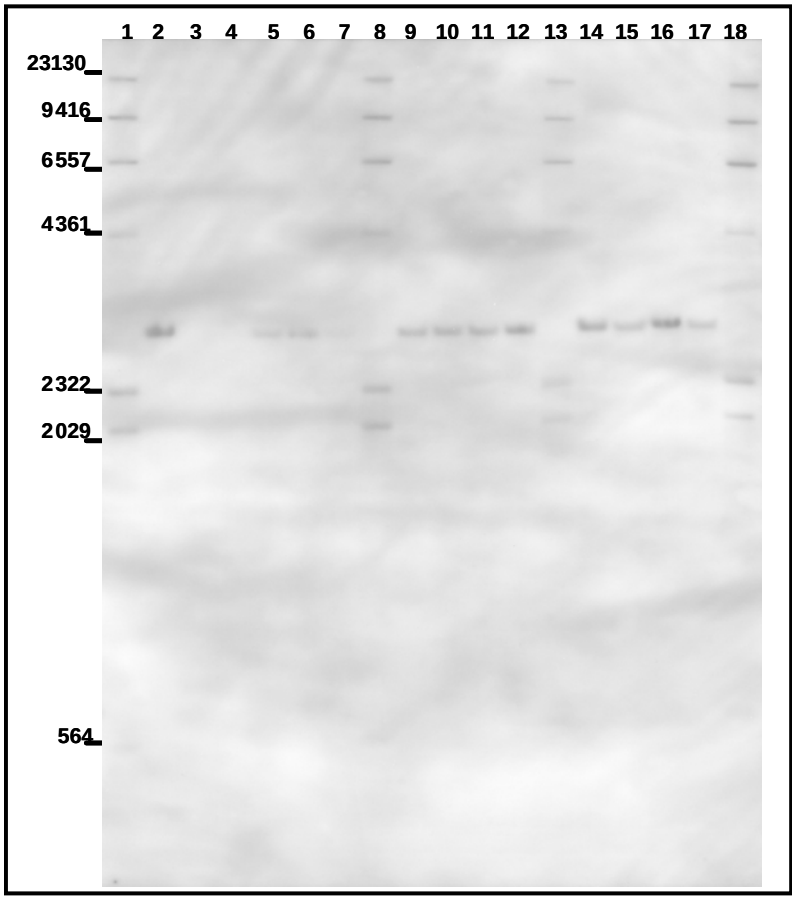


Figure 16. Southern blot analysis of *Nco* I digest with *pat* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 12 and 15 were due to the greater amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container).

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

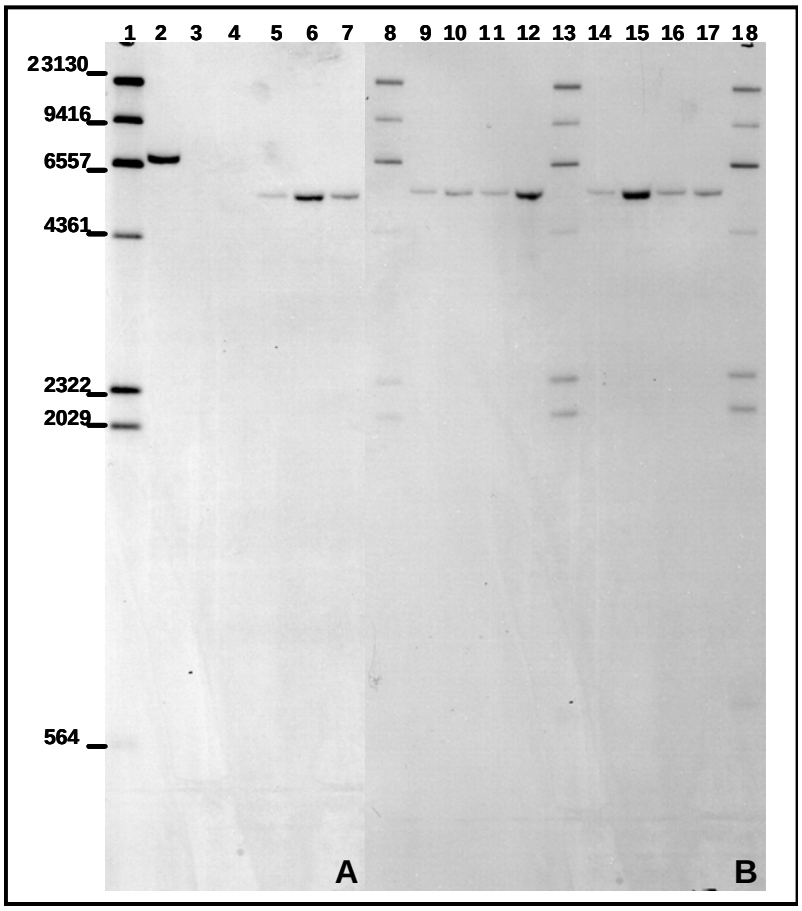


Figure 17. Southern blot analysis of *Sph* I/*Xho* I digest with *pat* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signals in Lane 10 and 15 were due to the greater amount of DNA recovered after digestion. Panel A and B were from the same blot and hybridized in the same container.)

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

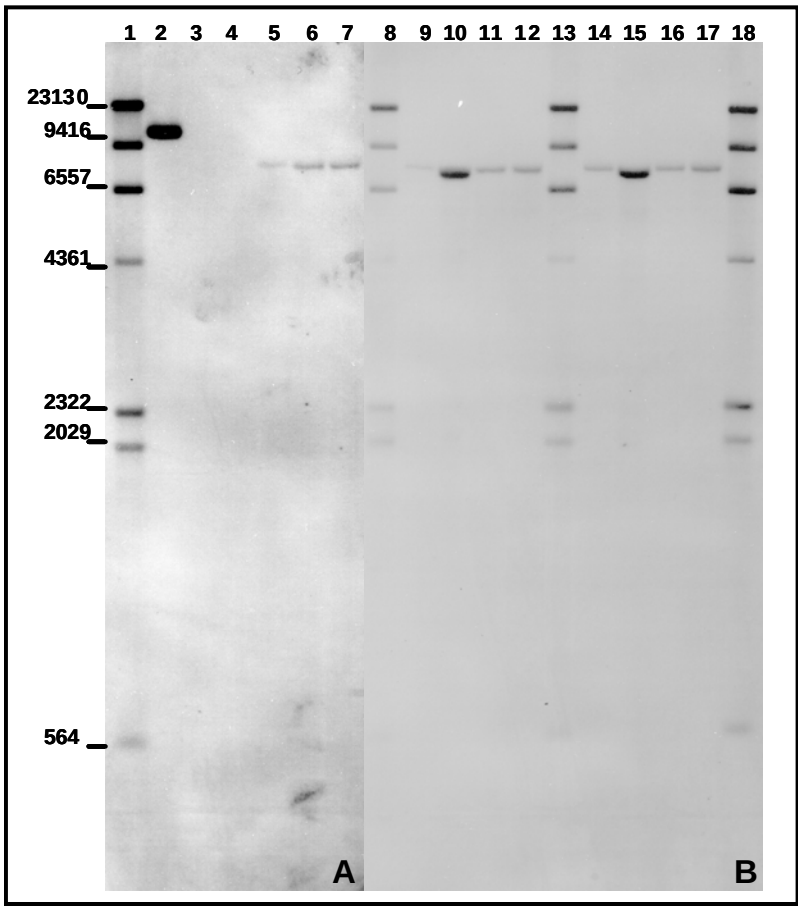


Figure 18. Southern blot analysis of *Nhe* I/*Xho* I digest with *pat* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Nhe* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively strong signal in Lane 9 was due to the greater amount of DNA recovered after digestion).

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

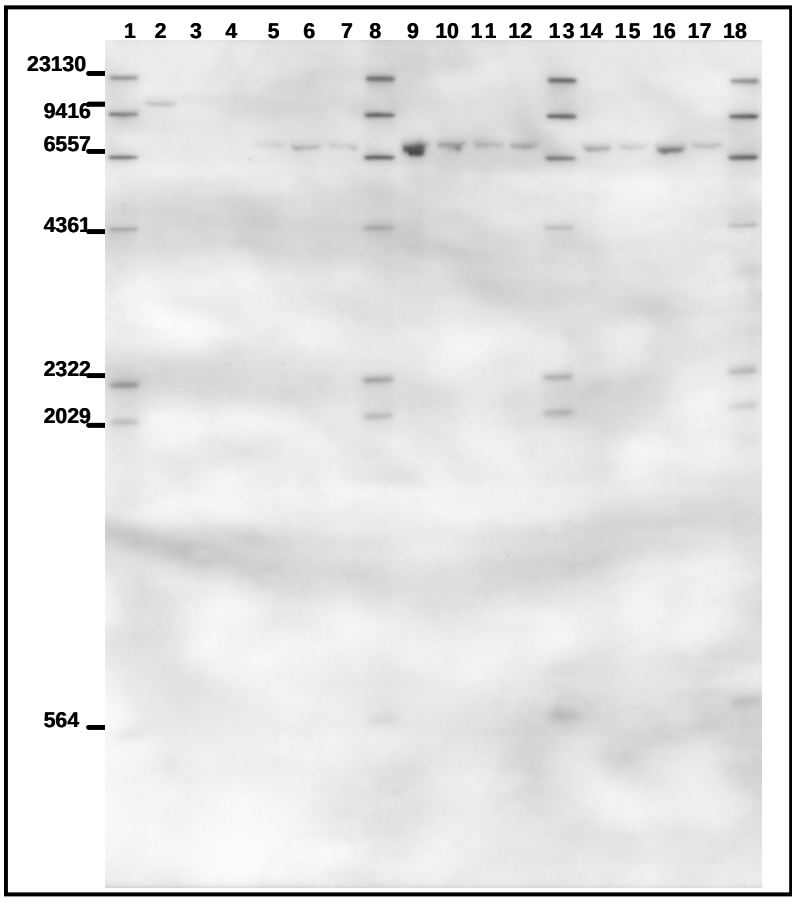


Figure 19. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with *pat* probe.

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

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

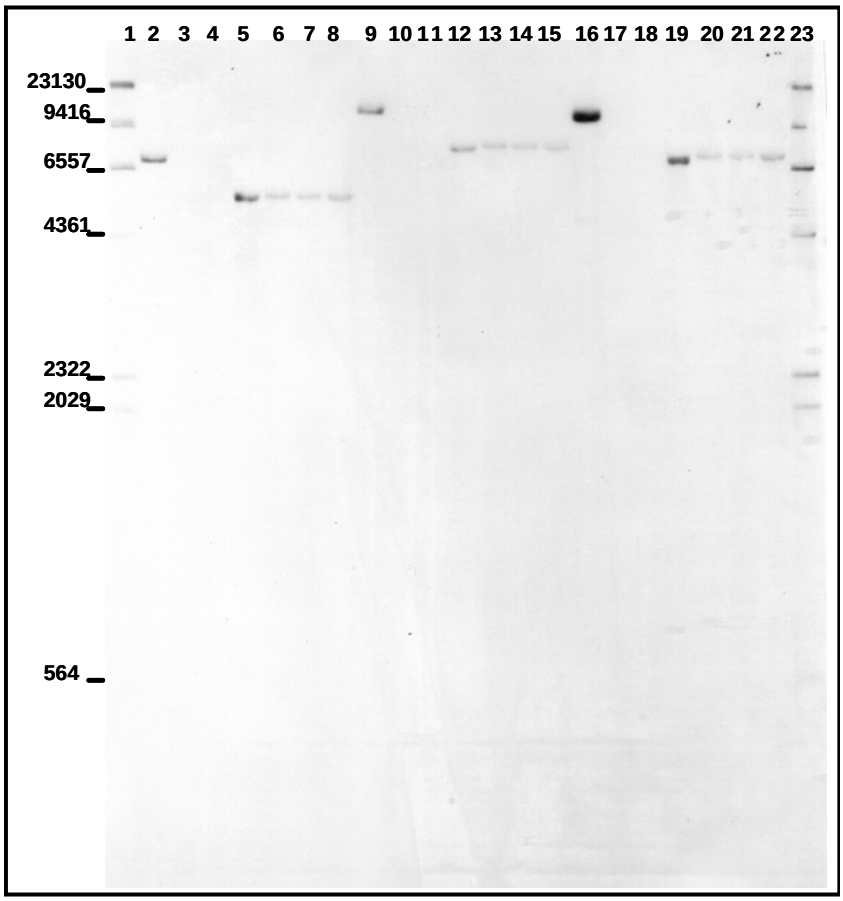


Figure 20. Southern blot analysis of *Pst* I/*Xho* I digest with *pat* probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with *pat* probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

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

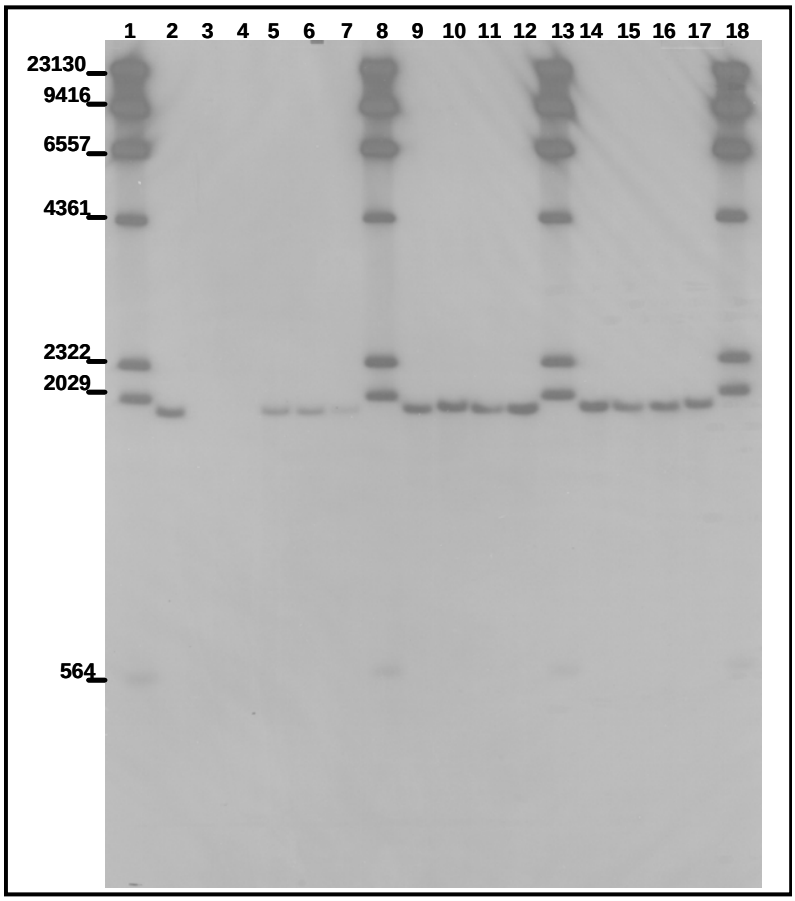


Figure 21. Southern blot analysis of *Pst* I/*Xho* I digest of T5 generation with *pat,* CsVMV, and AtuORF1 probes.

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

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

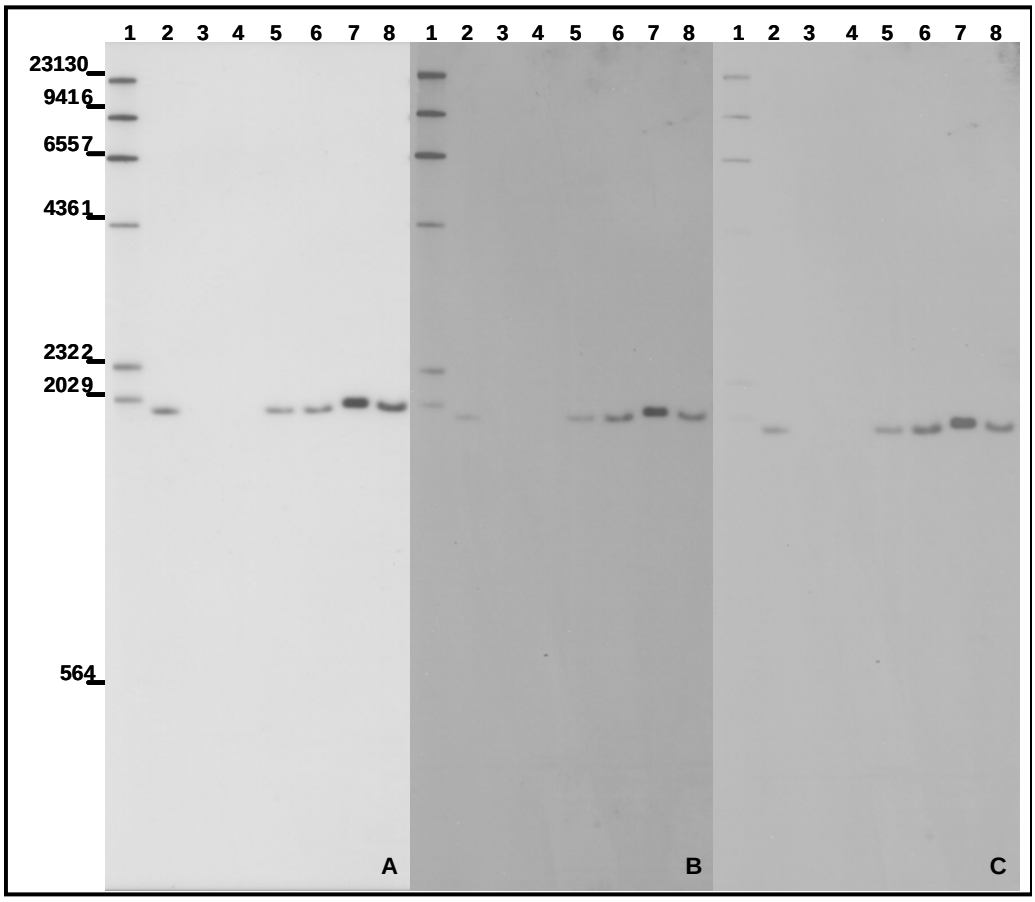


Figure 22. Southern blot analysis of *Pst* I/*Xho* I digest with CsVMV probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with CsVMV probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

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

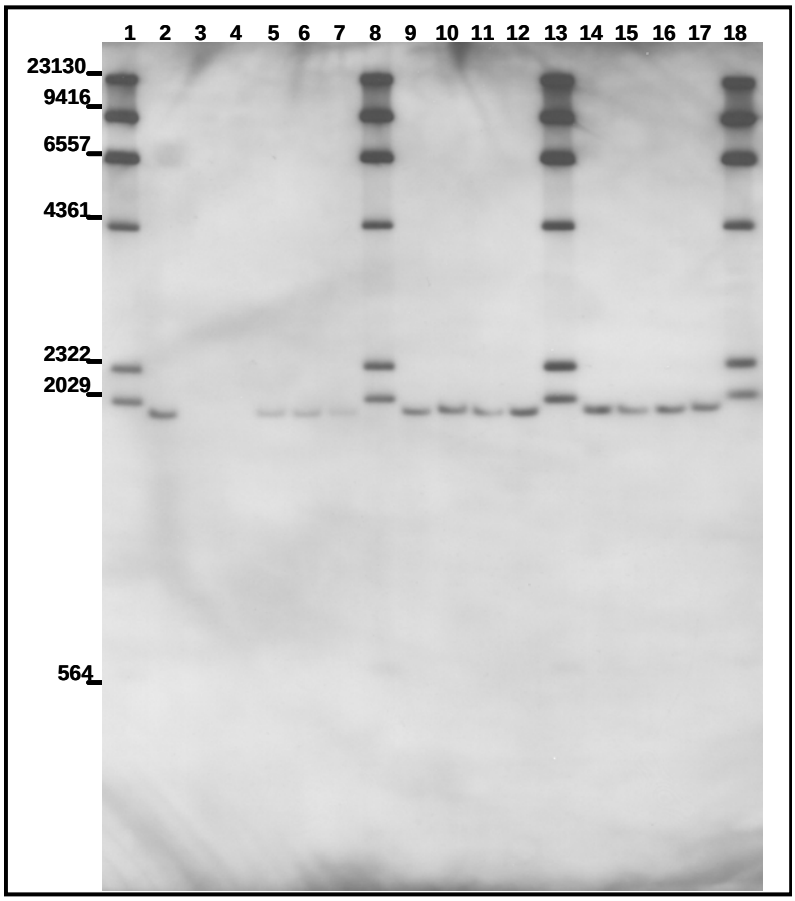


Figure 23. Southern blot analysis of *Pst* I/*Xho* I digest with AtuORF1 probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Pst* I/*Xho* I and hybridized with AtuORF1 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

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

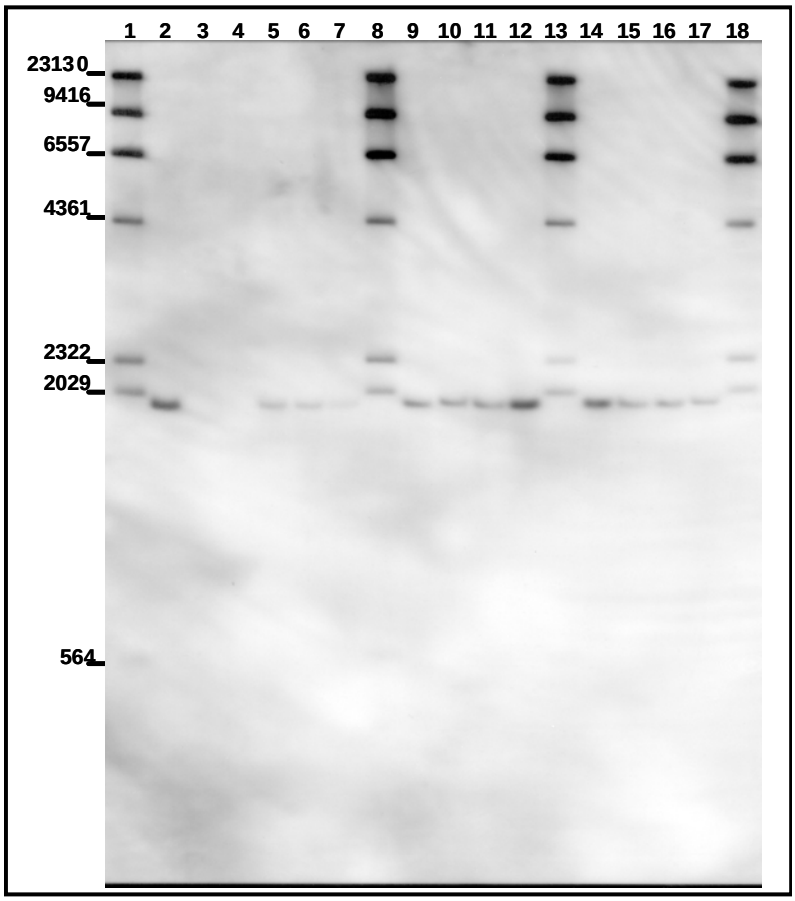


Figure 24. Southern blot analysis of *Bam*H I/*Nco* I digest with RB7 probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Bam*H I/*Nco* I and hybridized with RB7 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: The relatively weak signals in Lane 6 and 7 were due to the less amount of DNA recovered after digestion).

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

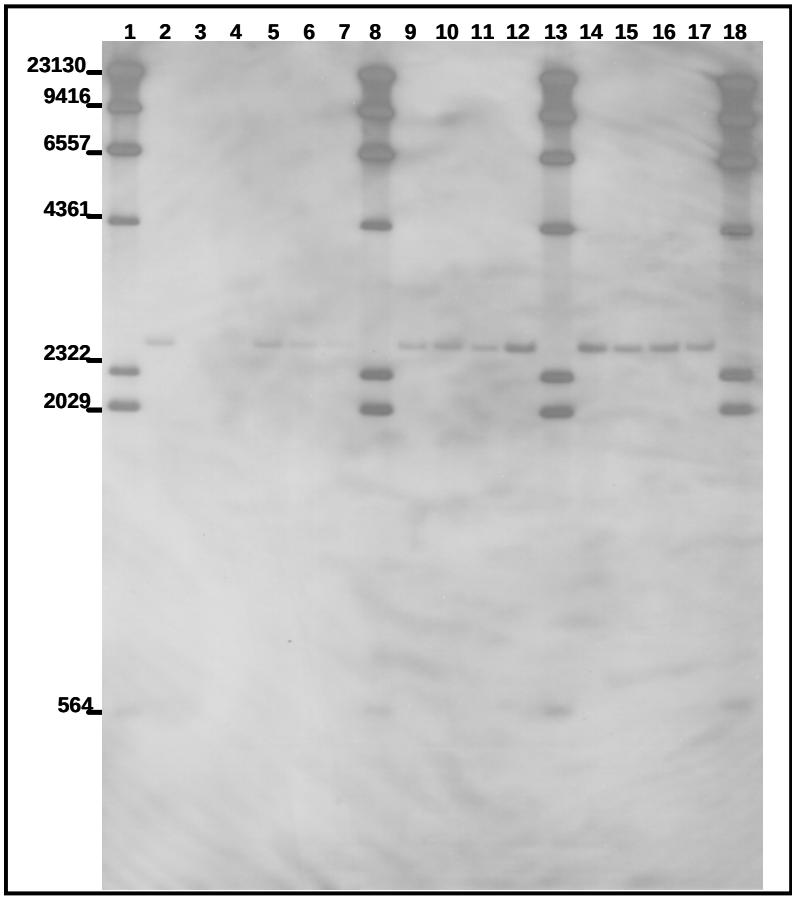


Figure 25. Southern blot analysis of *Bam*H I/*Nco* I digest with AtUbi10 probe.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2, T3, T4 and the non-transgenic control were digested with *Bam* HI/*Nco* I and hybridized with AtUbi10 probe. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome.

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

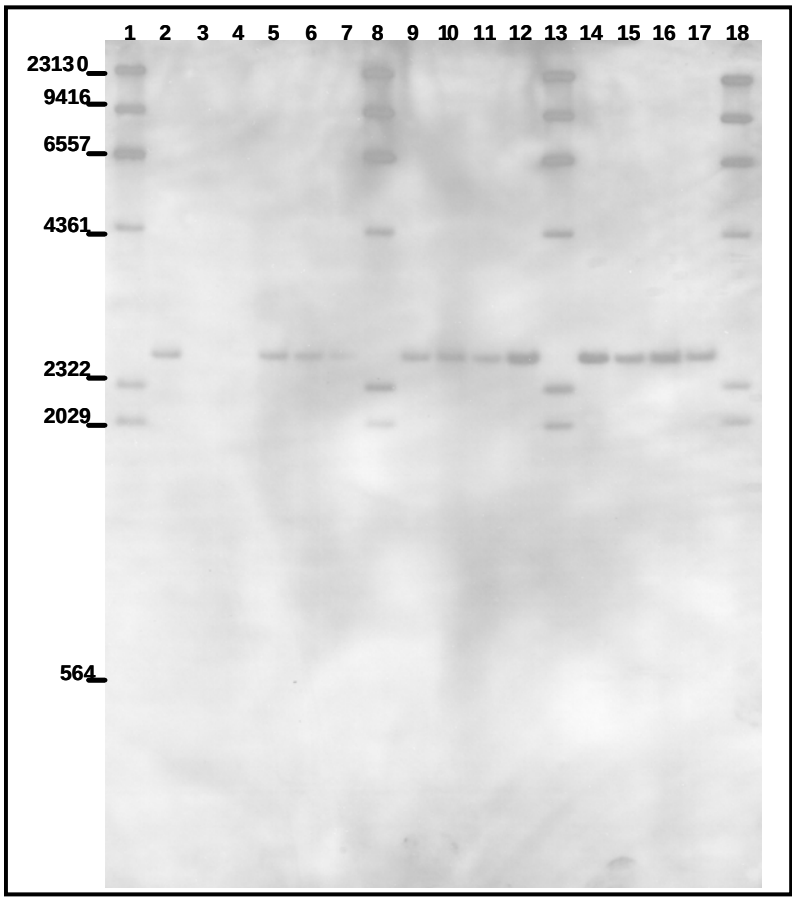


Figure 26. Southern blot analysis of *Bam*H I/*Nco* I digest of T5 generation with RB7 and AtUbi10 probes.

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

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

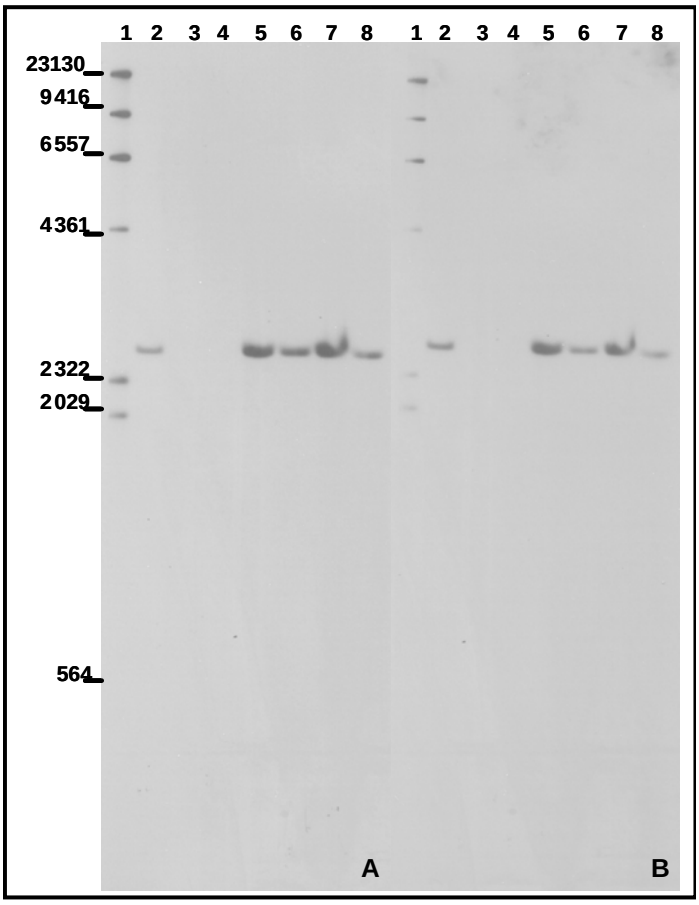


Figure 27. Southern blot analysis of *Nco* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).

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

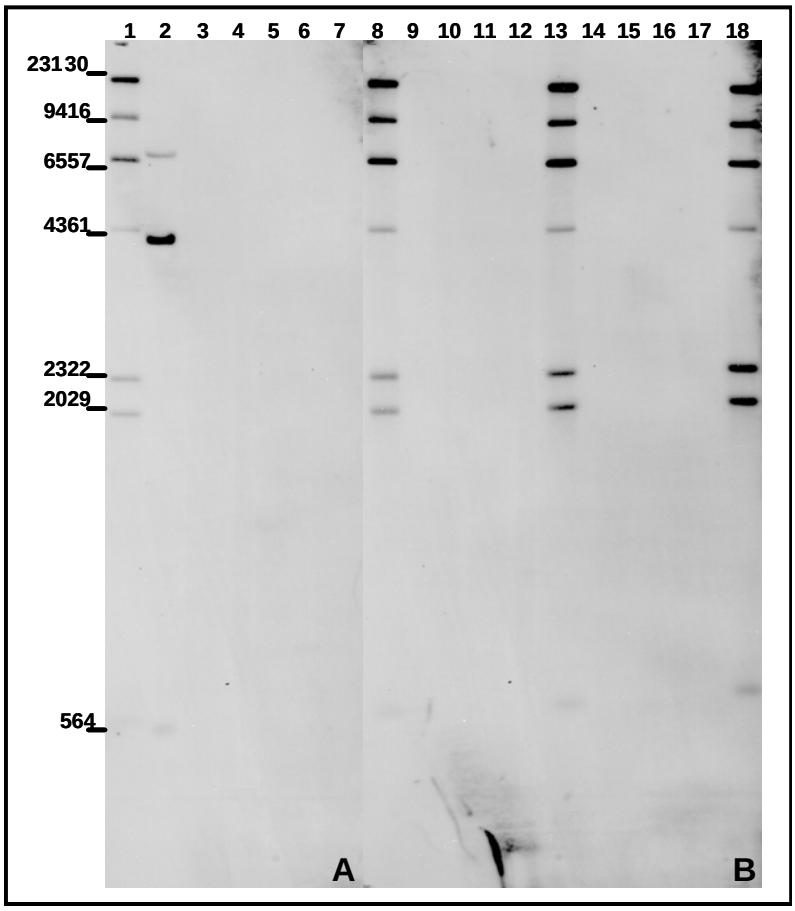


Figure 28. Southern blot analysis of *Nco* I digest with backbone probe set 2 from plasmid pDAB4468 vector backbone.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Nco* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*) probes. Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).

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

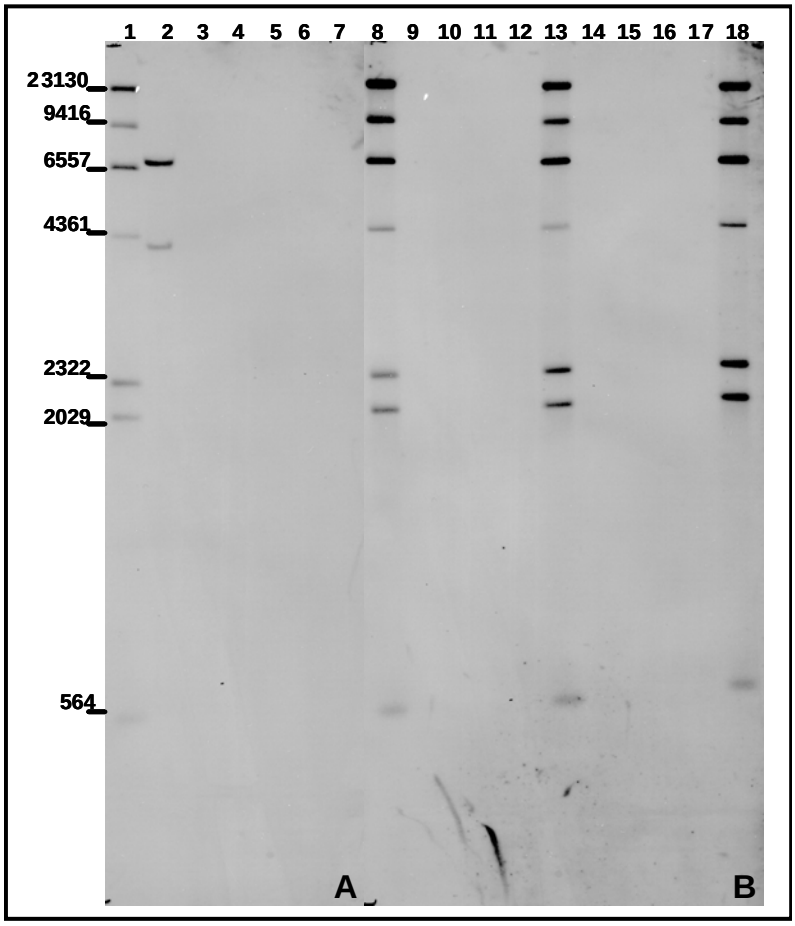


Figure 29. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 1 from plasmid pDAB4468 vector backbone.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with Backbone Probe Set 1 (Backbone1, Flanking A, and *SpecR*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).

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

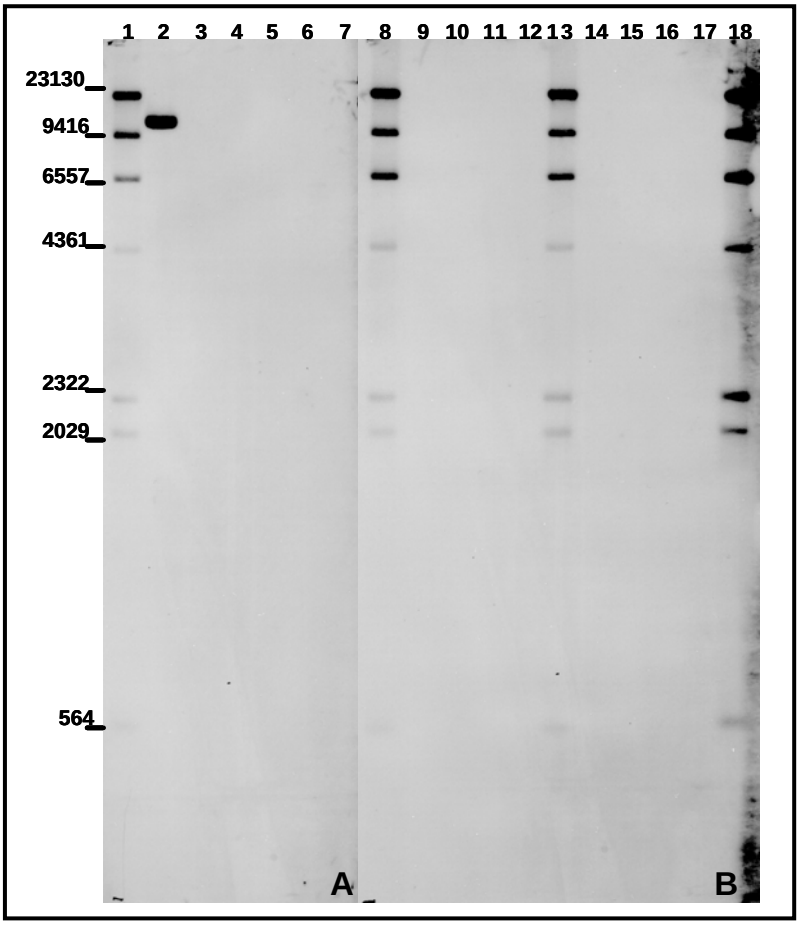


Figure 30. Southern blot analysis of *Sph* I/*Xho* I digest with backbone probe set 2 plasmid pDAB4468 vector backbone.

DNA isolated from individual plants of soybean event DAS-68416-4 generations T2 (Panel A), T3, T4 (Panel B) and the non-transgenic control were digested with *Sph* I/*Xho* I and hybridized with Backbone Probe Set 2 (Backbone2, Flanking B, and *Ori-Rep*). Nine (9) µg of DNA was digested and loaded per lane. The plasmid control contained plasmid pDAB4468 mixed with 9 µg of non-transgenic DNA at a ratio approximately equivalent to 1 transgene copy per soybean genome. (Note: Panel A and B were from the same blot and hybridized in the same container).

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

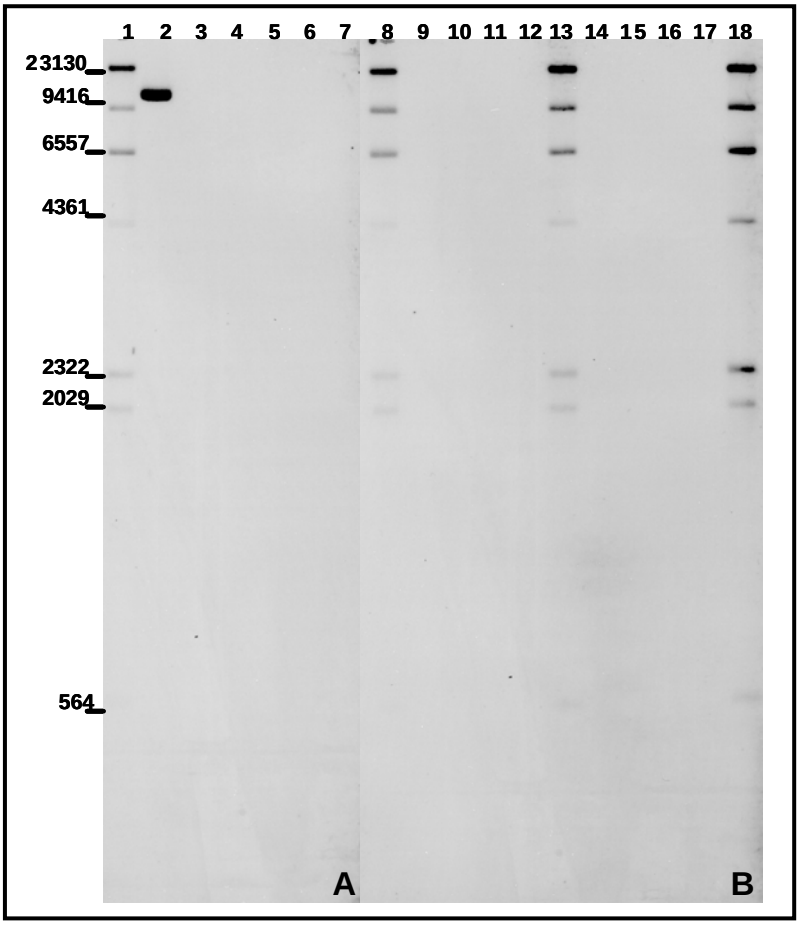


Figure 31. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generations with the backbone probe set 1 from pDAB4468 vector backbone.

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

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

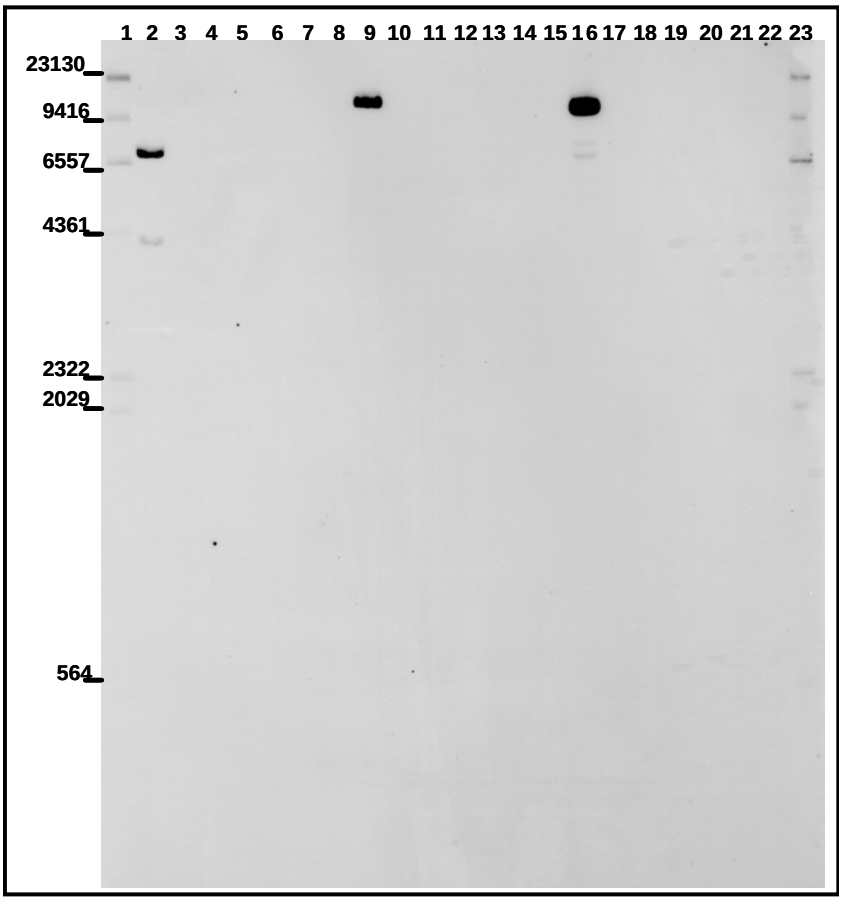
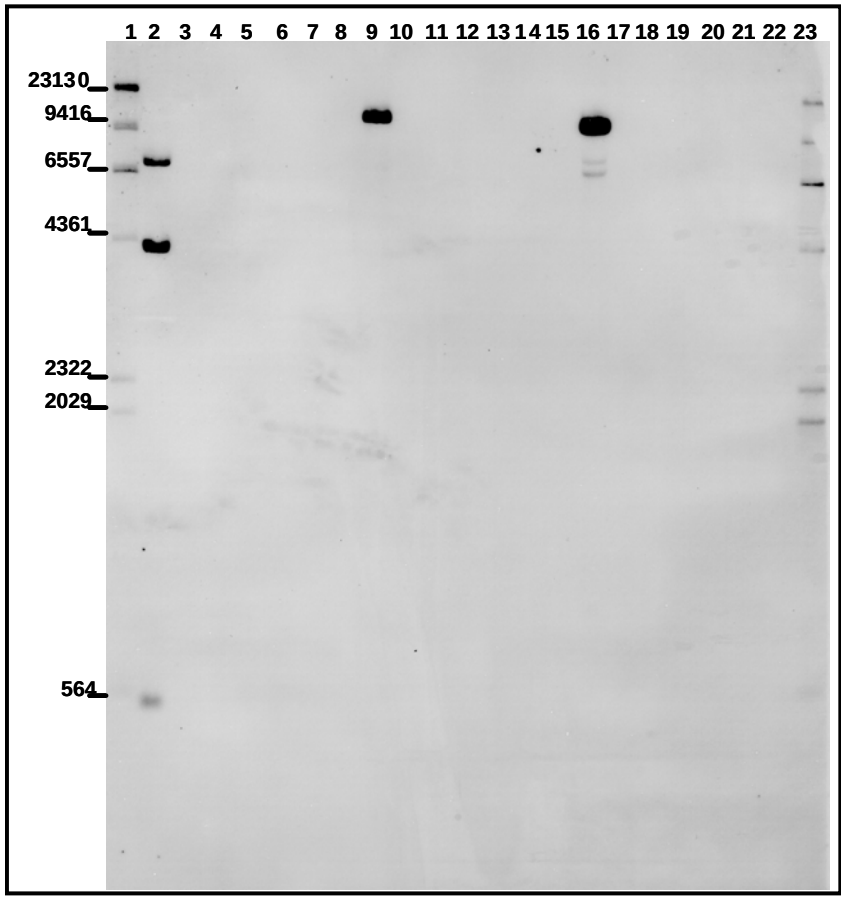


Figure 32. Southern blot analysis of *Nco* I, *Sph* I/*Xho* I, and *Nhe* I/*Xho* I digests of T5 generation with the backbone probe set 2 from pDAB4468 vector backbone.

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

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



V-E. Segregation Analysis

V-E.1. Genetic and Molecular Analysis of a Segregating Generation

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

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

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

Table 4. Results of F2 individual plants tested for of AAD-12 expression within a single segregating generation.

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

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

Table 5. Predicted and observed hybridizing fragments in Southern blot analysis of F2 population.

Restriction Enzymes	DNA Probe	Sample Source	Southern Blot Figure	Expected Fragment Sizes (bp) [1]	Observed Fragment Size (bp)[2]
Nco I	*aad-12*	pDAB4468	Figure 33	7429	7429
		Control (Maverick)	Figure 33	none	none
		AAD-12 positive plants in F2	Figure 33	>4043(border)	~5500
		AAD-12 negative plants in F2 (null segregants)	Figure 33	none	none
	pat	pDAB4468	Figure 34	7429	7429
		Control (Maverick)	Figure 34	none	none
		AAD-12 positive plants in F2	Figure 34	>4043(border)	~5500
		AAD-12 negative plants in F2 (null segregants)	Figure 34	none	none

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

Table 6. Results of F2 individual plants analyzed by Southern blot with *aad-12* and *pat* probes within a single segregating generation.

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

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

Figure 33. Southern blot analysis of *Nco* I digest with *aad-12* probe.

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

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

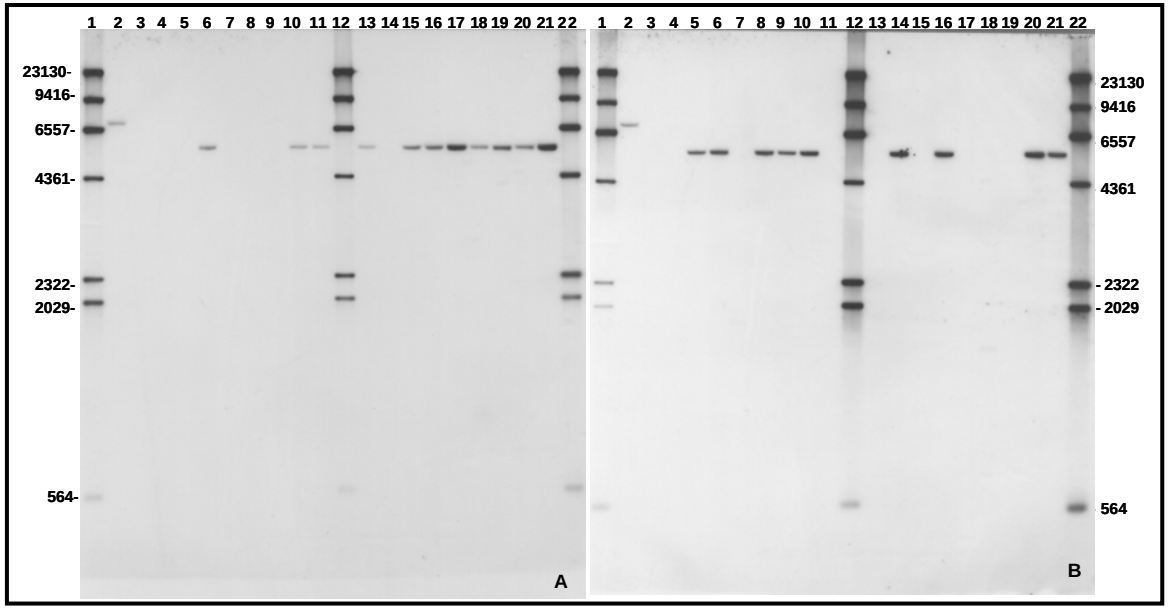
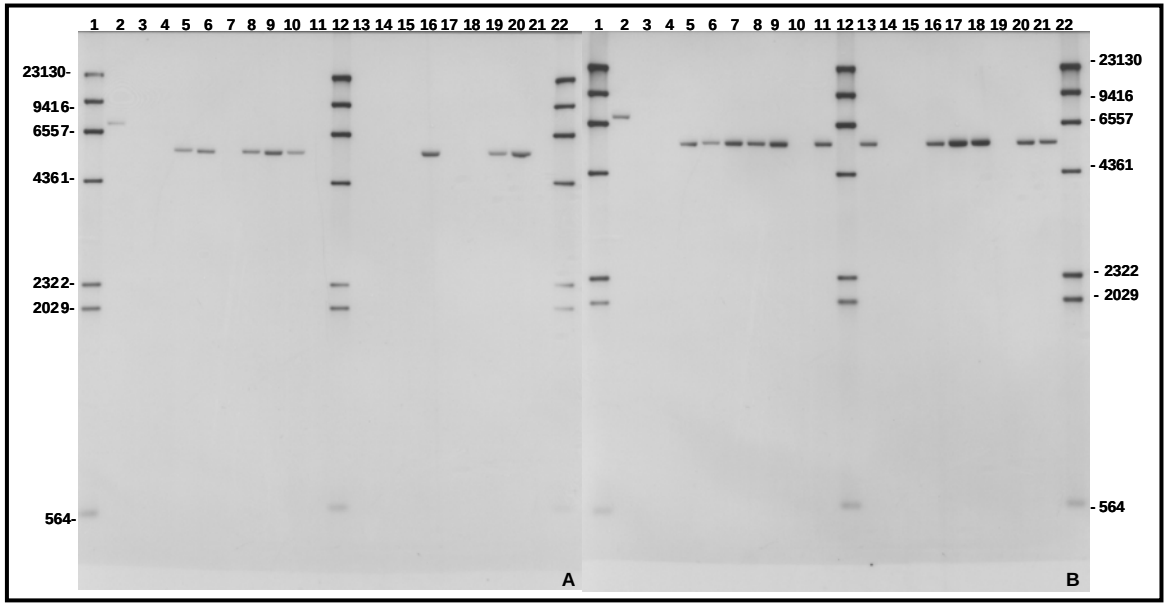


Figure 34. Southern blot analysis of *Nco* I digest with *pat* probe.

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

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



V-E.2. Segregation Analysis of Breeding Generations

Chi-square analysis of trait inheritance data from a F2 breeding generation was conducted to determine the Mendelian inheritance of *aad-12* in DAS-68416-4 soybeans. The presence or absence of *aad-12* was determined using a gene-specific fluorescence-based detection method specific for *aad-12*. The expected segregation ratio of 3:1 for plants containing *aad-12* (homozygous + hemizygous) versus plants not containing *aad-12* was observed (Table 7).

Table 7. Results of F2 individual plants tested for *aad-12* within a single segregating generation.

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

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

V-F. Summary of the Genetic Characterization

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

Molecular characterization of soybean event DAS-68416-4 by Southern blot analysis confirmed the insertion of a single intact copy of the *aad-12* and *pat* expression cassettes from the T-DNA insert of pDAB4468. No additional DNA fragments from the *aad-12* and *pat* expression cassettes were identified in DAS-68416-4 and no plasmid backbone sequences were present. DAS-68416-4 was also shown to be stably integrated across four distinct breeding generations (T2, T3, T4, and T5) and displayed the expected inheritance pattern of a single insert/locus in a generation (F2) that was segregating for the DAS-68416-4 event.

VI. Characterization of the Introduced Proteins

VI-A. AAD-12

VI-A.1. Identity of the AAD-12 Protein

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

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

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

VI-A.2. Biochemical Characterization of the AAD-12 Protein

Large quantities of purified AAD-12 protein are required to perform safety assessment studies. Because it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans, 2004), the AAD-12 protein was microbially-produced using *Pseudomonas fluorescens (Pf)*. Characterization studies were performed to confirm the equivalency of the AAD-12 protein expressed *in planta* in soybean line DAS-68416-4 with the *Pf* microbe-derived AAD-12 protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycoprotein detection, and protein sequence analysis by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray ionization-liquid chromatography mass spectrometry (ESI-LC/MS) were used to characterize the biochemical properties of the protein. Using these methods, the AAD-12 protein from *Pf* and the transgenic soybean event DAS-68416-4 were shown to be biochemically equivalent, thereby supporting the use of the microbially-produced protein in safety assessment studies.

The methods and results of the biochemical characterization of the DAS-68416-4 soybean- and microbe-derived AAD-12 proteins are described in detail in Appendix 2. Briefly, both the plant and *Pf*-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. There was no evidence of glycosylation of the DAS-68416-4 soybean-derived AAD-12 protein. Amino acid sequence was confirmed by enzymatic peptide mass fingerprinting using MALDI-TOF MS and ESI-LC/MS. The N-terminal methionine was found to be cleaved from

both protein sources and the N-terminal peptide of the plant AAD-12 was determined to be acetylated after the N-terminal methionine was cleaved. These two co-translational processes, cleavage of the N-terminal methionine residue and N-terminal acetylation, are common modifications that have been found to occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman, 2003).

VI-A.3. Expression of the AAD-12 Protein in Plant Tissues

A field expression study was conducted at six locations in U.S. and Canada during 2008. Six sites (Iowa, Illinois, Indiana, Nebraska and Ontario, Canada (2 sites)) were planted with DAS-68416-4 soybean and the conventional control (Maverick). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Four treatments of the DAS-68416-4 soybean (unsprayed, sprayed with 2,4-D, sprayed with glufosinate, or sprayed with both 2,4-D and glufosinate) were tested (see Appendix 4 for application rates and timings). Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at V5 and V10 stage, and root and forage were collected at the R3 stage of development. The grain was collected at the R8 stage of development (Gaska, 2006). The soluble, extractable AAD-1 protein was measured using a validated enzyme-linked immunosorbent assay (ELISA) method. AAD-12 protein levels for all tissue types were calculated on ng/mg dry weight basis. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed in Appendix 4.

A summary of the AAD-12 protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 8. Average expression values ranged from 15.48 ng/mg dry weight in R3 stage root to 66.08 ng/mg dry weight in V5 stage leaf tissue. Expression values were similar for the all sprayed treatments as well as for the plots sprayed and unsprayed with 2,4-D and glufosinate herbicides. No AAD-12 protein was detected in the control tissues across the six locations.

Table 8. Summary of AAD-12 protein levels in tissues collected from DAS-68416-4 produced in the U.S. and Canada during 2008.

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

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

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

VI-A.4. 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 in 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 Gravenitz 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; Ramachandra Rao and Ravishankar, 2000; Shetty *et al.*, 2006). This utility has led to a history of safe use for *Delftia acidovorans* in the food processing industry. For example, see US Patent 5,128,253 "Bioconversion process for the production of vanillin" issued on July 7, 1992 to Kraft General Foods (Labuda *et al.*, 1992).

Lack of allergenic potential

- The step-wise, weight-of-evidence approach (Codex, 2003) 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 9.00 allergen database, using the search criteria of either a match of eight or more contiguous identical amino acids, or >35% identity over 80 amino acid residues.
- The AAD-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.

Lack of toxic potential

- The AAD-12 protein does not share meaningful amino acid sequence similarities with known toxins. Amino acid homologies were evaluated using a global sequence similarity search against the GenBank non-redundant protein dataset. The search identified 618 similar proteins, which can be broken down into a few major subclasses. The largest subclass, containing 474 proteins, was identified as tauD or taurine dioxygenases. These are proteins involved in the degradation of taurine (Eichorn *et al.*, 2007). The next largest class, with 138 members, was clavaminic acid synthetases or "CAS-like"(Zhang *et al.*, 2000). There were 2 TolC proteins which are known efflux pumps (Koronakis *et al.*, 2000). The last four proteins were: 1) a (S)-2-(2,4-dichlorophenoxy)propionate, 2-oxoglutarate dioxygenase (Schleinitz *et al.*, 2004); 2) a pvcB protein which is a known CAS-like protein (see accession page of NP_968348); 3) an inosine-uridine preferring nucleoside hydrolase (Gopaul *et al.*, 1996); and

4) a hypothetical protein with no functional annotation. None of these protein classes are known toxins.

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

VI-A.5. Summary of AAD-12 Protein Characterization

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

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

VI-B. PAT

VI-B.1. Identity of the PAT Protein

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium. The *pat* transgene in DAS-68416-4 encodes a protein sequence that is identical to the native PAT protein (Accession number: Q57146). PAT is comprised of 183 amino acids and has a molecular weight of ~21 kDa (Figure 36).

Figure 36. Amino acid sequence of the PAT protein.

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

VI-B.2. Biochemical Characterization of the PAT Protein

Characterization of the biochemical properties of the plant-derived PAT protein was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), lateral flow strips and western blot analysis. Using these methods the PAT

protein produced in DAS-68416-4 soybean was shown to be equivalent to that produced in other transgenic crops (USDA 1996, USDA 2001, USDA 2004, USDA 2006).

VI-B.3. Expression of the PAT Protein in Plant Tissues

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

A summary of the PAT protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 9. Average expression values ranged from 1.73 ng/mg dry weight in R3 stage root to 11.76 ng/mg dry weight in V10 stage leaf tissue. Expression values were similar for the all sprayed treatments as well as for the plots sprayed and unsprayed with 2,4-D and glufosinate herbicides. No PAT protein was detected in the control tissues across the six locations.

Table 9. Summary of PAT protein levels in tissues collected from DAS-68416-4 produced in the U.S. and Canada during 2008.

Tissue	Treatment	PAT ng/mg Tissue Dry Weight		
		Mean	Std. Dev.	Range
V5 Leaf	DAS-68416-4 Unsprayed	9.17	2.99	4.33 - 13.75
	DAS-68416-4 + Glufosinate	9.83	2.66	3.67 - 13.78
	DAS-68416-4 + 2,4-D	9.01	3.03	4.87 - 13.92
	DAS-68416-4 + Glufosinate and 2,4-D	10.05	3.76	3.00 - 15.03
V10 Leaf	DAS-68416-4 Unsprayed	10.94	1.31	8.43 - 13.35
	DAS-68416-4 + Glufosinate	11.51	1.69	9.08 - 14.44
	DAS-68416-4 + 2,4-D	11.76	2.02	7.49 - 14.81
	DAS-68416-4 + Glufosinate and 2,4-D	11.58	1.45	9.26 - 14.15
Root	DAS-68416-4 Unsprayed	1.73	0.51	0.47 - 2.84
	DAS-68416-4 + Glufosinate	1.92	0.45	1.01 - 2.67
	DAS-68416-4 + 2,4-D	1.73	0.68	0.42 - 2.83
	DAS-68416-4 + Glufosinate and 2,4-D	1.93	0.55	0.36 - 2.68
Forage	DAS-68416-4 Unsprayed	3.63	2.88	0.06 - 12.54
	DAS-68416-4 + Glufosinate	4.81	3.75	0.40 - 12.10
	DAS-68416-4 + 2,4-D	5.28	4.20	0.12 - 12.13
	DAS-68416-4 + Glufosinate and 2,4-D	4.73	3.63	0.45 - 12.35
Grain	DAS-68416-4 Unsprayed	2.73	0.34	1.96 - 3.37
	DAS-68416-4 + Glufosinate	2.74	0.28	2.29 - 3.39
	DAS-68416-4 + 2,4-D	2.79	0.26	2.21 - 3.13
	DAS-68416-4 + Glufosinate and 2,4-D	2.82	0.23	2.43 - 3.25

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

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

VI-B.4. 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. Detailed biochemical characterization of the PAT protein derived from DAS-68416-4 soybean was conducted. Western blot analysis techniques were used to determine that the PAT protein expressed in soybean was the same molecular weight and

immunoreactivity as the native protein. Polyclonal and monoclonal antibodies were used that recognize antigenic epitopes on the protein. Any protein that was smaller (a degradation fragment) or larger (fusion protein or post-translationally modified version) in size would have been detected as a band of different molecular weight.

Characterization of PAT protein expression in DAS-68416-4 plants over the growing season was determined by analyzing leaf, root, whole plant, and grain tissues from DAS-68416-4 plants sprayed with 2,4-D, glufosinate, both 2,4-D and glufosinate, and non-sprayed.

VI-C. References

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

Eichhorn, E., Van Der Ploeg, J.R., Kertesz, M.A., Leisinger, T. (1997) Characterization of alpha-ketoglutarate-dependent taurine dioxygenase from Escherichia coli. Journal of Biological Chemistry 272:23031-23036.

Evans, S. L. (2004) Producing Proteins from Genetically Modified Organisms for Toxicology and Environmental Fate Assessment of Biopesticides. In: Parekh, S. R. (Ed), The GMO Handbook: Genetically Modified Animals, Microbes, and Plants in Biotechnology, Humana Press Inc., Totowa, New Jersey, pp. 53-84.

Gaska, J. (2006) Soybean growth and development (http://soybean.uwex.edu/library/soybean/morphology/documents/04_soy_growth_development.pdf)

Gopaul, D.N., Meyer, S.L., Degano, M., Sacchettini, J.C., Schramm, V.L. (1996) Inosine-uridine nucleoside hydrolase from Crithidia fasciculata. Genetic characterization, crystallization, and identification of histidine 241 as a catalytic site residue. Biochemistry 35:5963-5970.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., Hughes, C. (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature 405:914-9.

Labuda, I.M., Goers, S.K., Keon, K.A. (1992) Bioconversion Process for the Production of Vanillin. US Patent #5,128,253.

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

Ramachachandra Rao, S. and Ravishankar, G.A. (2000) Vanilla Flavor: Production by Conventional and Biotechnological Routes. Journal of the Science of Food and Agriculture 80:289-304.

Schleinitz, K.M., Kleinsteuber, S., Vallaeys,T., Babel, W. (2004) Localization and Characterization of Two Novel Genes Encoding Stereospecific Dioxygenases Catalyzing 2(2,4-Dichlorophenoxy)propionate Cleavage in Delftia acidovorans MC1. Applied and Environmental Microbiology 70:5357-65.

Shetty, K., Paliyath, G., Pometto, A., Levin, R.E. (2006) Food Biotechnology, CRC Press, ISBN 0824753291, pp. 1672-1673.

Tamaoka, J., Ha, D.M., Komagata, K. (1987) Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas.* International Journal of Systematic Bacteriology 37:52-59.

Toms, A., Wood, J.M. (1970) The Degradation of trans-Ferulic Acid by *Pseudomonas acidovorans*. Biochemistry 9:337-343.

USDA (1996) Availability of Determination of Nonregulatated status for Soybeans Genetically Engineered for Glufosinate Herbicide Tolerance. Federal Register Volume 61, Number 160:42581-42582.

USDA (2001) Availability of Determination of Non-regulatated status for Genetically Engineered Corn for Insect Resistance and Glufosinate Herbicide Tolerance (Corn line 1507). Federal Register Volume 66, Number 157:42624-42625.

USDA (2004) Availability of Determination of Nonregulated Statusfor Cotton Lines Genetically Engineered for Insect Resistance. Federal Register Volume 69, Number 156:50154-50155.

USDA (2005) Availability of Determination of Non-regulated Status for Genetically Engineered Corn (Corn line DAS-59122-7). Federal Register Volume 70, Number 194:58663-58664.

Von Gravenitz, A, (1985) Ecology, clinical significance, and antimicrobial susceptibility encountered glucose-non-fermenting gram-negative rods, p. 181-232. In G. L. Gilardi (ed.), Nonfermentative gram-negative rods, laboratory identification and clinical aspects. Marcel Dekker, Inc., New York. Winkelmann G, Drechsel H (1997).

Wen, A., Fegan, M., Hayward, C., Chakraborty, S., Sly, L.I. (1999) Phylogenetic relationships among members of the *Comamonadaceae*, and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka *et al.* 1987) gen. nov., comb. nov. International Journal of Systematic Bacteriology 49:567-576.

Zhang, Z., Ren, J.-S., Stammers, D.K., Baldwin, J.E., Harlos, K., Schofield, C.J. (2000) Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. Nature Structural and Molecular Biology 7:127-133.

VII. Agronomic Performance

VII-A. Phenotypic and Agronomic Characteristics

Agronomic trials were conducted with DAS-68416-4 soybeans in 2008 at 6 locations in the U.S. and Canada (Experiment 1) and in 2009 at 21 locations in the U.S (Experiment 2). These studies compared DAS-68416-4 soybeans with the non-transgenic control (Maverick). Experiment 1 used the same plots that were used for the protein expression (Sections VI-A.3 and VI-B.3) and nutrient composition (Section VIII) studies. In Experiment 1, DAS-68416-4 soybeans were evaluated with and without 2,4-D and glufosinate herbicide spray treatments. Experiment 2 was conducted at 21 locations to allow for testing in a broader range of environmental conditions. The results of both experiments showed that the DAS-68416-4 soybean was equivalent to non-transgenic soybean.

VII-A.1. Experiment 1

An agronomic study with DAS-68416-4 soybean and a non-transgenic control (Maverick) was conducted in 2008 at six sites located in Iowa (Keokuk county), Illinois (Clinton county), Indiana (Parke county), Nebraska (York county) and Ontario, Canada (2 sites). Agronomic determinants, including stand count, emergence, seedling vigor, plant height, lodging, days to flowering, and final population were evaluated to investigate the equivalency of the DAS-68416-4 transgenic soybeans (with and without herbicide treatments) to the control (Table 10).

The test and control soybean seed were planted at a seeding rate of approximately 112 seeds per 25 ft row with a row spacing of approximately 30 inches. At each site, 3 replicate plots of each treatment were established, with each plot consisting of 2-25 ft rows. 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 ft of a non-transgenic soybean of similar relative maturity. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop.

Herbicide treatments were applied with a spray volume of approximately 20 gallons per acre (187 L/ha). These applications were designed to replicate maximum label rate for commercial practices. 2,4-D was applied as 3 broadcast over-the-top applications for a seasonal total of 3 lb ae/A. Individual applications of 1.0 lb ae/A (1120 g ae/ha) were made at pre-emergence and approximately V4 and R2 growth stages. Glufosinate was applied as 2 broadcast over-the-top applications for a seasonal total of 0.74 lb ai/A (828 g ai/ha). Individual applications of 0.33 lb ai/A and 0.41 lb ai/A (374 and 454 g ai/ha) were made at approximately V6 and R1 growth stages.

Table 10. Agronomic parameters evaluated in Experiment 1.

Parameter	Evaluation Timing	Description	Scale or Units
Stand count	VC-V2	Number of plants emerged in rows of each plot	Actual count per plot
Emergence	VC-V2	Stand count divided by the number of seeds planted	%
Seedling vigor	VC-V2	Visual estimate of average vigor of emerged plants per plot	1-10 scaled based on growth of the non-transformed soybeans 10 = Growth equivalent to non-transformed 9 = Plant health is 90% as compared to non-transformed, etc.
Plant vigor / injury	After post-emergent herbicide applications	Injury from herbicide applications	1-10 scale based on growth of the non-transformed soybeans 10 = Growth equivalent to non-transformed 9 = Plant health is 90% as compared to non-transformed, etc.
Days to flower	R1	Number of days from planting to when 50% of plants reached R1	Days
Plant height	Approximately R6	Height from soil surface to the tip of the highest leaf when extended by hand	Height in cm (average of 10 plants per plot)
Lodging	Approximately R8	Visual estimate of lodging severity	Visual estimate on 0-100% scale based on the number of plants lodged
Final population	Approximately R8	The number of plants remaining in rows of each plot	Actual count per plot, including plants removed during previous sampling

Analysis of variance was conducted across the field sites for the agronomic data using a mixed model (SAS Version 8; SAS Institute 1999). Entry was considered a fixed effect, and location, block within location, location-by-entry, and entry-by-block within location were designated as random effects. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between DAS-68416-4 (unsprayed AAD-12), DAS-68416-4 sprayed with glufosinate (AAD-12 + glufosinate), DAS-68416-4 sprayed with 2,4-D (AAD-12 + 2,4-D), and DAS-68416-4 sprayed with both glufosinate and 2,4-D (AAD-12 + both herbicides), and the control entry using t-tests.

Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise p-values is very high ($1-0.95^{\text{number of comparisons}}$). In this study there

were four comparisons per analyte and 12 quantitated observation types (including disease incidence and insect damage, Section VII-B), resulting in 48 comparisons made in the across-site analysis. Therefore, the probability of declaring one or more false differences based on unadjusted p-values was >91% ($1-0.95^{48}$).

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

Experiment 1 Results

An analysis of the agronomic data collected from the control, AAD-12 unsprayed, AAD-12 + 2,4-D, AAD-12 + glufosinate, and AAD-12 + both herbicides was conducted. No statistically significant differences were observed for stand count, emergence, seedling vigor, days to flower, lodging, or final population (Table 11Table 11. Analysis of agronomic characteristics from Experiment 1.). For plant height, a significant paired t-test was observed between the control and the AAD-12 + 2,4-D spray. However, no significant overall treatment effect was observed, differences were very small between the AAD-12 treatment and the control, and differences were not shared among the different AAD-12 treatments. Based on these results, DAS-68416-4 soybean was agronomically equivalent to the non-transgenic control.

Table 11. Analysis of agronomic characteristics from Experiment 1.

Parameter	P-value for Overall Treatment Effect[a]	Control	Treatment Means (P-value[b], Adj. P[c])			
			Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Stand Count	0.774	170	172 (0.709,0.824)	175 (0.311,0.575)	173 (0.476,0.672)	175 (0.269,0.575)
Emergence	0.714	76.7	77.4 (0.738,0.824)	79.1 (0.301,0.575)	79.0 (0.327,0.575)	79.4 (0.256,0.575)
Seedling Vigor	0.547	9.72	9.39 (0.146,0.575)	9.50 (0.326,0.575)	9.44 (0.222,0.575)	9.39 (0.146,0.575)
Vigor/Injury App. 2[d]	0.511	10.0	9.86 (0.461,0.671)	9.89 (0.555,0.718)	9.83 (0.378,0.611)	9.67 (0.087,0.575)
Vigor/Injury App. 3[e]	0.462	10.0	10.0 (1.000,1.000)	9.89 (0.320,0.575)	9.83 (0.141,0.575)	9.89 (0.320,0.575)
Vigor/Injury App. 5[f]	0.431	9.94	9.89 (0.721,0.824)	9.78 (0.289,0.575)	9.67 (0.085,0.575)	9.78 (0.289,0.575)
Days to Flower	0.452	49.0	49.5 (0.261,0.575)	49.4 (0.395,0.611)	48.7 (0.568,0.718)	49.2 (0.668,0.801)
Plant Height	0.144	101	98.1 (0.145,0.575)	99.2 (0.390,0.611)	96.1 (**0.020**,0.575)	97.2 (0.062,0.575)
Lodging (%)	0.948	17.2	18.2 (0.885,0.904)	21.3 (0.551,0.718)	20.7 (0.606,0.746)	21.7 (0.511,0.700)
Final Population	0.268	156	154 (0.770,0.840)	161 (0.335,0.575)	155 (0.817,0.853)	163 (0.127,0.575)

[a] Overall treatment effect estimated using an F-test.
[b] Comparison of the sprayed and unsprayed treatments to the control using a t-test.
[c] P-values adjusted using a False Discovery Rate (FDR) procedure.
[d] Evaluated after application of 2,4-D at the V4 growth stage.
[e] Evaluated after application of 2,4-D at the R2 growth stage.
[f] Evaluated after application of glufosinate at the R1 growth stage.
Bolded P-values are significant (<0.05).

VII-A.2. Experiment 2

An agronomic study with DAS-68416-4 soybean and a non-transgenic control (Maverick) was conducted in 2009 at 21 sites in the U.S. (Table 12). Ten locations had both a 2,4-D sprayed and unsprayed component, while the remaining eleven locations had no 2,4-D applied.

Table 12. Locations for Experiment 2 agronomic and yield trials, 2009.

County	State	County	State
Sprayed and non-sprayed		*Non-sprayed only*	
Keokuk	IA	Madison	IA
Clinton	IL	Story	IA
Benton	IN	Ford	IL
LaGrange	IN	Saline	IL
Parke	IN	Stark	IL
Butler	MO	Benton	IN
Henry	MO	Newton	IN
Seward	NE	Queen Anne	MD
York	NE	Adair	MO
Fulton	OH	Jasper	MO
		Polk	NE

Agronomic determinants, including emergence, seedling vigor, days to flower, days to maturity, plant height, lodging, shattering, and yield were evaluated to investigate the equivalency between the non-sprayed and sprayed DAS-68416-4 soybeans, and between non-sprayed DAS-68416-4 soybeans and the control (Table 13).

Each plot consisted of 2 rows planted 0.762 meters apart. Plots were 5.3 or 3.8 meters in length depending on the location of the trial. Planting density was 27.5 seeds per meter. A pre-emergent herbicide typical of soybean production was applied at most locations. Hand weeding or conventional post-emergent herbicides were used for weeds that were not controlled by the pre-emergent herbicide or by 2,4-D application.

A randomized-complete-block design was used for the 21 experiments that did not have a 2,4-D spray component. Each location of the trial consisted of 4 replications of each treatment.

A modified split-plot design with 4 replications was used for the 10 trials that included the 2,4-D spray component. Whole plots were herbicide treatments and subplots were test lines. Treatments consisted of a non-2,4-D-sprayed treatment and 2,4-D applied at the V3 and R2 growth stages. For both applications, 2,4-D was applied at a rate of 2 lbs ae/A (2240 g ae/ha, equivalent to twice the maximum proposed use rate). Whole-plots which received the 2,4-D treatment were grouped together for ease of application. The non-transgenic control (Maverick) was forced to a set position within the herbicide treated whole-plots. This was done to reduce any chance of border effect which would have occurred had the control been placed beside a 2,4-D-tolerant entry. The control plots were expected to die after herbicide application and so would have left bare ground adjacent to resistant plots had they been randomized among the other entries.

Table 13. Data collected in Experiment 2 agronomic and yield trials, 2009.

Parameter	Evaluation Timing	Description	Scale or Units
Emergence	VC - V2	Stand count in 1 meter section of row divided by number of seeds planted per meter	%
Seedling vigor	V1 - V3	General seedling vigor	1 (low) to 10 (high)
Days to Flower	R1	Number of days from planting to when 50% of plants are at R1	days
Stand count	R1	Number of plants in one meter section of row	
Disease incidence	~R6	Opportunistic note on any disease that occurred at a location	%
Insect damage	~R6	Opportunistic note on any insect damage that occurred at a location	%
Plant Height	R8	Final height of plot at R8	cm
Days to Maturity	R8	Number of days from planting to when 95% of plants in plot have reached their mature color	days
Lodging	R8	Degree of lodging in a plot	1 (none) - 5 (flat)
Shattering	R8	Percent of pods shattered	%
Yield	R8	Quantity of seed produced by the plot	bu/acre
100 seed weight	R8	Weight of 100 random seeds from the harvested plot	g

Experiment 2 Results – Comparison of Sprayed vs. Non-Sprayed DAS-68416-4 Soybean

All agronomic observations for the 2,4-D sprayed and non-sprayed DAS-68416-4 soybeans were statistically indistinguishable across the ten field locations, indicating phenotypic equivalence even when sprayed with 2,4-D at twice the maximum proposed use rate (Table 14). Insect damage ratings were taken at seven of the ten locations and were identical for sprayed and non-sprayed plots, and all values were ≤5% damage.

Table 14. Agronomic parameters for sprayed (2,4-D) and unsprayed DAS-68416-4 soybean.

		Mean			
Parameter	**Unit**	**Sprayed**	**Unsprayed**	**SE[1]**	**Pr>F**
Emergence	%	78.4	79.8	3.6	0.5820
Seedling vigor	1-10 scale	8.8	8.9	0.4	0.4757
Days to flower	days	40.6	39.8	3.8	0.3822
Stand count	No./m	21.1	21.4	1.0	0.6601
Disease incidence	%	8.7	8.7	3.0	1.0000
Plant height at senescence	cm	79.8	83.1	8.7	0.0888
Days to maturity	days	133.1	132.8	5.3	0.2847
Lodging	1-5 scale	1.5	1.8	0.3	0.1866
Shattering	%	1.2	1.2	0.6	0.3434
Yield	bu/acre	48.2	51.3	6.6	0.2818
100 seed weight	g	15.1	15.3	0.4	0.6912

[1]SE = pooled standard error of the mean.

Experiment 2 Results - Comparison of DAS-68416-4 and Control (Maverick) Soybean

All agronomic observations for non-sprayed control (Maverick) and DAS-68416-4 soybeans were statistically indistinguishable across the 21 locations except for days to maturity and 100 seed weight (Table 15). On average, Maverick took less than one additional day to mature compared with event DAS-68416-4 soybean. This difference in maturity is not biologically significant. The weight per 100 seeds was statistically lower for event DAS-68416-4 soybean compared with Maverick soybean, but the difference was less than 3.5% and was not accompanied by reduced yield. Therefore this difference is not considered to be biologically significant. Overall, event DAS-68416-4 soybean was agronomically equivalent to the non-transgenic control (Maverick) soybean.

Table 15. Agronomic parameters for DAS-68416-4 and Maverick soybean.

		Mean			
Parameter	**Unit**	**Maverick**	**DAS-68416-4**	**SE[1]**	**Pr>F**
Emergence	%	79.4	77.6	2.7	0.3154
Seedling vigor	1-10 scale	8.7	8.7	0.2	1.0000
Days to flower	days	42.3	42.3	2.1	0.7961
Stand count	No./m	21.8	21.7	0.8	0.7482
Disease incidence	%	5.9	6.0	1.8	0.6703
Insect damage	%	2.4	2.4	0.7	0.3364
Plant height	cm	92.7	91.6	5.5	0.1389
Days to maturity	days	129.3	128.4	3.3	**0.0017**
Lodging	1-5 scale	2.0	2.1	0.2	0.3100
Shattering	%	0.8	0.8	0.3	0.6787
Yield	bu/acre	53.9	54.1	3.7	0.8043
100 seed weight	g	15.8	15.3	0.3	**0.0014**

[1]SE = pooled standard error of the mean.

VII-A.3. Conclusions

Results from experiments in 2008 and 2009 indicate that DAS-68416-4 soybean is agronomically equivalent to non-transgenic control (Maverick) soybean. Treatments of 2,4-D and/or glufosinate did not significantly change the agronomic performance of DAS-68416-4 soybean.

VII-B. Ecological Evaluations

The DAS-68416-4 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-68416-4 soybeans compared to the conventional soybean varieties in the same trials. As part of Experiment 1 described in Section VII-A.1, disease and insect damage was rated on a numerical scale of 0-100%, with 0% representing no damage due to disease incidence or insect resistance. Table 16 shows results across the 6 sites described in Experiment 1.

Table 16. Analysis of disease incidence and insect damage from Experiment 1.

Parameter	P-value for Overall Treatment Effect[a]	Control	Treatment Means (P-value[b], Adj. P[c])			
			Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Disease Incidence (%)[d]	0.422	13.1	12.6 (0.803,0.853)	11.8 (0.456,0.671)	11.1 (0.251,0.575)	10.1 (0.091,0.575)
Insect Damage[d]	0.332	24.1	21.8 (0.140,0.575)	22.1 (0.204,0.575)	22.3 (0.236,0.575)	20.9 (**0.044**,0.575)

[a] Overall treatment effect estimated using an F-test.
[b] Comparison of the sprayed and unsprayed treatments to the control using a t-test.
[c] P-values adjusted using a False Discovery Rate (FDR) procedure.
[d] Visual estimate on 0-100% scale; 0% = no damage.

No statistically significant differences were observed for disease incidence. For insect damage, a significant paired t-test was observed between the control and the AAD-12 + both herbicides. However, no significant overall treatment effect was observed, the difference between the AAD-12 treatment and the control was small, and differences were not shared among the different AAD-12 treatments.

Insect damage and disease incidence was also measured at 21 field sites in Experiment 2 (Section VII-A.2). No significant difference was observed between DAS-68416-4 soybeans that had been sprayed with 2,4-D and non-sprayed. No significant differences were observed between DAS-68416-4 soybeans and the non-transgenic control.

Ecological observations were also made from all USDA APHIS notified field trials conducted in 2006-2009 (Appendix 5). Incidence of disease and insect presence in trials of DAS-68416-4 soybeans were recorded and differences in incidence or response of DAS-68416-4 soybeans compared with the conventional control were examined. In all cases, no differences were seen in any of the trials of DAS-68416-4 soybeans compared with the conventional controls. The disease and insect stressors observed in trials of DAS-68416-4 and conventional soybeans are summarized in Table 17**.** These observations support the conclusion that the response of DAS-68416-4 soybean to ecological stressors does not differ from that of conventional soybean.

Table 17. Disease and insect stressors observed in trials of DAS-68416-4 and conventional soybean.

Year	USDA Notification Number	State (County)	Diseases	Insects
2007	06-292-105n	IN (Benton)	none	aphids, bean leaf beetles, corn rootworm beetles, Japanese beetles, lady bugs, leaf hoppers
2007-2008	07-242-107n	PR (Santa Isabel)	Carla virus	spider mites, whiteflies
2008	08-071-107n	CA (Tulare), IL (Clinton), IN (Benton, Parke), IA (Jefferson, Story), MN (Dakota), MS (Washington), NE (York)	brown spot, Cercospora leaf blight, rust, Septoria leaf spot	aphids, bean leaf beetles, grasshoppers, Japanese beetles, lady bugs, stink bugs, thrips, yellow-striped armyworms
2008	08-121-102n	IL (Schuyler, Stark), IN (LaGrange), MO (Adair, Henry), NE (Polk, Seward, York), OH (Fulton, Lucas)	Septoria brown	aphids, grasshoppers, lady bugs, thrips
2008	08-121-103n	IA (Boone)	none	none

VII-C. Germination and Dormancy Evaluations

Changes in seed dormancy characteristics were evaluated by looking at the germination of DAS-68416-4 seed compared with the non-transgenic control (Maverick) under warm and cold conditions.

For the warm germination test, event DAS-68416-4 and control soybean seeds were placed at 25 seeds/plate into Petri dishes containing germination pads saturated with water and excess water drained. The plates were placed at 25 ºC and held under these conditions for 5 days. Sixteen plates (400 seeds) were prepared per line. After five days, the number of non-germinated seeds was recorded.

For the cold germination test, seeds were planted at 100 seeds per half-flat filled with potting soil. Flats were sub-watered and held at 10 ºC for 7 days followed by exposure to 25 ºC for 5 days, after which the number of non-germinated seed was recorded.

Data from each test were analyzed by ANOVA using a completely randomized design with four replicates of 100 seeds per replicate. Data were transformed using the arcsine of the square root of the number of germinated seeds divided by 100 for statistical analysis. Percent germination is summarized in Table 18.

Table 18. Germination of DAS-68416-4 seeds under warm and cold conditions.

		Replicate				
Test	**Line**	**1**	**2**	**3**	**4**	**Mean**
Warm	DAS-68416-4	97	100	99	100	99.0
Warm	Control	100	100	99	97	99.0
Cold	DAS-68416-4	92	92	92	89	91.3
Cold	Control	98	88	98	96	95.0

There were no significant differences in germination between DAS-68416-4 and control soybeans in either the warm or cold germination experiments (Pr > F = 1.0 and 0.13, respectively). These results indicate that the seed dormancy characteristics have not been changed in DAS-68416-4 soybeans.

VII-D. Summary of Agronomic, Disease, and Pest Characteristics

Agronomic data evaluating plant growth characteristics throughout the growing season demonstrate the equivalence of DAS-68416-4 soybean with conventional non-transgenic soybean. 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-68416-4 soybeans and conventional soybeans across diverse environments. Therefore, these data support the conclusion that agronomic, disease and pest characteristics of DAS-68416-4 soybean are not significantly different from that of conventional soybeans, and there is no indication that DAS-68416-4 soybeans will pose an increased plant pest risk.

VII-E. References

Benjamini, Y., Hochberg, Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. Royal Statistical Soc. B, 57:289-300.

VIII. Grain and Forage Composition

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

The herbicide treatments were identical to those used in studies for protein expression analysis (Sections VI-A.3, VI-B.3) and agronomic Experiment 1 (Section VII-A.1). Herbicide treatments were applied with a spray volume of approximately 20 gallons per acre (187 L/ha). These applications were designed to replicate maximum label rate for commercial practices. 2,4-D was applied as 3 broadcast over-the-top applications for a seasonal total of 3 lb ae/A. Individual applications of 1.0 lb ae/A (1120 g ae/ha) were made at pre-emergence and approximately V4 and R2 growth stages. Glufosinate was applied as 2 broadcast over-the-top applications for a seasonal total of 0.74 lb ai/A (828 g ai/ha). Individual applications of 0.33 lb ai/A and 0.41 lb ai/A (374 and 454 g ai/ha) were made at approximately V6 and R1 growth stages.

Samples of soybean forage and grain were analyzed for nutrient content with a variety of tests (OECD 2001). The analyses performed for forage included protein, fat, ash, moisture, carbohydrate, acid detergent fiber (ADF), neutral detergent fiber (NDF), calcium and phosphorus. The analyses performed for grain included proximates (ash, total fat, moisture, protein, cholesterol, carbohydrate), fiber, minerals, amino acids, fatty acid, vitamins, anti-nutrients.

The results of the nutritional analysis for soybean forage and grain were compared with values reported in literature. A summary of the compositional data used for comparison can be found in Appendix 6. Analysis of variance was also conducted across the field sites using a mixed model. Entry was considered a fixed effect, and location, block within location, and location-by-entry were designated as random effects. The significance of an overall treatment effect was estimated using an F-test. Paired contrasts were made between DAS-68416-4 (unsprayed AAD-12), DAS-68416-4 sprayed with glufosinate (AAD-12 + glufosinate), DAS-68416-4 sprayed with 2,4-D (AAD-12 + 2,4-D), and DAS-68416-4 sprayed with both glufosinate and 2,4-D (AAD-12 + both herbicides), and the control entry using t-tests.

Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise p-values is very high ($1-0.95^{\text{number of comparisons}}$). In this study there were four comparisons per analyte (75 quantitated analytes), resulting in 300 comparisons made in the across-site composition analysis. Therefore, the probability of declaring one or more false differences based on unadjusted p-values was >99.99%.

One method to account for multiplicity is to adjust p-values to control the experiment-wise error rate (probability that all declared differences are significant), but when many comparisons are made in a study, the power for detecting specific effects can be reduced significantly. An alternative with much greater power is to adjust p-values to control the probability that each declared difference is significant. This can be accomplished using False Discovery Rate (FDR)

procedures (Benjamini and Hochberg, 1995). Therefore the p-values were adjusted using FDR to improve discrimination of true differences among treatments from random effects (false positives).

VIII-A. Compositional Analysis of Soybean Forage

An analysis of the protein, fat, ash, moisture, carbohydrate, acid detergent fiber (ADF), neutral detergent fiber (NDF), calcium and phosphorus in soybean forage samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 19 and Figure 37.

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

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

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

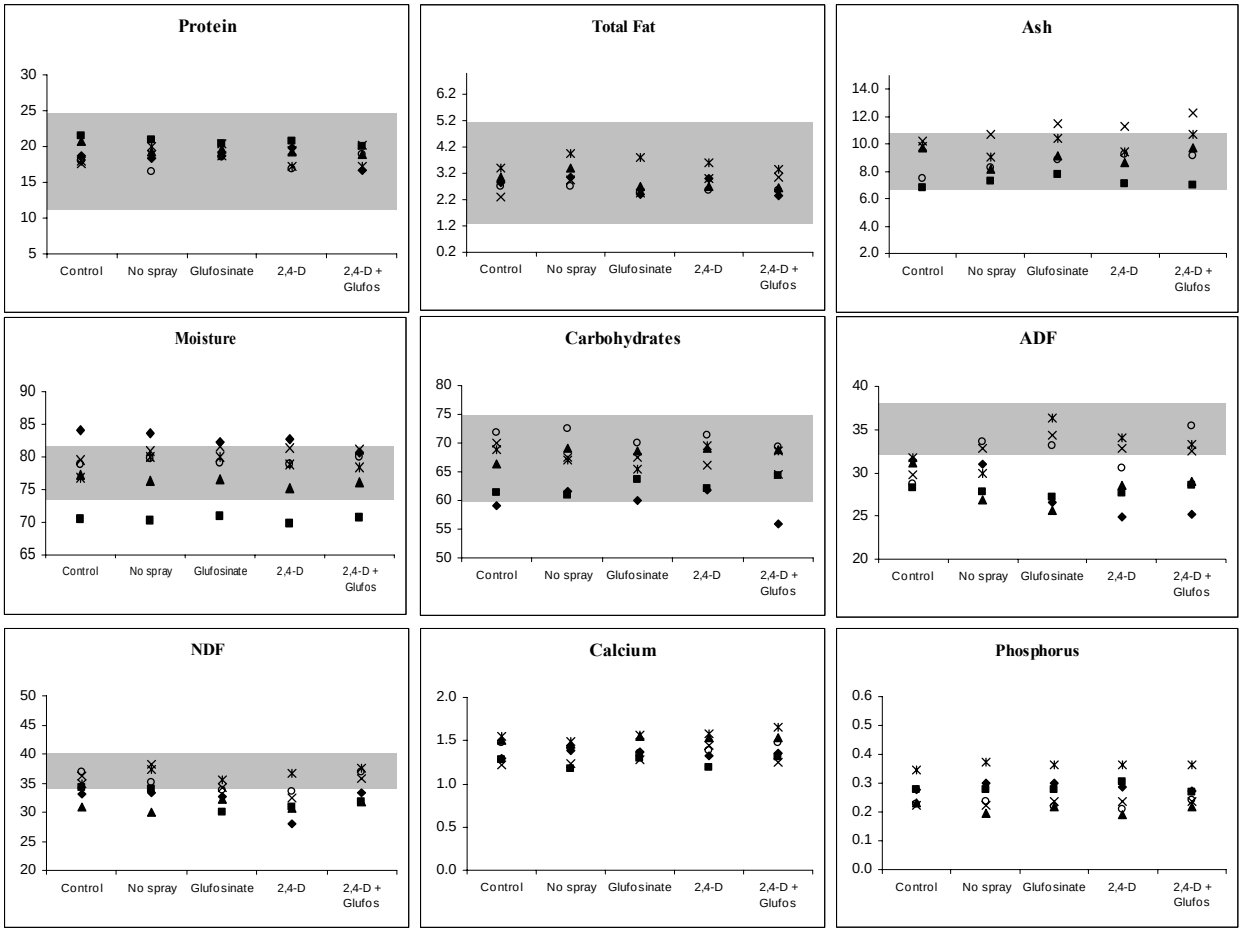
Table 19. Summary of the proximate, fiber and mineral analysis of soybean forage (% dry weight).

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

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
NR = not reported
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 37. Summary of the proximate, fiber and mineral analysis of soybean forage (% dry weight).

Percent dry-weight for all analytes, except moisture which was percent fresh-weight.
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded.



VIII-B. Compositional Analysis of Soybean Grain

VIII-B.1. Proximates and Fiber

An analysis of the protein, fat, ash, moisture, cholesterol, carbohydrate, ADF, NDF and total dietary fiber in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 20 and Figure 38.

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

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

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

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

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

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

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

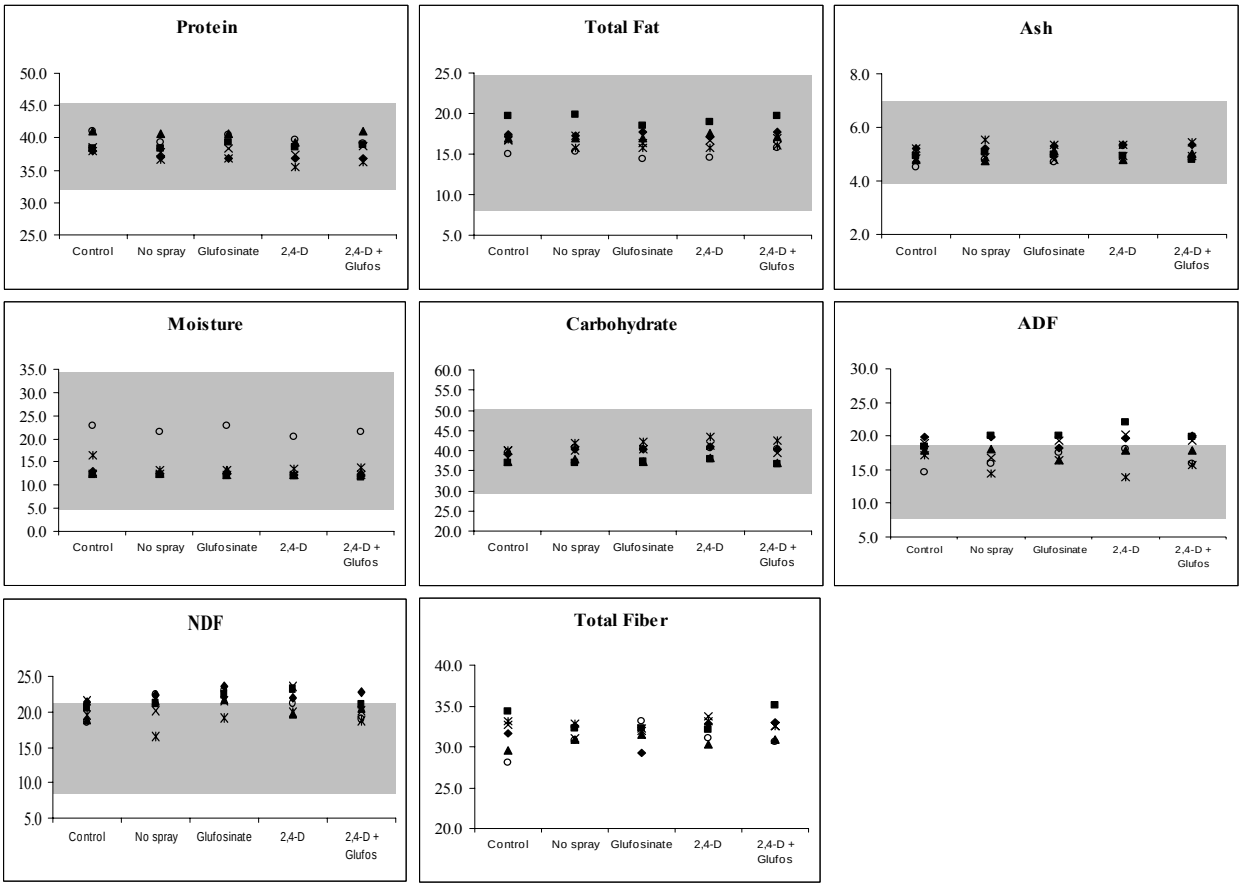
Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 20. Summary of the proximate and fiber analysis of soybean grain (% dry weight).

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

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
NA = statistical analysis was not performed since a majority of the data was < LOQ.
NR = not reported.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 38. Summary of the proximate and fiber analysis of soybean grain.
Percent dry-weight for all analytes, except moisture which was percent fresh-weight. Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded. Grain was also analyzed for cholesterol, but results were less than the limit of quantitation.



VIII-B.2. Minerals

The analysis of the calcium, chromium, copper, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 21 and Figure 39.

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

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

Magnesium levels were significantly different in the across-site analysis for the AAD12 + both herbicides and AAD-12 + glufosinate compared with the control based on the unadjusted and adjusted p-values, respectively, but the overall treatment effect was not significant. Magnesium across site mean values were slightly lower than the reported literature values, but the differences were small (<3%) in comparison to the control and all AAD-12 entries were closer to literature values compared with the control.

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

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

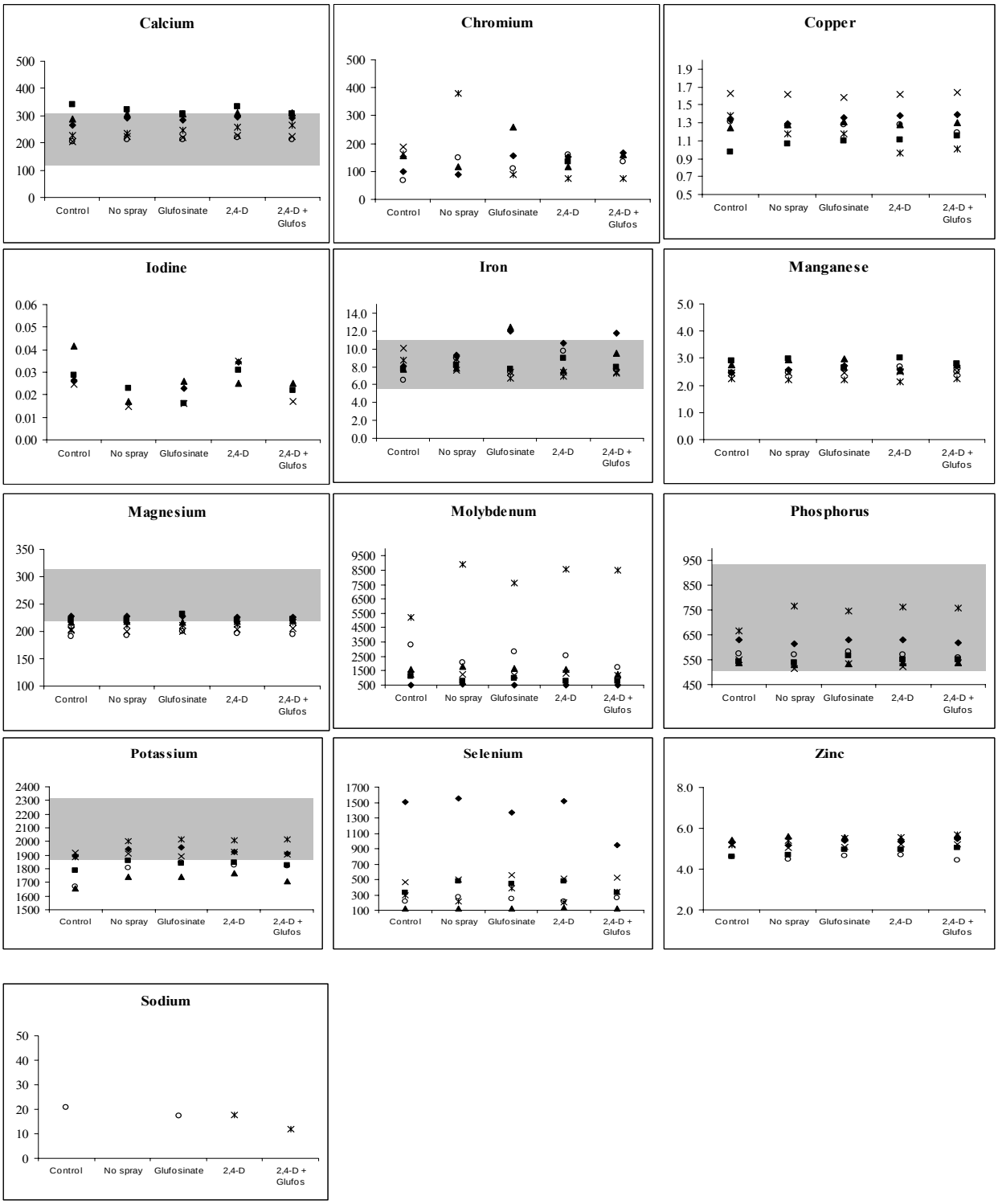
Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 21. Summary of the mineral analysis of soybean grain (mg/100g dry weight).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d]) Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Calcium	117-307	0.102	256	265 (0.174,0.351)	264 (0.237,0.432)	274 (**0.010**,0.057)	269 (**0.050**,0.148)
Chromium (ppb)	NR	0.775	145	149 (0.912,0.941)	175 (0.468,0.653)	126 (0.613,0.796)	137 (0.855,0.916)
Copper	NR	0.887	1.31	1.28 (0.534,0.728)	1.30 (0.788,0.873)	1.27 (0.367,0.564)	1.28 (0.461,0.649)
Iodine	NR	0.285	0.027	0.023 (0.430,0.632)	0.021 (0.182,0.358)	0.032 (0.348,0.551)	0.023 (0.348,0.551)
Iron	5.54-11.0	0.917	8.15	8.46 (0.719,0.853)	8.95 (0.353,0.552)	8.53 (0.656,0.819)	8.59 (0.608,0.796)
Magnesium	219-313	0.082	**210**	**212** (0.437,0.634)	**215** (**0.020**,0.087)	**213** (0.143,0.305)	**215** (**0.021**,0.088)
Manganese	NR	0.984	2.56	2.60 (0.608,0.796)	2.60 (0.618,0.799)	2.58 (0.781,0.873)	2.59 (0.698,0.831)
Molybdenum (ppb)	NR	0.845	2165	2557 (0.353,0.552)	2462 (0.479,0.665)	2563 (0.346,0.551)	2284 (0.722,0.853)
Phosphorus	507-935	0.675	583	589 (0.630,0.804)	599 (0.191,0.363)	596 (0.272,0.469)	594 (0.349,0.551)
Potassium	1868-2316	**0.0005**	**1801**	1876 (**0.0003**,**0.009**)	1882 (**0.0001**,**0.006**)	1883 (**0.0001**,**0.006**)	**1864** (**0.001**,**0.019**)
Selenium (ppb)	NR	0.490	490	523 (0.626,0.802)	520 (0.659,0.819)	511 (0.758,0.861)	418 (0.280,0.475)
Sodium	NR	NA	20.9	< LOQ	17.3	19.7	14.1
Zinc	NR	0.096	5.06	5.07 (0.868,0.926)	5.19 (0.117,0.268)	5.21 (0.074,0.197)	5.25 (**0.027**,0.105)

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
NR = not reported.
NA= statistical analysis was not performed since a majority of the data was < LOQ.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 39. Summary of the mineral analysis of soybean grain (mg/100 g dry weight). Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded. Grain was also analyzed for sodium, but results were less than the limit of quantitation.



VIII-B.3. Amino Acids

An analysis of the following amino acids: alanine, arginine, aspartic acid, cysteine, glutamic acid glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine; in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 22 and Figure 40Figure 40. Summary of the amino acid analysis of soybean grain (% dry weight).
.

No statistical differences were observed between the control and transgenic entries for cysteine, methionine, proline, tyrosine or tryptophan. The isoleucine level for AAD-12 + 2,4-D was significantly different from the control based on the unadjusted p-value, but this was not accompanied by a significant FDR-adjusted p-value or a significant overall treatment effect. The levels of the remaining 12 amino acids were slightly lower (<7%) for two or more of the AAD-12 entries compared with the control, but all fell within the literature range for non-transgenic soybean. All amino acids for all entries were within the literature ranges, indicating that the differences were not biologically meaningful.

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

Table 22. Summary of the amino acid analysis of soybean grain (% dry weight).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d])			
				Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Alanine	1.51-2.10	**0.003**	1.74	1.70 (**0.001**,**0.017**)	1.70 (**0.004**,**0.033**)	1.69 (**0.0003**,**0.009**)	1.71 (**0.014**,0.067)
Arginine	2.29-3.40	**0.007**	3.15	2.97 (**0.004**,**0.033**)	3.00 (**0.012**,0.066)	2.94 (**0.001**,**0.015**)	2.96 (**0.003**,**0.026**)
Aspartic Acid	3.81-5.12	**0.007**	4.52	4.41 (**0.004**,**0.033**)	4.44 (**0.037**,0.124)	4.38 (**0.0005**,**0.010**)	4.43 (**0.014**,0.067)
Cysteine	0.37-0.81	0.254	0.60	0.60 (0.637,0.808)	0.60 (0.787,0.873)	0.61 (0.900,0.936)	0.61 (0.110,0.260)
Glutamic Acid	5.84-8.20	**0.002**	6.98	6.76 (**0.001**,**0.015**)	6.83 (**0.019**,0.086)	6.70 (**0.0001**,**0.006**)	6.80 (**0.006**,**0.043**)
Glycine	1.46-2.00	**0.001**	1.74	1.69 (**0.0004**,**0.009**)	1.70 (**0.002**,**0.023**)	1.69 (**0.0001**,**0.006**)	1.70 (**0.001**,**0.017**)
Histidine	0.88-1.22	**0.003**	1.09	1.06 (**0.002**,**0.023**)	1.07 (**0.014**,0.067)	1.05 (**0.0002**,**0.007**)	1.07 (**0.013**,0.067)
Isoleucine	1.54-2.08	0.232	1.87	1.83 (0.100,0.241)	1.85 (0.450,0.642)	1.82 (**0.042**,0.134)	1.85 (0.514,0.708)
Leucine	2.20-4.00	**0.010**	3.06	3.00 (**0.007**,**0.046**)	3.02 (0.068,0.186)	2.98 (**0.001**,**0.011**)	3.01 (**0.037**,0.124)
Lysine	2.29-2.84	**0.005**	2.56	2.51 (**0.004**,**0.034**)	2.52 (**0.028**,0.105)	2.49 (**0.0003**,**0.009**)	2.52 (**0.022**,0.093)
Methionine	0.43-0.68	0.433	0.56	0.55 (0.377,0.575)	0.55 (0.245,0.438)	0.55 (0.089,0.225)	0.55 (0.742,0.860)
Phenylalanine	1.60-2.35	**0.008**	2.02	1.97 (**0.014**,0.067)	1.98 (**0.044**,0.138)	1.94 (**0.0004**,**0.009**)	1.97 (**0.027**,0.105)
Proline	1.69-2.28	0.374	1.91	1.85 (0.059,0.169)	1.88 (0.400,0.597)	1.87 (0.155,0.324)	1.87 (0.240,0.432)
Serine	1.11-2.48	0.063	1.99	1.95 (0.082,0.210)	1.95 (0.115,0.268)	1.91 (**0.006**,**0.043**)	1.93 (**0.021**,0.088)
Threonine	1.14-1.89	**0.001**	1.62	1.57 (**0.002**,**0.020**)	1.58 (**0.008**,**0.048**)	1.55 (**<0.0001**,**0.006**)	1.57 (**0.002**,**0.022**)
Tryptophan	0.36-0.67	0.330	0.43	0.43 (0.593,0.787)	0.43 (0.981,0.981)	0.43 (0.904,0.936)	0.42 (0.095,0.235)
Tyrosine	1.02-1.61	0.449	1.36	1.34 (0.275,0.471)	1.35 (0.517,0.708)	1.33 (0.096,0.235)	1.33 (0.153,0.321)
Valine	1.50-2.44	0.159	1.97	1.92 (**0.032**,0.116)	1.94 (0.279,0.475)	1.92 (**0.038**,0.124)	1.95 (0.346,0.551)

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 40. Summary of the amino acid analysis of soybean grain (% dry weight).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded.

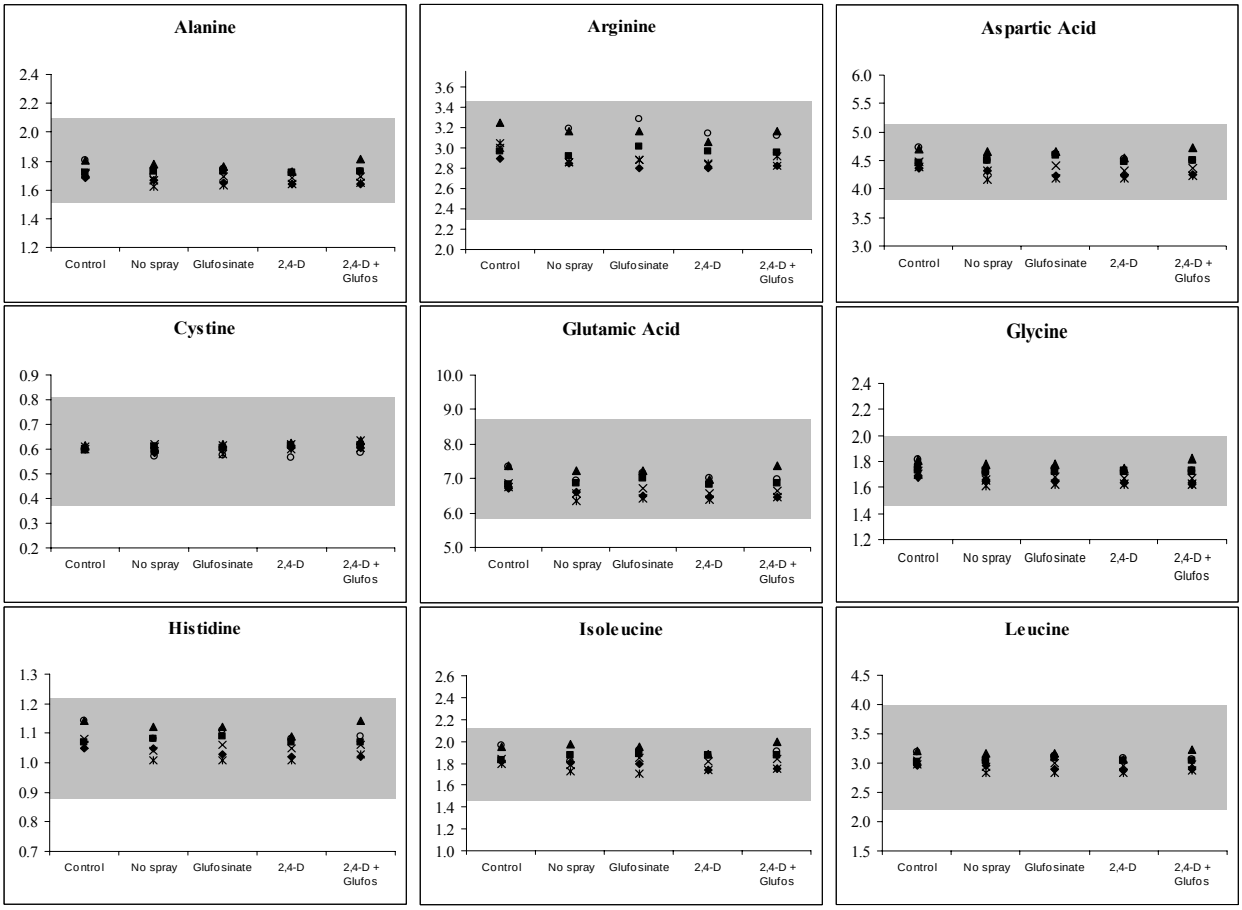
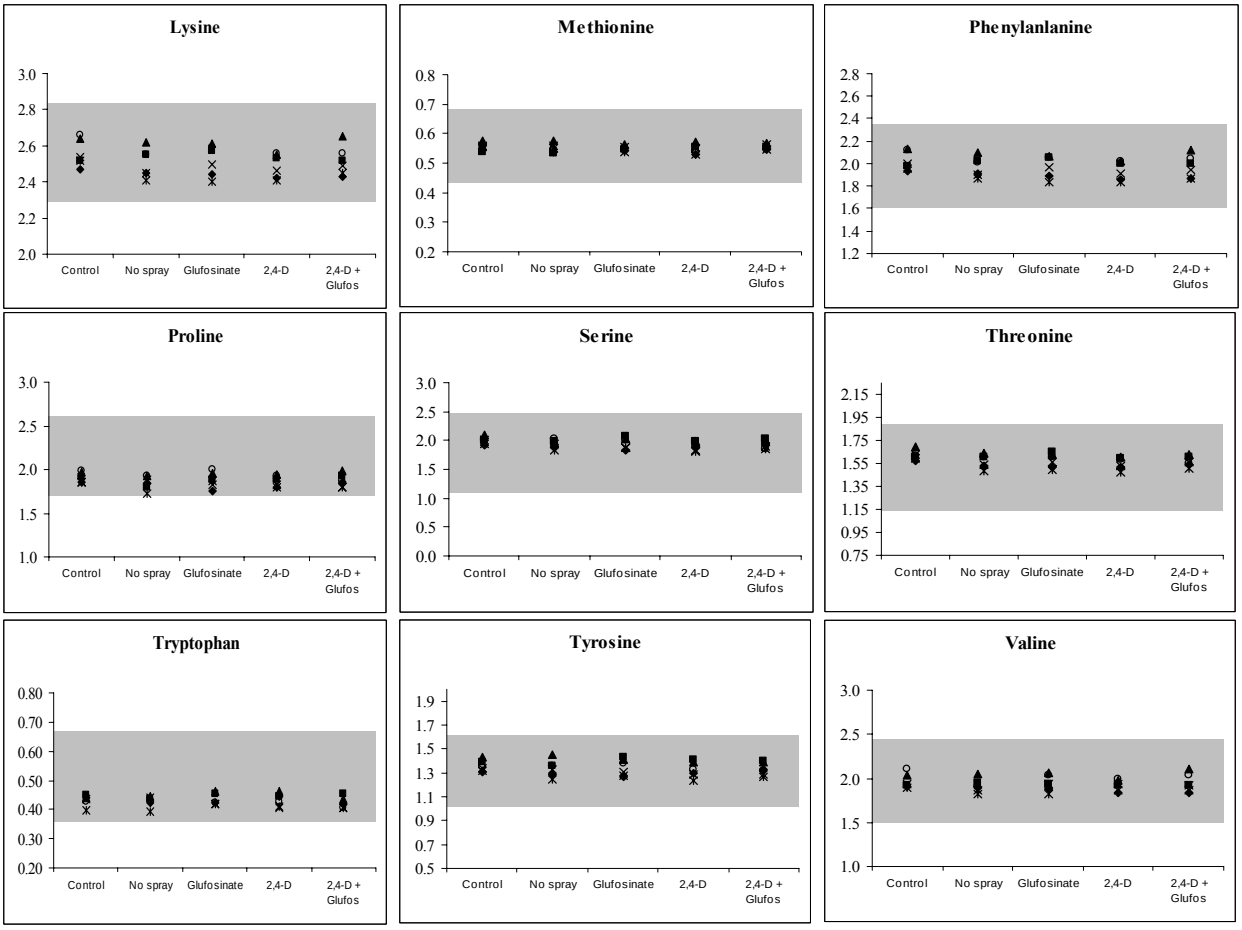


Figure 40. Summary of the amino acid analysis of soybean grain (% dry weight).

(cont.). Summary of the amino acid analysis of soybean grain (% dry weight).



VIII-B.4. Fatty Acids

An analysis of 22 fatty acids in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 23 and Figure 41.

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

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

The level of 18:0 stearic was significantly different between the control and the unsprayed and AAD-12 + glufosinate, based on unadjusted p-values. However, no significant differences were observed based on the adjusted p-values or the overall treatment effect, and all entries were within the reported literature values, indicating equivalence to non-transgenic soybean.

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

The level of 18:2 linoleic was significantly different between the control and the unsprayed and AAD-12 + 2,4-D, based on unadjusted p-values. However, no significant differences were observed in the adjusted p-values or the overall treatment effect, and 18:2 linoleic levels were within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

Levels of 18:3 linolenic were significantly different between each of the AAD-12 entries and the control based on unadjusted p-values, and the adjusted p-values were also significant between the unsprayed AAD-12 and AAD-12 + both herbicide treatment compared with the control. No literature values are available for 18:3 linolenic, however, differences between the AAD-12 and control treatment were small (<6%).

The level of 20:0 arachidic was significantly different between the control and the unsprayed, AAD-12 + glufosinate, AAD-12 + 2,4-D, and AAD-12 + both herbicides based on unadjusted p-values, and 20:0 arachidic also had significant differences in the across-site analysis in the adjusted p-value for the unsprayed and AAD-12 + glufosinate treatments. However, 20:0

arachidic levels were within the reported literature values for all treatments, indicating equivalence to non-transgenic soybean.

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

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

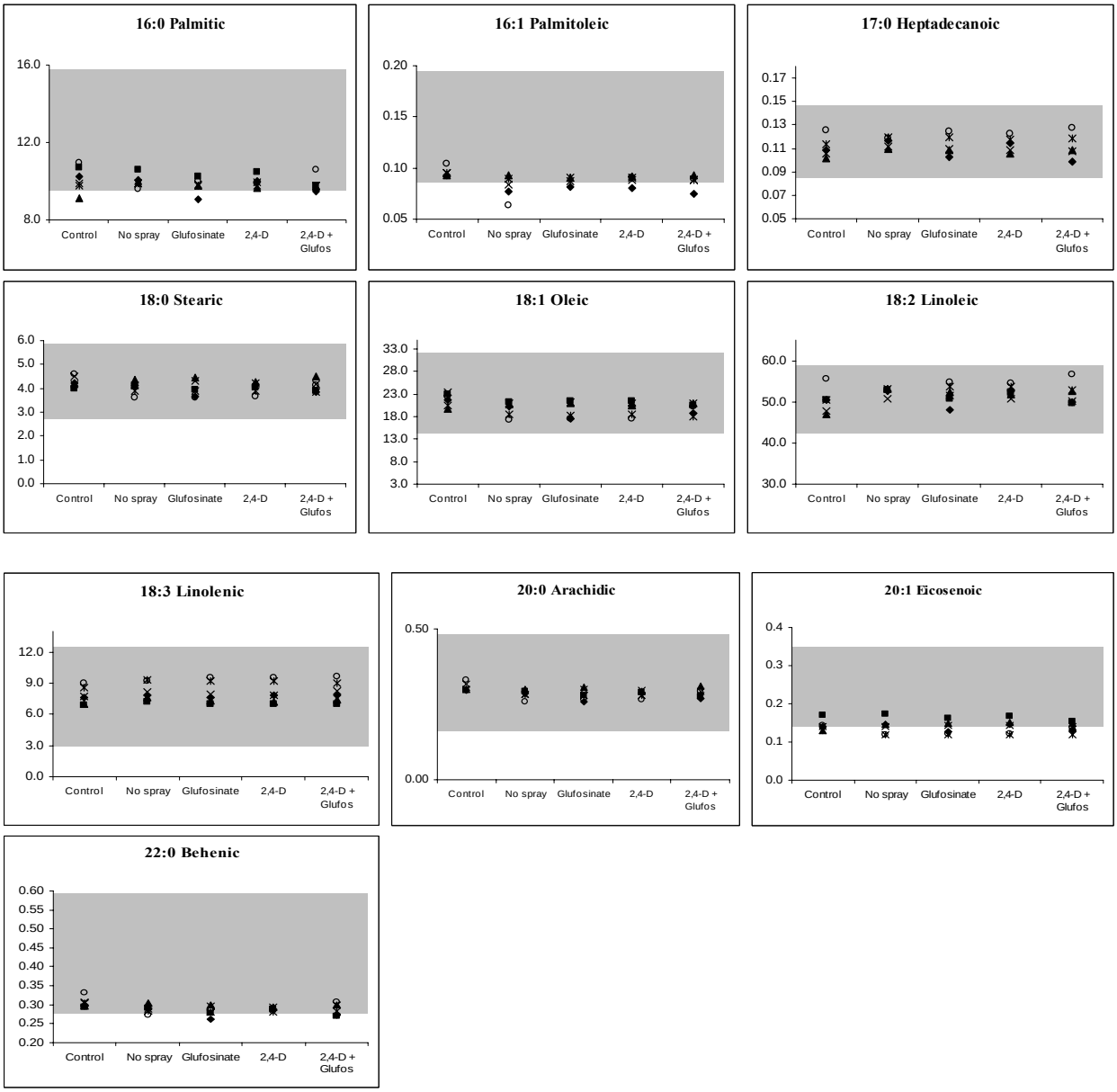
Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 23. Summary of the fatty acid analysis of soybean grain (% total fatty acids).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d]) Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
8:0 Caprylic	0.15	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
10:0 Capric	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
12:0 Lauric	0.08-0.13	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
14:0 Myristic	0.07-0.24	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
14:1 Myristoleic	0.12-0.13	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
15:0 Pentadecanoic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
15:1 Pentadecenoic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
16:0 Palmitic	9.55-15.77	0.607	10.1	10.0 (0.625,0.802)	9.78 (0.148,0.313)	9.94 (0.455,0.644)	9.85 (0.249,0.441)
16:1 Palmitoleic	0.09-0.19	**0.029**	0.097	**0.085** (**0.003**,**0.028**)	0.088 (**0.038**,0.124)	0.087 (**0.027**,0.105)	0.089 (**0.029**,0.109)
17:0 Heptadecanoic	0.09-0.15	0.640	0.111	0.114 (0.162,0.336)	0.113 (0.331,0.539)	0.114 (0.239,0.432)	0.113 (0.296,0.493)
17:1 Heptadecenoic	0.07-0.09	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
18:0 Stearic	2.70-5.88	0.136	4.28	4.03 (**0.048**,0.145)	3.98 (**0.018**,0.081)	4.05 (0.060,0.169)	4.06 (0.073,0.196)
18:1 Oleic	14.3-32.2	**0.010**	21.8	19.8 (**0.004**,**0.033**)	19.5 (**0.001**,**0.017**)	19.9 (**0.006**,**0.043**)	19.9 (**0.006**,**0.043**)
18:2 Linoleic	42.3-58.8	0.145	50.3	52.5 (**0.030**,0.109)	51.9 (0.116,0.268)	52.6 (**0.024**,0.095)	52.0 (0.087,0.222)
18:3 γ-Linolenic	3.00-12.52	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
18:3 Linolenic	NR	**0.022**	7.83	8.23 (**0.003**,**0.031**)	8.15 (**0.016**,0.073)	8.10 (**0.034**,0.119)	8.21 (**0.004**,**0.034**)
20:0 Arachidic	0.16-0.48	**0.023**	0.307	0.284 (**0.007**,**0.045**)	0.282 (**0.004**,**0.033**)	0.285 (**0.009**,0.052)	0.287 (**0.014**,0.067)
20:1 Eicosenoic	0.14-0.35	0.683	0.143	0.140 (0.582,0.779)	**0.136** (0.201,0.380)	0.141 (0.794,0.875)	**0.138** (0.327,0.538)
20:2 Eicosadienoic	0.08-0.25	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
20:3 Eicosatrienoic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
20:4 Arachidonic	NR	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
22:0 Behenic	0.28-0.60	0.053	0.305	0.288 (**0.023**,0.095)	0.285 (**0.008**,**0.048**)	0.288 (**0.020**,0.087)	0.288 (**0.020**,0.087)

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
NA = statistical analysis was not performed since a majority of the data was < LOQ.
NR = not reported.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 41. Summary of the fatty acid analysis of soybean grain (% total fatty acids). Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded. Grain was also analyzed for 8:0 Caprylic, 10:0 Capric, 12:0 Lauric, 14:0 Myristic, 14:1 Myristoleic, 15:0 Pentadecanoic, 15:1 Pentadecenoic, 17:1 Heptadecenoic, 18:3 gamma-Linolenic, 20:2 Eicosadienoic, 20:4 Arachidonic, 20:3 Eicosatrienoic, but levels were below the limit of quantitation.



VIII-B.5. Vitamins

An analysis of vitamins in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 24 and Figure 42.

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

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

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

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

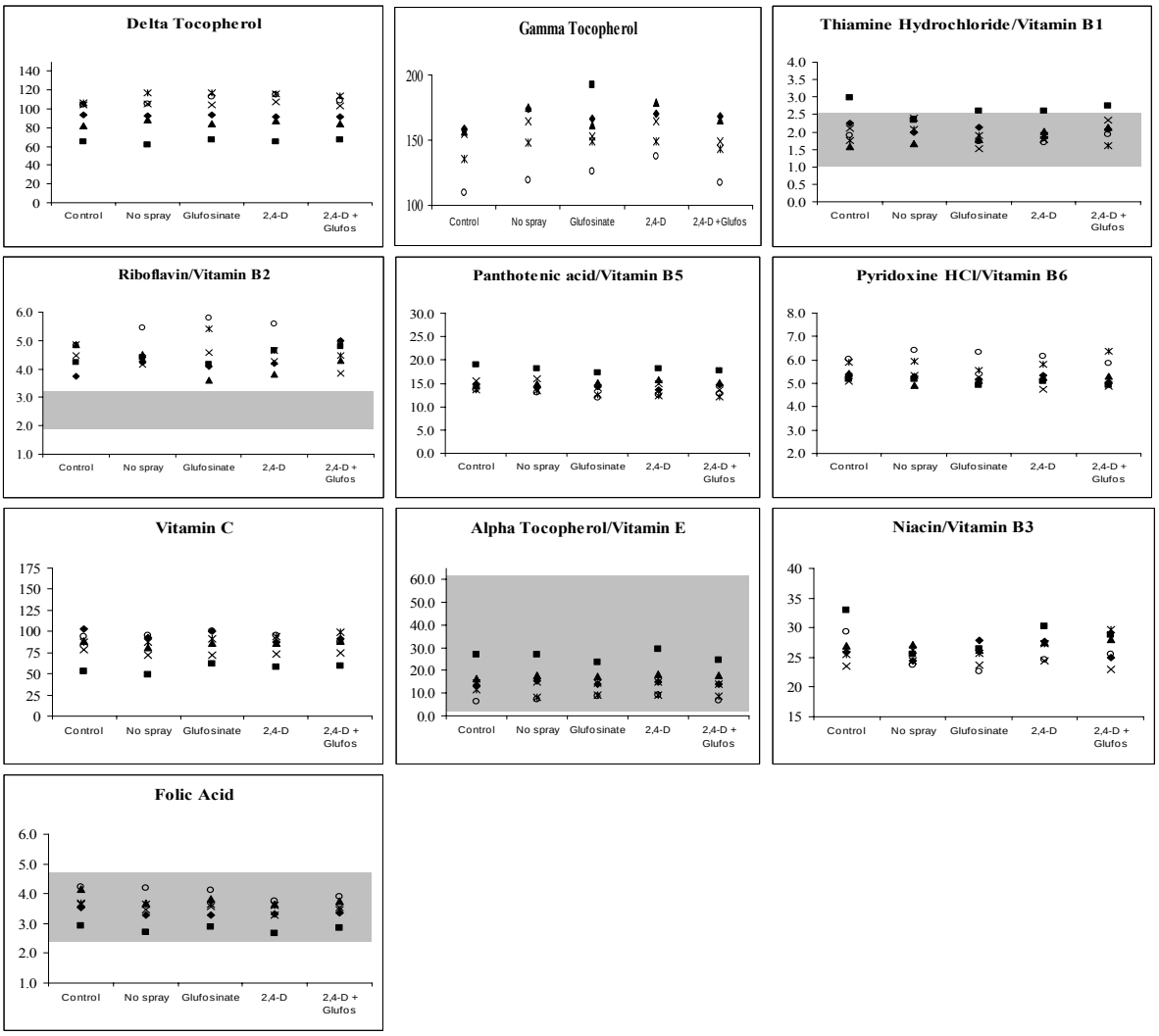
Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 24. Summary of vitamin analysis of soybean grain (mg/kg dry weight).

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

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
NR = not reported.
NA= statistical analysis was not performed since a majority of the data was < LOQ.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 42. Summary of vitamin analysis of soybean grain (mg/kg dry weight).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded. Grain was also analyzed for Beta-Tocopherol, Vitamin A, Vitamin B12, and Vitamin D, but results were less than the limit of quantitation.



VIII-B.6. Isoflavones

The analysis of isoflavones in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 25 and Figure 43.

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

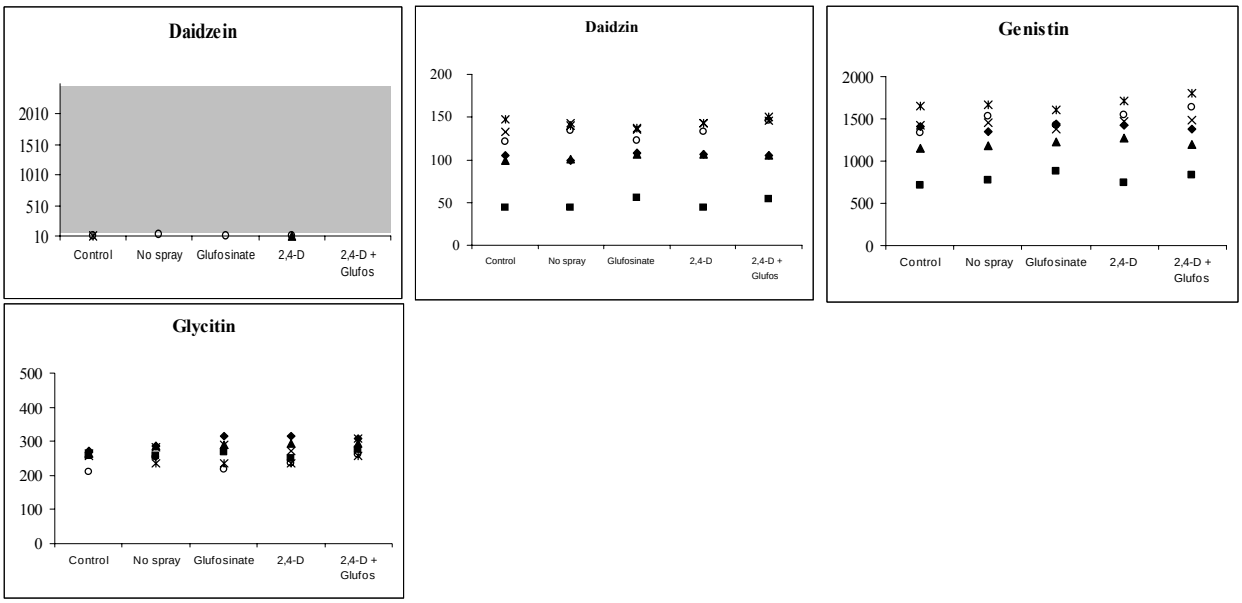
Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 25. Summary of isoflavone analysis of soybean grain (µg/g).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d]) Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Daidzein	60.0-2454	NA	**19.2**	**31.2**	**14.8**	**13.0**	< LOQ
Daidzin	NR	0.068	1085	1103 (0.584,0.779)	1112 (0.391,0.589)	1128 (0.187,0.360)	1179 (**0.007**,**0.045**)
Genistein	144-2837	NA	**22.9**	< LOQ	< LOQ	< LOQ	< LOQ
Genistin	NR	0.069	**1282**	**1321** (0.292,0.490)	**1327** (0.220,0.408)	**1357** (0.052,0.152)	**1389** (**0.007**,**0.044**)
Glycitein	15.3-1070	NA	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Glycitin	NR	**0.032**	253	267 (0.142,0.305)	270 (0.076,0.201)	268 (0.121,0.274)	285 (**0.002,0.021**)

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
NA= statistical analysis was not performed since a majority of the data was < LOQ.
NR = not reported.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 43. Summary of isoflavone analysis of soybean grain (µg/g).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded. Grain was also analyzed for genistein and glycitein, but results were less than the limit of quantitation.



VIII-B.7. Antinutrients

An analysis of anti-nutrients in soybean grain samples from the control, unsprayed AAD-12, AAD-12 + glufosinate, AAD-12 + 2,4-D and AAD-12 + both herbicides was performed. A summary of the results across all locations is shown in Table 26 and Figure 44.

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

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

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

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

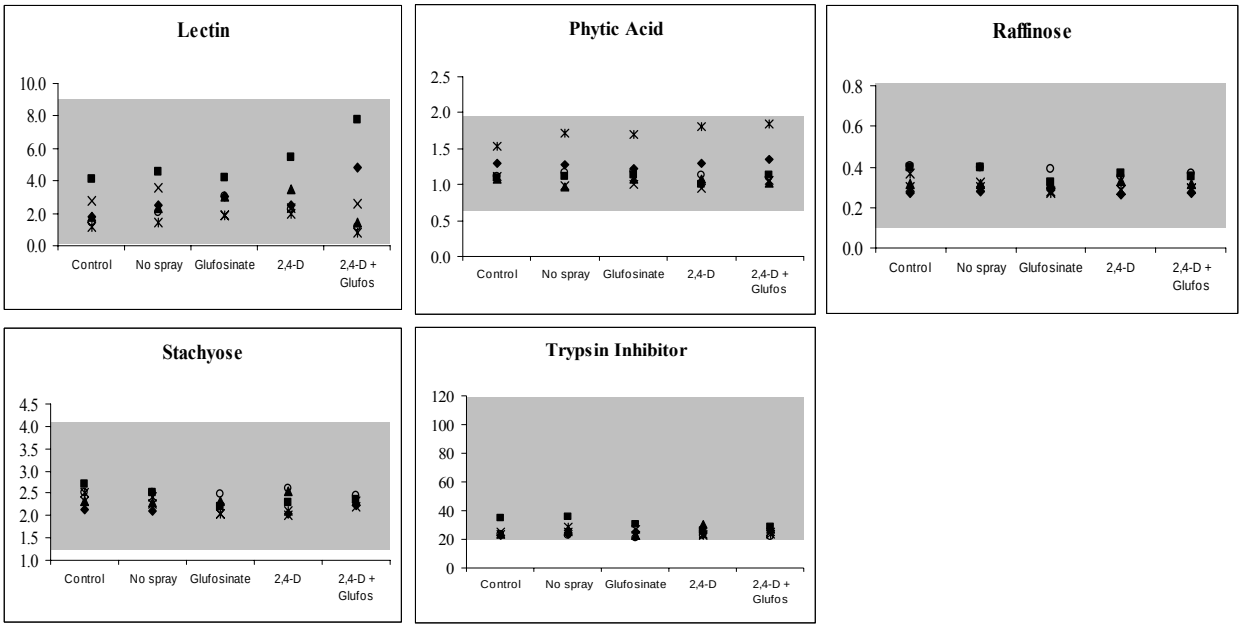
Based on these compositional constituents, the grain from DAS-68416-4 soybean was substantially equivalent to that of non-transgenic soybean.

Table 26. Summary of anti-nutrient analysis of soybean grain (% dry weight).

Analyte	Literature Values[a]	P-value for Overall Treatment Effect[b]	Control	Treatment Means (P-value,[c] Adj. P[d]) Unsprayed	Sprayed Glufosinate	Sprayed 2,4-D	Sprayed Both
Lectin (H.U./mg)	0.11-9.04	0.552	2.18	2.74 (0.333,0.540)	2.84 (0.254,0.444)	2.98 (0.176,0.351)	3.09 (0.124,0.277)
Phytic Acid	0.63-1.96	0.725	1.20	1.20 (0.949,0.962)	1.22 (0.673,0.819)	1.21 (0.896,0.936)	1.25 (0.253,0.444)
Raffinose	0.1-0.9	0.111	0.344	0.339 (0.753,0.860)	0.310 (**0.033**,0.118)	0.317 (0.082,0.210)	0.315 (0.062,0.173)
Stachyose	1.2-4.1	0.217	2.42	2.34 (0.378,0.575)	2.23 (**0.027**,0.105)	2.28 (0.105,0.253)	2.32 (0.231,0.425)
Trypsin Inhibitor (TIU/mg)	19.6-184	0.435	25.3	27.2 (0.204,0.383)	24.7 (0.657,0.819)	24.9 (0.748,0.860)	25.3 (0.973,0.979)

[a] Combined range from Appendix 6.
[b] Overall treatment effect estimated using an F-test.
[c] Comparison of the transgenic treatments to the control using t-tests.
[d] P-values adjusted using a False Discovery Rate (FDR) procedure.
Bolded mean values are outside of the reported literature range.
Bolded P-values are significant (<0.05).

Figure 44. Summary of anti-nutrient analysis of soybean grain (% dry weight).
Values at each location shown: diamond = IA, square = IL, triangle = IN, X = NE, star = ON1, and circle = ON2. Literature ranges (Appendix 6) are shaded.



VIII-C. Summary of Grain and Forage Composition

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

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

VIII-D. References

Benjamini, Y., Hochberg, Y. (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. Royal Statistical Soc. B, 57:289-300.

OECD (2001) Consensus Document on Compositional Considerations for New Varieties of Soybean: Key Food and Fee Nutrients and Anti-Nutrients.

IX. Environmental Consequences and Impact on Agronomic Practices

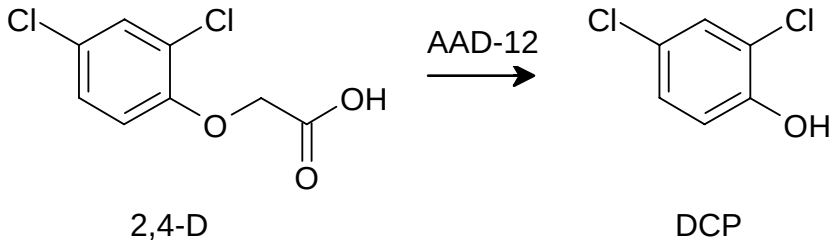
IX-A. Mode of Action of the AAD-12 Protein

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

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.

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

Figure 45. Degradation reaction of 2,4-D catalyzed by AAD-12.



IX-A.1. Field Efficacy

The efficacy of DAS-68416-4 soybean for the detoxification of 2,4-D and subsequent protection from injury caused by this compound has been characterized in field studies conducted in 2007 and 2008. Trials were designed to evaluate the preemergence (2008 only) and postemergence tolerance to 2,4-D. The preemergence trials were conducted as a randomized complete block design and postemergence trials were a split plot design with application timing as the main plots and herbicide rates as the sub plots. Experimental units consisted of single-row plots for postemergence trials and two-row plots for preemergence trials, approximately 3 m in length and all trials contained three replications. Postemergence trials received an application of glufosinate at 0.41 lb ae/A at the V1 growth stage on all DAS-68416-4 plots to remove unintended nulls prior to application of herbicide treatments. All herbicide applications were made with gas-pressurized small-plot spray equipment delivering approximately 140 – 190 l/ha of spray volume. The formulation of 2,4-D used was a 456 g ae/liter dimethylammonium salt (DMA). Visual injury ratings were taken approximately 7 days after crop emergence in the case of preemergence trials and 7 days after application of herbicide treatments in the case of postemergence trials. 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, with 0 = no injury as compared to untreated plots and 100 = death of all plants.

Tolerance to 2,4-D

In 2007, data from a field study in Indiana indicated excellent tolerance of DAS-68416-4 soybean to 2,4-D at rates ranging from 0.5 to 4.0 lb ae/A applied at either the V4 or R2 growth stages. At 7 days after the V4 application mean injury to DAS-68416-4 soybeans ranged from 0 to 8% as the 2,4-D rate increased from 0.5 to 4.0 lb ae/A. Injury to the non-transgenic Maverick soybean from the same treatments ranged from 58 to 85% injury. Similar evaluations after the R2 application produced mean injury ranging from 0 to 5% for DAS-68416-4 soybeans and 32 to 72% for non-transgenic Maverick. Injury symptoms to the non-transgenic Maverick soybean consisted mainly of stem and leaf malformation, growth inhibition, and tissue necrosis.

Further field evaluations of 2,4-D tolerance were conducted in 2008. Studies examining the response of DAS-68416-4 soybeans and non-transgenic Maverick soybeans to applications of 2,4-D DMA preemergence and at the V2-V3, V6 and R2 growth stages were conducted at locations in Mississippi, Indiana, and Minnesota.

Averaged across the three locations, preemergence application of 2,4-D DMA to DAS-68416-4 resulted in ≤2% injury regardless of application rate (Table 27). The same treatments caused 34 to 60% injury to non-transgenic Maverick. Injury from pre-emergence applications of 2,4-D consisted of stand loss and reduction in growth.

Table 27. DAS-68416-4 soybean tolerance to preemergence applications of 2,4-D, 2008.

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]			
			DAS-68416-4		Maverick	
2,4-D amine	1.0 lb ae/A	Pre-emergence	2	ns	34	b
2,4-D amine	2.0 lb ae/A	Pre-emergence	2	ns	58	b
2,4-D amine	4.0 lb ae/A	Pre-emergence	0	ns	63	a

[a] ae/A = acid equivalent/acre
[b] Application stage in terms of soybean plant growth development.
[c] Ratings were taken approximately 7 days after application. Means within each column followed by the same letter are not significantly different as determined by restricted maximum likelihood methods for mixed models and Tukey, or for unbalanced data, Tukey-Kramer HSD test (0.05). ns indicates no significant differences.

The most evident symptoms from post-emergence 2,4-D treatments to non-transgenic Maverick were stem twisting, leaf malformation, and necrosis at this evaluation timing, with overall injury ranging from 60 to 93% depending on application rate and timing. Injury to DAS-68416-4 soybeans (Table 28) was ≤5% and consisted almost entirely of small necrotic patches on the leaves observed at the 2.0 to 4.0 lb ae/A rates and was confined to the leaves near the top of the canopy a the time of application. No injury was observed on newly emerging leaves. Sequential applications of 2,4-D at 1.0 lb ae/A followed by a second application of 1.0 lb ae/A caused no injury to DAS-68416-4 soybeans, but caused 89% injury to non-transgenic Maverick when applied at V2 followed by V6, and 95% injury when applied at V6 followed by R2.

Table 28. DAS-68416-4 soybean tolerance to post-emergence applications of 2,4-D, 2008.

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]			
			DAS-68416-4		Maverick	
2,4-D amine	1.0 lbs ae/A	V2-V3	0	ns	81	b
2,4-D amine	2.0 lbs ae/A	V2-V3	1	ns	90	ab
2,4-D amine	4.0 lbs ae/A	V2-V3	5	ns	93	a
2,4-D amine	1.0 lbs ae/A	V6	0	b	67	cd
2,4-D amine	2.0 lbs ae/A	V6	0	b	81	abc
2,4-D amine	4.0 lbs ae/A	V6	4	a	86	ab
2,4-D amine	1.0 lbs ae/A	R2	0	b	60	a
2,4-D amine	2.0 lbs ae/A	R2	1	ns	69	c
2,4-D amine	4.0 lbs ae/A	R2	2	ns	74	bc
2,4-D amine	1.0 f/b 1.0 lbs ae/A	V2 f/b V6	0	ns	89	b
2,4-D amine	1.0 f/b 1.0 lbs ae/A	V6 f/b R2	0	ns	95	a

[a] ae/A = acid equivalent/acre
[b] Application stage in terms of soybean plant growth development.
[c] Ratings were taken approximately 7 days after application. Means within each columns followed by the same letter are not significantly different as determined by restricted maximum likelihood methods for mixed models and Tukey, or for unbalanced data, Tukey-Kramer HSD test (0.05). ns indicates no significant differences.

Tolerance to Glufosinate

Since DAS-68416-4 soybeans also contain the *pat* gene, the post-emergence tolerance to glufosinate as the 183 g ae/liter ammonium salt was also evaluated in trials during 2007 and 2008 at the same locations as the 2,4-D tolerance studies. Trial methods and evaluations were similar to those utilized for 2,4-D studies.

In 2007, field testing of applications of glufosinate to DAS-68416-4 soybeans at the V4 stage of development resulted in 3, 10, and 22% injury 7 days after application of 0.375, 0.75, and 1.5 lbs ae/A (420, 840, and 1680 g ae/ha), respectively. The same applications to non-transformed Maverick resulted in 78, 90, and 98% injury. Injury symptoms were mainly of chlorosis, necrosis, and growth inhibition, typical of injury normally observed from glufosinate.

Field trials with glufosinate in 2008 demonstrated results consistent with the 2007 data (Table 29). A similar dose response of DAS-68416-4 soybean to glufosinate was noted, regardless of soybean growth stage at application. Glufosinate injury to non-transformed Maverick ranged from 54 to 98% depending on application rate and timing.

Table 29. DAS-68416-4 soybean tolerance to postemergence applications of glufosinate, 2008.

Herbicide	Rate[a]	Application Stage[b]	Percent Plant Injury[c]			
			DAS-68416-4		Maverick	
glufosinate	0.366 lbs ae/A	V2-V3	2	b	91	ns
glufosinate	0.73 lbs ae/A	V2-V3	9	b	97	ns
glufosinate	1.46 lbs ae/A	V2-V3	19	a	98	ns
glufosinate	0.366 lbs ae/A	V6	3	b	78	b
glufosinate	0.73 lbs ae/A	V6	9	b	88	ab
glufosinate	1.46 lbs ae/A	V6	16	a	92	a
glufosinate	0.366 lbs ae/A	R2	2	b	54	a
glufosinate	0.73 lbs ae/A	R2	3	b	77	b
glufosinate	1.46 lbs ae/A	R2	18	b	90	ab
glufosinate	0.366 f/b 0.366 lbs ae/ha	V2 f/b V6	5	a	98	a
glufosinate	0.366 f/b 0.366 lbs ae/ha	V6 f/b R2	1	b	87	a

[a] ae/A= acid equivalent/acre
[b] Application stage in terms of soybean plant growth development.
[c] Ratings were taken approximately 7 days after application. Means within each column followed by the same letter are not significantly different as determined by restricted maximum likelihood methods for mixed models and Tukey, or for unbalanced data, Tukey-Kramer HSD test (0.05). ns indicates no significant differences.

Summary of Herbicide Tolerance

For DAS-68416-4 soybeans, the proposed maximum single application rate of 2,4-D will be 1.0 lb ae/A (see section IX-E.2). Results of field testing indicate that DAS-68416-4 soybeans provide acceptable tolerance to 2,4-D at rates of at least two times this proposed maximum use rate.

The maximum use rate of glufosinate on herbicide-tolerant soybeans is 2 applications at 0.366 lb ae/A. Results of field testing indicate that DAS-68416-4 soybeans provide acceptable tolerance to glufosinate at rates of at least two times this rate.

IX-B. Weediness Potential

Commercial soybean varieties in the United States are not considered weeds and are not effective in 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 colonisers (OECD, 2000).

The introduction of aryloxyalkanoate herbicide-tolerance into soybean will not alter the weediness characteristics of soybean. Agronomic properties of DAS-68416-4 soybean related to weediness, such as germination, emergence, seedling vigor, and response to environmental

stressors, have been shown to be identical to conventional soybean. These findings demonstrate that DAS-68416-4 soybean do not have increased weediness potential relative to conventional soybean.

IX-C. Gene Flow Assessment

IX-C.1. Vertical Gene Flow Assessment

Soybean is considered to be a self-pollinated species, although natural crossing can occur (OECD, 2000) at low rates. 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-68416-4 soybeans to wild soybean relatives in the United States.

IX-C.2. Horizontal Gene Flow Assessment

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Connor *et al.*, 2003). Even if such a transfer were to take place, transfer of the *aad-12* gene from DAS-68416-4 soybean would not present a human health or plant pest risk, based on the safety data presented in this petition. The gene encoding the AAD-12 protein is from a naturally occurring soil bacterium, *Delftia acidovorans,* and is already present in nature. Transfer recipients would, therefore, not pose a greater plant pest risk than the environmentally prevalent wild type microbes from which the genes originated.

IX-D. Current US Agronomic Practices for Soybeans

IX-D.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 2007/08 was $27 billion, the second-highest value among U.S.-produced crops, trailing only corn. Soybean and soybean product exports accounted for 43 percent of U.S. soybean production in 2007/08. 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, 2008).

A total of 75.7 million acres were planted to soybeans in the United States in 2008 (USDA NASS, 2008). Of these planted acres, about 74.6 million acres were harvested, valued at $27.4 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.

IX-D.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 (2000) 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 (see Table 30). Some fields are also infested with perennial weeds (quackgrass, Johnsongrass, Canada thistle, others) which are more difficult to control since they can reproduce from seed or underground rhizomes.

Table 30. Common troublesome weeds in soybeans in 2006-2008.
(Data from DMR-Kynetec).

Weed Species	Total Soybean Acres Treated[1]		
	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, 2008). 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, 2009-2010). Broadleaf and grass weeds compete at different levels of intensity depending upon the competitiveness of the crop, tillage system, environmental conditions, and other weeds present. Generally, broadleaf weeds are more damaging to a broadleaf crop, while grass weeds are more competitive in a grass crop. Crop yield loss information is available for certain single weed species growing with soybeans in the U.S. Midwest (Table 31).

Table 31. Yield reduction from specific weed species in soybeans.
(from Penn State Agronomy Guide, 2009-2010).

	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] Interference data are from Stoller *et al*., 1985, *Reviews of Weed Science*; E. L. Knake and F. W. Slife, 1962, *Weeds* 10:26; and E. L. Werner and W. S. Curran, 1995, Proc. NEWSS 49:23.

IX-D.3. Weed Management in Soybean

In 2005, USDA NASS surveyed 17 states (AR, IL, IN, IA, KS, KY, LA, MI, MN, MS, MO, NE, NC, OH, SD, TN, VA; about 90% 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, 2006). In 2006, 95% 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 always receive a post-emergence herbicide application. In 2005, the most widely used herbicide in soybeans was glyphosate, driven by the high adoption of glyphosate tolerant soybeans (USDA NASS, 2006). Soybeans received an average of 1.5 applications of glyphosate on 91% of the acres planted in 2005. Herbicides such as 2,4-D (6% of acres), chlorimuron (4% of acres), and trifluralin (23% 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, 2002). Herbicides are only one of several tools available for growers to consider using in an IWM approach. Herbicide-tolerant soybeans 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, 2002). Some of these concerns include selection for herbicide-resistant weeds, shifts in weed species, drift to sensitive non-target crops or non-agricultural habitats, herbicide persistence in the soil resulting in carryover to the next crop, herbicide misapplications, and over-reliance on herbicides to control weeds.

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

IX-D.4. Crop Rotation 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, 2006). 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 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 2002). 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, conservation tillage is the only system that seems to support sustainable soybean production from an environmental standpoint.

IX-E. Potential Impact on Agronomic Practices

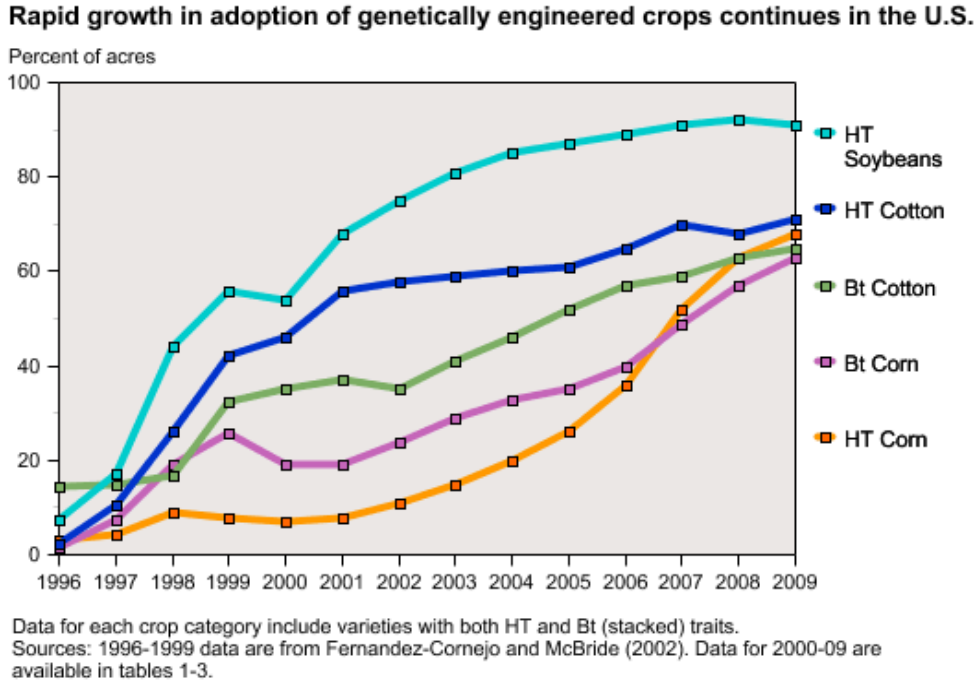
IX-E.1. Potential Impact on Cultivation and Management Practices

Soybean lines that contain herbicide-tolerance traits (glyphosate) have been on the market since 1996 and have experienced broad adoption (Figure 46). 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 91 percent in 2009 (USDA ERS 2009a). 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 91% of soybean acres in the U.S. in 2009 planted to glyphosate-tolerant soybeans, typical cultivation and management practices used by growers today already take into account the management of herbicide-tolerant traits. DAS-68416-4 soybeans are comparable to conventional soybeans phenotypically and agronomically (Section VII-D), 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-68416-4 soybeans (Section VII-B) and therefore, no impacts are expected on insect control practices for DAS-68416-4 soybeans. It is anticipated that the same management practices used today for soybeans with the glyphosate-tolerance trait will also be appropriate for DAS-68416-4 soybeans.

Figure 46. Adoption of genetically engineered crops in the U.S.
From USDA ERS, 2009b



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

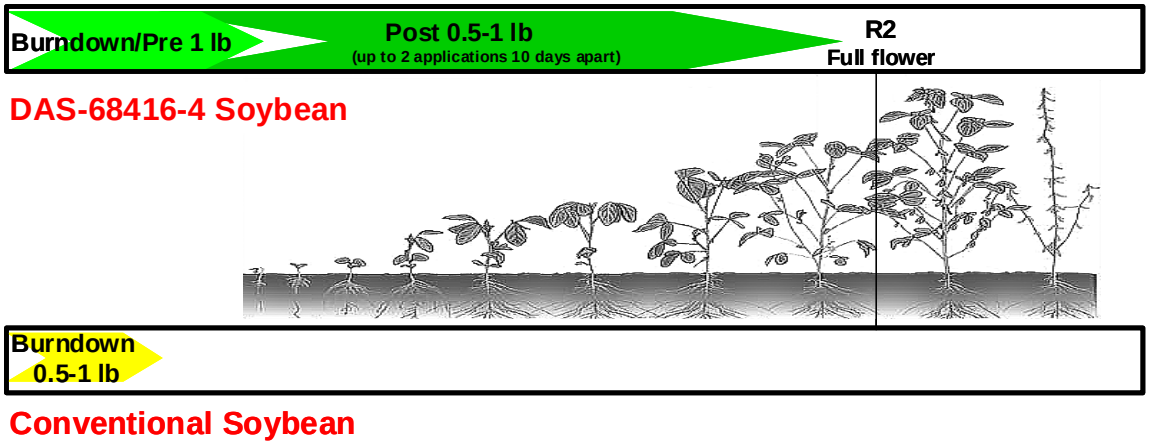
DAS-68416-4 soybeans confers tolerance to the herbicides 2,4-D and glufosinate, both of which will provide expanded weed management options in soybeans. Post-emergence applications of 2,4-D control a broad spectrum of broadleaf weeds. 2,4-D also has some short-lived soil residual activity (4-10 day soil half life) which provides limited residual control of later germinating broadleaf weeds. Post-emergence applications of glufosinate will control a broad spectrum of grass and broadleaf weeds. Thus, DAS-68416-4 soybeans will provide alternatives to glyphosate in weed management systems. 2,4-D and/or glufosinate 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-68416-4 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 10 days apart over-the-top of the soybeans up to the R2 stage (full flower) of development (Figure 47). Thus, the proposed maximum seasonal rate of 2,4-D on soybeans will increase from 1.0 lb ae/A (current) to 3.0 lbs ae/ha (DAS-68416-4 soybeans). DAS-68416-4 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.

Figure 47. 2,4-D herbicide application timing and rates for conventional and DAS-68416-4 soybeans.

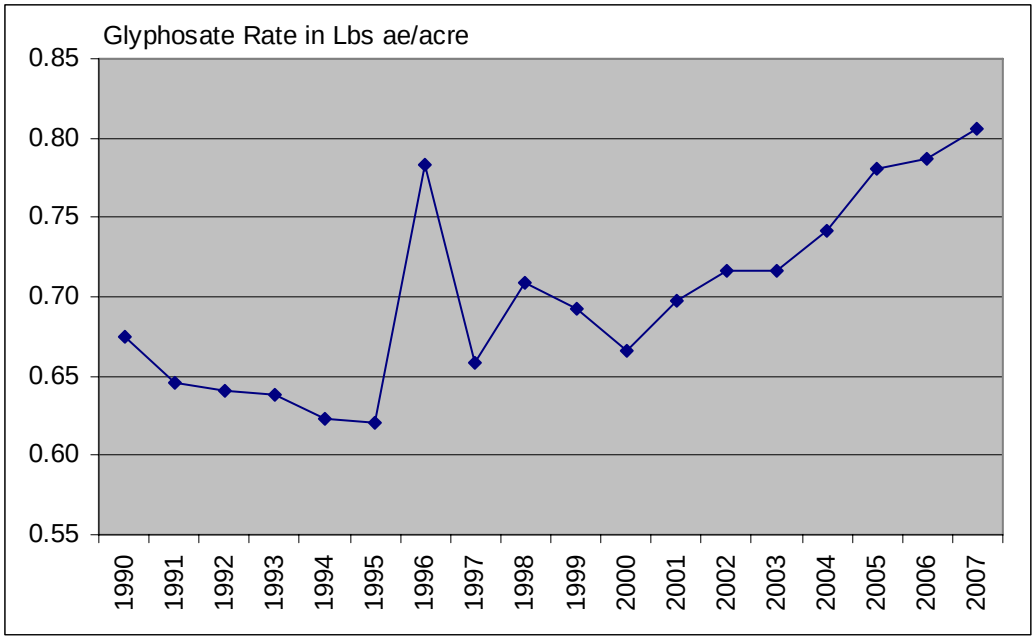


The use pattern for glufosinate on DAS-68416-4 soybeans will be consistent with the current use pattern of glufosinate on other soybean products that contain the *pat* gene.

While 2,4-D is currently registered as a pesticide, supporting information on proposed label changes for its use with DAS-68416-4 soybeans is being provided by Dow AgroSciences to U.S. EPA for review. Dow AgroSciences is also developing an extensive stewardship program that will include technological advancements in application and off-target movement, as well as utilizing several media venues to educate and facilitate adoption of the technology and decision management tools to ensure the proper use and stewardship of both the trait and chemical technologies.

In the future, DAS-68416-4 soybeans could be stacked with glyphosate-tolerance traits. Such combined trait products (stacks) would have the potential to improve weed control by allowing use of herbicide combinations or mixtures which can provide more consistent performance in post-emergence weed control programs, and counteract glyphosate "rate-creep" (steady increase in rates needed to obtain effective weed control; Figure 48) on hard-to-control weeds (Jaehnig, 2005). DAS-68416-4 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 48. Glyphosate application rates in U.S. corn and soybeans from 1990 thru 2007. (Data from DMR-Kynetec)



IX-E.3. Potential Impact on Volunteer Management

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 colonisers.

DAS-68416-4 soybeans are tolerant to 2,4-D and glufosinate. In the unlikely event that they would grow as volunteers in the year following cultivation, they can still be effectively controlled with 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 91% of all soybeans planted in the U.S. in 2009 were glyphosate-tolerant soybeans (USDA ERS 2009c). If DAS-68416-4 soybeans 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.

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

Based on substrate specificity of the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme activity, no effect on non-target organisms or endangered species is anticipated for DAS-68416-4 soybean. The *aad-12* gene and expressed protein are present in nature in the soil bacterium *Delftia acidovorans.* AAD-12 is not a potential food allergen or toxin (Section VI-A.4) and DAS-68416-4 soybean has been shown to be substantially equivalent to conventional soybean based on the compositional analysis of grain and forage (Section VIII). Observations made during field testing of DAS-68416-4 soybean revealed no effects on invertebrate populations (Section VII-B) and agronomic characteristics equivalent to conventional soybean (Section VII-A).

The Endangered Species Act (ESA) (16 USC 1531) is administered by the U.S. Fish & Wildlife Service (FWS). Under ESA, Section 6 requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact. It is not anticipated that DAS-68416-4 soybean will impact any currently listed species of concern (U.S. FWS, 2009) since it is not anticipated that DAS-68416-4 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-68416-4 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 *aad-12* gene conferring tolerance to 2,4-D. It is therefore reasonable to conclude that DAS-68416-4 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 proposed extension of the existing use pattern of 2,4-D in soybeans will be addressed by the EPA as part of their 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 proposed new post-emergent and seasonal use patterns in DAS-68416-4 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 that also provides 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-68416-4 soybean.

IX-F. Herbicide Resistance Management

IX-F.1. Herbicide Resistance

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

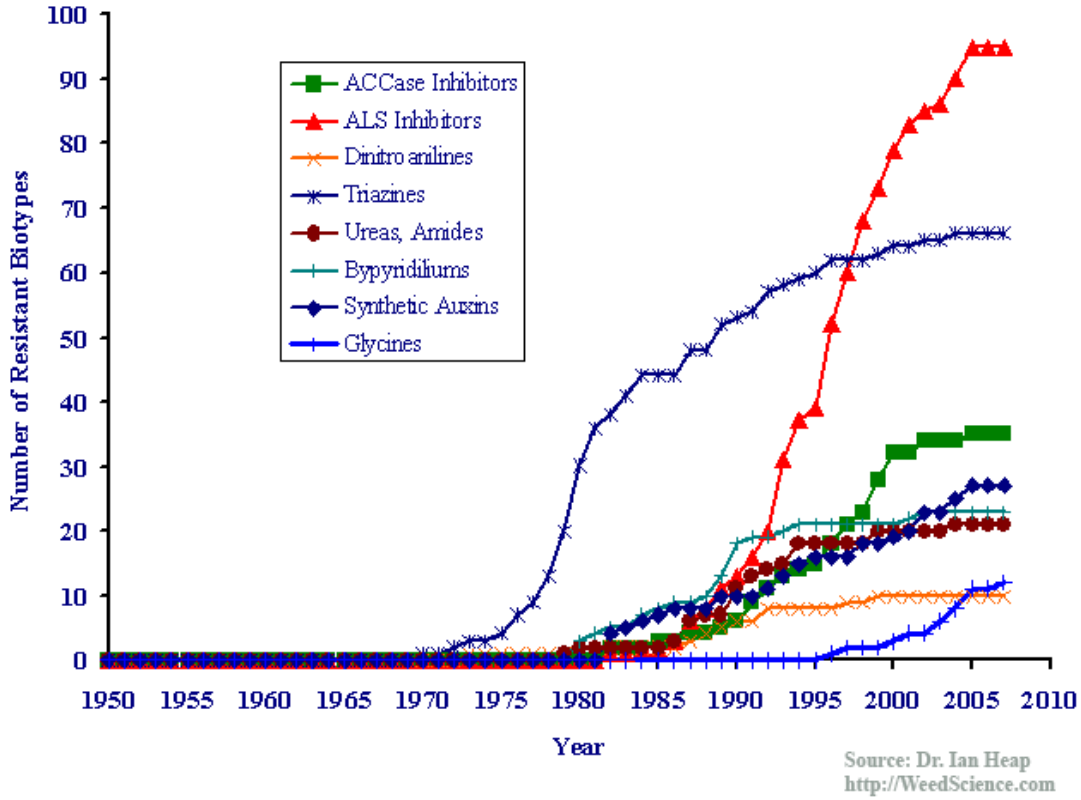
The Weed Science Society of America defines herbicide resistance as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type" (WSSA, 1998). Target plants with genes conferring resistance to a given herbicide can occur naturally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

Herbicide-resistant weeds have been a problem for growers for decades (Heap, 1997). The earliest documented reports of herbicide-resistant weeds were resistance to 2,4-D in wild carrot (*Daucus carota*) in 1952 and spreading dayflower (*Commelina diffusa*) in 1957 (Heap, 2009). During the 1970s, up to 30 different weed species were reported to be resistant to the triazine herbicides (Bandeen *et al*., 1982). Today, more than 330 weed biotypes around the world have been reported to have some populations that are resistant to one or more herbicides (Heap, 2009). A weed biotype is a sub-type or sub-population of a weed species, in this case one that has developed resistance to one or more herbicides. Report of a resistant biotype for a given weed species does not mean that weed resistance is common, widespread, or persistent in that species. There are generally many other options available to control these resistant biotypes. Table 32 shows a tabular summary of the total number of resistant species for each herbicide mode of action as of November 2009. Figure 49 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 7.

Table 32. Herbicide-resistant weeds.
(Heap, 2009)

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

Figure 49. Resistant weed biotypes per herbicide mode of action.



IX-F.2. Factors Impacting Development of Resistance

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

A matrix that can be used to evaluate the risk of selection for herbicide-resistant weeds based on cropping system practices in shown in Table 33. 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 33. Assessment of resistance risk by evaluation of cropping systems.
(Nevill *et al*., 1998)

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

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

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

IX-F.3. Herbicide Resistance Management

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

As the number of glyphosate-resistant weed species increases, it becomes increasingly important for growers to introduce greater diversity into their weed management programs (Powles, 2008a). This diversity could be achieved with herbicide rotations/sequences, mixtures of robust herbicides with different modes of action, and use of non-herbicide weed control tools. Glyphosate is increasingly being mixed with effective doses of other herbicides to manage these hard-to-control and resistant weed species. New herbicide-tolerant traits 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 34 shows that several common weeds in U.S. corn and soybeans which are resistant to or difficult to control with glyphosate or ALS herbicides and can be effectively controlled with 2,4-D.

Introduction of DAS-68416-4 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. DAS-68416-4 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-68416-4 soybeans can be extended to other herbicide-tolerant cropping systems, such as those with tolerance to glufosinate or ALS-inhibiting herbicides. Furthermore, DAS-68416-4 soybeans will allow use of glufosinate herbicides to significantly delay the selection for glyphosate-resistance in grass and broadleaf weed species.

Table 34. Glyphosate- and ALS-resistant weeds controlled by 2,4-D.
(Heap, 2009)

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

DAS-68416-4 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.

IX-G. Summary of Environmental Assessment

The AAD-12 protein is an enzyme with alpha-ketoglutarate-dependent dioxygenase activity which results in metabolic inactivation of several herbicides of the aryloxyalkanoate family. Field testing results confirm that AAD-12 protein expressed in DAS-68416-4 soybean provides robust tolerance to 2,4-D. There are no new phenotypic characteristics in DAS-68416-4 soybean to indicate it is any different from conventional soybean in weediness potential, and like conventional soybean, the risk of gene flow from DAS-68416-4 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-68416-4 soybean. The availability of DAS-68416-4 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-68416-4 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.

IX-H. References

Ahrent, D.K., Caviness, C.E. (1994) Natural cross-pollination of twelve soybean cultivars in Arkansas. Crop Science 34:376-378.

Bandeen, J.D., Stephenson, G.R., Cowett, E.R. (1982) Discovery and distribution of herbicide-resistant weeds in North America. In Lebaron H.M. and Gressel J., eds., Herbicide Resistance in Plants. John Wiley & Sons, Inc., New York, pp 9-30.

Beard, B.H., Knowles, P.F. (1971) Frequency of cross-pollination of soybeans after seed irradiation. Crop Science 11:489-492.

CAST (2009) Sustainability of U.S. Soybean Production: Conventional, Transgenic, and Organic Production Systems. Council for Agricultural Science and Technology - Special Publication No. 30, Ames, Iowa. June, 2009

Caviness, C.E. (1966) Estimates of natural cross-pollination in Jackson soybeans in Arkansas, Crop Science 6:211-212.

Connor, A.J., Glare, T.R., Nap, J.P. (2003) The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. The Plant Journal 33:19-46.

Dalley, C.D., Renner, K.A, Kells, J.J. (2002) Weed Competition in Roundup Ready Soybeans and Corn. Michigan State University, Integrated Pest Management Fact Sheet. (www.ipm.msu.edu/CAT05_fld/pdf/5-26FactSheet.pdf)

Duke, S.O. and Powles, S.B. (2008) Editorial: Glyphosate-Resistant Weeds and Crops. Pest Management Science 64:317-318.

Dyer, W.E. (1994) Resistance to glyphosate, in Herbicide Resistance in Plants: Biology and Biochemistry, ed. by S.B. Powles and J.A.M. Holtum. CRC Lewis Publishers, New York, NY, pp. 229-241.

Givens, W.A., Shaw, D.R., Kruger, G.R., Johnson, W.G., Weller, S.C., Young, B.G., Wilson, R.G., Owen, M.D.K., Jordan, D. (2009) Survey of Tillage Trends Following the Adoption of Glyphosate-Resistant Crops. Weed Technology 23:150-155.

Hartzler, B. and Pringnitz, B. (2000) Early Season Weed Competition - Integrated Crop Management IC-484 (8), Iowa State University. (http://www.ipm.iastate.edu/ipm/icm/2000/5-8-2000/earlyweed.html)

Hayes, R.M. (2000) Herbicide Resistant Crops and Weed Species Shifts. Proceedings: Third Annual National Conservation Tillage Cotton and Rice Conference. (http://www.btinternet.com/~nlpWESSEX/Documents/cottonrrproblems.htm)

Heap, I.M. (1997) The Occurrence of Herbicide-Resistant Weeds Worldwide. Pesticide Science 51:235-243.

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

HRAC (1998) Herbicide Resistance Action Committee. Guidelines to the management of herbicide resistance. (http://www.hracglobal.com/Publications/ManagementofHerbicideResistance/tabid/225/Default.aspx#guidelines)

Jaehnig, K.C. (2005) Farmers facing rise of herbicide resistant weeds. Southern Illinois University Carbondale Country Column. (http://news.siu.edu/news/April04/042704kj4032.jsp)

Knezevic, S. Z. (2002) Use of Herbicide Tolerant Crops as a Component of an Integrated Weed Management Program, University of Nebraska NebGuide, p.1484. (http://elkhorn.unl.edu/epublic/live/g1484/build/#concerns)

Krausz, R.F., B.G. Young, G. Kapusta, and J.L. Matthews. (2001) Influence of Weed Competition and Herbicides on Glyphosate-Resistant Soybean (*Glycine max*). Weed Technology 15:530-534.

Lauer, J. (2007) Continuous Corn or Rotate in 2008? The Principle of Crop Rotation. University of Wisconsin Agronomy Department, Agronomy Advice, Field Crops 28.426-51. (http://corn.agronomy.wisc.edu/AA/A051.aspx)

Loux, M., Stachler, J., Johnson, B, Nice, G. (2006) The Benefits of Preemergence Herbicides in Roundup Ready Soybean. Fact Sheet from The Ohio State University and Purdue University. (http://agcrops.osu.edu/weeds/documents/prefactfinal.pdf)

Mallory-Smith, C. (2008) Pacific Northwest Weed Management Handbook – Managing Herbicide-resistant Weeds. (http://weeds.ippc.orst.edu/pnw/weeds?06W_HRES01.dat)

Moss, S. (2002) Chapter 11 – Herbicide-Resistant Weeds. *Weed Management Handbook*, Ninth Edition, Edited by Robert Naylor, Blackwell Publishing, British Crop Protection Council

Müller, R.H., Jorks, S., Kleinsteuber, S., Babel, W. (1999) *Comamonas acidovorans* strain MC1: a new isolate capable of degrading the chiral herbicides dichlorprop and mecoprop and the herbicides 2,4-D and MCPA. Microbiological Research 154:241-246.

Nevill, D., Cornes, D. and Howar, S. (1998) The Role of HRAC in the Management of Weed Resistance. (http://www.hracglobal.com/Publications/HRACManagementandWeedResistance/tabid/228/Default.aspx)

OECD (2000) Consensus Document on the Biology of *Glycine max* (L.) Merr. (Soybean). Organisation for Economic Co-operation and Development, Paris, France. Series on Harmonization of Regulatory Oversight in Biotechnology No. 15. EVN/JM/MONO(2000)9. (http:/www.olis.oecd.org/olis/2000doc.nsf/LinkTo/env-jm-mono(2000)9)

Penn State Agronomy Guide. 2009-2010. Part 2, Section 1: Pest Management. (http://agguide.agronomy.psu.edu/pm/sec1/sec12f.cfm)

Powles, S.B. (2008a) Evolution in action: glyphosate-resistant weeds threaten world crops. Outlooks on Pest Management 19:256-259.

Powles, S.B. (2008b) Review: Evolved glyphosate-resistant weeds around the globe: lessons to be learnt. Pest Management Science 64:360-365.

Ray, J.D., Kilen, T.C., Abel, C.A., Paris, R.L. (2003) Soybean natural cross-pollination rates under field conditions. Environmental Biosafety Research 2:133-138.

Sankula, S., Blumenthal, E. (2004) Impacts on U.S. Agriculture of Biotechnology-Derived Crops Planted in 2003– An Update of Eleven Case Studies. National Center for Food and Agricultural Policy.
(http://www.ncfap.org/documents/2004finalreport.pdf.)

USDA ERS (2008) USDA Economic Research Service – Soybeans and Oil Crops Briefing Room
(http://www.ers.usda.gov/Briefing/SoybeansOilCrops/)

USDA ERS (2009a) USDA Economic Research Service Adoption of Genetically Engineered Crops in the U.S.: Extent of Adoption.
(http://www.ers.usda.gov/Data/BiotechCrops/adoption.htm)

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

USDA ERS (2009c) USDA Economic Research Service Data Sets – Adoption of Genetically Engineered Crops in the U.S. – Soybean Varieties.
(http://www.ers.usda.gov/Data/BiotechCrops/ExtentofAdoptionTable3.htm)

USDA ERS ARMS (2002) USDA Economic Research Service – Agricultural Resource Management Survey.
(http://www.ers.usda.gov/data/ARMS/app/Crop.aspx.)

USDA ERS ARMS (2006) USDA Economic Research Service – Agricultural Resource Management Survey.
(http://www.ers.usda.gov/data/ARMS/app/Crop.aspx.)

USDA NASS (2006) USDA National Agricultural Statistics Service – Agricultural Chemical Usage – 2005 Field Crops Summary.
(http://usda.mannlib.cornell.edu/usda/nass/AgriChemUsFC/2000s/2006/AgriChemUsFC-05-17-2006.pdf)

USDA NASS (2008) USDA National Agricultural Statistics Service – Quick Stats.
(http://www.nass.usda.gov/Data_and_Statistics/Quick_Stats/)

WSSA (1998) Resistance and Tolerance Definitions. Weed Science Society of America. (http://www.wssa.net/Weeds/Resistance/definitions.htm)

Westendorf, A.; Benndorf, D.; Müller, R.; Babel, W. (2002) The two enantiospecific dichlorprop/α-ketoglutarate-dioxygenases from *Delftia acidovorans* MC1-protein and sequence data of *RdpA* and *SdpA* Microbiological Research 157:317-22.

Westendorf, A., Müller, R.H., Babel, W. (2003) Purification and characterization of the enantiospecific dioxygenases from *Delftia acidovorans* MC1 initiating the degradation of phenoxypropionates and phenoxyacetate herbicides. Acta Biotechnologica 23: 3-17.

Wright, T.R.; Lira, J.M.; Walsh, T.; Merlo, D.J., Jayakumar, P., Lin, G. (2007) Novel Herbicide Resistance Genes. World Intellectual Property Organization International Publication Number WO 2007/053482 A2

York, A.C., Beam, J.B., Culpepper, A.S. (2005) Control of Glyphosate-resistant Soybean in Cotton. Journal of Cotton Science 9:102-109.

X. Adverse Consequences of Introduction

Field and laboratory testing of DAS-68416-4 soybean has demonstrated that it has no significant differences from non-transgenic conventional soybean apart from the intended change of herbicide-tolerance. DAS knows of no study results or other observations associated with DAS-68416-4 soybean that would be anticipated to result in adverse consequences from introduction. Soybean varieties derived from DAS-68416-4 will be the first soybean varieties to express tolerance to 2,4-D. As such, they will be an important tool to growers in areas where weeds have become resistant to other broad spectrum herbicides, such as glyphosate.

XI. Appendices

Appendix 1. Methods for Molecular Characterization of DAS-68416-4 Soybean

1.1. DAS-68416-4 Soybean Material

Transgenic soybean seeds from four distinct generations of soybean containing event DAS-68416-4 were planted in the greenhouse. After at least two weeks of growth, leaf punches were taken from each plant and were tested for AAD-12 protein expression using a rapid lateral flow test strip according to the manufacturer's instructions. Each plant was given a "+" or "-" for the presence or absence of the AAD-12 protein.

1.2. Control Soybean Material

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

1.3. Reference Materials

DNA of the plasmid pDAB4468 was added to samples of the Maverick control genomic DNA at a ratio approximately equivalent to 1 copy of the transgene per soybean genome and used as the positive control to verify probe hybridization and sizes of internal fragments.

1.4. DNA Probe Preparation

DNA probes specific to the genetic elements in the T-DNA insert of pDAB4468 and the vector backbone were produced via polymerase chain reaction (PCR) amplification using pDAB4468 plasmid DNA as a template, followed by purification.

1.5. Sample Collection and DNA Extraction

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

Method 2 is based on the modified CTAB method. Briefly, leaf samples were individually ground in liquid nitrogen followed by the addition of extraction buffer (~5:1 ratio milliliter CTAB extraction buffer: gram leaf tissue) and RNase-A (>10 µL) (Qiagen, Valencia, catalog # 1007885). After approximately 2 hours of incubation at ~65 °C with gentle shaking, samples were spun down and the supernatants were extracted with equal volume of chloroform:octanol = 24:1 (chloroform, Sigma, Catalog # 366922-4L; octanol, Sigma, catalog # O4504-100mL). DNA was precipitated by mixing the supernatants with equal volume of precipitation buffer (1% CTAB, Sigma, Catalog # H6269-2506; 50 mM Tris-HCl, Invitrogen, Catalog # 15568-025; 10

mM EDTA, AcruGene, Catalog # 51234). The precipitated DNA was dissolved in high salt TE buffer (1X TE pH8.0, thermo, Catalog # 17890; 1.0M NaCl, AccuGene, Catalog # 51202) followed by precipitation with isopropyl alcohol (Mallinckrot, Catalog # 3031-08). The precipitated DNA was rinsed with 70% ethanol, air-dried, then dissolved in appropriate volume of 1 X TE buffer (pH8.0).

To check the quality of the resultant genomic DNA, an aliquot of the DNA samples was electrophoretically separated on a 1% agarose gel containing ethidium bromide (~1 µg/mL) with 1X TBE buffer (89 mM Tris-Borate, 20 mM EDTA, pH 8.3). The gel was visualized under ultraviolet (UV) light to confirm that the DNA was not degraded and that the RNA had been removed by the RNase-A. The concentration of DNA in solution was determined by a picogreen kit (Invitrogen, Carlsbad, CA, catalog # P7589) in a fluorometer (Bio-TEK, FLX800).

1.6. DNA Digestion and Electrophoretic Separation of the DNA Fragments

Genomic DNA extracted from the soybean leaf tissue was digested with restriction enzymes by combining approximately 9 µg of genomic DNA with approximately 5-11 units of the selected restriction enzyme per µg of DNA in the corresponding reaction buffer. Each sample was incubated at 37°C overnight. The positive control sample was prepared by combining pDAB4468 plasmid DNA with genomic DNA from the Maverick control (at a ratio approximately equivalent to 1 copy of the transgene per soybean genome) and was digested using the same procedures and restriction enzymes as the transgenic DNA samples. DNA from the Maverick control was digested using the same procedures and restriction enzymes as the test samples to serve as the negative control.

The digested DNA samples were precipitated with Quick-Precip (Edge BioSystems) and re-suspended to achieve the desired volume for gel loading. The DNA samples and molecular size markers were then electrophoresed through 0.8% agarose gels with 1× TBE buffer (89mM Tris, 89mM Boric acid, 2mM EDTA) at 55-65 V for 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide and the DNA was visualized under UV light. A photographic record was made of each stained gel.

1.7. Southern Transfer

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

1.8. Probe Synthesis and Hybridization

The hybridization probes were generated using a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, from DNA fragments generated by primers specific to the gene elements and other regions from plasmid pDAB4468. The PCR synthesis of the probes was performed using PCR DIG Probe Synthesis Kit (Roche Diagnostics) and following the manufacturer's recommended procedures.

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

1.9. Detection

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

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

1.10. References

Guillemant, P. (1992) Isolation of Plant DNA: A Fast, Inexpensive, and Reliable Method. Plant Molecular Biology Reporter 10(1): 60-65.

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

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

2.1. DAS-68416-4 Transgenic Soybean Material

Greenhouse-grown DAS-68416-4 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 a rapid lateral flow test strip according to the manufacturer's instructions. Leaves (and some stems) from AAD-12 expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

2.2. Control Soybean Material

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

2.3. Reference Material

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

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

The AAD-12 protein was extracted from lyophilized leaf tissue in a PBST (Phosphate Buffered Saline with 0.05% Tween 20, pH 7.4) based buffer with added stabilizers, and the soluble proteins were collected by centrifugation. The supernatant was filtered and the soluble proteins were allowed to bind to Phenyl Sepharose (PS) beads (GE Healthcare). After an hour of incubation, the PS beads were washed with PBST and the bound proteins were eluted with Milli-Q water. Sodium chloride was added to increase the conductivity and the PS purified proteins were loaded onto an anti-AAD-12 immunoaffinity column which had been conjugated with an AAD-12 specific polyclonal antibody. The non-bound proteins were collected from the column and the column was washed extensively with pre-chilled PBS (phosphate buffered saline, pH 7.4). The bound proteins were eluted from the column with a 3.5 M NaSCN, 50 mM Tris, pH 8.0 buffer and examined by SDS-PAGE and western blotting.

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

Lyophilized leaf tissue from event DAS-68416-4 and Maverick was mixed with PBST buffer containing ~2.0% protease inhibitor cocktail (Sigma) and the protein was extracted by grinding with ball bearings in a Geno-Grinder. The samples were centrifuged and the supernatants were mixed with Laemmli sample buffer, heated, and briefly centrifuged. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived AAD-12, was also mixed with sample buffer and loaded on to the gel. Electrophoresis was conducted with Tris/glycine/SDS buffer (Bio-Rad). Following electrophoresis, the gel was cut in half, with one half stained with Pierce GelCode Blue protein stain and the other gel half

was electro-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was then probed with an AAD-12 specific polyclonal rabbit antibody. A chemiluminescent substrate was used to visualize the immunoreactive bands.

2.6. Detection of Post-Translational Glycosylation

The immunoaffinity-purified, plant-derived AAD-12 protein was analyzed for evidence of glycosylation by electrophoresis with microbe-derived AAD-12 protein, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase as controls. The control protein samples were adjusted to concentrations approximately equal with the plant-derived AAD-12 protein and mixed with Laemmli buffer. The proteins were heated, centrifuged, and applied directly to a Bio-Rad Criterion SDS-PAGE gel. Following electrophoresis, the gel was cut in half. One gel half was stained with Pierce GelCode Blue stain for total protein. The remaining half of the gel was stained with GelCode Glycoprotein Stain to visualize the glycoproteins. The glycoproteins present on the gel were visualized as magenta bands on a light pink background.

2.7. Mass Spectrometry Peptide Mass Fingerprinting and Sequence Analysis of Plant- and Microbe-Derived AAD-12 Protein

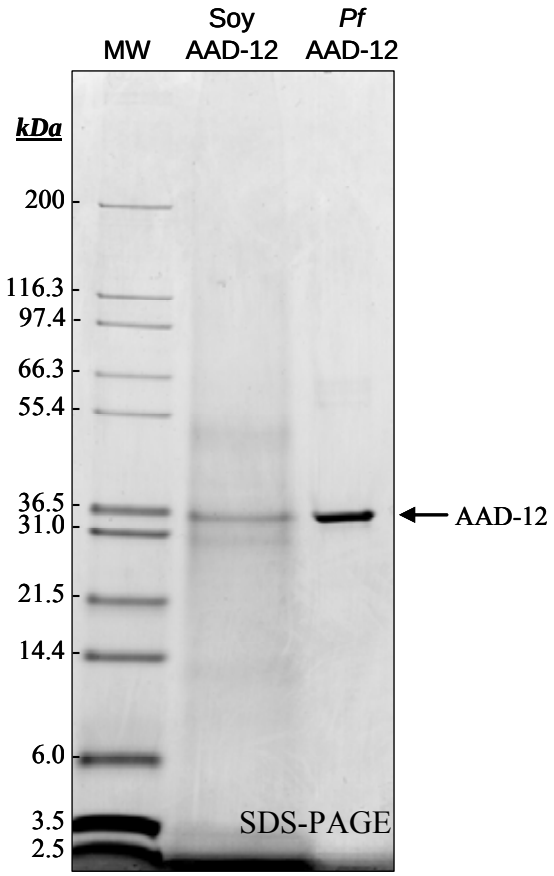
The immunoaffinity purified AAD-12 plant-derived protein was subjected to in-solution digestion by trypsin and Asp-N followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and electrospray-ionization liquid chromatography/mass spectrometry (ESI-LC/MS).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

2.8. 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 50). As expected, the corresponding plant-derived AAD-12 protein was identical in size to the microbe-derived protein. Predictably, the plant purified fractions contained a minor amount of non-immunoreactive impurities in addition to the AAD-12 protein. The co-purified proteins were likely retained on the column by weak interactions with the column matrix (Holroyde *et al*., 1976, Kennedy and Barnes, 1983 and Williams *et. al.*, 2006).

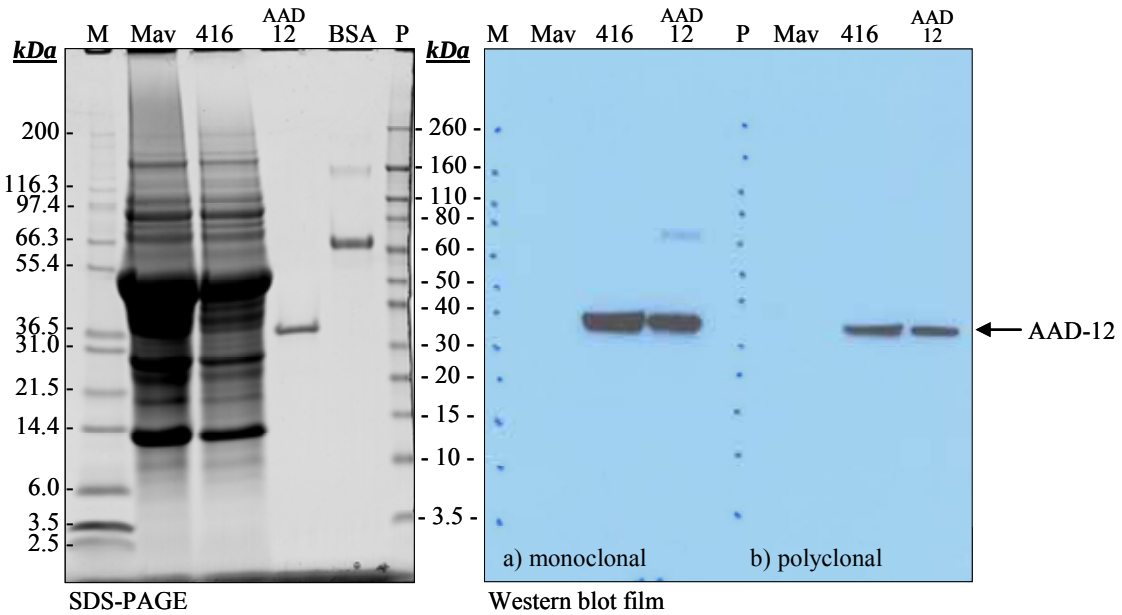
The microbe-derived AAD-12 and DAS-68416-4 plant tissue extract showed a positive signal of the expected size on the western blot using the anti-AAD-12 polyclonal antibody (Figure 51). In the AAD-12 western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the samples from the transgenic plant. The monoclonal antibody did detect a small amount of the AAD-12 dimer in the microbe-derived protein. These results add to the evidence that the protein expressed in soybean is not glycosylated which would add to the overall protein molecular weight.

Figure 50. SDS-PAGE of soybean- and microbe-derived AAD-12.



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

Figure 51. SDS-PAGE and western blot of soybean- and microbe-derived AAD-12 protein extracts.



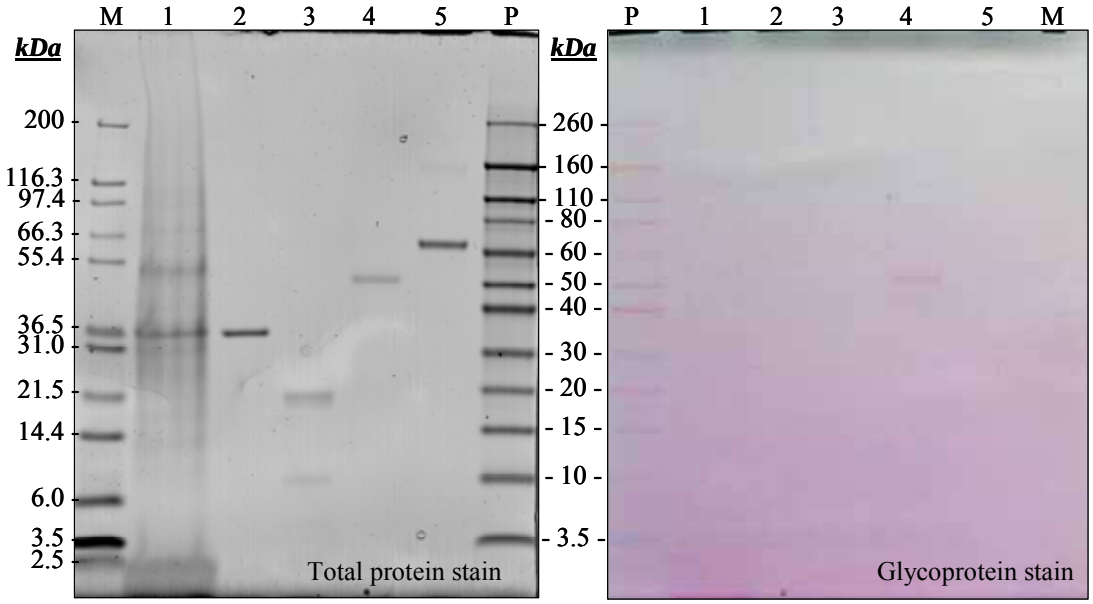
Lane	*Sample*	*Amount*
M	Invitrogen Mark12 MW markers	10 µL
Mav	Nontransgenic Soybean Extract	32 µL
416	Event DAS-68416-4 extract	32 µL
AAD-12	Microbe-Derived AAD-12	~785 ng
BSA	Bovine Serum Albumin (BSA)	~785 ng
P	Novex Prestained MW Markers	10 µL

2.9. Results of Detection of Glycosylation of AAD-12 Protein

No covalently-linked carbohydrates were detectable on the plant- or microbe-derived AAD-12 proteins (Figure 52). Horseradish peroxidase, a glycoprotein, was used as a positive indicator for glycosylation. Soybean trypsin inhibitor and bovine serum albumin, both non-glycoproteins, served as negative controls.

Figure 52. Glycosylation analysis of soybean- and microbe-derived AAD-12 proteins.

Note: The immunoaffinity-purified, soybean-derived AAD-12 protein, microbe-derived AAD-12, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted to a similar concentration prior to loading on the gel. After electrophoresis, the gel was cut in half and one half was stained with GelCode Blue stain for total protein, the other half of the gel was stained with a GelCode Glycoprotein Staining Kit to visualize the glycoproteins.



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

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

Following digestion of the plant-derived AAD-12 protein by trypsin and Asp-N, the masses of the detected peptides were compared with those deduced based on potential cleavage sites in the sequence of the AAD-12 protein. Figure 53 illustrates the theoretical peptide cleavage which was generated *in silico* using PAWs software (Proteometrics LLC).

The trypsin and Asp-N digestion of soybean-derived AAD-12 protein yielded high detection of the expected peptides, resulting in 73.4% coverage of the AAD-12 protein sequence (Figure 54). The analysis confirmed the plant-derived protein amino acid sequence matched that of the microbe-derived AAD-12 protein and that of the predicted amino acid sequence. Results of these analyses indicated that the amino acid sequence of the soybean-derived AAD-12 protein was equivalent to the *P. fluorescens*-expressed protein.

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

The N-terminal sequence of the first 27 residues of the plant-derived and all 292 residues of the microbe-derived AAD-12 protein was obtained by mass spectrometry. The amino acid sequences for N-terminus of both proteins was A^{2} H A A L S P L S Q I T P T G A T L G A T V T G V H L A T L^{27}, indicating the N-terminal methionine had been removed (Table 35 and Figure 54). These results suggest that during or after translation in the plant and *P. fluorescens*, the N-terminal methionine is cleaved by a methionine aminopeptidase. In addition to the methionine being removed, the N-terminal peptide of the AAD-12 protein was shown to be acetylated after the N-terminal methionine was cleaved. These two co-translational processes, cleavage of N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority (~85%) of eukaryotic proteins (Polevoda and Sherman, 2000; Polevoda and Sherman, 2002).

The C-terminal sequences of the plant- and microbe-derived AAD-12 proteins were determined to be identical to the expected sequences (Table 36 and Figure 54).

Figure 53. Theoretical trypsin (top panel) and Asp-N (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 trypsin digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

Digestion at K (lysine) and R (arginine)

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

Digestion at D (aspartate)

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

Figure 54. Sequence coverage in the tryptic and Asp-N peptide mapping analysis of plant-derived AAD-12 protein with MALDI-TOF and ESI/LC MS.

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

N-Ac

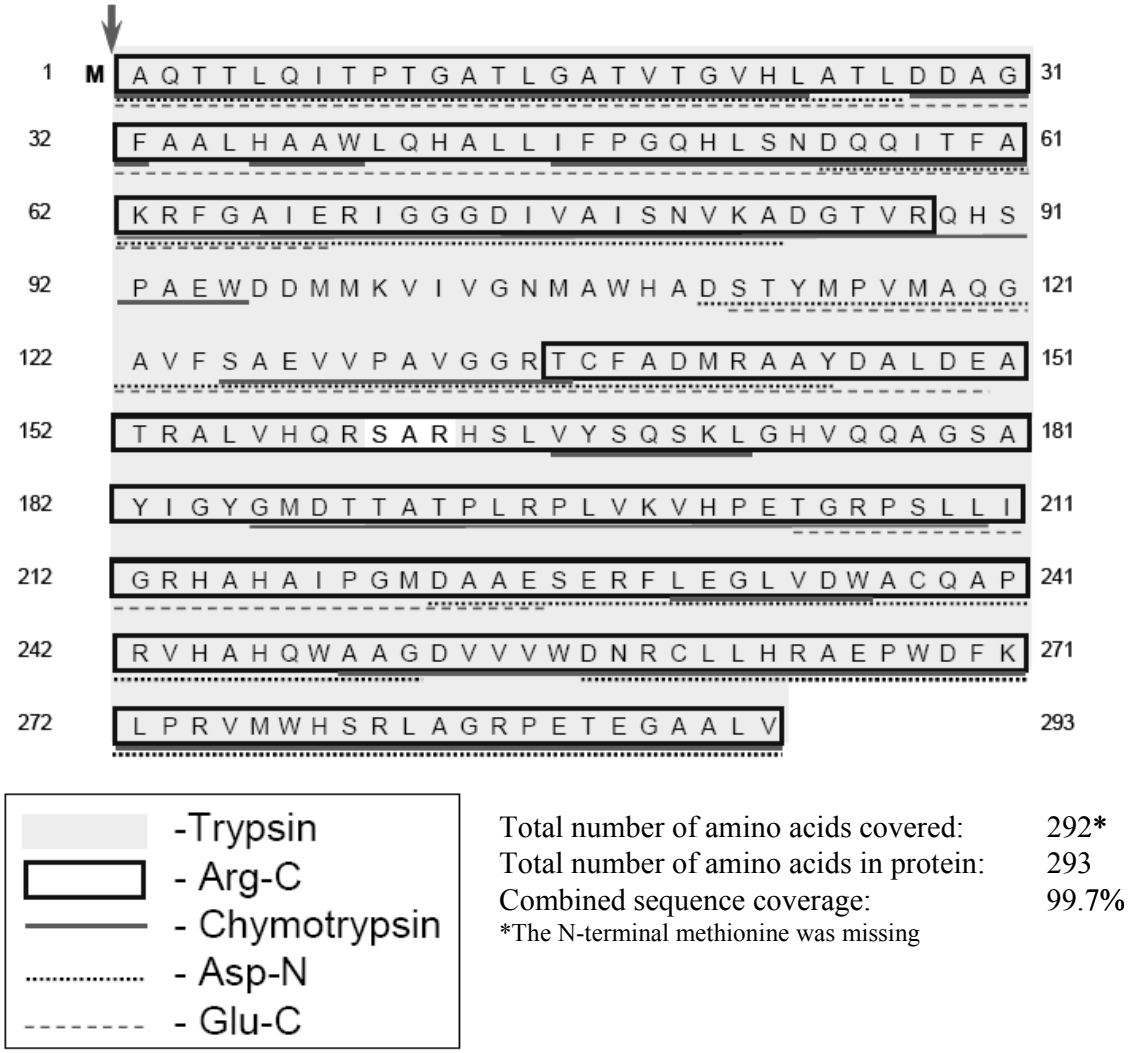
```
001 M A Q T T L Q I T P T G A T L G A T V T G V H L A T L D D A 30
031 G F A A L H A A W L Q H A L L I F P G Q H L S N D Q Q I T F 60
061 A K R F G A I E R I G G G D I V A I S N V K A D G T V R Q H 90
091 S P A E W D D M M K V I V G N M A W H A D S T Y M P V M A Q 120
121 G A V F S A E V V P A V 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
```

- Trypsin
____ - Asp N

Total number of amino acids covered: 215*
Total number of amino acids in protein: 293
Combined sequence coverage: 73.4%
*The N-terminal methionine was missing

Figure 55. Sequence coverage in the peptide mapping analysis of microbe-derived AAD-12 protein with MALDI-TOF and ESI/LC MS.

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



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

*The N-terminal methionine was missing

Table 35. Summary of N-terminal sequence data of AAD-12 soybean- and microbe-derived proteins.

Source	Expected N-terminal Sequence[1]
P. fluorescens	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}
Soybean Event DAS-68416-4	M^{1} A Q T T L Q I T P T G A T L G A T V T G V H L A T L D^{27}

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

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

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

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

Notes:

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

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

Table 36. Summary of C-terminal sequence data of AAD-12 soybean- and microbe-derived proteins.

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

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

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

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

Notes:

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

Amino acid residue abbreviations:

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

2.12. Conclusions

The biochemical identity of microbe-derived AAD-12 protein was equivalent to the protein purified from leaf tissue of event DAS-68416-4. 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 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-68416-4 soybean are biochemically equivalent.

2.13. References

Holroyde, M. J., Chesher, J. M. E., Trayer, I. P., and Walker, D. G. (1976) Studies on the Use of Sepharose –*N*-(6-Aminohexanoyl)-2-Amino-2-Deoxy-D-Glucopyranose for the Large-Scale Purification of Hepatic Glucokinase. Journal of Biochemistry 153:351-361.

Kennedy J. F. and Barnes J. A. (1983) Immunochemical studies of the non-specific interactions of cyanogen bromide-activated macroporous agarose-based immunoadsorbents. Journal of Chromatography 281, 83-93.

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

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

Williams, A. J. K., Norcross, A. J., Chandler, K. A. and Bingley, P. J. (2006) Non-specific binding to protein A Sepharose and protein G Sepharose in insulin autoantibody assays may be reduced by pre-treatment with glycine or ethanolamine. Journal of Immunological Methods 314, 170–173.

Appendix 3. Methods and Results for Characterization of PAT Protein

3.1. DAS-68416-4 Transgenic Soybean Material

Greenhouse-grown DAS-68416-4 T4 plants were used as the plant source of the PAT protein. Prior to use, individual plants were leaf tested to confirm expression of the PAT protein using a rapid lateral flow test strip according to the manufacturer's instructions. Leaves (and some stems) from PAT expressing plants were harvested, lyophilized, ground to a fine powder, and stored frozen until needed.

3.2. Control Soybean Material

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

3.3. Reference Material

Recombinant PAT microbial protein was produced in *Pseudomonas fluorescens* (*Pf*) and purified to homogeneity (Snodderley, 2006). The microbe-derived PAT protein preparation was aliquoted and stored at -80 ºC to maintain activity.

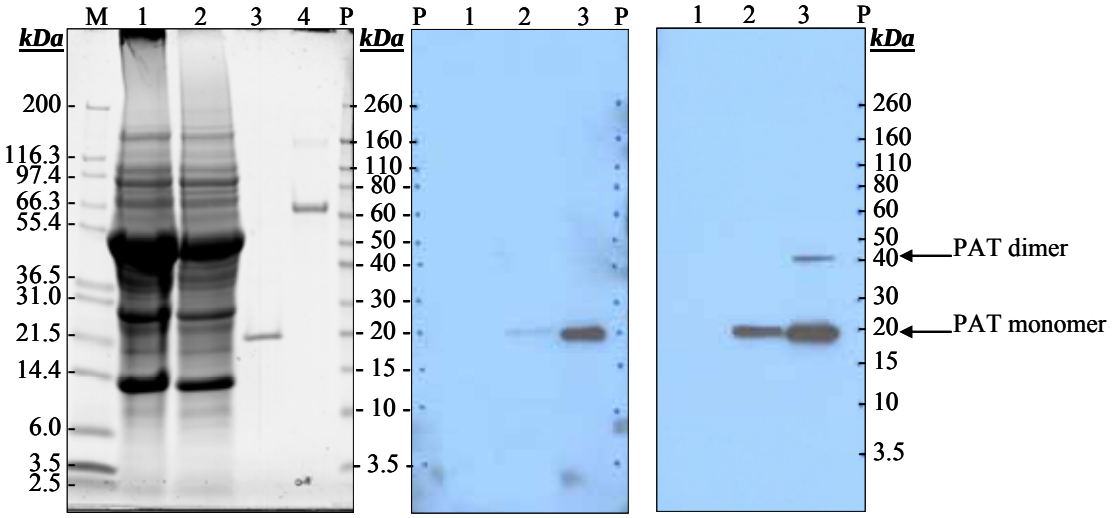
3.4. SDS-PAGE and Western Blot Analysis of Crude Extracts

Lyophilized leaf tissue from event DAS-68416-4 and Maverick was mixed with PBST buffer containing ~2.0% protease inhibitor cocktail (Sigma) and the protein was extracted by grinding with ball bearings in a Geno-Grinder. The samples were centrifuged and the supernatants were mixed with Laemmli sample buffer, heated and briefly centrifuged. The samples were loaded directly on to a Bio-Rad Criterion SDS-PAGE gel. The positive reference standard, microbe-derived PAT, was also mixed with sample buffer and loaded on to the gel. Electrophoresis was conducted with Tris/glycine/SDS buffer (Bio-Rad). Following electrophoresis, the gel was cut in half, with one half stained with Pierce GelCode Blue protein stain and the other gel half was electro-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was then cut in half with one probed with a PAT specific polyclonal rabbit antibody and the remaining half probed with a PAT specific monoclonal antibody. A chemiluminescent substrate was used to visualize the immunoreactive bands.

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

The soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal antibodies and showed the expected band at approximately 21 kDa (Figure 56, Panel B and C). In the PAT western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the transgenic soybean extract. This result adds to the evidence that the protein expressed in soybean is not post-translationally modified which would have added to the overall protein molecular weight.

Figure 56. SDS-PAGE and western blots of DAS-68416-4 and non-transgenic Maverick soybean.



Panel A: SDS-PAGE **Panel B:** Polyclonal antibody western blot **Panel C:** Monoclonal antibody western blot

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

3.6. Conclusions

The PAT protein produced in DAS-68416-4 soybean was shown to be equivalent to that produced in other transgenic crops (USDA 1996, USDA 2001, USDA 2004, USDA 2006).

3.7. References

USDA (1996) Availability of Determination of Nonregulatated status for Soybeans Genetically Engineered for Glufosinate Herbicide Tolerance. Federal Register Volume 61, Number 160:42581-42582.

USDA (2001) Availability of Determination of Non-regulatated status for Genetically Engineered Corn for Insect Resistance and Glufosinate Herbicide Tolerance (Corn line 1507). Federal Register Volume 66, Number 157:42624-42625.

USDA (2004) Availability of Determination of Nonregulated Statusfor Cotton Lines Genetically Engineered for Insect Resistance. Federal Register Volume 69, Number 156:50154-50155.

USDA (2005) Availability of Determination of Non-regulated Status for Genetically Engineered Corn (Corn line DAS-59122-7). Federal Register Volume 70, Number 194:58663-58664.

Appendix 4. Methods for AAD-12 and PAT Protein Expression Analysis

4.1. Experimental Design

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

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

4.2. Sample Collection

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

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

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

<u>Root (R3)</u>

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

<u>Forage (R3)</u>

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

<u>Grain (R8 – Maturity)</u>

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

4.3. Determination of AAD-12 Protein Concentration

The AAD-12 protein was extracted from soybean tissues except grain with a phosphate buffered saline solution with Tween-20 (PBST) and 0.75% ovalbumin (OVA). For grain, the protein was extracted with a PBST buffer containing 0.1% Triton-100. The plant tissue and grain extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analyzed using an AAD-12 ELISA kit in a sandwich format. Briefly, an aliquot of the diluted sample and a horseradish peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate are incubated in the wells of a microtiter plate coated with an immobilized anti-AAD-12 polyclonal antibody. These antibodies bind with AAD-12 protein in the wells and form a "sandwich" with AAD-12 protein bound between soluble and the immobilized antibodies. The unbound samples and conjugate are then removed from the plate by washing with PBST. Subsequent addition of an enzyme substrate generated a 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 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 minus 650 nm was measured using a Molecular Devices Spectra Max 190 or Spectra Max M2 plate reader. A calibration curve was generated and the AAD-12 concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

4.4. Determination of PAT Protein in Soybean Tissue Samples

The PAT protein was extracted from soybean tissues with a phosphate buffered saline solution with Tween-20 (PBST) and 1% polyvinylpyrrolidone (PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted with PBST/1% PVP, and analyzed using a PAT ELISA kit. Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT antibody and anti-PAT antibodies coated in the wells of a 96-well plate in a sandwich ELISA format. At the end of the incubation period, the unbound reagents were removed from the plate by washing. Subsequent addition of an enzyme substrate generated a 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 was related to the concentration of PAT in the sample (i.e., lower residue concentrations result in lower color development). The absorbance at 450 minus 650 nm was measured using a Molecular Devices Spectra Max 190 or Spectra max M2 plate reader. A calibration curve was generated and the PAT concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ software which was compatible with the plate reader. Samples were analyzed in duplicate wells with the average concentration of the duplicate wells being reported.

Appendix 5. USDA Notifications for DAS-68416-4 Soybean

USDA Notification Number	Notification Authorization Date	Notification Expiration Date	State(s)	Total Number of Trials Planted[1]	Status of Trial[2]
09-259-105n	9/25/2009	9/25/2010	PR	TBD	Pending
09-086-101n	5/30/2009	5/30/2010	IL, IN, IA, MN, MO, NE, WI	10	Pending
09-084-110n	4/15/2009	4/15/2010	AL, AR, CA, GA, IL, IN, IA, MI, MN, MO, MS, NE, OH	45	Pending
09-075-105n	4/15/2009	4/15/2010	HI, IN, IA, PR	0	Pending
09-068-101n	4/13/2009	4/13/2010	AR, IL, IN, IA, MD, MI, MO, ND, NE, OH, PR, WI	77	Pending
09-061-104n	4/6/2009	4/6/2010	AR, IL, IN, IA, MN, MS, NY, OH, TN	29	Pending
09-005-108n	1/1/2009	1/1/2010	HI	2	Pending
08-323-102n	12/3/2008	12/3/2009	PR	0	Pending
08-254-110n	9/26/2008	9/26/2009	PR	10	Pending
08-170-103n	6/26/2008	6/26/2009	MO	1	Pending
08-137-103n	6/5/2008	6/5/2009	MD	1	Pending
08-121-103n	5/14/2008	5/14/2009	IA	1	Submitted
08-121-102n	5/15/2008	5/15/2009	IL, IN, MO, NE, OH	11	Submitted
08-071-107n	4/14/2008	4/14/2009	CA, IL, IN, IA, MN, MN, NE	22	Submitted
07-242-107n	9/30/2007	9/30/2008	PR	4	Submitted
06-292-105n	12/1/2006	12/1/2007	IN	2	Submitted

[1]Trials not yet planted as of October 15, 2009 are indicated as TBD (to be determined).
[2]Pending reports as of December 1, 2009 to be submitted within 6 months of the notification expiration date.

Appendix 6. Literature Ranges for Compositional Analysis

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

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

OECD (2001) Consensus Document on Compositional Considerations for New Varieties of Soybean: Key Food and Fee Nutrients and Anti-Nutrients.

Table 37. Summary of literature values for proximates in soybean forage.

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

NA – Literature Values Not Available
FW=Fresh Weight; DW=Dry Weight

Table 38. Summary of literature values for proximates in soybean grain.

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

NA – Literature Values Not Available
FW=Fresh Weight; DW=Dry Weight

Table 39. Summary of literature values for amino acids in soybean grain.

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

NA – Literature Values Not Available
DW=Dry Weight

Table 40. Summary of literature values for isoflavones in soybean grain.

Isoflavones (µg/g)	OECD	ILSI
Daidzein	202-2060	60-2453.5
Glycitein	109-1070	15.3-310.4
Genistein	315-2680	144.3-2837.2
Daidzin	NA	NA
Glycitin	NA	NA
Genistin	NA	NA

NA – Literature Values Not Available

Table 41. Summary of literature values for fatty acids in soybean grain.

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

NA – Literature Values Not Available
FA – Fatty Acids

Table 42. Summary of literature values for vitamins in soybean grain.

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

NA – Literature Values Not Available

Table 43. Summary of literature values for minerals in soybean grain.

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

NA – Literature Values Not Available

Table 44. Summary of literature values for anti-nutrients in soybean grain.

Anti-Nutrients	OECD	ILSI
Phytic Acid (% DW)	NA	0.63-1.960
Raffinose (% DW)	0.1-0.9	0.212-0.661
Stachyose (% DW)	1.4-4.1	1.21-3.50
Lectin (H.U./mg)*	NA	0.105-9.038
Trypsin Inhibitor (TIU/mg)**	100-184	19.59-118.68

NA – Literature Values Not Available
*H.U. - Hemagglutinating Unit
**TIU - Trypsin Inhibitor Unit
DW – dry weight

Table 45. Summary of literature values for tocopherols in soybean grain.

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

NA – Literature Values Not Available

Appendix 7. Glyphosate, 2,4-D, Glufosinate and Herbicide Resistant Weeds

7.1. Herbicide Tolerant Crops

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

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 available to farmers in 2005 are listed in Table 46 (Duke, 2005). Transgenes were only used to confer tolerance to bromoxynil, glufosinate, and glyphosate. The bromoxynil-tolerant crops are no longer sold. This leaves only glyphosate- and glufosinate-tolerant transgenic crops, and of those, glyphosate has had a strongest impact on weed management (Duke, 2005).

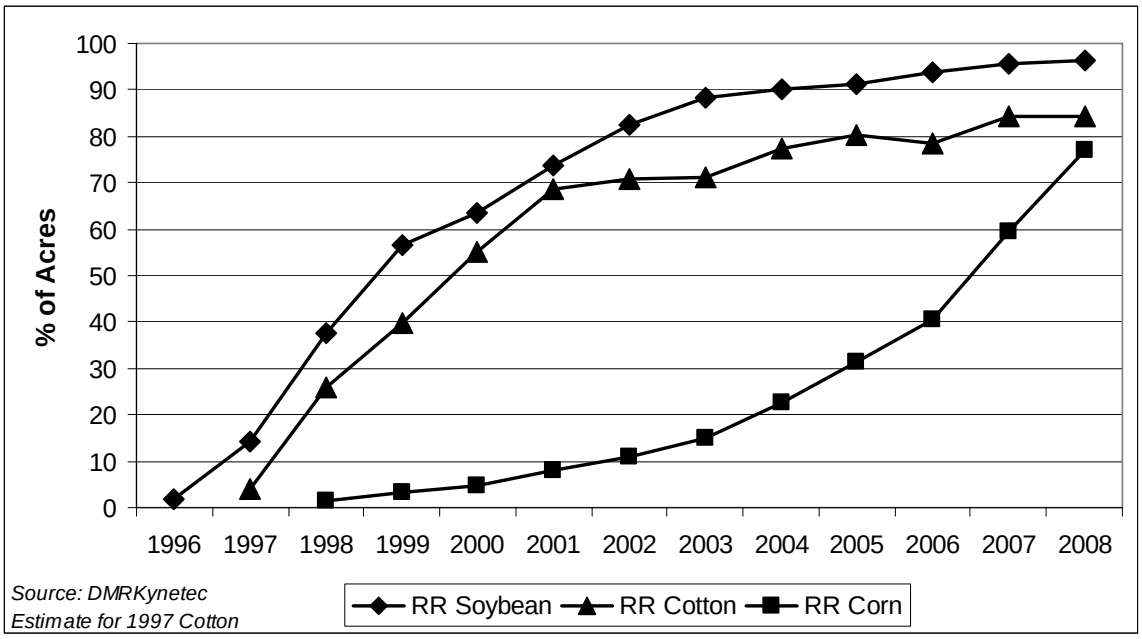
Table 46. Herbicide-tolerant crops available to farmers in North America in 2005.

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

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

Growers choose glyphosate-tolerant crops because it makes weed control easier and more effective, increases profit, requires less tillage, and does not restrict crop rotations (Green, 2009). Thus, glyphosate-tolerant corn, soybeans, and cotton have experienced an unprecedented rapid adoption rate by U.S. farmers (Figure 57. U.S. adoption rates of glyphosate-tolerant soybean, cotton and corn.). 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 US).

Figure 57. U.S. adoption rates of glyphosate-tolerant soybean, cotton and corn.



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

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

<u>Glyphosate</u>

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

it can be foliar applied over-the-top of recent glyphosate-tolerant transgenic crops, including corn, soybeans, cotton, canola, and sugarbeets.

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

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

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

<u>2,4-Dichlorophenoxyacetic Acid (2,4-D)</u>

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

controls many broadleaf weeds including carpetweed, dandelion, cocklebur, horseweed, morning glory, pigweed sp., lambsquarters, ragweed spp., shepherd's-purse and velvetleaf. It has little to no activity on grasses (Industry Task Force II, 2005).

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

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

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

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

<u>Glufosinate</u>

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 of 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.

7.3. 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 47 shows a summary of the sixteen glyphosate-resistant weed species that have been reported from 1996 to November 2009. These data clearly show that glyphosate-resistance in weeds is expanding around the globe. Most notably, there have been reports of nine new weed species with some biotypes resistant to glyphosate in the U.S. since 2000 (Figure 58). Two of these glyphosate-resistant weed species have already become a significant problem for farmers across a large geographic area. *Conyza canadensis* infests at least two million hectares of glyphosate-tolerant crops in the U.S. (Main *et al*., 2004) and glyphosate-resistance in Palmer amaranth has serious ramifications for future weed management in the Southeast U.S. due to its rapid growth rate, extremely competitive nature, and resistance to other herbicide modes of action (Culpepper *et al*., 2008). Researchers have also reported that individual biotypes of seven of the 16 glyphosate-resistant species are also resistant to herbicides with other modes of action (Table 48). The Palmer amaranth, common waterhemp, and *Conyza canadensis* biotypes were reported in the corn and soybean growing states. Although herbicide options to control these biotypes with multiple herbicide resistance will be more limited, 2,4-D is still a viable control option for the broadleaf weeds.

Table 47. Weed species with reported glyphosate-resistant biotypes.
(Heap, 2009)

Common Name	Species Name	First Confirmed Report		Later Confirmed Reports
		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	
Liverseedgrass	*Urochloa panicoides*	2008	Australia	

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

Figure 58. Number of glyphosate-resistant weeds reported globally by year from 1996 to 2008.
(Compiled from Heap, 2009)

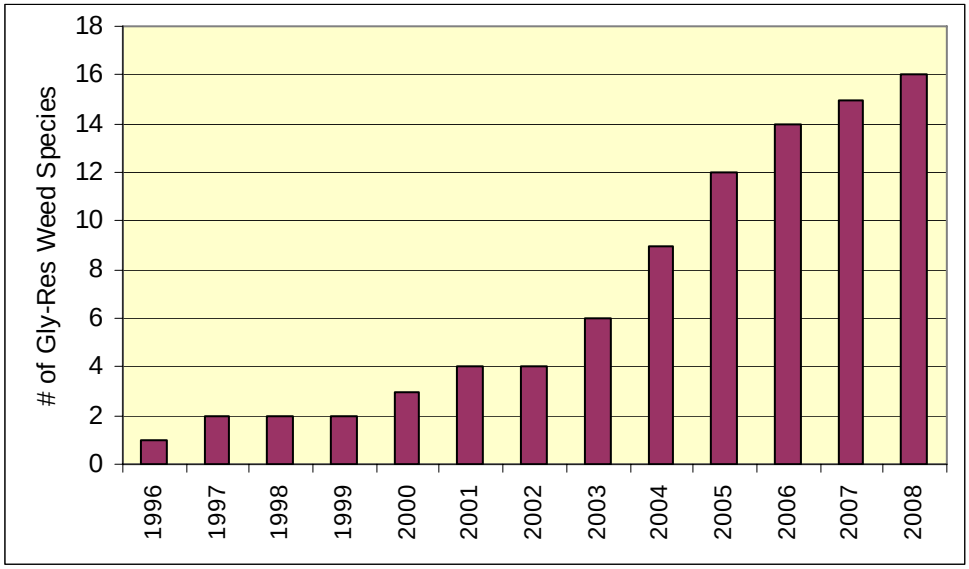


Table 48. Global reports of glyphosate-resistant weed biotypes with resistance to other herbicide modes of action.
(Heap, 2009)

Common Name	Species Name	Year – Country (State)	Multiple Resistance to Other Herbicide MOAs
Palmer amaranth	*Amaranthus palmeri*	2008 – US (MS)	ALS
Common waterhemp	*Amaranthus rudis*	2005 – US (MO)	ALS, PPO
		2006 – US (IL)	ALS
Horseweed	*Conyza Canadensis*	2003 – US (OH)	ALS
		2007 – US (MS)	Bipyridiliums
Goosegrass	*Eleusine indica*	1997 – Malaysia	ACCase
Wild poinsettia	*Euphorbia heterophylla*	2006 – Brazil	ALS
Italian ryegrass	*Lolium multiflorum*	2002 – Chile	ALS
Rigid ryegrass	*Lolium rigidum*	1999 - Australia	ACCase, ALS, Dinitroanilines
		2003 – S.Africa	ACCase, 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*., 2007). Weed scientists in Ohio and Indiana have also identified a biotype of common lambsquarters in at least a dozen fields that appears to have low-level glyphosate-resistance (Curran *et al*., 2007). The increased reports of glyphosate-resistant species, plus the geographic spread of their infestations, have caused some to raise concerns about the long term sustainability for glyphosate. Some researchers have stated that applying glyphosate alone over wide areas on highly variable and prolific weeds made the evolution of resistant weeds inevitable (Owen, 2001; Thill and Lemerle, 2001).

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 49.

Table 49. 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, 2009). Today, a total of sixteen weed species have documented reports of 2,4-D resistant biotypes someplace around the globe (Table 50). Wild carrot in soybeans and roadsides, field bindweed in cropland, and prickly lettuce in cereals are the only ones reported on the U.S. mainland (Heap, 2009). Wild carrot, yellow bur-head, wild radish, musk thistle, and corn poppy are the only 2,4-D resistant weeds that have reported infestations in more than 1,000 acres. Some of these 2,4-D resistant biotypes have documented cross resistance to other auxin herbicides or multiple resistance to some ALS-inhibiting herbicides. It is notable that most of these resistant species do not appear to be spreading, as indicated by few reports of additional sites after the initial report.

Table 50. Weed species with reported 2,4-D-resistant biotypes.
(Heap, 2009)

Common Name	Species Name	Herbicide(s)	Year	Country or State
Wild carrot	*Daucus carota*	2,4-D 2,4-D 2,4-D	1952 1993 1994	Ontario Michigan 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 2,4-D	1975 1975	France United Kingdom
Gooseweed	*Spenoclea zeylanica*	2,4-D 2,4-D 2,4-D	1983 1995 2000	Philippines Malaysia 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 2,4-D, bensulfuron-methyl	1995 1998	Indonesia 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 IX-F.1, Figure 49). 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-68416-4 soybeans should not result in 2,4-D resistant weeds becoming 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 are currently no reports of weed biotypes which have developed resistance to glufosinate (Heap, 2009). Thus, glufosinate is an excellent tool to include in a weed management program.

7.4. References

Bourdot, G.W., Saville, D.J., and Hurrell, G.A. (1996) Ecological fitness and the decline of resistance to the herbicide MCPA in a population of *Ranunculus acris*. Journal of Applied Ecology 33:151-160.

Bradshaw, L.D., Padgette, S.R., Kimball, S.L. and Wells, F.H. (1997) Perspectives on Glyphosate Resistance. Weed Technology 11:189-198.

Coble, H.D. and Warren, L.S. (1997) Weed control investigations in corn, cotton, crop rotations, soybean, small grain. *Annual Report*, Department of Crop Science, North Carolina State University 28:103-113.

Cranston, H.J., Kern, A.J., Hackett, J.L., Miller, E.K., Maxwell, B.D., and Dyer, W.E. (2001) Dicamba resistance in kochia. Weed Science 49:164-170.

Culpepper, A.S., Whitaker, J.R., MacRae, A.W. and York, A.C. (2008) Distribution of Glyphosate-Resistant Palmer Amaranth (*Amaranthus palmeri*) in Georgia and North Carolina during 2005 and 2006. Journal of Cotton Science 12:306-310.

Curran, B., Sprague, C., Stachler, J. and Loux, M. (2007) The Glyphosate, Weeds, and Crops Series: Biology and management of common lambsquarters (GWC-11). (http://www.glyphosateweedscrops.org/Info/GWC-11.pdf)

Duke, S.O. (2005) Taking stock of herbicide-resistant crops ten years after introduction. Pest Management Science 61:211-218.

Duke, S.O. and Powles, S.B. (2008a) Editorial: Glyphosate-resistant weeds and crops. Pest Management Science 64:317-318.

Duke, S.O. and Powles, S.B. (2008b). Mini-review – Glyphosate: a once-in-a-century herbicide. Pest Management Science 64:319-325.

FAO (1997) Pesticide Residues in Food – Glyphosate report. (http://www.fao.org/docrep/W8141E/w8141e0u.htm)

Foresman, C. and Glasgow, L. (2008) Grower perception and experiences with glyphosate-resistant weeds. Pest Management Science 64:388-391.

Franz J.E., Mao, M.K, Sikorski, J.A. (1997) Glyphosate: A Unique and Global Herbicide. ACS Monograph No. 189. American Chemical Society, Washington, DC, 653 pp.

Green, J.M. (2009) Evolution of glyphosate-resistant crop technology. Weed Science 57:108-117.

Gustafson, D.I. (2008) Sustainable use of glyphosate in North American cropping systems. Pest Management Science 64:409-416.

Heap, I.M. (1997) The occurrence of herbicide-resistant weeds worldwide. Pesticide Science 51:235-243.

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

Hite, G.A., King, S.R., Hagood, E.S. (2007) Response of selected Virginia common lambsquarters (*Chenopodium album*) biotypes to glyphosate. Weed Science Society of America Abstracts 47:22.

Industry Task Force II on 2,4-D Research Data: Issue Backgrounder – What is 2,4-D (2005) (http://www.24d.org/background/Backgrounder-What-is-24D-Dec-2005.pdf)

Jasieniuk, M., Brule-Babel, A.L. and Morrison, I.N. (1996) The evolution and genetics of herbicide resistance in weeds. Weed Science 44:176-193.

Kelley, K.B. and Riechers, D.E. (2007) Recent developments in auxin biology and new opportunities for auxinic herbicide research. Pesticide Biochemistry and Physiology 89:1-11.

Kern, A.J., Chaverra, M.E. and Dyer, W.E. (2005) Dicamba-responsive genes in herbicide-resistant and susceptible bioytpes of kochia (*Kochia scoparia*). Weed Science 53:139-145.

Main, C., Mueller, T.C., Hayes, R.M., and Wilkerson, J.B. (2004) Response of selected horseweed (*Conyza canadensis*) populations to glyphosate. Journal of Agricultural and Food Chemistry 52:879-883.

Mockaitis, K. and Estelle, M. (2008) Auxin receptors and plant development: A new signaling paradigm. Annual Review of Cell Developmental Biology 24:55-80.

Monsanto (2005). Backgrounder: History of Monsanto's Glyphosate Herbicides. (http://www.monsanto.com/monsanto/content/products/productivity/roundup/back_history.pdf)

OECD (2002) Module II - Herbicide Biochemistry, Herbicide Metabolism and the Residues in Glufosinate-Ammonium (Phosphinothricin)-Tolerant Transgenic Plants. (http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/NT00002CCE/$FILE/JT00125605.PDF)

Owen, M.D.K. (2001) World maize/soybean and herbicide resistance. Pages 101-163 *in* S. B. Powles and D. L. Shaner, eds. *Herbicide Resistance and World Grains*. New York: CRC.

Owen, M.D.K. (2008) Review: Weed species shifts in glyphosate-resistant crops. Pest Management Science 64:377-387.

Powles, S.B., Lorraine-Colwill, D.F. , Dellow, J.J. and Preston, C. (1998) Evolved resistance to glyphosate in rigid ryegrass (*Lolium rigidum*) in Australia. Weed Science 46:604-607.

Shaner, D.L. (2000) The impact of glyphosate-tolerant crops on the use of other herbicides and on resistance management. Pest Management Science 56:320-326.

Siehl, D.L. (1997) Inhibitors of EPSP synthase, glutamine synthase and histidine synthesis, in Herbicide Activity: Toxicology, Biochemistry and Molecular Biology, ed. by Roe R.M., Burton J.D. and Kuhr R.J. IOS Press, Amsterdam, The Netherlands, pp. 37-67.

Thill, D.C. and Lemerle, D. (2001) World wheat and herbicide resistance. Pages 61-70 *in* S. B. Powles and D. L. Shaner, eds. *Herbicide Resistance and World Grains*. New York: CRC.

Tu, M., Hurd, C. and Randall, J.M. (2001) Weed Control Methods Handbook, The Nature Conservancy. (http://www.invasive.org/gist/handbook.html)

US EPA (1993) Glyphosate Reregistration Eligibility Decision (RED). EPA-738-F-93-011. (http://www.epa.gov/oppsrrd1/REDs/old_reds/glyphosate.pdf)

US EPA (2006) Pesticides Reregistration, 2,4-D RED Facts. 738-R-05-002 (http://www.epa.gov/oppsrrd1/REDs/factsheets/24d_fs.htm.)

Van Eerd, L.L., Stephenson, G.R., Kwiatkowski, J., Grossmann, K., and Hall, C. (2005) Physiological and biochemical characterization of quinclorac resistance in a false cleavers (Galium spurium L.) biotype. Journal of Agriculture and Food Chemistry 53:1144-1151.

Vencill, W.K. (2002) Herbicide Handbook (8th edition) ed. Lawrence, KS: Weed Science Society of America.

Walsh, T.A., Neal, R., Merlo, A.O., Honma, M., Hicks, G.R., Wolff, K., Matsumura, W. and Davies, J.P. (2006) Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-Dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. Plant Physiology 142:542-552.

Appendix 8. Stewardship of Herbicide Tolerant DAS-68416-4 Soybeans

Dow AgroSciences (DAS) takes product stewardship seriously and recognizes the importance of ongoing stewardship regarding both our agricultural chemical and biotechnology trait products. Dow AgroSciences will effectively steward DAS-68416-4 soybeans through both the agricultural chemical and seed business units using a variety of means with our sales force, commercial channels and grower customers.

8.1. Communication to Agricultural Chemical and Seed Customers

Technical Bulletins

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

Direct Mail

Dow AgroSciences uses direct mail newsletters sent on a monthly basis through both our chemical products business and seed brands. The Mycogen seed brand sends six "Let's Talk Agronomy" newsletters per year. These newsletters go directly to our farmer customers highlighting agronomics and production practices, as well as information regarding traits and other new technologies. The DAS chemical products newsletters contain product usage guidelines, positioning and best use practices. DAS has highlighted weed resistant management strategies and proper stewardship, and in the future will highlight proper stewardship of herbicide programs compatible with DAS-68416-4 soybeans through both chemical products and seed brands newsletters.

Sales Literature

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

Information on Websites

The Dow AgroSciences seed brands websites focus on agronomic and proper hybrid placement. The websites also contain a specific section devoted to trait stewardship and following specific guidelines in planting transgenic traits (see the Mycogen Seed website, http://www.mycogen.com). In the future, websites can also provide information on DAS-68416-4 soybeans that will feature trait and herbicide stewardship programs. The websites will also contain links to technical bulletin and herbicide resistance management strategies to provide a comprehensive resource.

Dow AgroSciences agricultural chemicals website (http://www.dowagro.com/usag/) features commercial and technical information and today contains information on pest resistance management such as rotating insecticides for effective stewardship. DAS can feature herbicide resistance management and effective stewardship of herbicide information labeled for DAS-68416-4 soybeans upon commercial launch. Industry and university links will be included to expose the reader to a wider range of technical information.

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

8.2. Trait and Herbicide Field Testing

Dow AgroSciences uses a wide variety of field trial collaborators including universities, consultants, other biotech commercial partners and internal resources in reviewing hundreds of field trials every year, including DAS-68416-4 soybean trials. These field trials allow Dow AgroSciences to precisely characterize, position, and recommend the proper herbicide approach for best long term success and efficacy for the trait and herbicide usage. These also allow us to monitor a consistent set of field trial data over a long period of time to observe any efficacy trends and adjust our herbicide recommendations if necessary to mitigate resistance threats.

8.3. Agricultural Chemical Labeling

Key Dow AgroSciences herbicides carry a weed resistance management statement. Figure 59 is an example of the weed resistance management statement printed on DAS glyphosate brand labels. Dow AgroSciences can place a similar statement on all products labeled for use in conjunction with DAS-68416-4 soybeans to help communicate proper weed resistant management strategies.

Figure 59. Example weed resistance management statement on DAS glyphosate brand labels.

Weed Resistance Management

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

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

Herbicide Selection:

- Rotate the use of glyphosate with non-glyphosate herbicides.
- Avoid using more than two applications of a glyphosate-based herbicide in a given field over a two-year period. Utilize tank mixes or sequential applications of herbicides with alternative modes of action if this is not possible.
- Use herbicides with alternative modes of action for burndown applications prior to planting Roundup Ready® crops that are likely to require more than one over-the-top application of glyphosate.

- Apply full rates of glyphosate at the specified time (correct weed size) to minimize escapes of tolerant weeds.

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

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

8.4. Training and Education of Sales Representatives and Agronomists

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

8.5. Tracking Customer Satisfaction and Managing Issues

Dow AgroSciences is directly involved with farmer and distributor/retail customers on a daily basis throughout the U.S. Customer satisfaction is of ultimate importance to the continued success of DAS, so continual positioning and follow up on the usage of the DAS-68416-4 soybeans and associated herbicides will be important in our launch and subsequent sales. As a result of our continual follow up directly with customers, we are able to implement and monitor the proper usage and stewardship of this technology.

DAS also electronically tracks any weed control non-performance issues and crop injury as serviced directly by our sales representatives. All new DAS sales representatives receive

extensive in-field training on weed control issues and handling customer complaints. This complaint handling and data entry helps us track any emerging issues specific to products, pests (including resistant weeds) or crops and address them on a broad scale.

8.6. Involvement in Industry Groups

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

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

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