Dow AgroSciences

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

OECD Unique Identifier: DAS-8191Ø-7

DAS-8191Ø-7 Cotton

Volume 1 of 3

Submitting Company:

Dow AgroSciences Australia Pty Ltd.

Submitted by:

____

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Glossary of Acronyms and Scientific Terms

°C	Degree Celsius
2,4-D	2,4-Dichlorophenoxyacetic acid
AA	Amino Acid
aad-12	Gene encoding the AAD-12 protein
AAD-12	Aryloxyalkanoate dioxygenase-12 protein
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ADF	Acid detergent fibre
ae	Acid equivalent
ae/ha	Acid equivalent per hectare
ai	Active ingredient
ai/ha	Active ingredient per hectare
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service, USDA
AtUbi10	Ubiquitin promoter from Arabidopsis thaliana
AtuORF1	3′ untranslated region from Agrobacterium tumefaciens
AtuORF23	3′ untranslated region from Agrobacterium tumefaciens
BLASTp	Basic Local Alignment Search Tool protein
bp	Base pair
BSA	Bovine Serum Albumin
CFR	Code of Federal Regulations
CFSAN	Center for Food Safety and Nutrition, US FDA
CsVMV	Promoter from cassava vein mosaic virus
DAS	Dow AgroSciences, LLC
DAS-8191Ø-7	OECD identifier for the cotton event expressing the AAD-12 and PAT proteins
DCP	2,4-Dichlorophenol
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (US)
ESI-LC/MS	Electrospray ionization-liquid chromatography mass spectrometry
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration (US)
FDR	False Discovery Rate
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FSANZ	Food Standards Australia New Zealand
g	gram
GS	Glutamine synthetase
ha	Hectare

HED	Health Effects Division
IAA	Indole acetic acid
ILSI	International Life Sciences Institute
Kb	Kilobase pair
kDa	Kilodalton
kPa	Kilopascals
L	Litre
LFS	Lateral Flow Strip
LOD	Limit of Detection
LOQ	Limit of Quantitation
M	Molar
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MARC	Metabolism Assessment Review Committee - US EPA
MCPA	((4-chlro-2-methyphenoxy) acetic acid)
µg	microgram
µL	microliter
µM	micromolar
mg	Milligrams
min	minute
mL	Milliliter
mM	millimolar
MOE	Margin of exposure
MW	Molecular Weight
NA	Not Applicable
ng/mg	Nanogram / milligram
NOAEL	No observed adverse effect level
NOEL	No observed effect level
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
Ori Rep	replication Origin Sequence
pat	Gene from Streptomyces viridochromogenes which encodes the PAT protein

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1. GENERAL INFORMATION ON THE APPLICATION

1.1 The Applicant

This application is submitted by:

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

Mr Peter Dryden

1.2 Summary

Dow AgroSciences LLC is submitting an application to amend the Australia New Zealand Food Standards Code Standard 1.5.2 to approve the use of DAS-8191Ø-7 Cotton; a new food produced using gene technology.

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

This submission contains sufficient supporting information to address the requirements identified by FSANZ to approve the use a new food produced using gene technology, as specified in section 3.5.1 of the FSANZ Application Handbook, 1st September, 2013. Only reports produced by Dow AgroSciences or The Dow Chemical Company are provided. All other citations are available; however, given that these are from published literature, they have not been copied. Any or all of these citations will be forwarded if requested.

The references provided in section 6.1 of this dossier are proprietary information which is owned by and has value to The Dow Chemical Company and their subsidiary companies. These reports may not be used or referenced by any other company or person without our express agreement. A list of the DAS studies relevant to the information parts in this application is indicated at the beginning of each section.

Dow AgroSciences has developed DAS-8191Ø-7 cotton which is tolerant to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate-ammonium. DAS-8191Ø-7 cotton will provide growers in approved regions 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-8191Ø-7 cotton plants have been genetically modified to express aryloxyalkanoate dioxygenase-12 (AAD-12). AAD-12 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 which can be used to transform ferulic acid into vanillin and related flavour metabolites.

In addition to AAD-12, DAS-8191Ø-7 cotton plants have also been genetically modified to express the phosphinothricin acetyltransferase (PAT) protein. The *pat* gene expressing the PAT protein was derived from *Streptomyces viridochromogenes* and provides tolerance to the herbicide glufosinate-ammonium in DAS-8191Ø-7 cotton plants. The PAT enzyme acetylates the primary amino group of glufosinate-ammonium, also known as phosphinothricin, rendering the herbicide inactive. The *pat* gene has been extensively reviewed by regulatory authorities in over eleven countries, including Australia and New

Zealand and has a long history of safe use, being used in over eight crop species representing over twenty-two biotechnology plant events globally.
The *aad-12* and *pat* expression cassettes introduced into DAS-8191Ø-7 cotton are the same as those introduced into DAS-68416-4 soybean and DAS-444Ø6-6 soybean; both have been evaluated and approved by FSANZ.

Australia is the third largest exporter of cotton in the world behind USA and India (USDA 2013). In the period 2011-2012, the Australian cotton industry produced approximately 5 million bales cotton from a planted area of more than 600,000 hectares, generating ca. $2.75 billion AUD in export revenue (ABARES 2012). Only very small quantities of cotton imports to Australia are reported in 2011- 2012, with less than 200 tonnes cotton lint from the USA; and 3,658 tonnes cottonseed oil imported predominantly from Malaysia (FAOSTAT). Only cottonseed oil and linters (fibre) from cottonseed are used in food applications; seeds are mainly used to obtain edible oil and as livestock feed. Due to the harsh conditions used in cottonseed processing, cottonseed oil and linters contain undetectable or negligible amounts of protein, therefore, oil and other products produced from DAS-8191Ø-7 cotton will contain extremely low levels of AAD-12 and PAT protein.

As with DAS-68416-4 soybean and DAS-444Ø6-6 soybean, the data and information presented in this application support the conclusion that food and feed derived from DAS-8191Ø-7 cotton is as safe and nutritious as those derived from non-transgenic cotton. The conclusion was based on 1) detailed molecular characterization of DAS-8191Ø-7 cotton; 2) safety assessment of the introduced AAD-12 and PAT proteins; and 3) nutrient composition analysis of DAS-8191Ø-7 cotton.

Molecular Characterisation of DAS-8191Ø-7 Cotton

The *aad-12* and *pat* genes were introduced into DAS-8191Ø-7 cotton using *Agrobacterium* mediated transformation. Molecular characterization by Southern blot analyses of DAS-8191Ø-7 cotton confirmed that a single, intact DNA insert containing the *aad-12* and *pat* gene expression cassettes was stably integrated into the cotton genome. Southern blot analyses also confirmed the absence of the plasmid backbone DNA in DAS-8191Ø-7 cotton. The integrity of the inserted DNA was demonstrated in five different breeding generations. Data from segregating generations confirmed the predicted Mendelian inheritance pattern. These data confirmed the stability of DAS-8191Ø-7 cotton during traditional breeding procedures.

Biochemical Characterization of DAS-8191Ø-7 Cotton

Microbial-derived AAD-12 and PAT have been extensively assessed to establish the safety of the protein. DAS-8191Ø-7 cotton-derived AAD-12 and PAT proteins were determined to be biochemically equivalent to the corresponding proteins from microbial expression host organisms. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 and PAT proteins. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-12 or PAT amino acid sequences. Both proteins hydrolysed rapidly in

simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-12 protein and 5000 mg/kg body weight of PAT protein.

The AAD-12 protein from cotton and soybean event DAS-68416-4 has identical amino acid sequence and N-terminal acetylation. Digestion samples of the transgenic soybean (event DAS-68416-4) leaf extracts were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot; the AAD-12 protein contained in crude soybean leaf extracts (in both transgenic tissue and nontransgenic tissue fortified with microbe-derived AAD-12) was readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C). Hence, the digestibility of the acetylated AAD-12 protein is established.

AAD-12 and PAT expression levels in DAS-8191Ø-7 cotton were measured using a protein-specific enzyme-linked immunosorbent assay (ELISA). Protein expression was analysed in multiple tissues collected throughout the growing season from DAS-8191Ø-7 cotton plants untreated and treated with 2,4-D plus glufosinate-ammonium. Glycosylation analysis revealed no detectable covalently linked carbohydrates in either AAD-12 or PAT proteins expressed in DAS-8191Ø-7 cotton plants. The low level expression of these proteins presents a low exposure risk to humans and animals, and the results of the overall safety assessment of AAD-12 and PAT indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

Compositional Assessment of DAS-8191Ø-7 Cotton

A composition assessment was conducted in which levels of key nutrients and anti-nutrients of DAS-8191Ø-7 cottonseed were compared with the appropriate non-transgenic near-isogenic control and non-transgenic reference lines. Samples were analysed for proximates, fibre, minerals, amino acids, fatty acids, vitamins, and anti-nutrients. Fifty-nine cotton analytes were assayed and the analyses conclude that DAS-8191Ø-7 cotton is compositionally equivalent to non-transgenic cotton.

Dow AgroSciences is seeking an amendment of the Australia New Zealand Food Standards Code - Standard 1.5.2 Food Produced Using Gene Technology by inserting: “food derived from herbicide-tolerant DAS-8191Ø-7 Cotton line”, into column 3 of the Schedule of Permitted Foods produced using Gene Technology, immediately after the last cotton entry.

STATUTORY DECLARATION – AUSTRALIA

The information provided in Parts 1 to 3 must be attested to by a statutory declaration in some suitable form along the following lines:

STATUTORY DECLARATION

Statutory Declarations Act 1959

I, [redacted] Regulatory Specialist ANZ, Dow AgroSciences Australia Ltd.

make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Declared at Frenchs Forest, NSW, Australia on 14th of November 2013
Before me,

Registration No. 150978.

* A statutory declaration must be made before a prescribed person under the *Statutory Declarations Act 1959*, available online at
http://www.frli.gov.au/ComLaw/Legislation/ActCompilation1.nsf/current/bytitle/7E3AE20F8329B422CA256F71004DB642?OpenDocument&mostrecent=1.

Checklist for General requirements

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

General requirements (3.1)

☑	3.1.1 Form of application ☑ *Application, abstracts and other key documents in English* ☑ *Executive Summary (separated from main application electronically and in hard copy)* ☑ *Relevant sections of Part 3 clearly identified* ☑ *Pages sequentially numbered* ☑ *Electronic copy (searchable)* ☑ *1 hard copy* ☑ *Electronic and hard copy identical* ☑ *Hard copy capable of being laid flat* ☑ *All references provided (in electronic and hard copy)*	☑	3.1.6 Assessment procedure ☑ *General* ☐ *Major* ☐ *Minor* ☐ *High level health claim variation*
		☑	3.1.7 Confidential Commercial Information ☐ *Confidential material separated in both electronic and hard copy* ☐ *Formal request including reasons* ☑ *Non-confidential summary provided*
☑	3.1.2 Applicant details	☑	3.1.8 Exclusive Capturable Commercial Benefit ☐ *Justification provided*
☑	3.1.3 Purpose of the application	☑	3.1.9 International and other national standards ☐ *International standards* ☐ *Other national standards*
☑	3.1.4 Justification for the application ☑ *Regulatory impact information* ☑ *Impact on international trade*	☑	3.1.10 Statutory Declaration
☑	3.1.5 Information to support the application ☑ *Data requirements*	☑	3.1.11 Checklist/s provided with application ☑ *3.1 Checklist* ☑ *Any other relevant checklists for Parts 3.2-3.7*

Checklist for Standards related to new foods

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

Foods Produced using Gene Technology (3.5.1)

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

Novel Foods (3.5.2)

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

C – Information on the safety of the novel food

(I) Plant or animal extracts

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

(II) Plant and animal extracts

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

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

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

1.3 Purpose of the application

Dow AgroSciences LLC (herein referred to as "DAS") has developed herbicide tolerant DAS-8191Ø-7 cotton to 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate. DAS-8191Ø-7 cotton is the unique identifier of these plants, in accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2002).

This application to the Food Standards Australia New Zealand has been based on the submission generated for other overseas agencies. This dossier contains sufficient supporting information to address the requirements identified by FSANZ to approve the use a new food produced using gene technology, as per section 3.5.1 of the FSANZ Application Handbook, 1st September 2013.

This application is a component of the Dow AgroSciences global approval process, especially for export destinations of cotton commodities and is consistent with Dow AgroSciences' corporate policy of ensuring full regulatory compliance. As a result, Dow AgroSciences is seeking an amendment of the Australia New Zealand Food Standards Code -Standard 1.5.2 Food Produced Using Gene Technology by inserting: "food derived from herbicide-tolerant DAS-8191Ø-7 cotton line", into column 3 of the Schedule of Permitted Foods produced using Gene Technology, immediately after the last cotton entry.

1.4 Justification for application

1.4 a Costs and Benefits of the genetically modified food

(i) Costs and benefits to the industry and business in general

DAS-8191Ø-7 cotton was developed using *Agrobacterium*-mediated transformation to stably incorporate the *aad-12* gene from *Delftia acidovorans* and the *pat* gene from *Streptomyces viridochromogenes* into cotton. The *aad-12* gene encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme which, when expressed in plants, degrades 2,4-diclorophenoxyacetic acid (2,4-D) to herbicidally-inactive 2,4-dichlorophenol (DCP). The *pat* gene encodes the enzyme phosphinothricin acetyltransferase that inactivates glufosinate-ammonium. DAS-8191Ø-7 cotton will provide growers in the USA 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.

With the introduction of genetically engineered glyphosate tolerant crops in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds that was unparalleled in agriculture. Consequently, producers were quick to adopt glyphosate tolerant crops, and in many instances, abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, and incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, alfalfa, sugar beets, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. More glyphosate tolerant crops (*e.g.,* wheat, rice, turf, *etc.*) are poised for introduction pending global market acceptance. Many other glyphosate tolerant species are in experimental or development stages (*e.g.*, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias) (Information Systems for Biotechnology 2011). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate tolerant crop systems.

Extensive use of glyphosate-only weed control programs has resulted in the selection of glyphosate resistant weeds, and continues to select for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (*i.e.*, weed shifts). Although glyphosate has been widely used globally for more than 30 years, the vast majority of resistant weeds have been identified in the past 5-8 years.

Resistant weeds in the USA 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. According to a 2012 survey across 31 states in the USA, 49% of growers reported the presence of glyphosate resistant weed on their farms (Pucci 2013). In 2011, the number was 34%. The problem is more pronounced in Southern regions the USA, with 92% of growers reporting glyphosate resistant weeds. 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 70 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 cotton provides an excellent option for controlling glyphosate resistant (or highly tolerant and shifted) broadleaf weed species for in-crop applications, allowing the grower to focus applications at the critical weed control stages and extending the application window.

The availability of DAS-8191Ø-7 cotton is expected to have a beneficial impact on weed control practices in the USA by providing growers with an advanced tool to address their weed control needs. The availability of DAS-8191Ø-7 cotton 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.

DAS-8191Ø-7 cotton will be commercialised in combination with other herbicide tolerant (*e.g.* glyphosate) and insect resistance cotton events in the future. Stacked varieties provide growers with built-in, sustainable resistance management tools to address glyphosate resistant and hard to control weeds, as well as incorporate the recognized environmental benefits of insect resistant traits. The combination of herbicide tolerance traits will allow the use of multiple herbicides in an integrated weed management program to control a broad spectrum of grass and broadleaf weed species in cotton. These herbicides will provide distinct modes of actions for use in conjunction with other herbicide active ingredients and mode of action for an effective weed management program in cotton. 2,4-D will provide improved in-crop post-emergence control of hard to control glyphosate resistant broad-leaf weeds, such as pigweed, waterhemp, horseweed, and morning glory.

DAS does not plan to cultivate or commercialise DAS-8191Ø-7 cotton in Australia and New Zealand. DAS-8191Ø-7 cotton will be marketed and sold as an alternative weed management tool throughout the cotton growing regions of the USA and will be grown for the same commercial uses as current transgenic and non-transgenic commercial cotton varieties.

The primary use of cotton is for fibres which are mainly used in the manufacturing of a large number of textiles. Only the cotton boll (consisting of fibres and seeds) is useful for either textile fibres or food or feed. The boll is processed to remove the fibre for textile use; the remaining cottonseed is processed into four major food and feed products: oil, meal, hulls and linters. Only cottonseed oil and linters (fibre) from cottonseed are used in food applications in which the seeds are mainly used to obtain edible oil and used as livestock feed. Linters are used in food, mainly in the production of meat casings (*e.g.* sausage) or as thickeners in ice cream and salad dressing, however, the total amount of linters used is very small (OECD 2009a).

Nutritional composition analysis and safety assessments of DAS-8191Ø-7 cotton indicate no biologically significant differences from non-transgenic cotton, supporting the conclusion that there will be no significant impact on commodity food and feed cotton products resulting from the presence of AAD-12 or PAT in cotton.

(ii) Costs and benefits to the consumer

The information presented in this submission support the conclusion that food and feed derived from DAS-8191Ø-7 cotton will be as safe and nutritious as those derived from conventional cotton. Because DAS-8191Ø-7 cotton has been demonstrated to be compositionally similar to conventional cotton, and AAD-12 and PAT proteins have a history of safe use, no significant impact is expected on human or animal health via commodity food and feed cotton products. DAS is unaware of any food or feed use of cotton for which DAS-8191Ø-7 cotton would be unsuitable.

(iii) Costs and benefits to the government

The local cost implications are made up of DAS personnel time both locally and globally as well as the direct fees associated with the submission. The assessment of DAS-8191Ø-7 by FSANZ constitutes a requirement in accordance with Division 1 of Standard 1.5.2 contained in the Australia New Zealand Food Standard Code, which mandates pre-market approval, including safety assessment, of food derived from an organism which has been modified by gene technology. There are few price or employment implications which are directly related to the FSANZ assessment of DAS-8191Ø-7. The trade implications however are considerable since non-approval by FSANZ would impose a trade restriction on DAS-8191Ø-7 and products derived from these lines.

1.4 b Potential impact on international trade

This application to the Food Standards Australia New Zealand has been based on the submission generated for other overseas agencies. It is a component of the Dow AgroSciences global approval process, especially for export destinations of cotton commodities and is consistent with Dow AgroSciences corporate policy of ensuring full regulatory compliance. It is a necessary component of the global approval process since without such food import approvals, the cultivation and marketing of DAS-8191Ø-7 in USA will be significantly hampered. DAS intends to submit dossiers to the regulatory authorities of trade partners for import clearance and production approval including, but not limited to, USA, Canada, Mexico, EU and Korea. The benefit and market share implication are difficult to quantify, however, freedom to operate in the marketplace is a market requirement and will have an impact on these factors.

2. TECHNICAL INFORMATION ON THE GM FOOD

2.1 Nature and identity of the genetically modified food

2.1 a Description of the GM organism

DAS-8191Ø-7 cotton plants have been genetically modified to express aryloxyalkanoate dioxygenase-12 (AAD-12). AAD-12 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 which can be used to transform ferulic acid into vanillin and related flavour metabolites.

In addition to AAD-12, DAS-8191Ø-7 cotton plants have also been genetically modified to express the phosphinothricin acetyltransferase (PAT) protein. The PAT enzyme acetylates the primary amino group of the herbicide phosphinothricin, also known as glufosinate-ammonium, rendering the herbicide inactive. The *pat* gene expressing the PAT protein was derived from *Streptomyces viridochromogenes* and provides tolerance to the herbicide glufosinate-ammonium in DAS-8191Ø-7 cotton plants.

The *aad-12* and *pat* genes were introduced into DAS-8191Ø-7 cotton using *Agrobacterium* mediated transformation. Molecular characterization by Southern blot analyses of DAS-8191Ø-7 cotton confirmed that a single, intact DNA insert containing the *aad-12* and *pat* gene expression cassettes was stably integrated into the cotton genome. Southern blot analyses also confirmed the absence of the plasmid backbone DNA in DAS-8191Ø-7 cotton. The integrity of the inserted DNA was demonstrated in five different breeding generations. Data from segregating generations confirmed the predicted Mendelian inheritance pattern. These data confirmed the stability of DAS-8191Ø-7 cotton during traditional breeding procedures.

2.1 b GM organism identification

This transformed cotton is known as event DAS-8191Ø-7. No commercial name has yet been identified.

2.1 c Food Identity

There is currently no intention to market food items containing cotton derived from DAS-8191Ø-7 with specific brands or names.

2.1 d Products containing the food or food ingredients

Cotton is primarily grown as fibre crop. Only the cotton boll (also known as 'seed cotton', which consists of fibres and seeds) is useful for fibre, food and feed with the main use being fibres which are mainly used in the manufacturing of a large number of textiles.

The remainder of the plant is left in the field for decomposition. The seed cotton is processed to remove the fibre for textile use; the remaining fuzzy seed is processed into four major food and feed products: oil, meal, hulls and linters.

Only cottonseed oil and linters (fibre) from cottonseed are used in food applications. Cottonseeds are mainly used to obtain edible oil and used as livestock feed while linters are mainly used in the production of meat casings (*e.g.* sausage) or as a viscosity enhancer (thickener) in ice cream salad dressings and toothpaste; however, the total amount of linters used in such products is very small.

For details on the food and feed uses of cotton refer to The OECD Consensus Document on the Compositional Considerations for New Varieties of Cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key Food and Feed Nutrients and Anti-Nutrients (OECD 2009a) and the series of publications from the Australian Office of Gene Technology Regulator (OGTR) on the biology of cotton in Australia (OGTR 2008).

2.2 History of Use of the Host and Donor Organisms

2.2 a Donor Organism

DAS-8191Ø-7 cotton was developed using *Agrobacterium* mediated transformation to introduce the *aad-12* and *pat* expression cassettes into cotton (*Gossypium hirsutum)*. The *aad-12* expression cassette consists of the AtUbi10 promoter, *aad-12* gene and AtuORF23 3' UTR terminator. The *aad-12* gene was isolated from *Delftia acidovorans* and the synthetic version of the gene was optimised for plant expression by modifying the G+C content bias to the plant system. The native and plant-optimized DNA sequences of *aad-12* are 80% identical. The *aad-12* gene is designed to express the Aryloxyalkanoate Dioxygenase-12 (AAD-12) protein which consists of 293 amino acids with a molecular weight of approximately 32 kDa. Expression of AAD-12 protein in plants confers tolerance to herbicides such as 2,4-D.

Delftia acidovorans, which has previously been identified as *Pseudomonas acidovorans* and *Comamonas acidovorans*, is a non glucose-fermenting, gram-negative, non spore-forming rod-shaped bacterium present in soil, fresh water, activated sludge, and clinical specimens (Tamaoka et al 1987, Von Graevenitz 1985, Wen et al 1999). *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavour metabolites (Rao & Ravishankar 2000, Shetty et al 2006, Toms & Wood 1970). 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* is controlled by the AtUbi10 promoter from *Arabidopsis thaliana* which is known to drive constitutive expression of the genes it controls (Norris et al 1993). The *aad-12* AtuORF23 3′ UTR terminator sequence is derived from *Agrobacterium tumefaciens* plasmid pTi15955 (Barker et al 1983).

The *pat* expression cassette consists of the cassava vein mosaic virus (CsVMV) promoter, *pat* and AtuORF1 3′ UTR terminator and designed to express the phosphinothricin *N*-acetyltransferase (PAT) protein. The *pat* gene was isolated from the common soil bacterium *Streptomyces viridochromogenes* (Wohlleben et al 1988) and the synthetic version of the gene was optimized for plant expression by modifying the G+C content bias to the plant system.

The presence of PAT protein in plants confers tolerance to glufosinate-ammonium. The *pat* gene encodes a protein of 183 amino acids that has a molecular weight of approximately 20 kDa. The *pat* gene has been widely used both as a selectable marker and herbicide tolerance trait in plants and products, previously approved by FSANZ.

Expression of the *pat* gene is controlled by the CsVMV promoter from cassava vein mosaic virus and the 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).

2.2 b Host Organism

Refer to The OECD Consensus Document on the Biology of Cotton (*Gossypium*, spp.) (OECD 2008), for information related to the following aspects of cotton biology:

- General description and morphology of cotton *(p 11 – 12)*
- Uses of cotton as a crop plant *(p 13)*
- Taxonomy *(p 14 – 15)*
- Centres of origin, diversity and domestication *(p 15 – 18)*
- Agronomic practices *(p 19 – 23)*
    - Biotic Environment (pest, diseases, weeds) *(p 21 – 23)*
    - Harvest, processing and crop rotation *(p23)*
- Reproductive biology *(p 24 – 27)*
    - Seed dormancy and germination *(p 26)*
    - Weediness and naturalization *(p 26-27)*
- Genetics & Hybridization *(p 28 – 29)*
    - Ability to cross intra and inter-species/genus *(p 29)*
- Toxin and Allergen potential *(p 34– 35)*
    - Gossypol *(p 34-35)*
    - Cyclopropenoid fatty acids *(p 35)*
    - Allergens *(p 35)*

(i) Characterisation of the recipient cotton cultivar

The publically available cotton variety Coker 310 (*G. hirsutum*) was used as the recipient lines for the generation of event DAS-8191Ø-7. The variety Coker 310 was developed by the cotton division of Coker's Pedigreed Seed Company and is an older commercial variety of upland cotton generated from a cross of Coker 100 Staple and Deltapine 15 and selected through successive generations of line selection (Bowman et al 2006, Smith et al 1999).

2.3 Nature of the Genetic Modification

Mo J, Ring S, 2012. Molecular Characterization of DAS-81910-7 CottonStudy ID 120456, Dow AgroSciences, LLC, Indianapolis, IN

Rapier K, 2012. Molecular characterization of DAS-81910-7 cotton within a single segregating generation. Study ID: 120457 Dow AgroSciences, LLC. Indianapolis, IN.

2.3 a Transformation Method

Cotton (*Gossypium hirsutum* L.) event DAS-8191Ø-7 was developed through *Agrobacterium*-mediated transformation of pDAB4468. The disarmed *A. tumefaciens* strain LBA4404 (Ooms et al 1982), carrying the binary vector, pDAB4468, was used to initiate transformation of cotton hypocotyl segments.

Agrobacterium-mediated transformation was carried out using a modified procedure based on Umbeck *et al.* (Umbeck 1991). Briefly, cotton seeds (cv. Coker 310) were germinated on basal media and hypocotyl segments were isolated and infected with *Agrobacterium* strain LBA4404 carrying pDAB4468. Following infection, the hypocotyl segments were cultured on a sequence of media containing carbenicillin and glufosinate-ammonium to inhibit the growth of *Agrobacterium* and untransformed cells, respectively.

Gene-specific PCR analyses were performed on embryogenic callus to identify transgenic lines containing the target genes (*aad-12* and *pat*). Selected calli were transferred to culture medium containing plant growth regulators to stimulate root regeneration. Rooted plants (T_0) were transferred to soil mixtures under high humidity in growth chambers for 2-4 weeks. The hardened plants were then transferred to greenhouse facilities.

Following the transfer to the greenhouse, young leaves of T_0 plants were painted with glufosinate-ammonium (1.5% w/v) to screen for putative transformants. Those glufosinate-ammonium tolerant plants were sampled and analysed at a molecular level to confirm the presence of the target genes and the absence of the vector backbone. Specifically, for T_0 plants, PCR analyses were performed to verify the absence of the bacterial selectable marker sequence, spectinomycin, in the vector backbone of pDAB4468 as well as the presence of the *aad-12* and *pat* genes.

PCR and Invader assays (Kwiatkowski et al 1999), were carried out to determine the copy number of the *pat* and *aad-12* genes. T_0 plants conferring the desirable copy number were self-pollinated to produce T_1 seed. For T_1 plants, Invader assay and Southern blot analyses were performed to identify plants containing a single *pat* and *aad-12* gene insertion. DAS-8191Ø-7 cotton was selected as the lead event based on molecular and phenotypic characteristics. Genetic characterization studies on DAS-8191Ø-7 cotton were initiated to further characterize the transgenic insert and the expressed proteins (Section 2.3 c). The major steps described in the development of DAS-8191Ø-7 cotton are described in Figure 1 with the breeding diagram for DAS-8191Ø-7 cotton in Figure 2.

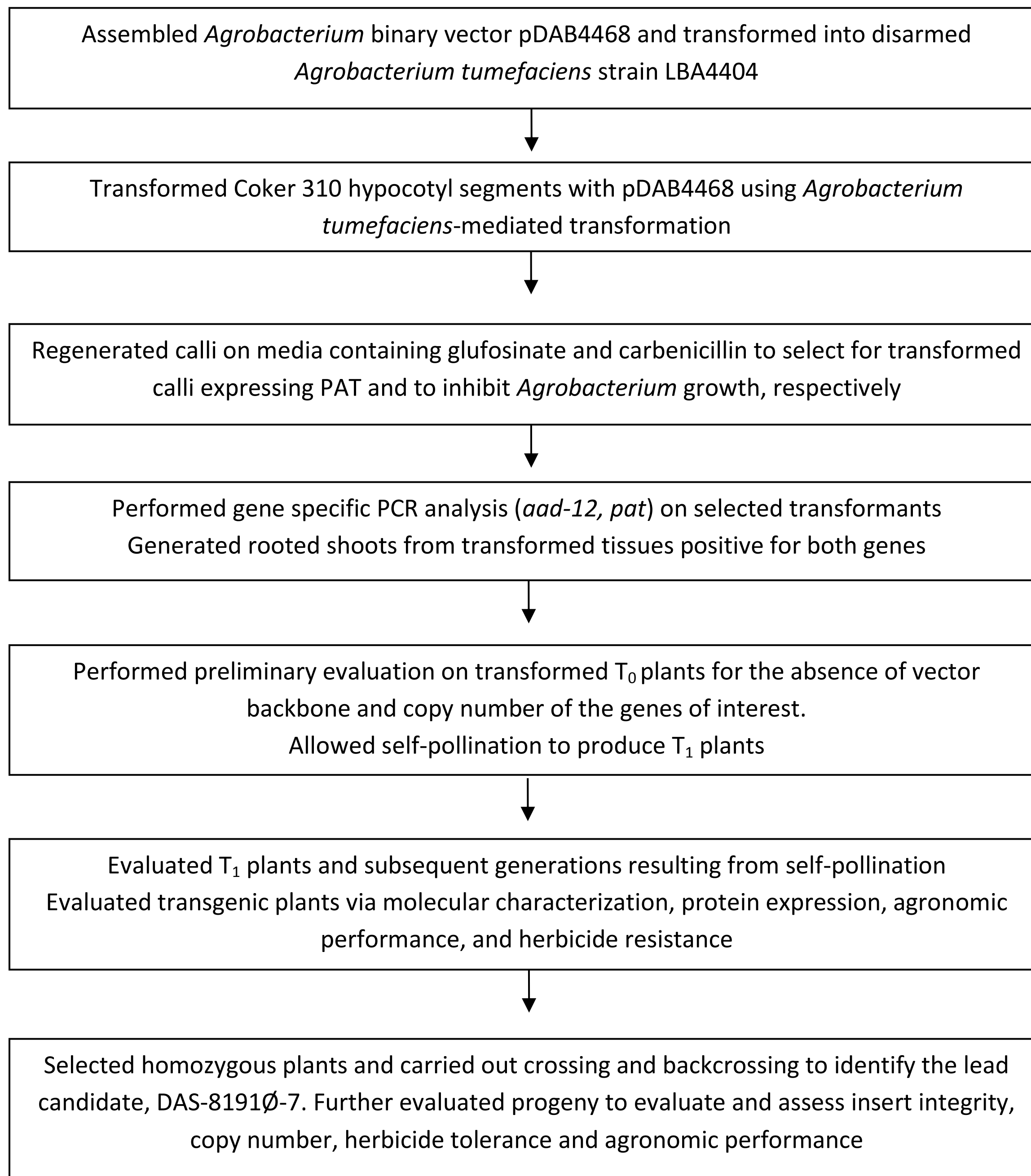
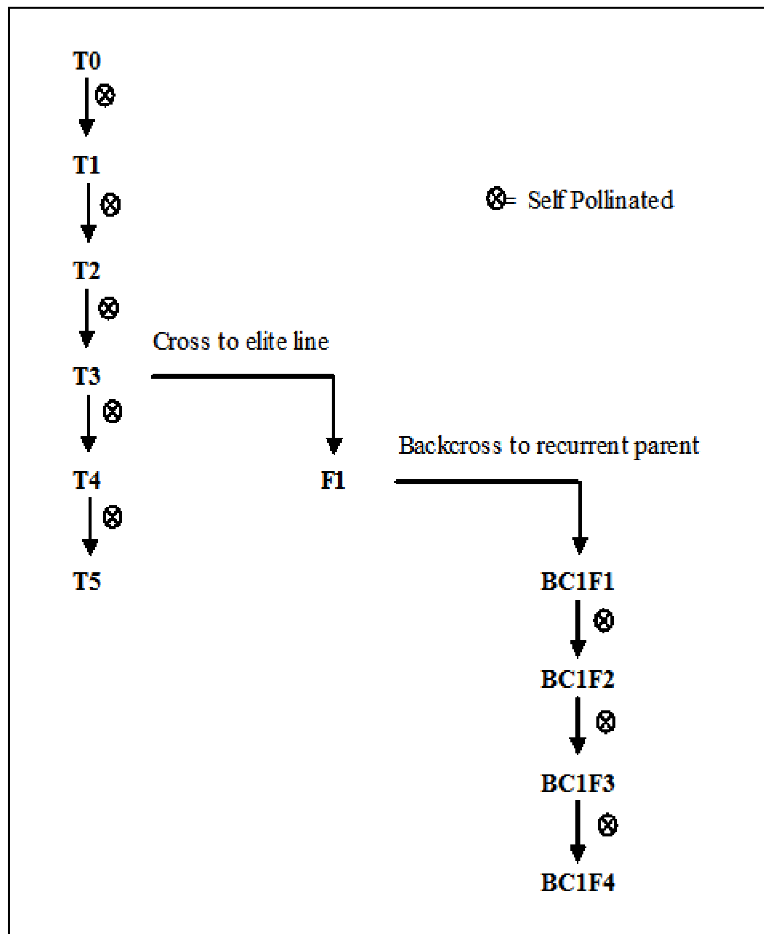


Figure 1. Schematic of the Development of DAS-8191Ø-7 Cotton



Analysis	DAS-8191Ø-7 Cotton Generations	Control
Molecular Analysis	T_2, T_3, T_4, T_5, BC_1F_2	Non-transgenic cotton
Segregation Analysis (single generation)	BC_1F_2	None
Segregation Analysis (breeding generations)	T_1, BC_1F_2	None
Protein Characterization	T_3	Non-transgenic cotton
Protein Expression	BC_1F_3	Non-transgenic cotton
Composition	BC_1F_3	Non-transgenic cotton

Figure 2. Breeding Diagram of DAS-8191Ø-7 Cotton

2.3 b Bacteria used for manipulation

A standard laboratory strain of *E.coli* was used for all vector manipulations and for amplification of the plasmid DNA (pDAB4468) that was used for the transformation.

2.3 c Gene Construct and Vectors

The plasmid vector, pDAB4468, was used in the transformation of cotton to generate DAS-8191Ø-7. A vector map and the summary of the genetic elements in pDAB4468 are provided in Figure 3 and Table 1, respectively. pDAB4468 is approximately 12-kb and contains one T-DNA that is delineated by T-DNA borders B and A (Figure 4). The T-DNA contains the *aad-12* and *pat* expression cassettes along with the RB7-MAR sequence. A gene expression cassette is comprised of sequences to be transcribed (the gene coding sequence) and the regulatory elements necessary for the expression of those sequences (*e.g.* promoter, terminator). Details of the expression cassettes in pDAB4468 are described in sections 2.3c (i) and (ii), and in Table 1.

The backbone region of pDAB4468, located outside the T-DNA region, contains two origins of replication (OriRep and Trf A) for the maintenance of the plasmid vector in bacteria and a bacterial selectable marker gene (*SpecR*). Details of the genetic elements are in Table 1.

(i) aad-12 expression cassette

The *aad-12* expression cassette consists of the AtUbi10 promoter, *aad-12* gene and AtuORF23 3' UTR terminator (Figure 4, Table 1) and is identical to the *aad-12* expression cassette in DAS-68416-4 soybean and DAS-444Ø6-6 soybean (FSANZ applications A1046 and A1073, respectively). The *aad-12* gene was isolated from *Delftia acidovorans* and the synthetic version of the gene was optimized for plant expression by modifying the G+C content bias to the plant system. The native and plant-optimised DNA sequences of *aad-12* are 80% identical. The *aad-12* gene is designed to express the Aryloxyalkanoate Dioxygenase-12 (AAD-12) protein, which consists of 293 amino acids with a molecular weight of approximately 32 kDa. Expression of AAD-12 protein in plants confers tolerance to herbicides such as 2,4-D.

D. acidovorans, which was previously described as *Pseudomonas acidovorans* and *Comamonas acidovorans*, is a non glucose-fermenting, gram-negative, non spore-forming rod-shaped bacterium present in soil, fresh water, activated sludge, and clinical specimens (Tamaoka et al 1987, Von Graevenitz 1985, Wen et al 1999). *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavour metabolites (Rao & Ravishankar 2000, Shetty et al 2006, Toms & Wood 1970). 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).

aad-12 expression is controlled by the AtUbi10 promoter from *Arabidopsis thaliana*, which is known to drive constitutive expression of the genes that it controls (Norris et al 1993). The terminator sequence, AtuORF23 3' UTR, is derived from *Agrobacterium tumefaciens* plasmid pTi15955 (Barker et al 1983).

(ii) pat expression cassette

The *pat* expression cassette consists of the cassava vein mosaic virus CsVMV promoter, *pat* gene and AtuORF1 3' UTR terminator (Figure 4) and is identical to the *pat* expression cassette in DAS-68416-4 soybean and DAS-444Ø6-6 soybean (FSANZ 2011, FSANZ 2013). The *pat* expression cassette is designed to express the PAT protein. The *pat* gene was isolated from the common soil bacterium *Streptomyces viridochromogenes* (Wohlleben et al 1988) and the synthetic version of the gene was optimized for plant expression by modifying the G+C content bias to the plant system.

The presence of PAT protein in plants confers tolerance to glufosinate-ammonium. The *pat* gene encodes a protein of 183 amino acids that has a molecular weight of approximately 20 kDa. The *pat* gene was used both as a selectable marker and herbicide tolerance trait in products previously approved in Australia & New Zealand, Canada and deregulated in USA.

Expression of the *pat* gene is controlled by the CsVMV promoter and the AtuORF1 3' UTR sequence from *A. tumefaciens* plasmid pTi15955. The CsVMV 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 that it controls (Verdaguer et al 1996).

(iii) RB7 MAR

In addition to the two expression cassettes, a matrix attachment region (MAR) of RB7 from *Nicotiana tabacum* was included at the 5' end of the T-DNA. 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 hypothesised that MARs may act as a buffer to protect transgenes from neighbouring chromosomal sequences that could destabilize their expression (Allen et al 1993, Allen et al 2000).

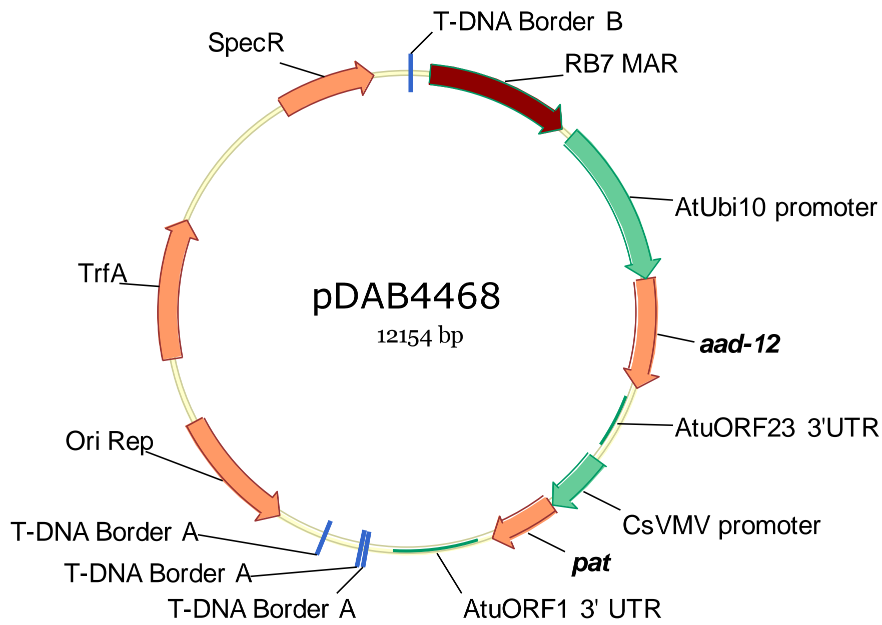


Figure 3. Plasmid map of pDAB4468

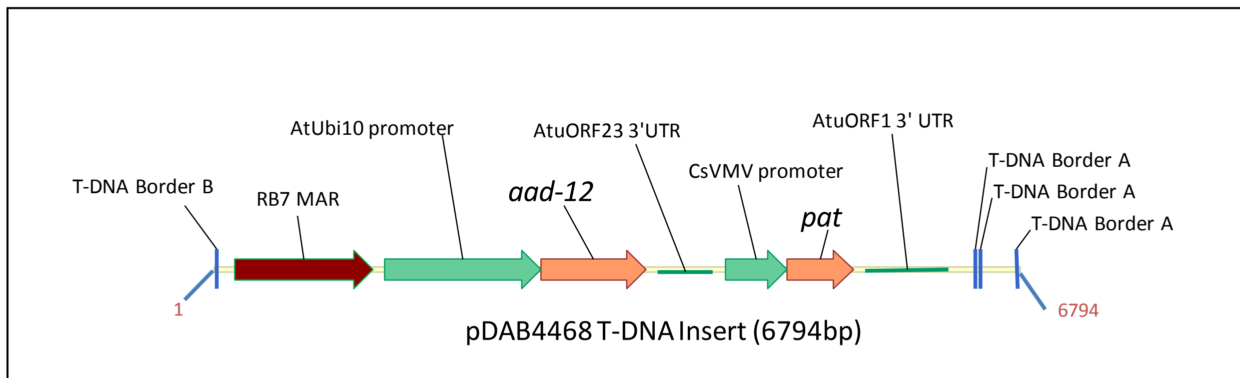


Figure 4. Diagram of T-DNA region in plasmid pDAB4468

Table 1. Genetic elements from plasmid pDAB4468

Feature Name	Feature Start	Feature Stop	Feature Size	Description
				T-DNA Region
T-DNA Border B	1	24	24	Transferring DNA sequences
Intervening sequence	25	160	136	Sequence from Ti plasmid pTi15955 (Barker et al 1983)
RB7-MAR	161	1326	1166	Matrix attachment region (MAR) from *Nicotiana tobacum* (Hall et al 1991)
Intervening sequence	1327	1421	95	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
AtUbi10	1422	2743	1322	*Arabidopsis thaliana* polyubiquitin UBQ10 gene comprising the promoter, 5' untranslated region and intron (Norris et al 1993)
Intervening sequence	2744	2751	8	Sequence used for DNA cloning
aad-12	2752	3633	882	Synthetic, plant-optimized version of an aryloxyalkanoate dioxygenase from *Delftia acidovorans* (Wright et al 2009)
Intervening sequence	3634	3735	102	Sequence used for DNA cloning
AtuORF23	3736	4192	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)
Intervening sequence	4193	4306	114	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
CsVMV	4307	4823	517	Promoter and 5' untranslated region derived from the cassava vein mosaic virus (Verdaguer et al 1996)
Intervening sequence	4824	4830	7	Sequence used for DNA cloning
pat	4831	5382	552	Synthetic, plant-optimized version of phosphinothricin *N*-acetyl transferase (PAT) gene, isolated from *Streptomyces viridochromogenes* (Wohlleben et al 1988)
Intervening sequence	5383	5484	102	Sequence from plasmid pCRI2.1(Invitrogen Cat. No. K205001) and multiple cloning sites
AtuORF1	5485	6188	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)
Intervening sequence	6189	6416	228	Sequence from Ti plasmid C58 (Wood et al 2001, Zambryski et al 1982)

Feature Name	Feature Start	Feature Stop	Feature Size	Description
T-DNA border A	6417	6440	24	Transferring DNA sequences
intervening sequence	6441	6459	19	Sequence from Ti plasmid C58 (Wood et al 2001, Zambryski et al 1982)
T-DNA border A	6460	6483	24	Transferring DNA sequences
intervening sequence	6484	6770	287	Sequence from Ti plasmid pTi15955 (Barker et al 1983)
T-DNA border A	6771	6794	24	Transferring DNA sequences
		Plasmid Backbone Region		
Plasmid backbone sequences	6795	7173	379	Plasmid backbone sequences from RK2 plasmid (Stalker et al 1981)
Ori Rep	7174	8193	1020	Replication origin sequences from RK2 plasmid (Stalker et al 1981)
Plasmid backbone sequences	8194	8738	545	Plasmid backbone sequences from RK2 plasmid (Stalker et al 1981)
Trf A	8739	9887	1149	Plasmid replication sequences for Trf A protein from RK2 plasmid (Stalker et al 1981)
Plasmid backbone sequences	9888	11091	1204	Plasmid backbone sequences from RK2 plasmid (Stalker et al 1981)
Spec R	11092	11880	789	Sequences for Spectinomycin resistance gene (Fling et al 1985)
Plasmid backbone sequences	11881	12154	274	Plasmid backbone sequences for cloning

2.3 d Molecular Characterization

Characterization of DAS-8191Ø-7 cotton was conducted by Southern blot (Mo & Ring 2012) and DNA sequence analyses. The sequence of the insert in DAS-8191Ø-7 cotton was confirmed (Figure 5) and the genetic elements identified in DAS-8191Ø-7 cotton are provided in Table 2.

Results demonstrate that the transgene insert in DAS-8191Ø-7 cotton occurred as a single integration of the respective T-DNA regions from plasmid pDAB4468, including a single, intact copy of each of the *aad-12* and *pat* gene expression cassettes along with a RB7 MAR element. The transgene insert is stably integrated and inherited across breeding generations, and no plasmid backbone sequences are present in DAS-8191Ø-7 cotton.

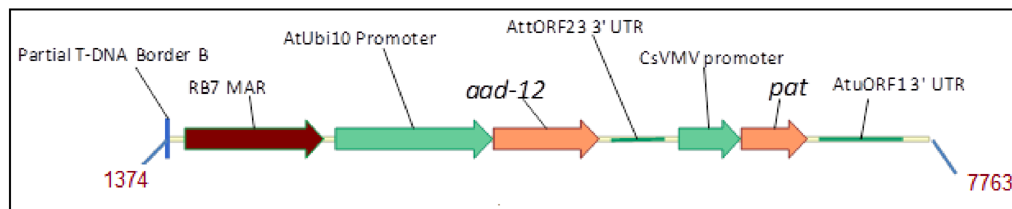


Figure 5. Diagram of Sequenced Transgene Insert in DAS-8191Ø-7 Cotton

Southern blot analyses were used to determine the copy and insertion number of the integrated DNA as well as the presence or absence of plasmid vector backbone sequences. The Southern analysis was designed to ensure that all potential transgenic segments from pDAB4468 would be identified in DAS-8191Ø-7 cotton. Locations of each probe on plasmid pDAB4468 are described in Figure 6 and Table 3 respectively.

Restriction enzymes were specifically chosen to fully characterise the transgene insert and detect any potential fragments of the T-DNA and backbone sequences in DAS-8191Ø-7 cotton. The expected and observed fragment sizes for DAS-8191Ø-7 cotton genomic DNA, generated by specific restriction enzyme and probe combinations, based on the known restriction enzyme sites of plasmid pDAB4468 and the intended T-DNA insert from pDAB4468, are shown in Figure 7 and Figure 8, respectively.

The Southern blot analyses described here consist of two types of DNA restriction fragments: a) internal fragments generated by known restriction enzyme recognition sites located within the T-DNA insert of pDAB4468, and b) border fragments generated by one known restriction enzyme recognition site located within the T-DNA insert and another site located in the cotton genome flanking the insert (Figure 8). Border fragment sizes vary by event because they rely on the location of the restriction enzyme recognition sites within the DNA sequence flanking the transgene insert. Since integration sites are unique for each event, border fragments provide a means to determine the number of transgene insertions and to specifically identify the event.

Table 2. Genetic Elements in DAS-8191Ø-7 Cotton

Feature Name	Feature Start	Feature Stop	Feature Length	Description
5' Flanking border	1	1373	1373	Cotton genomic DNA flanking the 5' end of the transgene insert in DAS-8191Ø-7 cotton
				Transgene Insert
Partial T-DNA Border B	1374	1375	2	Partial sequence from T-DNA Border Bs
Intervening sequence	1376	1511	136	Sequence from Ti plasmid pTi15955 (Barker et al 1983)
RB7-MAR	1512	2677	1166	Matrix attachment region (MAR) from *Nicotiana tobacum* (Hall et al 1991)
Intervening sequence	2678	2772	95	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
AtUbi10	2773	4094	1322	*Arabidopsis thaliana* polyubiquitin UBQ10 gene comprising the promoter, 5' untranslated region and intron (Norris et al 1993)
Intervening sequence	4095	4102	8	Sequence used for DNA cloning
aad-12	4103	4984	882	Synthetic, plant-optimized version of an aryloxyalkanoate dioxygenase from *Delftia acidovorans* (Wright et al 2009)
Intervening sequence	4985	5086	102	Sequence used for DNA cloning
AtuORF23	5087	5543	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)
Intervening sequence	5544	5657	114	Sequence from plasmid pENTR/D-TOPO (Invitrogen Cat. No. A10465) and multiple cloning sites
CsVMV	5658	6174	517	Promoter and 5' untranslated region derived from the cassava vein mosaic virus (Verdaguer et al 1996)
Intervening sequence	6175	6181	7	Sequence used for DNA cloning
pat	6182	6733	552	Synthetic, plant-optimized version of phosphinothricin *N*-acetyl transferase (PAT) gene, isolated from *Streptomyces viridochromogenes* (Wohlleben et al 1988)
Intervening sequence	6734	6835	102	Sequence from plasmid pCRI2.1 (Invitrogen Cat. No. K205001) and multiple cloning sites

Feature Name	Feature Start	Feature Stop	Feature Length	Description
AtuORF1	6836	7539	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)
Intervening sequence	7540	7763	224	Sequence from Ti plasmid C58 (Wood et al 2001, Zambryski et al 1982)
		Cotton Genomic DNA		
3′ Flanking border	7764	8834	1071	Cotton genomic DNA flanking the 3′ end of the transgene insert in DAS-8191Ø-7 cotton

Genomic DNA for Southern blot analysis was prepared from leaf material of individual DAS-8191Ø-7 cotton plants from five distinct breeding generations (Figure 2). Genomic DNA from leaves of non-transgenic variety Coker 310 was used as a control material. Plasmid DNA of pDAB4468 added to genomic DNA from the non-transgenic variety Coker 310 served as the positive control for Southern blot analysis. Materials and methods used for Southern analyses are further described in Appendix 1.

The expected restriction fragments of the inserted DNA are shown in Table 4 and Figure 8. Southern blot analysis results are shown in Figure 9 through Figure 35.

Table 3. List of Probes and their Positions in Plasmid pDAB4468

Probe Name	Position in pDAB4468	Length (bp)
RB7	25-1432	1408
AtUbi10	1433-2750	1318
aad-12	2752-3633	882
AtuORF23 3′ UTR	3607-4300	694
CsVMV	4301-4871	571
pat	4831-5382	552
AtuORF1 3′ UTR	5361-6411	1051
Ori	6412-8193	1782
Backbone2	8160-9887	1728
Backbone1	9857-11110	1254
SpecR	11092-24	1087

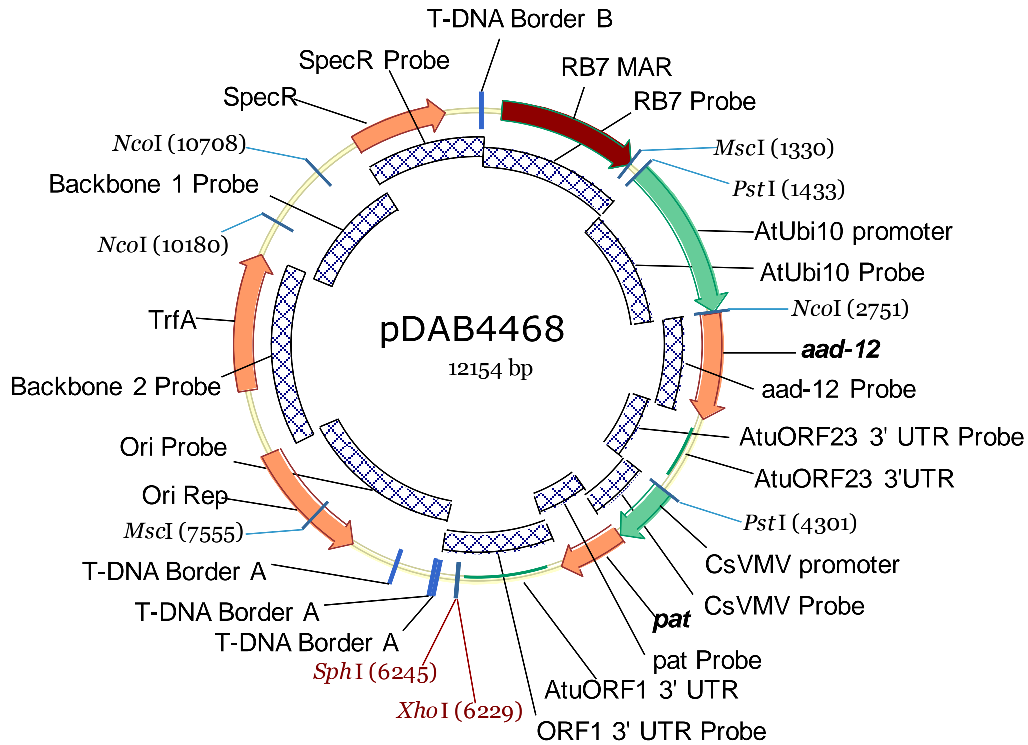


Figure 6. Probe Locations on pDAB4468 used in Southern Blot Analysis

The eleven probes described in Table 3 are shown as hashed boxes in the inner circle of the pDAB4468 plasmid map (outer circle).

Table 4. Predicted & Observed Sizes of Hybridizing Fragments in Southern Blot Analyses

Probe	Restriction enzyme	Sample	Lane	Expected fragment sizes (bp)[1]	Observed fragment sizes (bp)[2]	Figure
aad-12	*Nco*I	pDAB4468	2	7429	~7400	Figure 9
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>4043	~9500	
	*Sph*I	pDAB4468	2	12154	~12200	Figure 10
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
pat	*Nco*I	pDAB4468	2	7429	~7400	Figure 11
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>4043	~9500	
	*Sph*I	pDAB4468	2	12154	~12200	Figure 12
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
AtUbi10	*Msc*I	pDAB4468	2	6225	~6200	Figure 13
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>5464	~15000	
	*Sph*I	pDAB4468	2	12154	~12200	Figure 14
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
AtuORF23 3'UTR	*Nco*I	pDAB4468	2	7429	~7400	Figure 15
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>4043	~9500	
	*Sph*I	pDAB4468	2	12154	~12200	Figure 16
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
CsVMV	*Nco*I	pDAB4468	2	7429	~7400	Figure 17
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>4043	~9500	
	*Sph*I	pDAB4468	2	12154	~12200	Figure 18
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
AtuORF1 3'UTR	*Nco*I	pDAB4468	2	7429	~7400	Figure 19
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>4043	~9500	

	*Sph*I	pDAB4468	2	12154	~12200	Figure 20
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
aad-12	*Pst*I (Release *aad-12* expression cassette)	pDAB4468	2	2868	~2900	Figure 21
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	2868	~2900	
AtUbi10		pDAB4468	2	2868	~2900	Figure **22**
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	2868	~2900	
AtORF23 3'UTR		pDAB4468	2	2868	~2900	Figure 23
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	2868	~2900	
pat	*Pst*I/*Xho*I (Release *pat* expression cassette)	pDAB4468	2	1928	~1900	Figure 24
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	1928	~1900	
CsVMV		pDAB4468	2	1928	~1900	Figure 25
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	1928	~1900	
AtuORF1 3'UTR		pDAB4468	2	1928	~1900	Figure 26
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	1928	~1900	
RB7	*Sph*I	pDAB4468	2	12154	~12200	Figure 27
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	>6245	~7000	
Ori	*Msc*I	pDAB4468	2	5929, 6225	~5900, ~6200	Figure 28
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	
	*Pst*I	pDAB4468	2	9286	~9300	Figure 29
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	
Backbone 2	*Msc*I	pDAB4468	2	5929	~5900	Figure 30
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	
Backbone 2	*Pst*I	pDAB4468	2	9286	~9300	Figure 31
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	

Backbone 1	*Msc*I	pDAB4468	2	5929	~5900	Figure 32
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	
	*Pst*I	pDAB4468	2	9286	~9300	Figure 33
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	
SpecR	*Msc*I	pDAB4468	2	5929	~5900	Figure 34
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	
	*Pst*I	pDAB4468	2	9286	~9300	Figure 35
		Coker 310	3	none	none	
		DAS-8191Ø-7	4-18	none	none	

[1]Expected fragment sizes are based on the plasmid map of pDAB4468 (Figure 7) and the intended T-DNA insert in DAS-8191Ø-7 cotton Figure 8

[2]Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labelled DNA Molecular Weight Marker fragments. Due to the incorporation of DIG molecules for visualization, the Marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

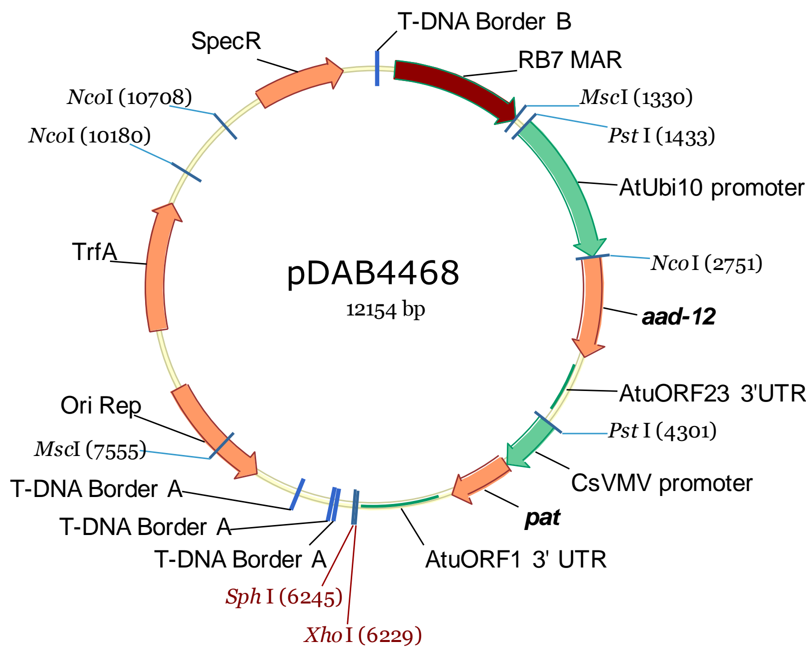
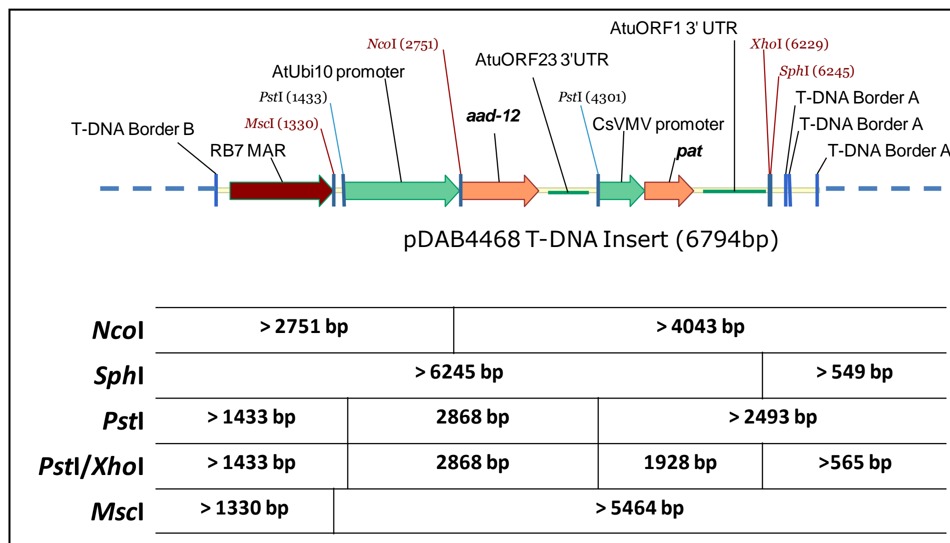


Figure 7. **pDAB4468 Showing Location of Restriction Enzymes used for Southern Analysis**



Enzyme				
NcoI	> 2751 bp	> 4043 bp		
SphI	> 6245 bp	> 549 bp		
PstI	> 1433 bp	2868 bp	> 2493 bp	
PstI/XhoI	> 1433 bp	2868 bp	1928 bp	>565 bp
MscI	> 1330 bp	> 5464 bp		

Figure 8. T-DNA Insert Restriction Enzyme Map of DAS-8191Ø-7 Cotton

Top: Intended T-DNA insert map of DAS-8191Ø-7 cotton showing restriction enzymes used for DNA digestion. Dashed blue lines on either end of insert represent cotton genomic DNA flanking T-DNA insert. Bottom: table of restriction enzymes (left) showing expected Southern blot hybridization band sizes for each restriction enzyme (right). A greater than symbol (>) denotes *border fragments* in which the hybridization band size is expected to be greater than the indicted size shown.

(i) Analysis of the Insert and Its Genetic Elements

Number of Transgenic Insertion Sites & Copy Number

To determine the number of pDAB4468 transgenic insertion sites as well as the copy numbers of the transgenes in DAS-8191Ø-7 cotton, detailed Southern blot analysis was conducted on genomic DNA from five distinct breeding generations of DAS-8191Ø-7 cotton (Figure 2). Restriction enzymes *Nco*I, *Sph*I, and *Msc*I were chosen to determine the number of inserts in DAS-8191Ø-7 cotton since each restriction enzyme cuts only once in the T-DNA insert (Figure 8) and at an undefined location in the cotton genome to generate a border fragment. Since integration sites are unique for each event, border fragments provide a means to determine the insertion and copy numbers to specifically identify the event.

To determine insertion and copy number, probes specific to the T-DNA insert (*aad-12*, *pat*, AtUbi10 promoter, AtuORF23 3′ UTR, CsVMV promoter, AtuORF1 3′UTR, and RB7 MAR) were used to screen Southern blots containing digested DAS-8191Ø-7 cotton genomic DNA.

When genomic DNA from DAS-8191Ø-7 cotton was digested with *Nco*I and hybridized with the *aad-12*, *pat,* ORF23, CsVMV, and AtuORF1 3′ UTR probes a single hybridization band of >4043 bp was expected (Figure 8). As anticipated, all DAS-8191Ø-7 cotton samples displayed a single band of ~9500 bp, consistent with the expected result of >4043 bp (Figure 9, Figure 11, Figure 15, Figure 17, Figure 19).

When digested with *Msc*I and hybridized with the AtUbi10 promoter probe, all DAS-8191Ø-7 cotton samples displayed a single band of ~15000 bp, consistent with the expected result of >5464 bp (Figure 8, Figure 13).

When digested with *Sph*I and hybridized with *aad-12*, *pat*, AtUbi10, ORF23 3′ UTR, CsVMV, and AtuORF1 3′ UTR and RB7probes, all DAS-8191Ø-7 cotton samples displayed a single band of ~7000 bp, consistent with the expected result of >6245 bp (Figure 8, Figure 10, Figure 12, Figure 14, Figure 16, Figure 18, Figure 20 and Figure 27).

In addition to DAS-8191Ø-7 cotton DNA, genomic DNA from leaves of non-transgenic variety Coker 310 was used as a negative control material. Plasmid DNA of pDAB4468 added to genomic DNA from the non-transgenic variety Coker 310 served as the positive control for Southern blot analysis. As expected, specific hybridization bands were detected in all of the positive control samples at the expected sizes for each restriction enzyme and probe combinations tested (Figure 9 - Figure 20 and Figure 27). For negative control plants, in which genomic DNA from non-transgenic variety Coker 310 was used, no hybridization bands were detected, as expected (Figure 9 - Figure 20 and Figure 27).

These data confirm that DAS-8191Ø-7 cotton contain a single integration and a single copy of the respective T-DNA sequence from pDAB4468 plasmid (including the AtUbi10 promoter, *add-12*, AtuORF23 3' UTR, CsVMV promoter, *pat*, AtuORF1 3' UTR along with the RB7 MAR element).

Structure of the Insert and Genetic Elements

To further characterize the structure of transgene insert and confirm that DAS-8191Ø-7 cotton contains a single intact copy of the *aad-12* expression cassette (AtUbi10 promoter, *add-12*, AtuORF23 3' UTR) and *pat* expression cassette (CsVMV promoter, *pat*, AtuORF1 3' UTR), DAS-8191Ø-7 cotton genomic DNA was digested with *Pst*I and *Pst*I/*Xho*I restriction enzymes and hybridized with the cassette element-based probes. As shown in Figure 7 and Figure 8, *Pst*I allows for the release of the full length *aad-12* expression cassette, while *Pst*I/*Xho*I allows for the release of the full length *pat* expression cassette.

Southern blot analysis was conducted on genomic DNA from five distinct breeding generations of DAS-8191Ø-7 cotton (Figure 2). When digested with *Pst*I and separately hybridized with the *aad-12*, AtUbi10, and AtuORF23 3' UTR probes, all DAS-8191Ø-7 cotton samples along with the positive control displayed a single band of ~2900 bp, consistent with the predicted size of 2868 bp for the *aad-12* expression cassette (Figure 8, Figure 21, Figure 22, and Figure 23). These data indicate that an intact *aad-12* expression cassette is present in all tested generations of DAS-8191Ø-7 cotton.

When digested with *PstI/XhoI* and separately hybridized with the *pat* probe, CsVMV, and AtuORF1 3' UTR probes, all DAS-8191Ø-7 cotton samples along with the positive control displayed a single band of ~1900 bp, consistent with the predicted size of 1928 bp for *pat* expression cassette (Figure 8, Figure 24, Figure 25, and Figure 26). These data indicate that an intact *pat* expression cassette is present in all tested generations of DAS-8191Ø-7 cotton.

Hybridization bands of the expected sizes were detected in all positive samples, while no specific hybridization band was detected in the non-transgenic cotton samples; as expected (Figure 21 - Figure 26). The hybridization pattern is consistent across all generations with all the tested restriction enzyme and probe combinations.

Taken together, the Southern blot analyses reveal that DAS-8191Ø-7 cotton contains a single intact insert of *aad-12* expression cassette and the *pat* expression cassette.

(ii) Absence of Plasmid Backbone Sequences

To verify that no plasmid vector backbone sequences were inserted in DAS-8191Ø-7 cotton, four probes (Backbone 1, Backbone 2, *Ori*, and *Spec*R) covering the entire backbone region of pDAB4468 plasmid DNA were generated and hybridized to *Msc*I and *Pst*I digested DAS-8191Ø-7 cotton DNA samples.

When digested with *Msc*I and independently hybridized with Backbone 1, Backbone 2, and *Spec*R probes, it would be anticipated that the positive control samples would have a band of ~5900 bp, while

in the absence of plasmid backbone, DAS-8191Ø-7 cotton samples and the negative control would be expected to have no hybridization bands (Figure 8).

When digested with *Msc*I and hybridized with Backbone 1, Backbone 2, and *Spec*R probes, no specific hybridization bands were detected in any DAS-8191Ø-7 cotton samples, except for the positive controls. A single band of ~5900 bp was detected in the positive control sample, which was consistent with the predicted size of 5929 bp (Figure 30, Figure 32 and Figure 34).

When blots containing the same digested genomic DNA and hybridized with *Ori* probe, two expected bands at ~5900 and ~6200 bp were detected only in the positive control sample and not in the DAS-8191Ø-7 cotton samples (Figure 28). This is consistent with the expected fragment sizes of 5929 and 6225 bp, since the Ori probe binds to DNA sequences on both sides of *MscI* digested pDAB4468 plasmid DNA (Figure 6).

When digested with *Pst*I and hybridized with Backbone 1, Backbone 2, *Ori*, and *Spec*R probes, no specific hybridization bands were detected in any DAS-8191Ø-7 cotton samples, except for the positive controls. A single band of ~9300 bp was detected in positive control sample, consistent with the predicted size of 9286 bp (Figure 6, Figure 29, Figure 31, Figure 33 and Figure 35).

These Southern analysis data, along with the positive and negative control results confirm that no backbone sequences from pDAB4468 are incorporated into DAS-8191Ø-7 cotton. The spectinomycin resistance gene (SpecR) in plasmid pDAB4468 (Figure 3) was therefore not inserted into the genome of DAS-8191Ø-7.

(iii) Stability of the Insert Across Generations

All DAS-8191Ø-7 cotton Southern hybridization samples, across all five generations (T2, T3, T4, T5, and BC1F2 see Figure 2) revealed an intact, single copy *aad-12* expression cassette, *pat* expression cassette and RB7 MAR insertion. These data clearly show stable integration and inheritance of the intact, single copy transgene insert across multiple generations of DAS-8191Ø-7 cotton.

(iv) Southern Blot analysis Conclusions

Southern blot analysis confirms that DAS-8191Ø-7 cotton contains a single copy of the transgene insert from pDAB4468, which includes the two gene expression cassettes, *aad-12* and *pat*, along with a RB7 MAR element (Section 2.3 d(i)). No plasmid backbone sequences were detected in DAS-8191Ø-7 cotton (Section 2.3 d(ii)). The hybridization patterns across five generations of DAS-8191Ø-7 cotton (T2, T3, T4, T5, and BC1F2) were identical, indicating that the insert is stably integrated and inherited in the DAS-8191Ø-7 cotton genome (Section 2.3 d(iii)).

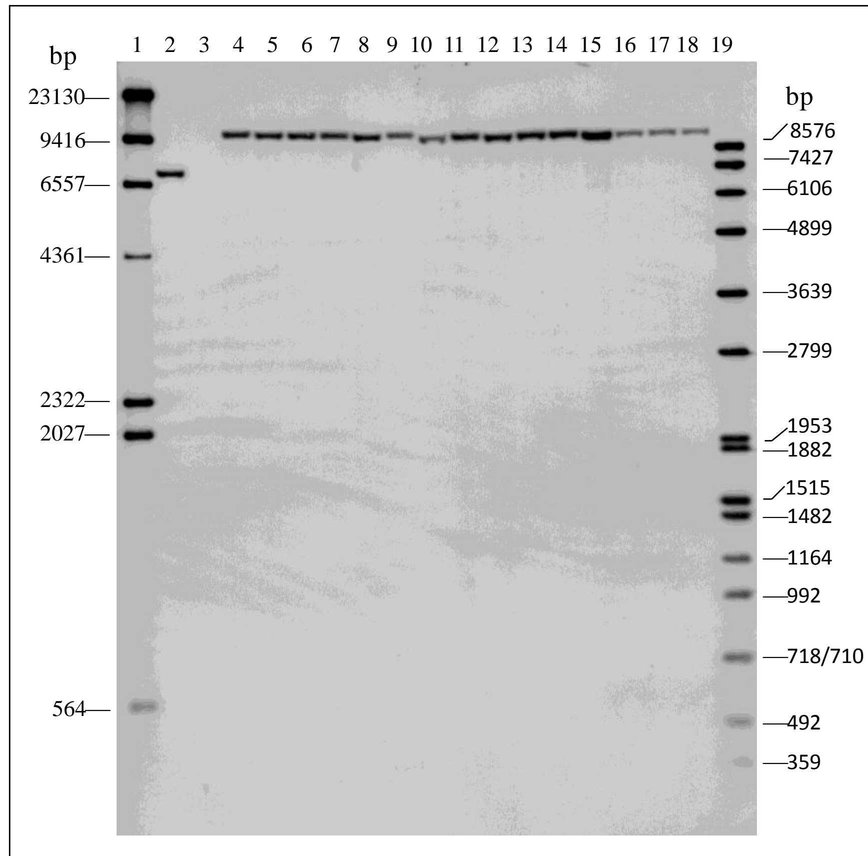


Figure 9. Southern analysis of DAS-8191Ø-7 cotton digested with *Nco*I; *aad-12* Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-03
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-03
6	DAS-8191Ø-7-T2-03	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-06
9	DAS-8191Ø-7-T3-03	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

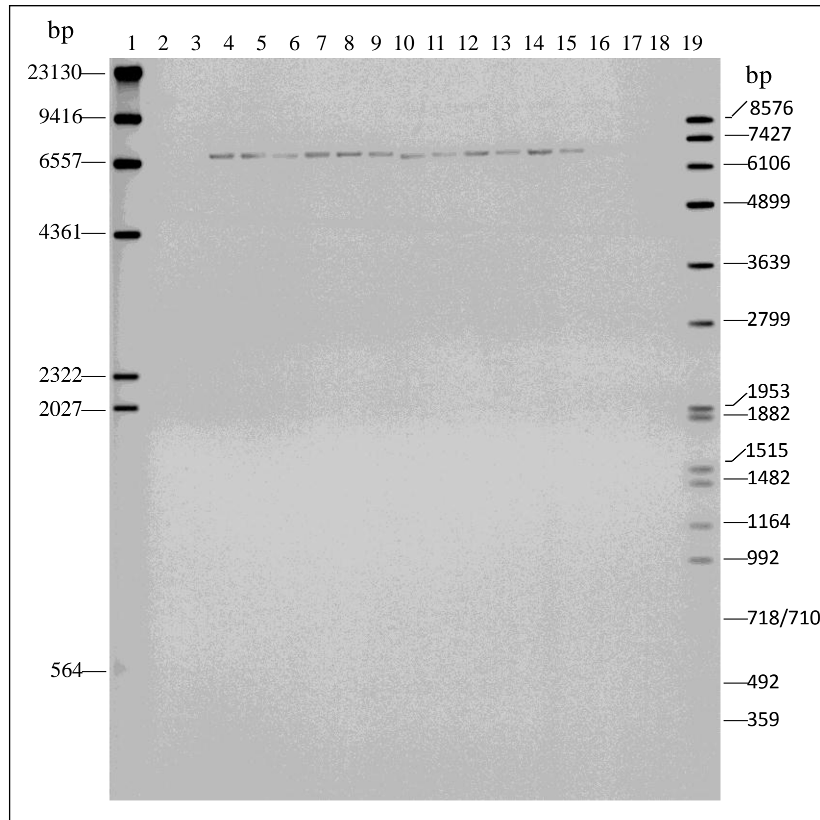


Figure 10. Southern analysis of DAS-8191Ø-7 cotton digested with *Sph*I; *aad-12* Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

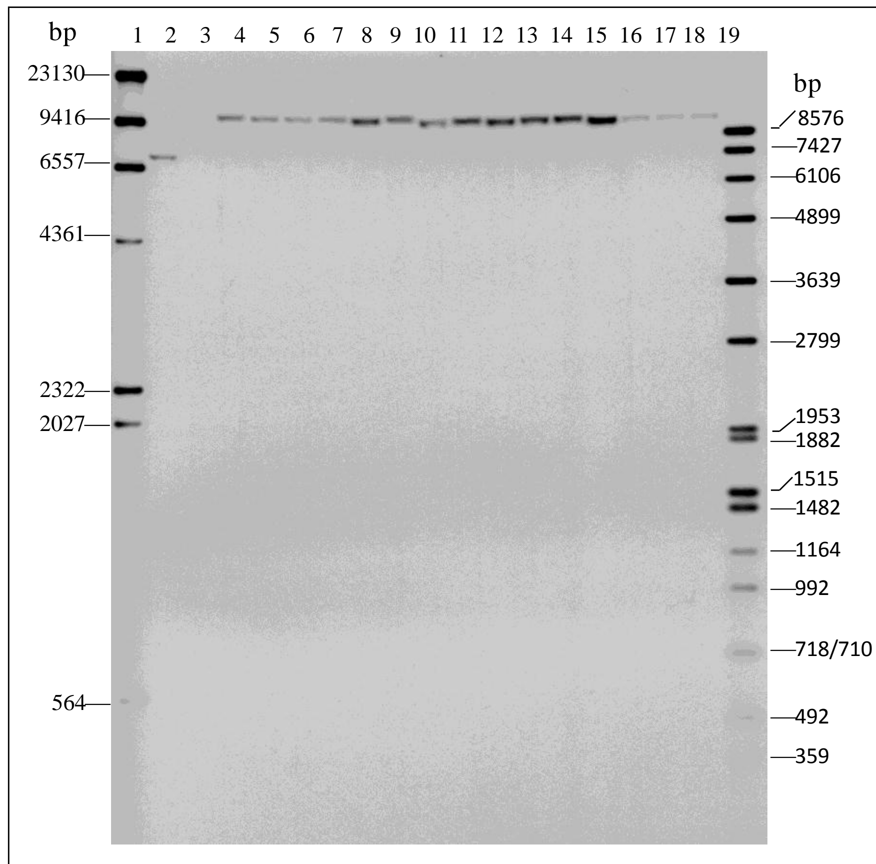


Figure 11. Southern analysis of DAS-81910-7 cotton digested with *Nco*I; *pat* Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81910-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-81910-7-T4-03
3	Coker 310	13	DAS-81910-7-T5-01
4	DAS-81910-7-T2-01	14	DAS-81910-7-T5-02
5	DAS-81910-7-T2-02	15	DAS-81910-7-T5-03
6	DAS-81910-7-T2-03	16	DAS-81910-7-BC1F2-04
7	DAS-81910-7-T3-01	17	DAS-81910-7- BC1F2-05
8	DAS-81910-7-T3-02	18	DAS-81910-7- BC1F2-06
9	DAS-81910-7-T3-03	19	DIG Molecular Weight Marker VII
10	DAS-81910-7-T4-01		

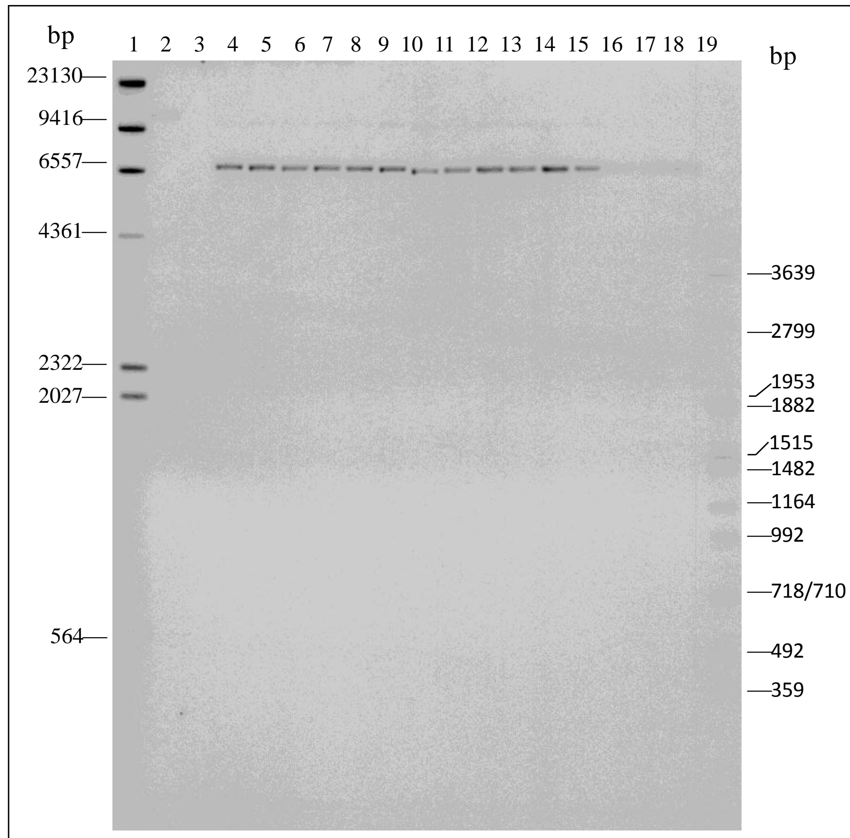


Figure 12. Southern analysis of DAS-8191Ø-7 cotton digested with *Sph*I; *pat* Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

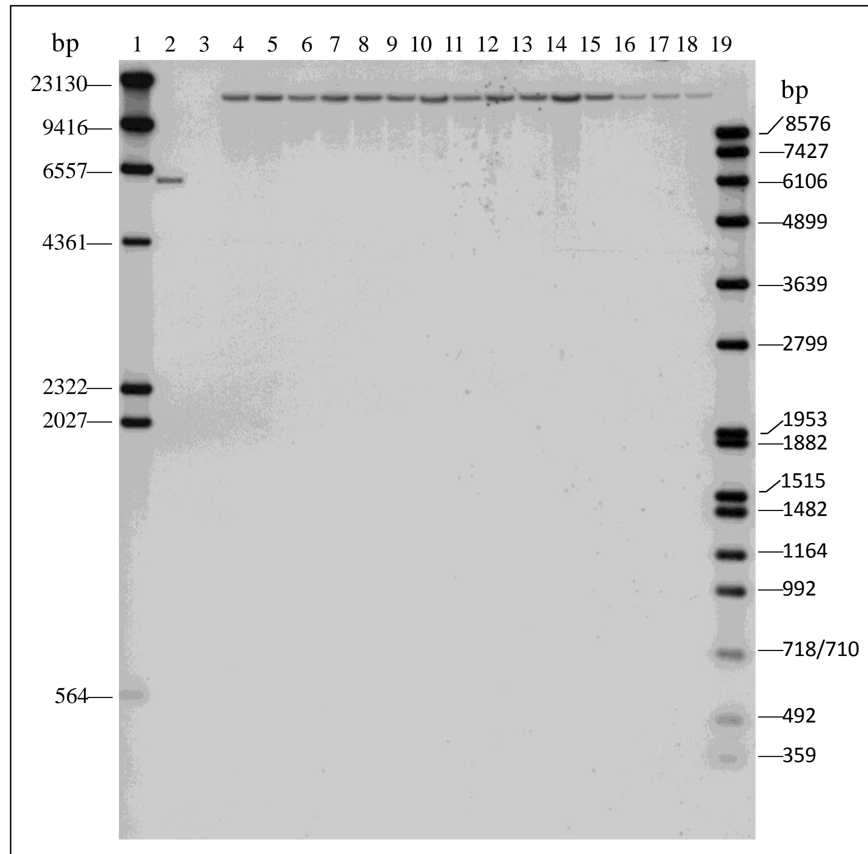


Figure 13. Southern analysis of DAS-8191Ø-7 cotton digested with *Msc*I; AtUbi10 Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

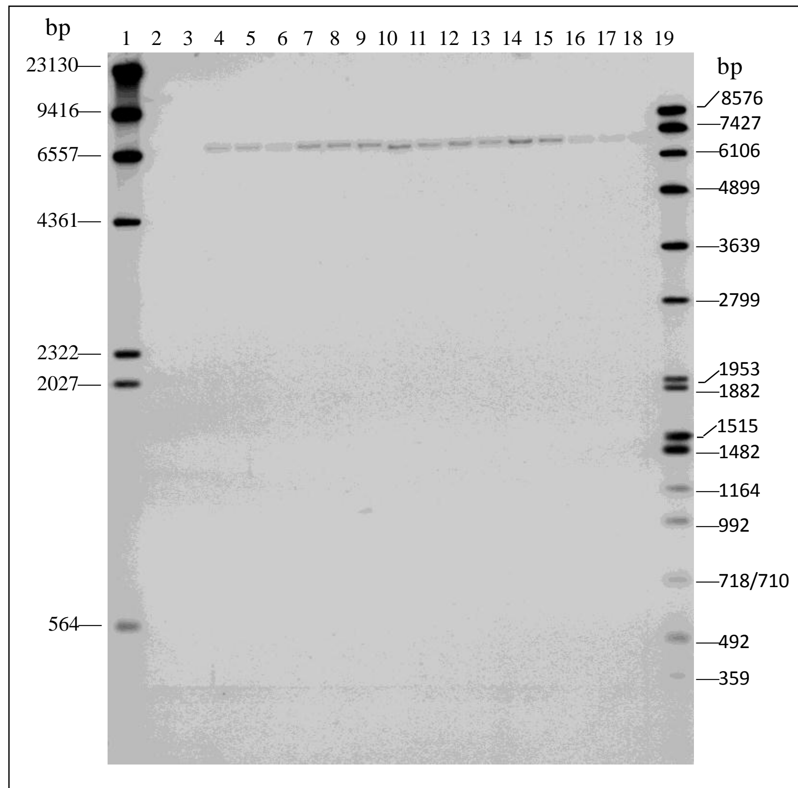


Figure 14. Southern analysis of DAS-81910-7 cotton digested with *Sph*I; AtUbi10 Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81910-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-81910-7-T4-05
3	Coker 310	13	DAS-81910-7-T5-01
4	DAS-81910-7-T2-01	14	DAS-81910-7-T5-03
5	DAS-81910-7-T2-03	15	DAS-81910-7-T5-04
6	DAS-81910-7-T2-04	16	DAS-81910-7-BC1F2-05
7	DAS-81910-7-T3-02	17	DAS-81910-7- BC1F2-06
8	DAS-81910-7-T3-03	18	DAS-81910-7- BC1F2-07
9	DAS-81910-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-81910-7-T4-01		

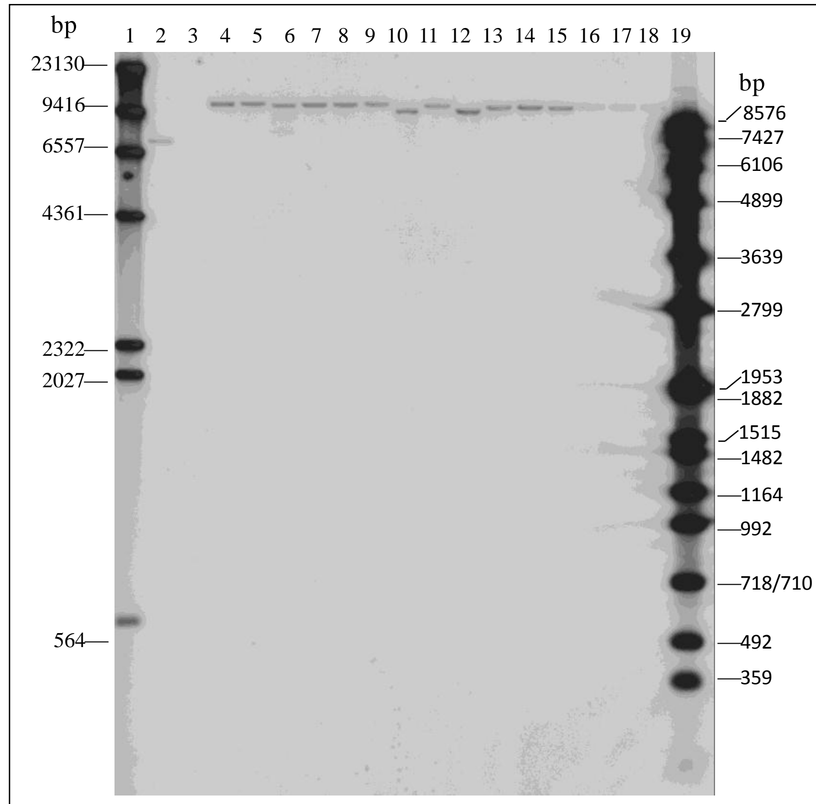


Figure 15. Southern analysis of DAS-8191Ø-7 cotton digested with *Nco*I; AtuORF23 3' UTR Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs. (*Note: Differential migration of hybridization bands in lanes 10 and 12 are attributable to minor impurities in DNA samples.)*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-03
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-03
6	DAS-8191Ø-7-T2-03	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-06
9	DAS-8191Ø-7-T3-03	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

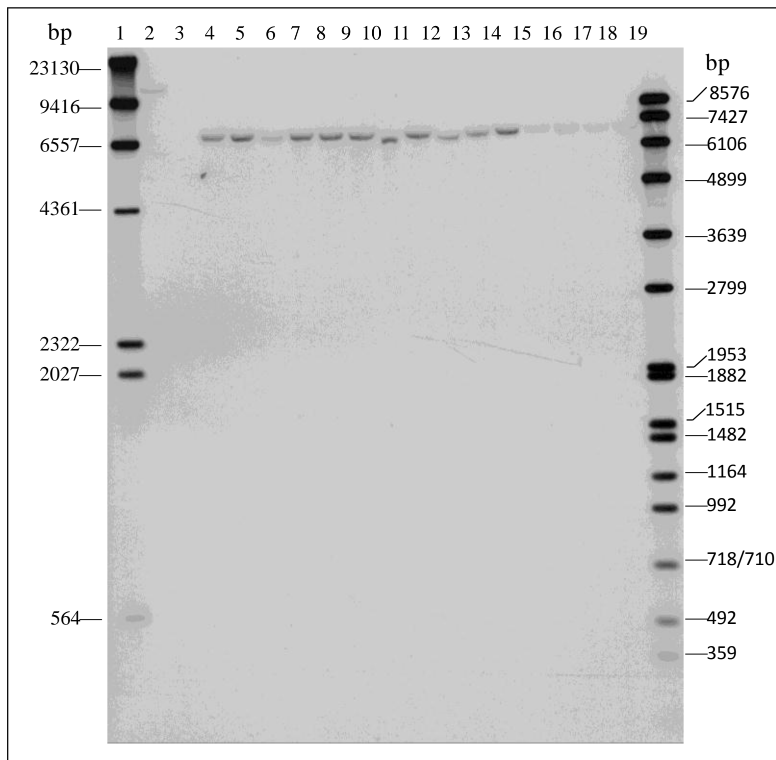


Figure 16. Southern analysis of DAS-81910-7 cotton digested with *Sph*I; AtuORF23 3' UTR Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs. (*Note: Differential migration of hybridization bands in lanes 10 and 12 are attributable to minor impurities in DNA samples)*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81910-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-81910-7-T4-05
3	Coker 310	13	DAS-81910-7-T5-01
4	DAS-81910-7-T2-01	14	DAS-81910-7-T5-03
5	DAS-81910-7-T2-03	15	DAS-81910-7-T5-04
6	DAS-81910-7-T2-04	16	DAS-81910-7-BC1F2-04
7	DAS-81910-7-T3-02	17	DAS-81910-7- BC1F2-05
8	DAS-81910-7-T3-03	18	DAS-81910-7- BC1F2-06
9	DAS-81910-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-81910-7-T4-01		

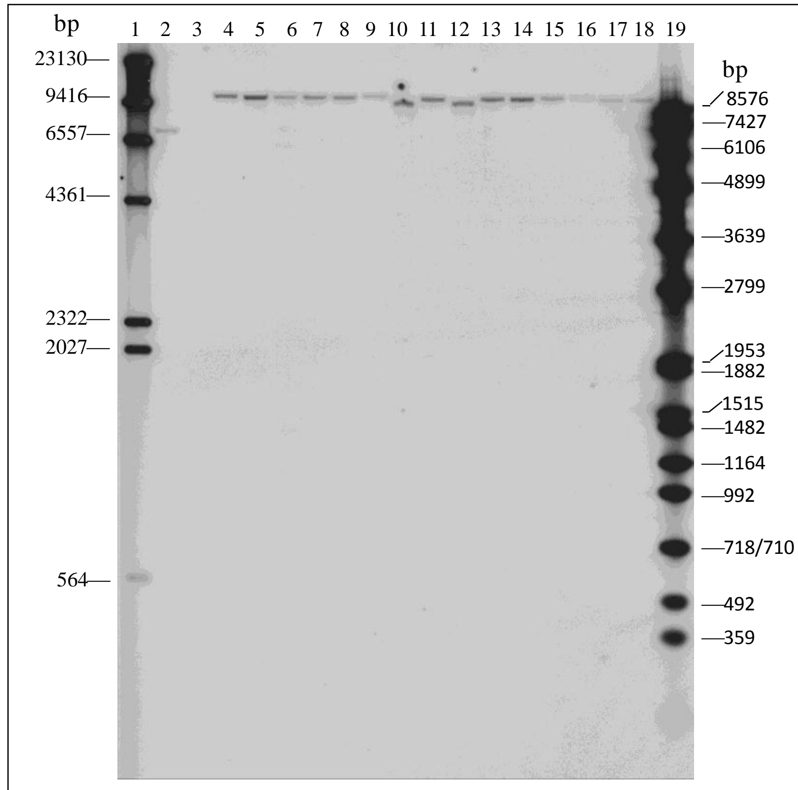


Figure 17. Southern analysis of DAS-8191Ø-7 cotton digested with *Nco*I; CsVMV Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs. (*Note: Differential migration of hybridization bands in lane 10 and 12 are attributable to minor impurities in DNA samples.)*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-03	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

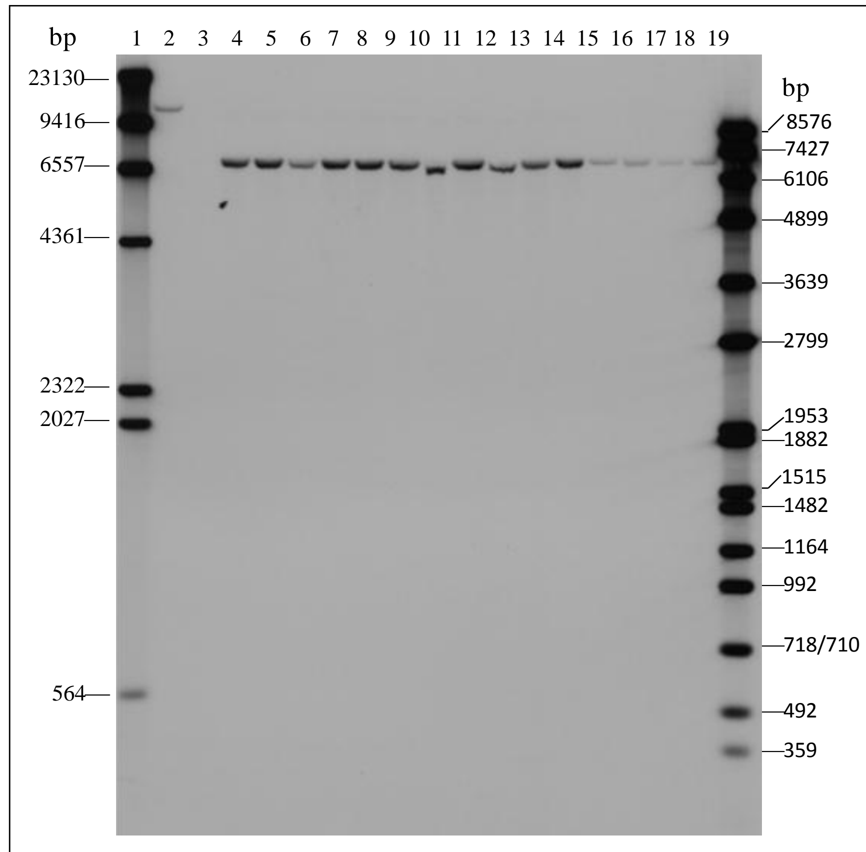


Figure 18. Southern analysis of DAS-8191Ø-7 cotton digested with *Sph*I; CsVMV Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

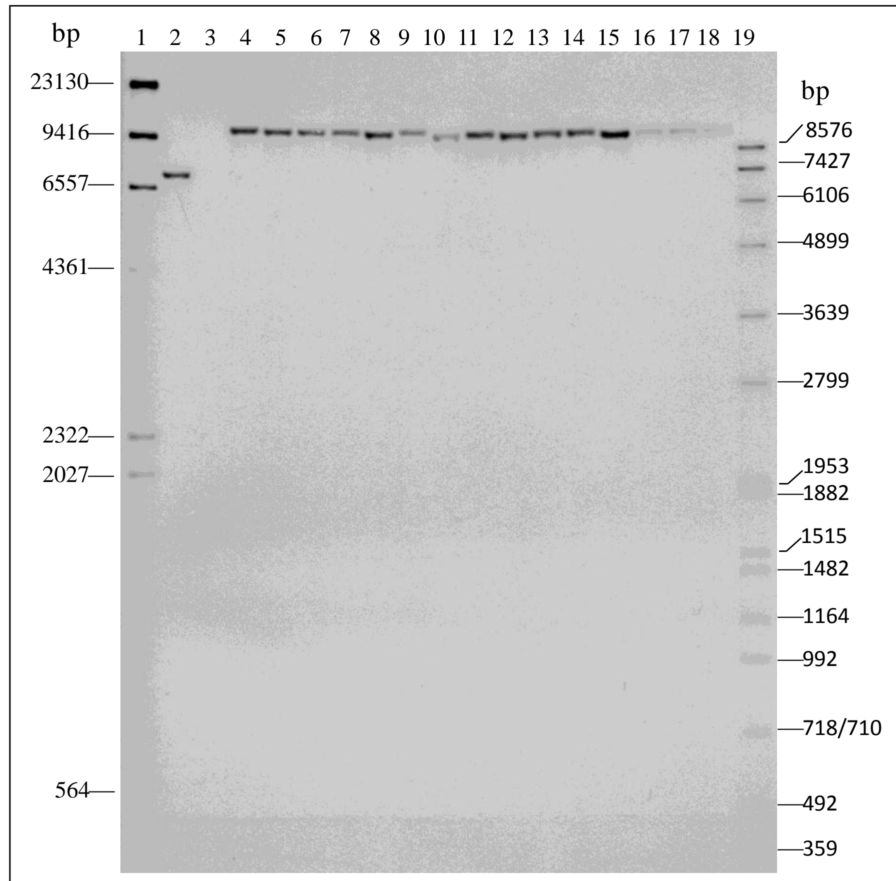


Figure 19. Southern analysis of DAS-8191Ø-7 cotton digested with *Nco*I; AtuORF1 3' UTR Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-03
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-03
6	DAS-8191Ø-7-T2-03	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-06
9	DAS-8191Ø-7-T3-03	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

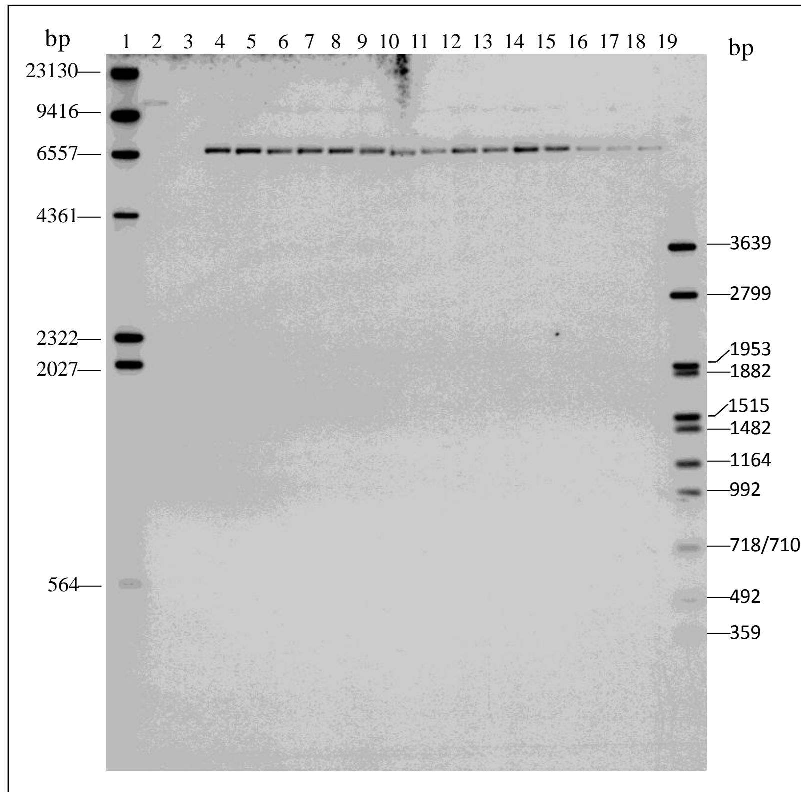


Figure 20. Southern analysis of DAS-8191Ø-7 cotton digested with *Sph*I; AtuORF1 3' UTR Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7 BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

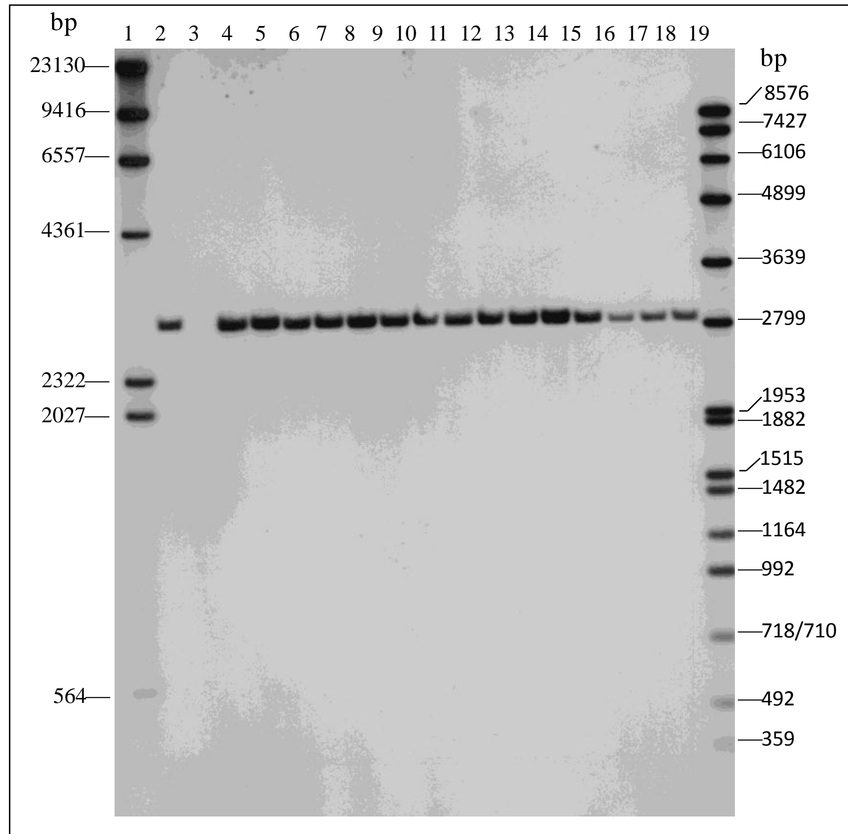


Figure 21. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I to release *aad-12* expression cassette; *aad-12* Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7-BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

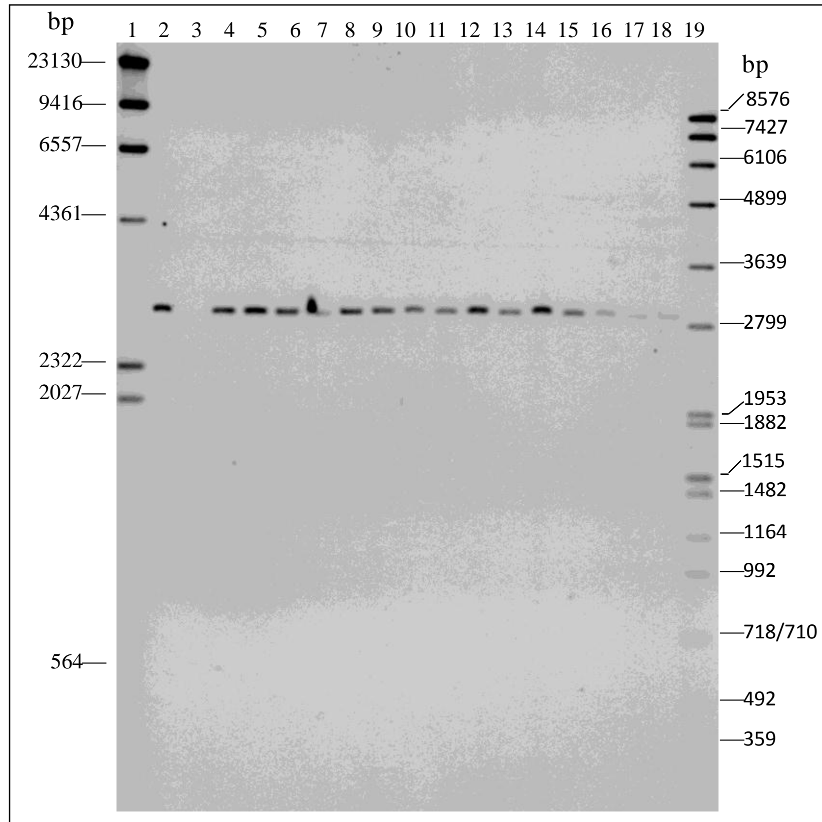


Figure 22. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I to release *aad-12* expression cassette; AtUbi10 Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7-BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

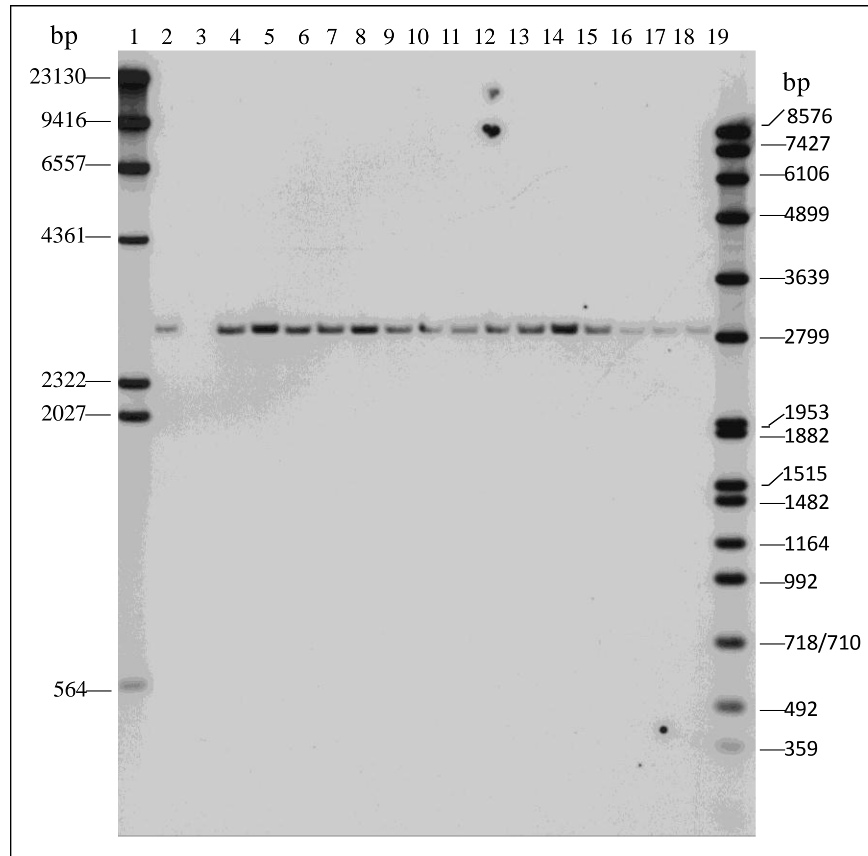


Figure 23. Southern analysis of DAS-81910-7 cotton digested with *Pst*I to release *aad-12* expression cassette; AtuORF23 3' UTR Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81910-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-81910-7-T4-05
3	Coker 310	13	DAS-81910-7-T5-01
4	DAS-81910-7-T2-01	14	DAS-81910-7-T5-02
5	DAS-81910-7-T2-02	15	DAS-81910-7-T5-04
6	DAS-81910-7-T2-04	16	DAS-81910-7-BC1F2-04
7	DAS-81910-7-T3-01	17	DAS-81910-7- BC1F2-05
8	DAS-81910-7-T3-02	18	DAS-81910-7- BC1F2-07
9	DAS-81910-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-81910-7-T4-01		

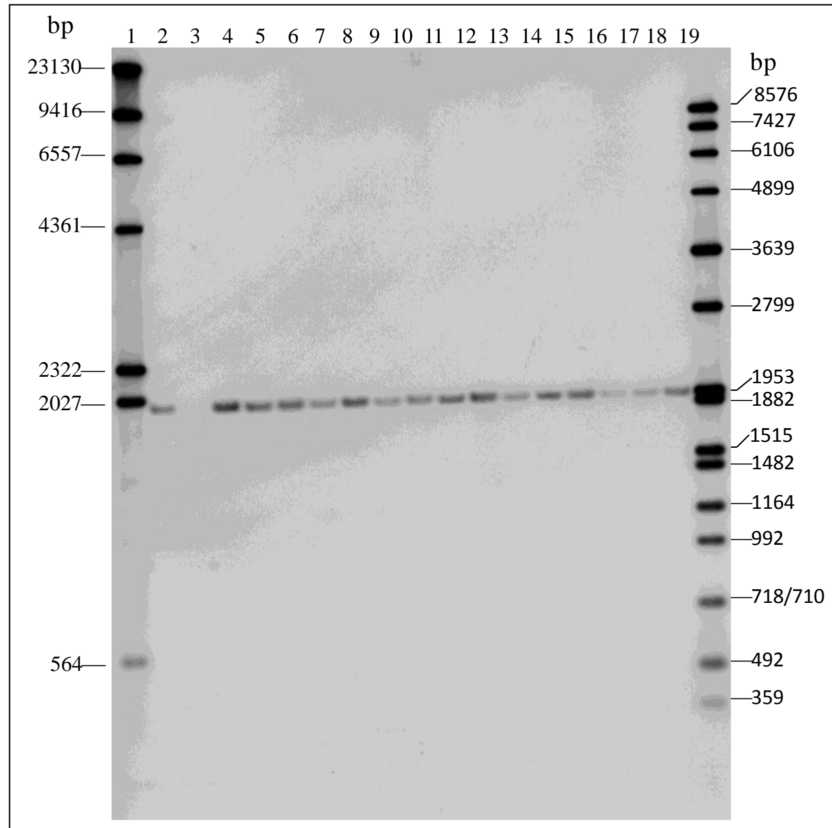


Figure 24. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I/*Xho*I to release *pat* expression cassette; *pat* Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7-BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

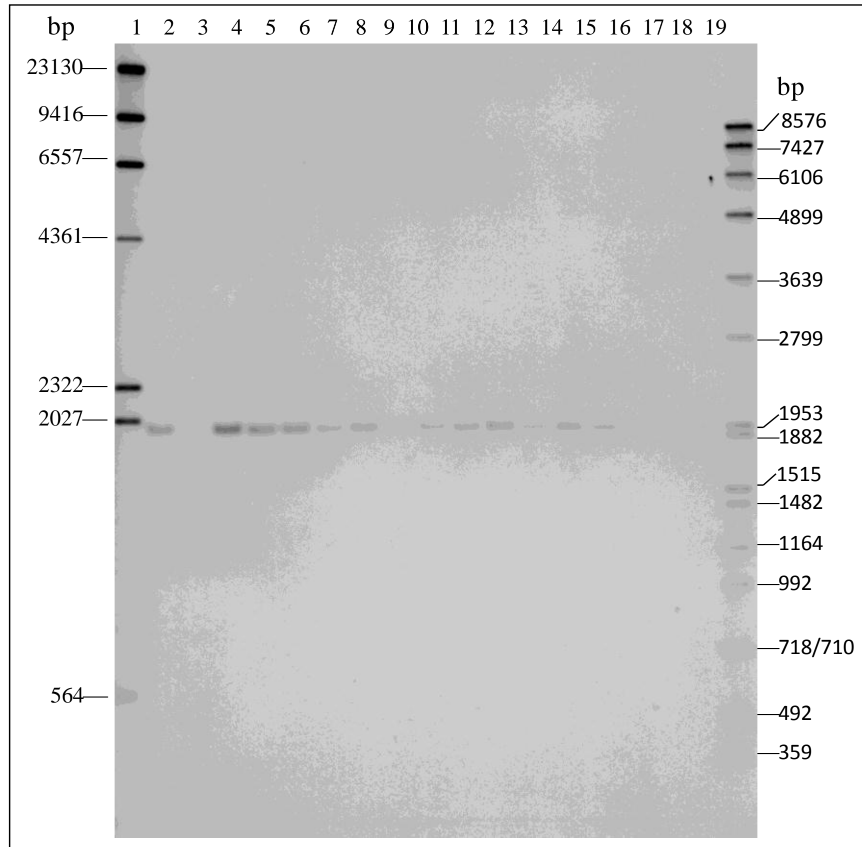


Figure 25. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I/*Xho*I to release *pat* expression cassette; CsVMV Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7- BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

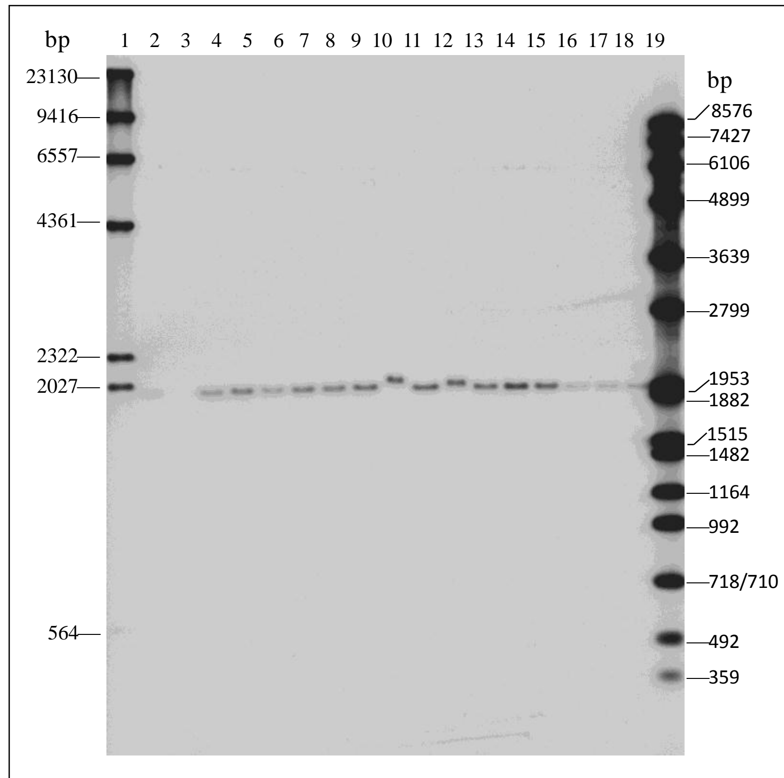


Figure 26. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I/*Xho*I to release *pat* expression cassette; AtuORF1 3' UTR Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs. *(Note: Differential migration of hybridization bands in lanes 10 and 12 are attributable to minor impurities in DNA samples.)*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7- BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7 -T3-02	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

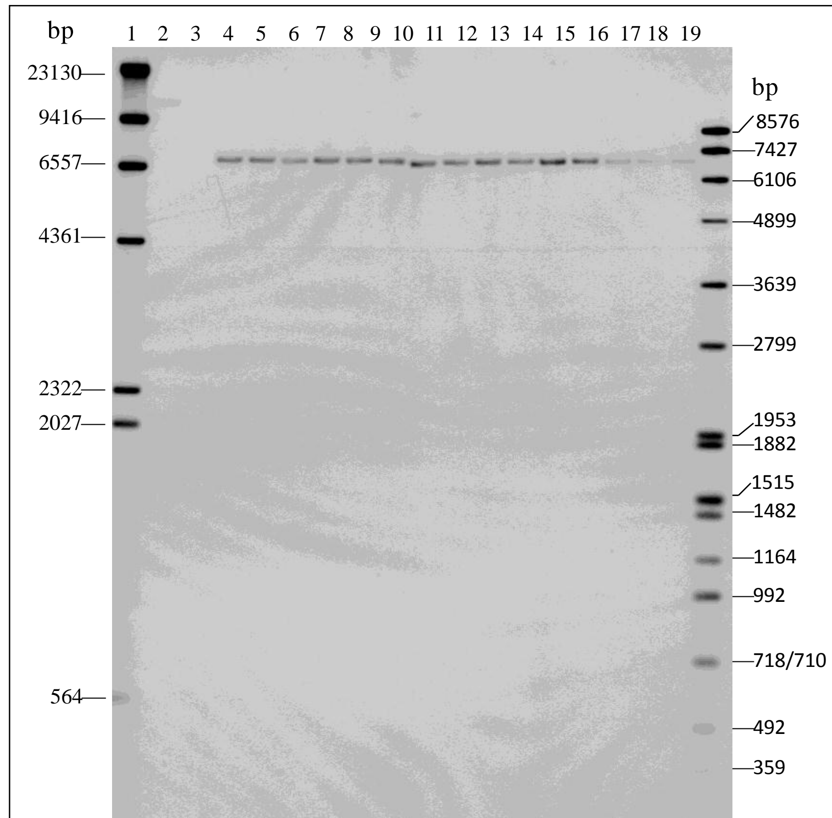


Figure 27. Southern analysis of DAS-8191Ø-7 cotton digested with *Sph*I; RB7 Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7- BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

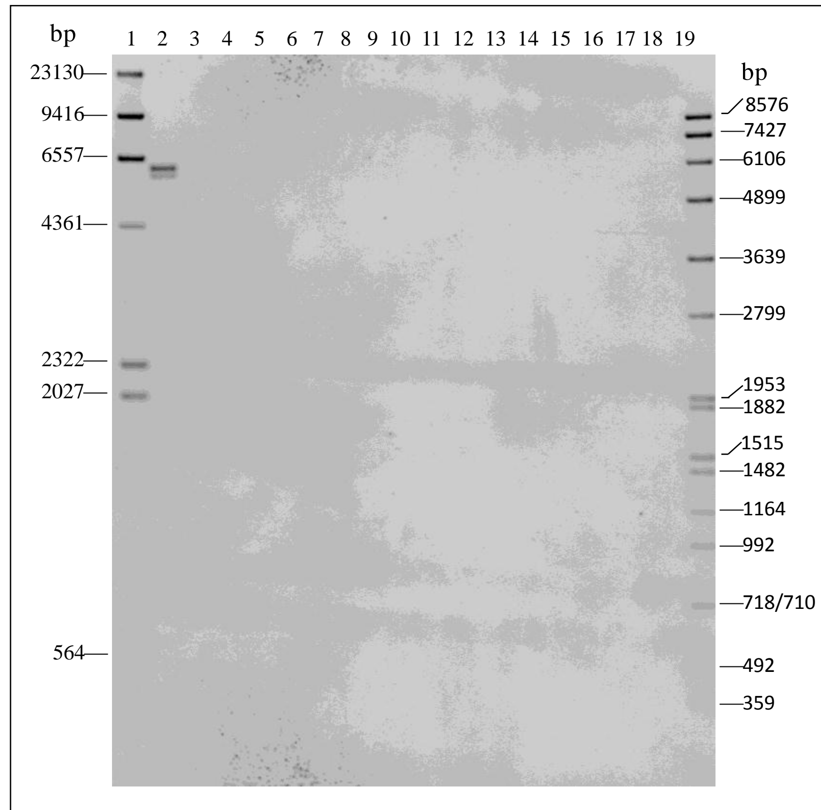


Figure 28. Southern analysis of DAS-8191Ø-7 cotton digested with *Msc*I; *Ori* Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7- BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

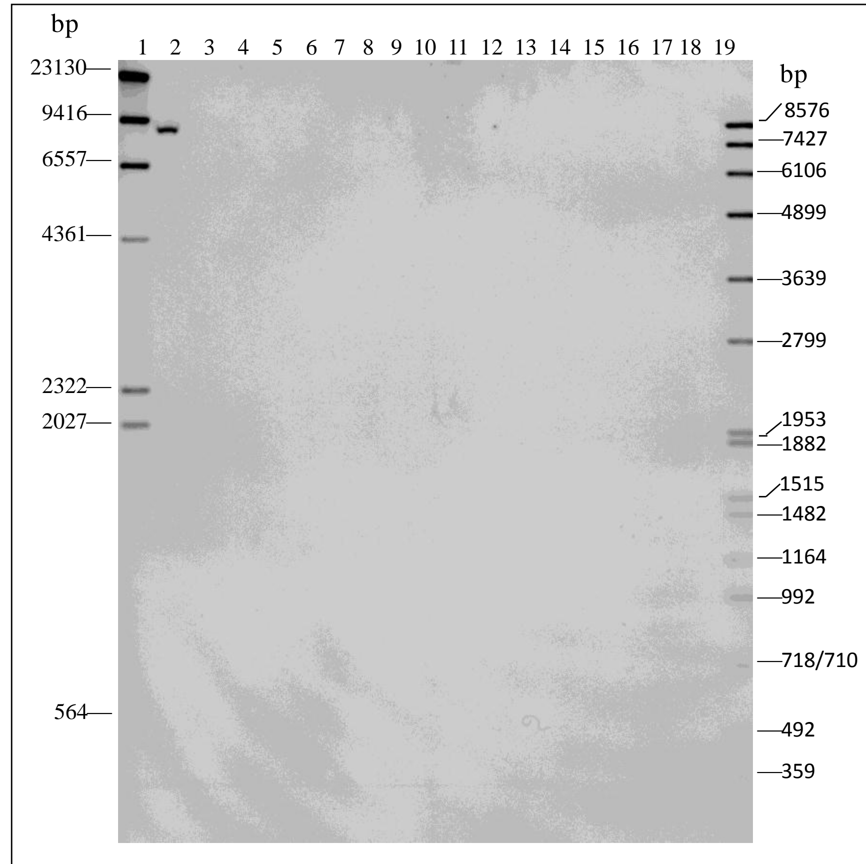


Figure 29. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I; Ori Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7- BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

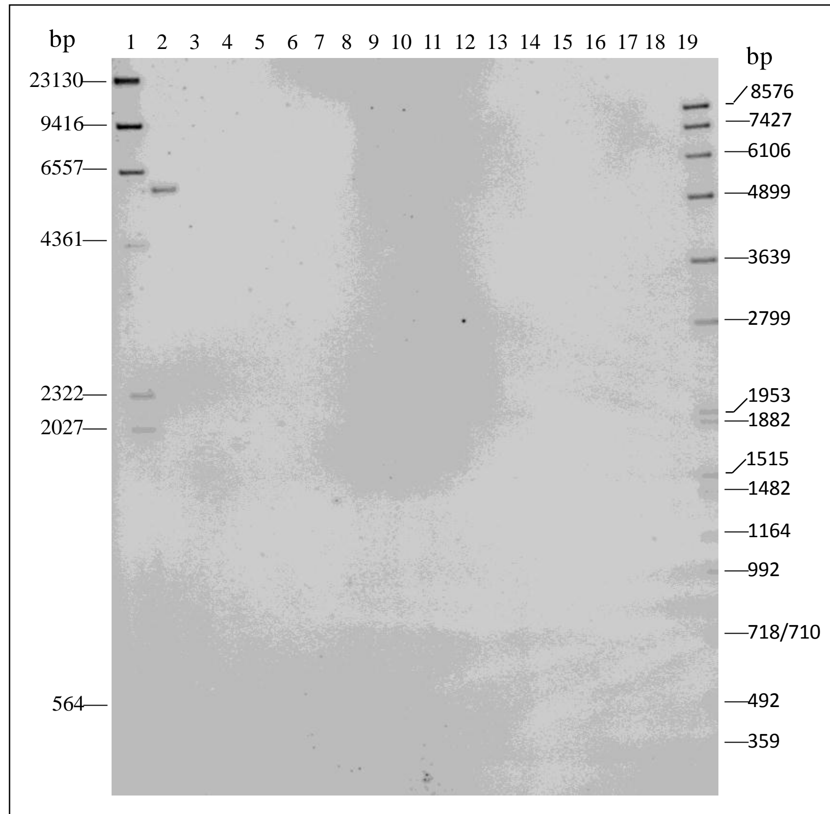


Figure 30. Southern analysis of DAS-8191Ø-7 cotton digested with *Msc*I; Backbone2 Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

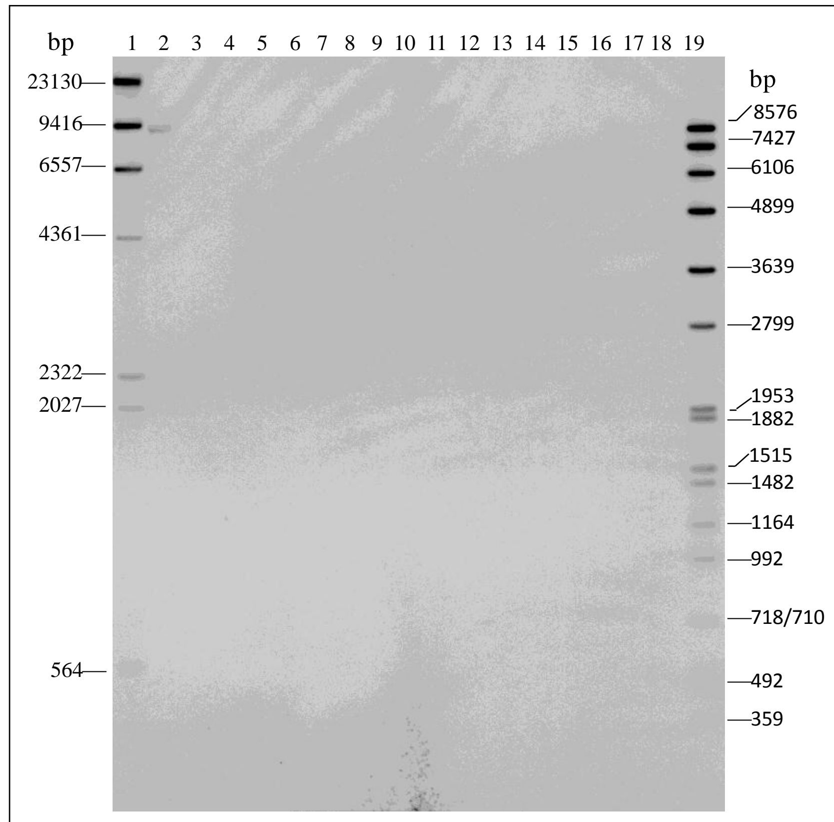


Figure 31. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I; Backbone2 Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

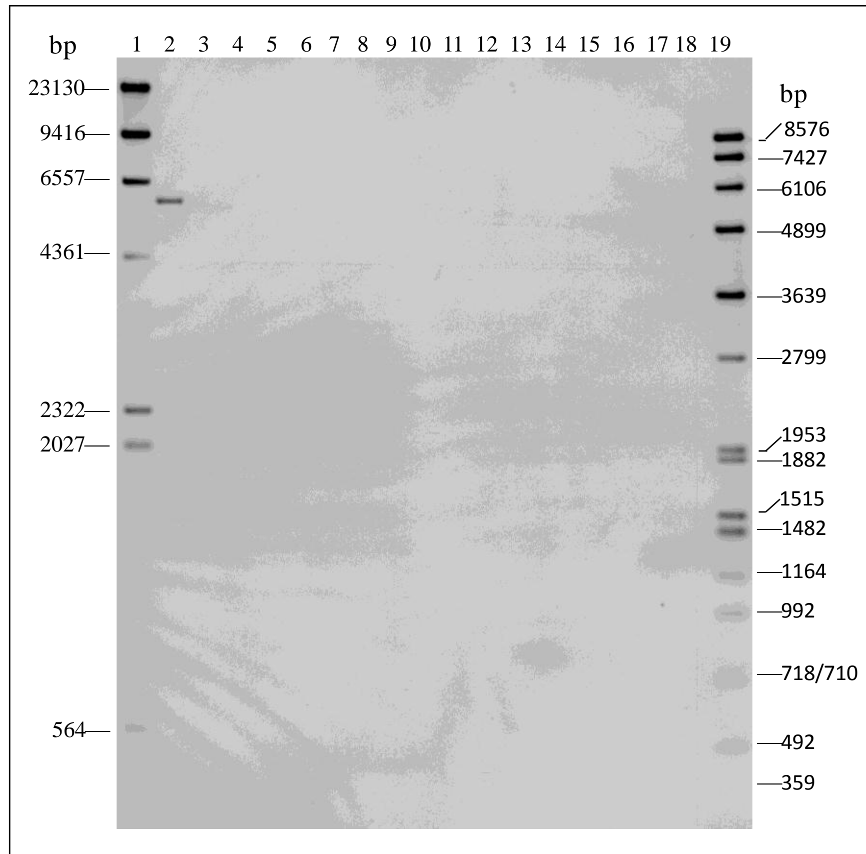


Figure 32. Southern analysis of DAS-8191Ø-7 cotton digested with *Msc*I; Backbone1 Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

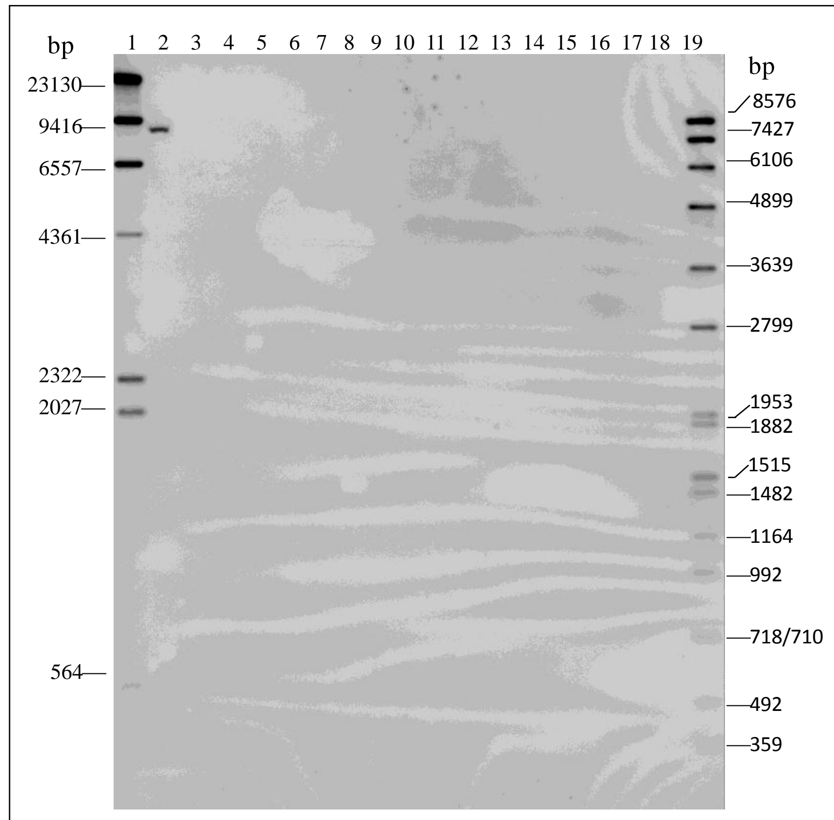


Figure 33. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I; Backbone1 Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

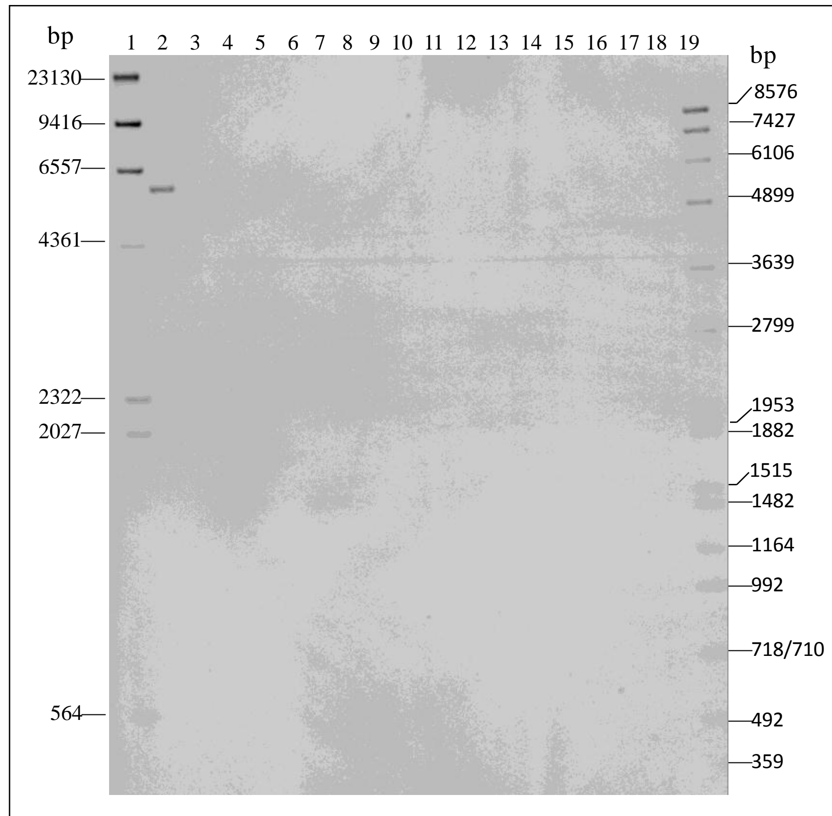


Figure 34. Southern analysis of DAS-8191Ø-7 cotton digested with *Msc*I; *Spec*R Probe

Approximately 10 μg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 μg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-03
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-03
5	DAS-8191Ø-7-T2-03	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-05
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-06
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

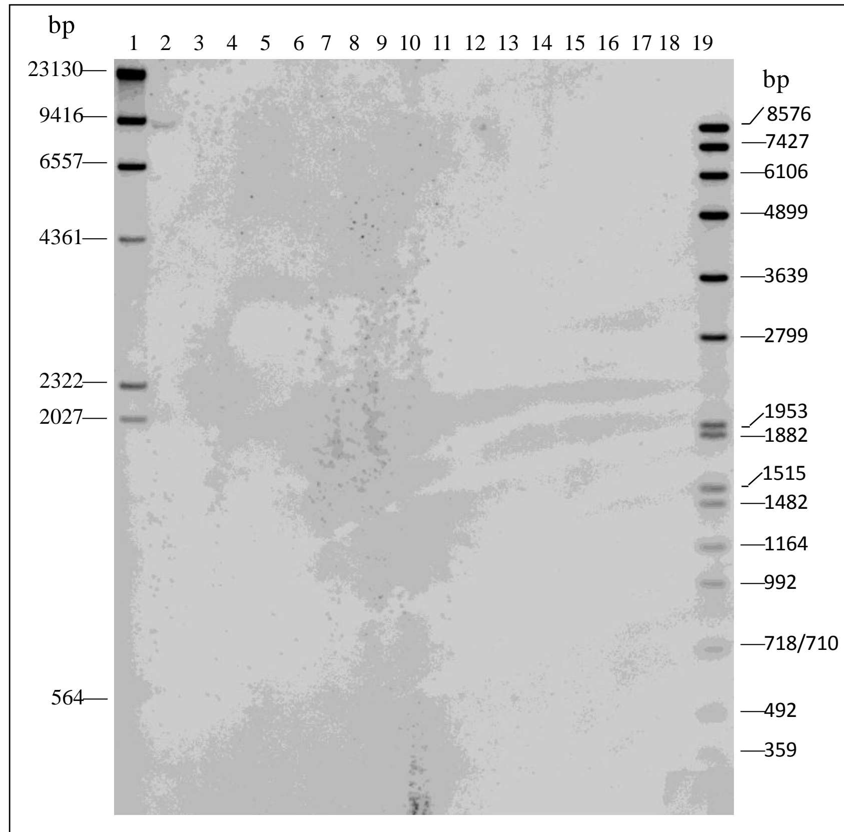


Figure 35. Southern analysis of DAS-8191Ø-7 cotton digested with *Pst*I; *Spec*R Probe

Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labelled DNA Molecular Weight Marker II and VII were indicated adjacent to membrane image. The last two digits in Sample Description indicate individual sample IDs.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-8191Ø-7-T4-02
2	Coker 310 + pDAB4468	12	DAS-8191Ø-7-T4-05
3	Coker 310	13	DAS-8191Ø-7-T5-01
4	DAS-8191Ø-7-T2-01	14	DAS-8191Ø-7-T5-02
5	DAS-8191Ø-7-T2-02	15	DAS-8191Ø-7-T5-04
6	DAS-8191Ø-7-T2-04	16	DAS-8191Ø-7-BC1F2-04
7	DAS-8191Ø-7-T3-01	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-02	18	DAS-8191Ø-7- BC1F2-07
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

2.3 e Stability of the genetic changes

(i) Genetic and Molecular Analysis of a Segregating Generation

DAS-8191Ø-7 cotton transgene insert resides at a single locus within the cotton genome and is inherited according to Mendelian inheritance principles. Chi-square goodness of fit analyses of trait inheritance data within a segregating generation was conducted to confirm the Mendelian inheritance of the transgene insert in DAS-8191Ø-7 cotton.

The inheritance pattern of the transgene insert within a segregating generation was demonstrated with Lateral Flow Strip (LFS) and event specific PCR analyses of individual plants within a BC_1F_2 generation of DAS-8191Ø-7 cotton (Figure 2).

A total of 136 plants from the BC_1F_2 generation of DAS-8191Ø-7 cotton were tested for AAD-12 protein expression by LFS assay. Of the 136 BC_1F_2 plants tested, 104 plants were positive and 32 were negative (segregated nulls) for AAD-12 protein expression. Genomic DNA was extracted from all these samples and followed by event-specific PCR analysis to determine the presence or absence of the DAS-8191Ø-7 transgene insert. Similarly, of the 136 plants tested by event-specific PCR, 104 plants were positive for the presence of DAS-8191Ø-7 transgene insert and the remaining 32 plants were negative (segregated null). All plants that tested positive for AAD-12 protein expression by LFS were also positive for the DAS-8191Ø-7 transgene insert by event-specific PCR analysis, and all plants that tested negative for AAD-12 protein expression were also negative for the presence of the DAS-8191Ø-7 transgene insert by event-specific PCR (Table 5).

This result confirmed that the phenotypic segregation matched the genotypic makeup of the tested plants in the $BC1F_2$ generation. Statistical analysis using a *chi*-square goodness of fit test indicated that the ratio of 104 positive to 32 null segregants did not significantly differ from the expected Mendelian 3:1 segregation pattern for a single independent locus (Rapier 2012).

Table 5. Segregating Generation Results of BC_1F_2 Individual Plants

Tested Method	Total plants tested	Positive	Negative	Expected ratio	P-value [a]
AAD-12 LFS	136	104	32	3:1	0.69
Event-Specific PCR	136	104	32	3:1	0.69

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

(ii) Segregation Analysis of Breeding Generations

Chi-square goodness of fit analyses of trait inheritance data from a population of T_1 and BC_1F_2 breeding generations was also conducted to determine the Mendelian inheritance of the transgene insert in DAS-8191Ø-7 cotton (Figure 2). The presence or absence of the transgene insert was determined using a PAT protein assay, a *pat* gene zygosity assay, or an *aad-12* gene zygosity assay for DAS-8191Ø-7 cotton. The expected segregation ratio of 3:1 for plants containing the transgene insert versus plants that do not contain the transgene insert (segregated nulls) was observed (Table 6). The observed segregation ratio does not significantly differ from the expected Mendelian 3:1 segregation pattern for a single independent locus. These results support the conclusion that DAS-8191Ø-7 transgene insert resides at a single locus within the cotton genome and is inherited according to Mendelian principles.

Table 6. Segregating Breeding Generation Results of DAS-8191Ø-7 Cotton Plants

Generation	Total Plants Tested	Tested Method	Positive	Null	Expected Ratio	P-value[a]
T1	191	PAT LFS	146	45	3:1	0.65
T1	196	*pat* gene zygosity	153	43	3:1	0.32
BC1F2	2571	*aad-12* gene zygosity	1916	655	3:1	0.58

[a] Based on a *chi*-square goodness of fit test

(iii) Stability of the Insert Across Generations

All DAS-8191Ø-7 cotton Southern hybridization samples, across all five generations (T2, T3, T4, T5, and BC1F2 see Figure 2) revealed an intact, single copy *aad-12* expression cassette, *pat* expression cassette and RB7 MAR insertion. These data clearly show stable integration and inheritance of the intact, single copy transgene insert across multiple generations of DAS-8191Ø-7 cotton.

Summary of the Genetic Characterization

Molecular characterization of DAS-8191Ø-7 cotton by Southern blot analysis confirmed that a single transgene insert containing each of the intact expression cassettes for *aad-12* and *pat*, along with the RB7 MAR element at the 5′ end, were integrated into DAS-8191Ø-7 cotton. No transformation plasmid backbone sequence was found in DAS-8191Ø-7 cotton as demonstrated by Southern blot analysis using probes covering the entire region of the plasmid flanking the T-DNA insert.

Southern blot analysis of five generations (T_2, T_3, T_4, T_5, and BC_1F_2) demonstrated the inserted DNA was stably inherited through multiple generations. Moreover, the transgene insert displayed the expected Mendelian inheritance pattern for a single independent insert/locus in segregating generations (T_1 and BC_1F_2), confirming that the transgene insert in DAS-8191Ø-7 cotton occurs at a single chromosomal locus with expected inheritance patterns.

2.4 Analytical Method for Detection

Theoharis NT, 2013. Determination of AAD-12 Protein in Cotton Tissues Using an Enzyme-Linked Immunosorbent Assay (ELISA).Study ID 120999, Dow AgroSciences, Indianapolis, IN

Smith-Drake JK,, 2011. Determination of phosphinothricin acetyltransferase (PAT) protein in cotton tissues and cottonseed processed products by enzyme-linked immunosorbent assay. GRM 07.26 Dow AgroSciences LLC. Indianapolis IN.

2.4 a Determination of AAD-12 Protein in Cotton Tissue Samples

ELISA method DAS 120999 was used to determine AAD-12 protein concentration in cotton tissue samples (Theoharis 2013).

The AAD-12 protein was extracted from cotton tissues except grain and pollen with a phosphate buffered saline solution containing 0.05% (v/v) Tween-20 (PBST), 0.75% ovalbumin (OVA) and 1.0% polyvinylpyrrolidone (PVP). For grain and pollen, the protein was extracted with a phosphate buffered saline solution containing 0.30% (v/v) Tween-20 (PBST), 0.75% OVA and 1.0% PVP.

The plant tissue and grain extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analysed using an AAD-12 ELISA kit. Briefly, an aliquot of the diluted sample and a horseradish peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate were incubated in the wells of a microtiter plate coated with an immobilized anti-AAD-12 polyclonal antibody. These antibodies bind with AAD-12 protein in the wells and form a "sandwich" with AAD-12 protein bound between soluble and the immobilized antibodies. The unbound samples and excess conjugate were then removed from the plate by washing with PBST. Subsequent addition of an enzyme substrate generated a coloured product. The reaction was stopped by adding a dilute acid solution.

Since the AAD-12 protein was bound in the antibody sandwich, the level of colour development, determined by measuring the absorbance of the solution, was related to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower colour development). The absorbance at 450 nm with a background subtraction at 650 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the AAD-12 concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analysed in duplicate wells with the average concentration of the duplicate wells being reported.

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

Tissue	AAD-12 (ng/mg)	
	LOD	LOQ
Bolls	0.5	1
Flower		
Leaf		
Pollen		
Root		
Seed		
Squares		
Whole Plant		

2.4 b Determination of PAT Protein in Cotton Tissue Samples

ELISA method GRM07.26 was used to determine PAT protein concentration in cotton tissue samples (Smith-Drake 2011).

The PAT protein was extracted from cotton tissues with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 1.0% polyvinylpyrrolidone (PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted with PBST/1.0% PVP if necessary, and analysed using a PAT ELISA kit. Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT monoclonal antibody and anti-PAT polyclonal antibodies coated in the wells of a 96-well plate in a sandwich ELISA format. At the end of the incubation period, the unbound reagents were removed from the plate by washing. Subsequent addition of an enzyme substrate generated a coloured product. The reaction was stopped by adding a dilute acid solution.

Since the PAT protein was bound in the antibody sandwich, the level of colour development, determined by measuring the absorbance of the solution, was related to the concentration of PAT in the sample (*i.e.*, lower residue concentrations result in lower colour development). The absorbance at 450 nm with a background subtraction at 650 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the PAT concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analysed in duplicate wells with the average concentration of the duplicate wells being reported.

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

Tissue	**PAT (ng/mg)**	
	LOD	LOQ
Bolls	0.025	0.06
Flower		
Leaf		
Pollen		
Root		
Seed		
Squares		
Whole Plant		

3. SAFETY OF THE GENETICALLY MODIFIED FOOD

3.1 Antibiotic Resistance Marker Genes

As described in section 2.3 d of this dossier, Southern blot analysis confirms that the expression cassette insertion into DAS-8191Ø-7 cotton does not contain any vector backbone from plasmid pDAB4468. Therefore the spectinomycin resistance gene (SpecR) in plasmid pDAB4468 (Figure 3) was not inserted into the genome of DAS-8191Ø-7.

3.1 a Clinical Relevance

Not applicable

3.1 b Therapeutic Efficacy

Not applicable

3.1 c Safety of the Gene Product

Please refer to section 3 *Safety of the Genetically Modified Food* in this application

3.1 d End Use Viability (micro-organisms)

Not applicable

3.2 Characterization of the Novel Proteins

Clement JM, Oman TJ, Juba AN, Singletary L, 2013. Characterization of the AAD-12 protein derived from transgenic cotton event DAS-8191Ø-7Study ID 110819, Dow AgroSciences LLC, Indianapolis, IN
Embrey SK, Juba AN, 2012. Certificate of Analysis for Phosphinothricin Acetyl Transferase (PAT, TSN303589) lyophilized protein standard Dow AgroSciences, LLC, Indianapolis, IN
Hill RC, 2013. Protein Expression of a Transformed Cotton Line Containing Aryloxyalkanoate Dioxygenase (AAD-12) and Phosphinothricin Acetyltransferase (PAT) - Event DAS-81910-7Study ID 120040.02, Dow AgroSciences LLC., Indianapolis IN
Karnoup A, Kuppannan K, 2008. Characterization of AAD-12: Batch TSN030732-002. ML-AL MD-2008-003833 The Dow Chemical Company. Midland, MI.
Oman TJ, Clement JM, Juba AN, Singletary LJ, 2013. Characterization of the Phosphinothricin Acetyltransferase (PAT) protein derived from transgenic cotton event DAS-8191Ø-7Study ID 120051, Dow AgroSciences, Indianapolis, IN

As described in Section 2.3 c , DAS-8191Ø-7 cotton contains the *aad-12* and *pat* expression cassettes which, when transcribed and translated, result in the expression of the AAD-12 and PAT proteins, respectively. This section summarizes: 1) the identity and mode of action of the AAD-12 and PAT proteins expressed in DAS-8191Ø-7 cotton; 2) demonstration of equivalence between the plant-produced and microbially-produced AAD-12 and PAT proteins, which were used in various protein safety studies; 3) the expression levels of AAD-12 and PAT proteins in DAS-8191Ø-7 plant tissues; 4) the assessment of the potential allergenicity and toxicity of AAD-12 and PAT proteins; 5) the food and feed safety assessment of AAD-12 and PAT proteins. Based on several lines of evidence described below, the data support a conclusion that these two proteins produced in DAS-8191Ø-7 cotton are safe for the environment and safe for human and animal consumption.

3.2 a Biochemical function and phenotypic effect of novel protein

(i) AAD-12

<u>Identity of the AAD-12 Protein</u>
The expressed DAS-8191Ø-7 cotton AAD-12 protein is comprised of 293 amino acids and has a molecular weight of 32 kDa (Figure 36). The wild-type aryloxyalkanoate dioxygenase (AAD-12) protein coding sequence was derived from *Delftia acidovorans*, a gram-negative soil bacterium (Wright et al 2010). The wild-type AAD-12 amino acid sequence and the DAS-8191Ø-7 cotton AAD-12 amino acid sequence (encoded by the plant-optimized *aad-1* gene (see section 2.3 c(i)) are greater than 99% identical, differing only by a single amino acid addition, alanine (A), at position number two (Figure 36). The additional alanine codon encodes part of an *Nco*I restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon of *aad-12*. 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 AAD-12 protein produced in DAS-8191Ø-7 cotton is expressed from the identical genetic *aad-12* sequence present in DAS-68416-4 soybean and DAS-444Ø6-6 soybean; these have been assessed and approved by FSANZ for applications A1046 and A1073, respectively.

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

Figure 36. **Amino Acid Sequence of Expressed AAD-12 in DAS-8191Ø-7**
DAS-8191Ø-7 cotton AAD-12 amino acid sequence was designed to have a single amino acid addition, alanine, at position number two (underlined in figure above) as compared to the native *D. acidovorans* AAD-12 amino acid sequence. See text for details.

Mode of Action of the AAD-12 Protein
Expression of the AAD-12 protein in transgenic crops has been shown to provide tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) by catalyzing the conversion of 2,4-D to 2,4-dichlorophenol (DCP), a herbicidally inactive compound, through an Fe(II)/α-keto acid-dependent dioxygenase reaction (Figure 37 and Figure 38) (Müller et al 1999, Westendorf et al 2002, Westendorf et al 2003, Wright et al 2009, Wright et al 2010).

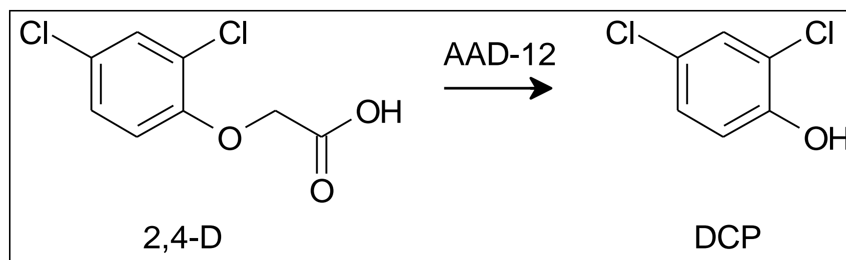


Figure 37. Degradation Reaction of 2,4-D Catalyzed by AAD-12

Enzyme Specificity
AAD-12 is able to degrade related achiral phenoxyacetate herbicides such as MCPA ((4-chloro-2-methylphenoxy) acetic acid) and pyridyloxyacetate herbicides such as triclopyr and fluroxypyr to their corresponding inactive phenols and pyridinols, respectively (Figure 38). 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 (Wright et al 2010).

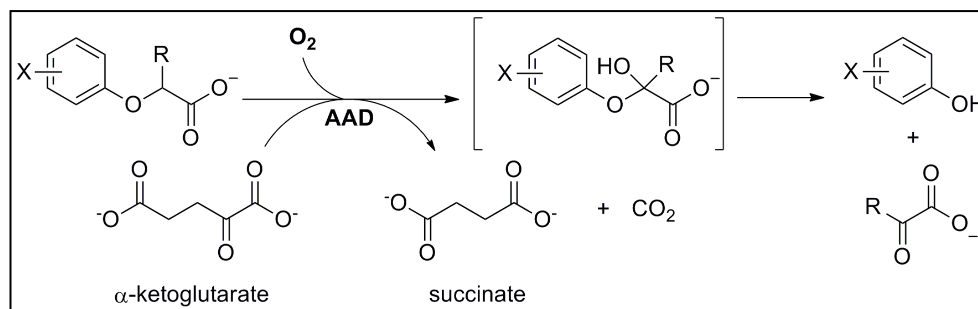


Figure 38. **General Reaction Catalyzed by AAD-12 (R=H or CH_3)**

The AAD-12 enzyme was screened for the ability to utilize endogenous plant substrates using a sensitive coupled *in vitro* enzyme assay (Luo et al 2006). Potential plant substrates were determined based on chemical structure, similar physiological function to known AAD-12 substrates, and abundance within primary/secondary metabolic pathways of plants. The substrates tested were separated into three groups; natural plant hormones (indole acetic acid, abscisic acid, gibberellin, and aminocyclopropane-1-carboxylate), phenylpropanoid intermediates (cinnamate, coumarate, and sinapate), and L-amino acids. 2,4-dichlorphenoxyacetic acid (2,4-D), the positive control substrate, showed a high level of activity in the enzyme assay. Under the same reaction conditions, the plant compounds identified and tested were not oxidized upon incubation with AAD-12, resulting in values at or below the background limit of detection (<3% positive control rate). Based on this survey of potential substrates, there is no indication that AAD-12 has activity on endogenous plant substrates.

Biochemical Characterisation 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, Raybould et al 2012), large quantities of AAD-12 protein was produced in *Pseudomonas fluorescens*. Characterization studies were performed to confirm the equivalency of the AAD-12 protein produced in *P. fluorescens* with the AAD-12 protein produced *in planta* in DAS-8191Ø-7 cotton. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycoprotein detection, enzymatic assay, and protein sequence analysis by matrix assisted laser desorption/ionization time-of-flight mass spectrometry/mass spectrometry (MALDI-TOF MS/MS) were used to characterize the biochemical properties of the proteins (Clement 2013, Karnoup & Kuppannan 2008). Using these methods, the AAD-12 protein isolated from *P. fluorescens* and DAS-8191Ø-7 cotton were shown to be biochemically and biologically equivalent, thereby supporting the use of the microbe-derived protein in safety assessment studies.

The methods and results of the biochemical characterization of the DAS-8191Ø-7 cotton and microbe-derived AAD-12 proteins are described in detail in Appendix 2 and in the attached reports (Clement

2013, Karnoup & Kuppannan 2008). Both the plant and *P. fluorescens*-derived AAD-12 proteins were observed at the expected molecular weight of 32 kDa by SDS-PAGE and were immunoreactive to AAD-12 protein-specific antibodies by western blot analysis. There was no evidence of glycosylation of either the DAS-8191Ø-7 cotton or *P. fluorescens*-derived AAD-12 proteins. Cotton- and *P. fluorescens*-derived AAD-12 were equally active using S-dichloroprop as a substrate and displayed similar kinetic parameters, indicating that the proteins are enzymatically equivalent. In addition, greater than 88% of the cotton-derived protein amino acid sequence was confirmed by either enzymatic peptide mass fingerprinting or MS/MS sequence analysis by MALDI-TOF MS/MS. The N-terminal methionine was found to be cleaved from both protein sources the N-terminal peptide of the plant-derived AAD-12 was determined to be acetylated after the N-terminal methionine was cleaved. These two post-translational processes, cleavage of the N-terminal methionine residue and N-terminal acetylation, are common modifications that have been found to occur on the vast majority (~85%) of eukaryotic proteins (Polevoda & Sherman 2003). The C-terminal peptides from DAS-8191Ø-7 cotton and *P. fluorescens* were intact and empirically determined to be identical.

Summary of AAD-12 Protein Characterization

Detailed biochemical characterization of the AAD-12 protein derived from DAS-8191Ø-7 cotton was conducted. SDS-PAGE, western blot, glycoprotein detection, enzymatic assay, and protein sequence analysis by MALDI-TOF MS/MS were used to characterize the biochemical properties of the proteins. Using these methods, the AAD-12 protein isolated from *P. fluorescens* and DAS-8191Ø-7 cotton were shown to be biochemically and biologically equivalent.

(ii) PAT Protein

Identity of the PAT Protein

The expressed DAS-8191Ø-7 cotton PAT protein is comprised of 183 amino acids and has a molecular weight of ~20 kDa (Figure 39). The PAT protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium (OECD 1999, Strauch et al 1988). The *pat* transgene in DAS-8191Ø-7 encodes a protein sequence that is identical to the native *P. viridochromogenes* PAT protein (UniProt Accession Number: Q57146) and is identical to that produced in DAS-444Ø6-6 and DAS-68416-4 soybeans; already approved by FSANZ (FSANZ 2011, FSANZ 2013).

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

Figure 39. Amino Acid Sequence of PAT

Mode of Action of the PAT Protein

The L-isomer of phosphinothricin (PPT) is a potent inhibitor of glutamine synthetase (GS) in plants and is used as a non-selective herbicide (OECD 1999). Inhibition of GS by PPT causes rapid accumulation of intracellular ammonia which leads to cessation of photorespiration and results in the death of the plant cell (Duan et al 2009). PAT acetylates the free NH_2 (amine) group of PPT (in the presence of acetyl coenzyme A) which is unable to bind to GS and thereby does not disrupt photorespiration, preventing autotoxicity (Figure 40, (Duke 1996)).

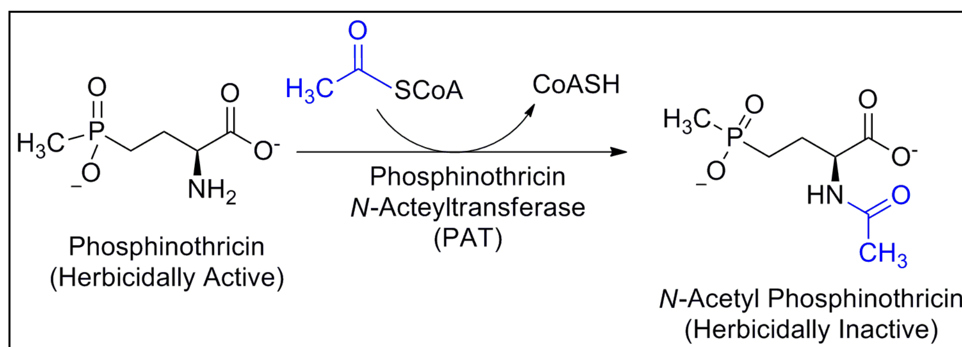


Figure 40. Mode of Action of the PAT Protein

Biochemical Characterization of the PAT Protein

Large quantities of purified PAT 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, Raybould et al 2012), the PAT protein was heterologously-expressed in *Pseudomonas fluorescens*. Characterization studies were performed to confirm the equivalency of the PAT protein produced in *P. fluorescens* with the PAT protein produced *in planta* in DAS-8191Ø-7 cotton. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, glycoprotein detection, enzymatic assay, and protein sequence analysis by matrix assisted laser desorption / ionization time-of-flight mass spectrometry/mass spectrometry (MALDI-TOF MS/MS) were used to characterize the biochemical properties of the proteins (Oman 2013). Using these methods, the PAT protein isolated from *P. fluorescens* and DAS-8191Ø-7 cotton were shown to be biochemically and biologically equivalent, thereby supporting the use of the microbe-derived protein in safety assessment studies.

The methods and results of the biochemical characterization of the DAS-8191Ø-7 cotton- and microbe-derived PAT proteins are described in detail in Appendix 3 and in the attached reports (Embrey & Juba 2012, Oman 2013). Both the DAS-8191Ø-7 cotton and *P. fluorescens*-derived PAT proteins were observed at the expected molecular weight of ~20 kDa by SDS-PAGE and were immunoreactive to PAT protein-specific antibodies by western blot analysis. There was no evidence of glycosylation of either the DAS-8191Ø-7 cotton or *P. fluorescens*-derived PAT proteins. Cotton and *P. fluorescens*-derived PAT were equally active using glufosinate as a substrate and displayed similar kinetic parameters, indicating that the proteins are enzymatically equivalent. In addition, greater than 90% of the cotton-derived protein amino acid sequence was confirmed by enzymatic peptide mass fingerprinting and MS/MS sequence analysis by MALDI-TOF MS/MS. The N-terminal methionine was found to be cleaved from both protein sources which is a common modification that has been found to occur on many proteins (Li & Chang 1995). The C-terminal peptides from DAS-8191Ø-7 cotton and *P. fluorescens* were intact and empirically determined to be identical.

Summary of PAT Characterisation

Detailed biochemical characterization of the PAT protein derived from DAS-8191Ø-7 cotton was conducted. SDS-PAGE, western blot, glycoprotein detection, enzymatic assay, and protein sequence analysis by MALDI-TOF MS/MS were used to characterize the biochemical properties of the proteins. Using these methods, the PAT protein isolated from *P. fluorescens* and DAS-8191Ø-7 cotton were shown to be biochemically and biologically equivalent with no glycosylation.

3.2 b Other Potential Novel Substances

To determine if any putative reading frames were created by the integration of the T-DNA insert into DAS-8191Ø-7 cotton, DNA sequences of the insert and its flanking border regions were analysed. Putative reading frames are conservatively defined as sequences that begin and end with stop codons and are greater than or equal to eight amino acids in length.

A total of eleven putative reading frames spanning the junctions between the insert and its flanking border regions in DAS-8191Ø-7 cotton were identified and subjected to BLASTp search for sequence similarity with known toxin proteins. The search against the GenBank non-redundant protein sequence dataset (updated March 07, 2013) did not detect any significant protein sequence similarity with toxic proteins harmful to humans or animals.

For evaluation of potential allergenicity two criteria were used. The first criterion is a search over 80-amino-acid or longer stretches to detect >35% identity between a query protein and known allergens. The window size of 80 amino acids was selected to correspond with a typical domain size in a protein, and recognizes that single protein domains may contain epitopes that mediate antibody binding (Codex Alimentarius Commission 2003, Ladics 2008). To ensure that high identity over a short stretch (for example, 80% over 60 amino acids) was not overlooked, a calculation, (Identity% × number of overlapped amino acids)/80, was implemented as a conversion to check the criteria of >35% over 80 amino acids when the alignment (overlapped amino acids) is less than 80 amino acids. The minimum reading frame for this analysis is greater than or equal to 29 amino acids. Five of the eleven putative reading frames spanning the junctions were less than 29 amino acids and thereby incapable of meeting this analysis threshold. None of the remaining six putative reading frames generated any significant amino acid sequence similarity with known allergens (FARRP Allergen Database Version 13, released February 2013).

The second criterion involves evaluating short amino acid stretches for identity between the query protein and known allergens (Silvanovich et al 2006, Stadler & Stadler 2003). Each putative reading frame was analysed for any matches of eight contiguous amino acids and compared with the FARRP allergen database. Since all eleven putative reading frames identified in DAS-8191Ø-7 cotton were greater than or equal to eight amino acids in length, all were subjected to this analysis. None of the eleven putative reading frames analysed against the peer reviewed FARRP allergen database generated a significant amino acid sequence similarity to known allergens.

3.2 c Site of expression of new substances

(i) Expression of the AAD-12 in Plant Tissues

A field expression study was conducted in the USA during 2012. Six sites (Alabama, Georgia, Louisiana, Missouri, North Carolina, and Texas) were planted with DAS-8191Ø-7 cotton and the non-transgenic control. The test sites represented regions of diverse agronomic practices and environmental conditions for cotton in North America. Two treatments of the DAS-8191Ø-7 cotton (unsprayed or sprayed with 2,4-D and glufosinate) were tested and plant tissue was collected from leaf, squares, bolls, pollen, flower, whole plant, root, and seed (Table 7).

Table 7. DAS-8191Ø-7 Cotton AAD-12 Expression Analysis Tissue Samples

Expression Tissue	Growth Stage[a]	Sample Size	No. of Samples per Entry/Site
Bolls	Peak Bloom	10-14 Bolls	4
Flower	Peak Bloom	14-18 Flowers	4
Leaf	4-Leaf	10-14 Leaves	4
Leaf	1st White Bloom	10-14 Leaves	4
Leaf	1st Open Boll	10-14 Leaves	4
Pollen	Early Bloom	0.2-0.5 mL	4
Root	Maturity	1-2 Plants	4
Seed	Maturity	175-250 grams	4
Squares	1st White Bloom	10-14 Squares	4
Whole Plant	Maturity	1-2 Plants	4

[a] Approximate growth stage

AAD-12 protein was extracted from DAS-8191Ø-7 cotton tissue and the soluble, extractable AAD-12 protein from each tissue was measured using a draft enzyme-linked immunosorbent assay (ELISA) method (Theoharis 2013). AAD-12 protein levels for all tissue types were calculated on ng/mg dry weight basis. The details of the materials and methods are described in Appendix 4

A summary of the AAD-12 protein concentrations (averaged across sites) in the various cotton matrices is shown in Table 8.

The mean AAD-12 protein levels were highest in the leaf at 71.17 ng/mg followed by pollen at 70.71 ng/mg, squares at 38.33 ng/mg, flower at 30.63 ng/mg, seed at 18.75 ng/mg, bolls at 17.17 ng/mg, whole plant at 16.42 ng/mg, and root at 10.74 ng/mg. AAD-12 expression levels were comparable for both sprayed and unsprayed treatments. No AAD-12 protein was detected in the control tissues (Hill 2013).

In summary, AAD-12 protein expression analysis was conducted using DAS-8191Ø-7 cotton tissue samples collected over the growing season. The low level expression of the AAD-12 protein in the various assayed tissue samples in DAS-8191Ø-7 cotton presents a low exposure risk to humans and animals.

Table 8. Summary of AAD-12 Protein Expression

Matrix (Growth Stage)	**AAD-12 ng/mg Tissue Dry Weight[a b]**		
	Overall Mean	Std. Dev.[c] (n = 48)	Min/Max Range[d]
Bolls (Peak Bloom)	17.17	7.91	4.36-33.39
Flower (Peak Bloom)	30.63	8.36	18.76-52.28
Leaf (4-Leaf)	71.17	46.63	7.78-180.72
Leaf (1st white Bloom)	17.53	8.6	1.45-40.56
Leaf (1st Open Boll)	51.26	19.63	17.38-89.23
Pollen (Early Bloom)	70.71	19.58	35.15-107.1
Root (Maturity)	10.74	5.27	ND[e]-22.89
Seed (Maturity)	18.75	4.81	6.75-27.77
Squares (1st White Bloom)	38.33	12.21	16.42-66.72
Whole Plant (Maturity)	16.42	12.18	ND[e]-46.98

[a]Calculated from AAD-12 expression raw data.
[b]Table represents overall results for unsprayed and sprayed cotton tissue across all sites.
[c]Standard deviation of individual cotton samples across all sites.
[d]Represents the min and max for individual cotton samples across all sites.
[e]ND = Not Detected, expression level below LOD (Limit of Detection). A zero value was used for ND results for mean calculation.

(ii) Expression of the PAT Protein in Plant Tissues

A field expression study was conducted in the USA during 2012. Six sites (Alabama, Georgia, Louisiana, Missouri, North Carolina, and Texas) were planted with DAS-8191Ø-7 cotton and the non-transgenic control. The test sites represented regions of diverse agronomic practices and environmental conditions for cotton in North America. Two treatments of the DAS-8191Ø-7 cotton (unsprayed, or sprayed with 2,4-D and glufosinate) were tested with PAT protein levels being determined in nine tissue types including leaf, squares, bolls, pollen, flower, whole plant, root, and seed (Table 9).

Table 9. **DAS-8191Ø-7 Cotton PAT Expression Analysis Tissue Samples**

Expression Tissue	Growth Stage[a]	Sample Size	No. of Samples per Entry/Site
Bolls	Peak Bloom	10-14 Bolls	4
Flower	Peak Bloom	14-18 Flowers	4
Leaf	4-Leaf	10-14 Leaves	4
Leaf	1st White Bloom	10-14 Leaves	4
Leaf	1st Open Boll	10-14 Leaves	4
Pollen	Early Bloom	0.2-0.5 mL	4
Root	Maturity	1-2 Plants	4
Seed	Maturity	175-250 grams	4
Squares	1st White Bloom	10-14 Squares	4
Whole Plant	Maturity	1-2 Plants	4

[a] Approximate growth stage

PAT protein was extracted from DAS-8191Ø-7 cotton tissue and the soluble, extractable PAT protein was measured using an enzyme-linked immunosorbent assay (ELISA) method (Smith-Drake 2011). PAT protein levels for all tissue types were calculated on ng/mg dry weight basis. The details of the materials and methods are described in Appendix 4**.**

A summary of the PAT protein concentrations (averaged across sites) in the various cotton matrices is shown in Table 10. The mean PAT protein levels were highest in the leaf at 13.29 ng/mg, followed by squares at 7.91 ng/mg, flower at 5.30 ng/mg, seed at 3.85 ng/mg, bolls at 3.16 ng/mg, root at 1.67 ng/mg, whole plant at 0.97 ng/mg and pollen at 0.11 ng/mg. PAT expression levels were comparable for both sprayed and unsprayed treatments (Hill 2013). No PAT protein was detected in the control tissue above the limit of quantitation (0.06 ng/mg) with the exception of a single seed sample which was most likely attributed to sampling error and/or contamination.

In summary, PAT protein expression analysis was conducted using DAS-8191Ø-7 cotton tissue samples collected over the growing season. The low level expression of the PAT protein in the various assayed tissue samples in DAS-8191Ø-7 cotton presents a low exposure risk to humans and animals.

Table 10. Summary of PAT Protein Expression in DAS-8191Ø-7 Cotton

Matrix (Growth Stage)	**PAT ng/mg Tissue Dry Weight[a b]**		
	Overall Mean	Std. Dev.[c] (n = 48)	Min/Max Range[d]
Bolls (Peak Bloom)	3.16	1.11	1.62-6.27
Flower (Peak Bloom)	5.3	1.09	2.92-8.20
Leaf (4-Leaf)	13.29	4.76	1.11-20.06
Leaf (1st white Bloom)	8.18	2.57	1.64-14.34
Leaf (1st Open Boll)	9.14	3.92	4.11-18.56
Pollen (Early Bloom)	0.11	0.22	ND[e]-0.99
Root (Maturity)	1.63	0.7	ND[e]-3.11
Seed (Maturity)	3.85	0.79	2.37-5.71
Squares (1st White Bloom)	7.91	2.39	4.38-14.48
Whole Plant (Maturity)	0.97	1.02	ND[e]-3.97

[a] Calculated from PAT expression raw data.
[b] Table represents overall results for unsprayed and sprayed cotton tissue across all sites.
[c] Standard deviation of individual cotton samples across all sites.
[d] Represents the min and max for individual cotton samples across all sites.
[e] ND = Not Detected, expression level below LOD (Limit of Detection). A zero value was used for ND results for mean calculation.

3.2 d Post-Translational Modification in the New Host

The methods and results of the biochemical characterization of the DAS-8191Ø-7 cotton and microbe-derived AAD-12 and PAT proteins are described in detail in Appendix 2 -3 and in (Clement 2013, Karnoup & Kuppannan 2008) (Embrey & Juba 2012, Oman 2013).

Both the plant and *P. fluorescens*-derived AAD-12 proteins were observed at the expected molecular weight of 32 kDa by SDS-PAGE and were immunoreactive to AAD-12 protein-specific antibodies by western blot analysis. There was no evidence of glycosylation of either the DAS-8191Ø-7 cotton or *P. fluorescens*-derived AAD-12 proteins. Cotton- and *P. fluorescens*-derived AAD-12 were equally active using S-dichloroprop as a substrate and displayed similar kinetic parameters, indicating that the proteins are enzymatically equivalent. In addition, greater than 88% of the cotton-derived protein amino acid sequence was confirmed by either enzymatic peptide mass fingerprinting or MS/MS sequence analysis by MALDI-TOF MS/MS. The N-terminal methionine was found to be cleaved from both protein sources the N-terminal peptide of the plant-derived AAD-12 was determined to be acetylated after the N-terminal methionine was cleaved. These two post-translational processes, cleavage of the N-terminal methionine residue and N-terminal acetylation, are common modifications that have been found to occur on the vast majority (~85%) of eukaryotic proteins (Polevoda & Sherman 2003). The C-terminal peptides from DAS-8191Ø-7 cotton and *P. fluorescens* were intact and empirically determined to be identical.

Both the DAS-8191Ø-7 cotton and *P. fluorescens*-derived PAT proteins were observed at the expected molecular weight of ~20 kDa by SDS-PAGE and were immunoreactive to PAT protein-specific antibodies by western blot analysis. There was no evidence of glycosylation of either the DAS-8191Ø-7 cotton or *P. fluorescens*-derived PAT proteins. Cotton and *P. fluorescens*-derived PAT were equally active using glufosinate as a substrate and displayed similar kinetic parameters, indicating that the proteins are enzymatically equivalent. In addition, greater than 90% of the cotton-derived protein amino acid sequence was confirmed by enzymatic peptide mass fingerprinting and MS/MS sequence analysis by MALDI-TOF MS/MS. The N-terminal methionine was found to be cleaved from both protein sources which is a common modification that has been found to occur on many proteins (Li & Chang 1995). The C-terminal peptides from DAS-8191Ø-7 cotton and *P. fluorescens* were intact and empirically determined to be identical.

3.2 e Novel Protein Silencing

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

3.2 f Novel Protein History of Consumption

The *aad-12* gene was isolated from *Delftia acidovorans* and the synthetic version of the gene was optimized for plant expression by modifying the G+C content bias to the plant system. The native and plant-optimized DNA sequences of *aad-12* are 80% identical.

Delftia acidovorans, which has previously been identified as *Pseudomonas acidovorans* and *Comamonas acidovorans*, is a non glucose-fermenting, gram-negative, non spore-forming rod-shaped bacterium present in soil, fresh water, activated sludge, and clinical specimens (Tamaoka et al 1987, Von Graevenitz 1985, Wen et al 1999). *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavour metabolites (Rao & Ravishankar 2000, Shetty et al 2006, Toms & Wood 1970). 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).

The PAT protein produced in DAS-8191Ø-7 Cotton was shown to be equivalent to that produced in other transgenic crops that have been previously approved by FSANZ (A1074, A1046, A481, A446, A543 and A518). The food and feed safety of PAT was assessed in these products and shown to present no food or feed safety risk.

The safety of phosphinothricin acetyltransferase (PAT) in biotech crops has been extensively studied and environmental release of biotech crops containing PAT have been issued by regulatory agencies in eleven different countries, involving over thirty eight events in eight plant species including cotton, maize and soybean, among other crops.

3.3 Potential Toxicity of the Novel Protein

Guttikonda S, 2012. Sequence Similarity of PAT Protein to Known Toxins by Bioinformatics Analysis (Update, February, 2012) Study ID 120480, Dow AgroSciences LLC, Indianapolis IN
Mo J, 2012. Sequence Similarity of AAD-12 Protein to Known Toxins by Bioinformatics Analysis (Update, February, 2012) Study ID 120142, Dow AgroSciences LLC, Indianapolis IN
Schafer BW, Embrey SK, 2008. In Vitro Simulated Gastric Fluid Digestibility of Aryloxyalkanoate Dioxygenase-12Study ID 080064, Dow AgroSciences LLC, Indianapolis, IN

The primary food source from cotton produced for human consumption is refined oil from cottonseed, which contains undetectable amounts of protein (Reeves III & Weihrauch 1979), therefore, assessing the allergenicity and toxicity of the expressed proteins in cotton is less relevant compared with other crops such as soybean, since little to no protein is present in consumed cotton products. Regardless, a detailed safety assessment of AAD-12 was conducted to assess any potential adverse effects to humans or animals resulting from the environmental release of crops containing AAD-12 (Codex Alimentarius Commission 2009). The conclusion from the assessment is that AAD-12 is unlikely to cause allergic reaction in humans or be a toxin to humans or animals.

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

3.3 a Assessment of Toxicity Potential of AAD-12

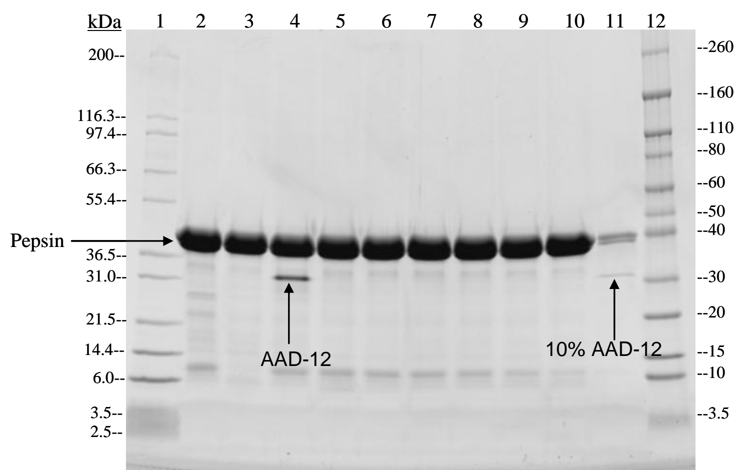
(i) Amino Acid Sequence Comparison to Known Toxins

The AAD-12 protein does not share meaningful amino acid sequence similarities with known toxins. Sequence homologies between the AAD-12 protein and known protein toxins were evaluated using BLASTp search algorithm against the GenBank non-redundant protein sequences (up to date as of February 18, 2012). By their annotations, the proteins returned by BLASTp search can be grouped into the following 10 categories: 2,4-D/alpha-ketoglutarate dioxygenase, putative alkylsulfatase, alpha-ketoglutarate (dependent) dioxygenase, alpha-ketoglutarate-dependent sulfonate dioxygenase, taurine catabolism dioxygenase, taurine dioxygenase, dioxygenase, oxidoreductase, pyoverdine biosynthesis protein, and hypothetical (putative) or unnamed proteins. AAD-12 (aryloxyalkanoate dioxygenase-12) itself is an alpha-ketoglutarate dependent dioxygenase. Hypothetical and unnamed proteins are derived from conceptual translation of DNA sequences generated from massive genome sequencing projects of various fungi and bacteria. Those proteins have functional annotations such as “probable taurine catabolism dioxygenase”, “clavaminic acid synthetase (CAS) –like”, and “putative alpha-ketoglutarate dependent dioxygenase”. None of those proteins are associated with protein toxins that are harmful to humans or animals (Mo 2012).

(ii) Lability in Simulated Gastric Fluid

The digestibility of the *P. fluorescens* derived AAD-12 protein was tested *in vitro* using simulated gastric fluid (SGF) (Schafer & Embrey 2008). The AAD-12 protein (3.7 µM) was incubated in SGF (0.32% w/v pepsin at pH 1.2; (The United States Pharmacopeia 2005)) for various periods of time. At each time point (0.5, 1, 2, 4, 8, and 16 minutes), 0.1 mL of the reaction mixture was removed and placed into a microcentrifuge tube containing 0.04 mL stop solution (200 mM Na_2CO_3, pH ~11.0). For the zero time point samples, 2.85 mL SGF solution was neutralized with 1.2 mL stop solution and then the AAD-12 protein sample was added. All samples were kept on ice after the stop solution was added. After all digestion time points were completed, the samples were mixed with Laemmli sample buffer and heated to 95 °C for 5 min. The samples were then analysed via SDS-PAGE and western blot analysis using a polyclonal antibody specific to AAD-12 (Figure 41 and Figure 42). The results demonstrated the AAD-12 protein was readily digested (not detectable at 30 seconds) in SGF.

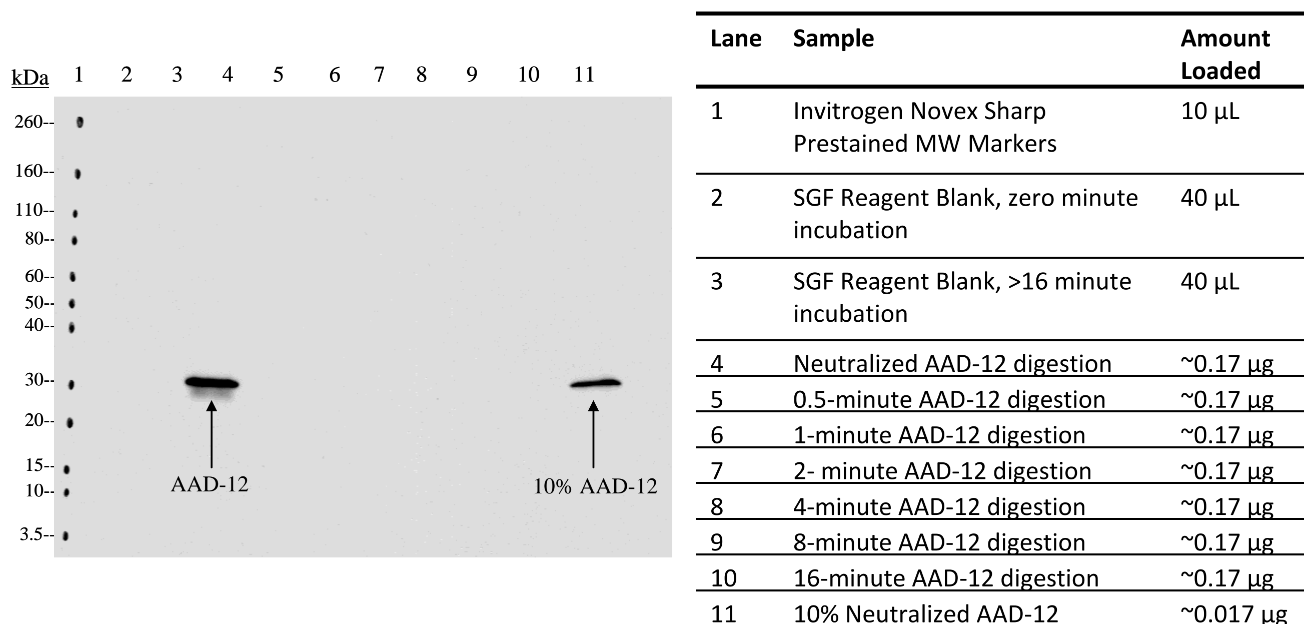
Furthermore, the stability of the AAD-12 protein in transgenic soybean leaf extracts (event DAS-68416-4, FSANZ application A1046) upon exposure to simulated gastric fluid (SGF) was also examined in a recent study (Schafer & Embrey 2011). In this study, digestion samples of the transgenic soybean leaf extracts were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot; the AAD-12 protein contained in crude soybean leaf extracts (in both transgenic tissue and nontransgenic tissue fortified with microbe-derived AAD-12) was readily digested by pepsin (not detectable at 30 seconds) under simulated gastric conditions (pH 1.2, 37 °C) (Schafer & Embrey 2011). The AAD-12 protein from cotton and soybean has the identical amino acid sequence and N-terminal acetylation. Therefore, the digestibility of the acetylated AAD-12 protein is established.



Lane	Sample	Amount Loaded
1	Invitrogen Mark 12 MW Markers	10 µL
2	SGF Reagent Blank, Ø min.	40 µL
3	SGF Reagent Blank, >16 min.	40 µL
4	Neutralized AAD-12 digestion	~1.68
5	0.5-minute AAD-12 digestion	~1.68
6	1-minute AAD-12 digestion	~1.68
7	2- minute AAD-12 digestion	~1.68
8	4-minute AAD-12 digestion	~1.68
9	8-minute AAD-12 digestion	~1.68
10	16-minute AAD-12 digestion	~1.68
11	10% Neutralized AAD-12	~0.17
12	Invitrogen Novex Sharp Prestained MW Markers	10 µL

Figure 41. **Simulated Gastric Fluid Analysis of AAD-12 Protein (SDS-PAGE)**

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



Lane	Sample	Amount Loaded
1	Invitrogen Novex Sharp Prestained MW Markers	10 µL
2	SGF Reagent Blank, zero minute incubation	40 µL
3	SGF Reagent Blank, >16 minute incubation	40 µL
4	Neutralized AAD-12 digestion	~0.17 µg
5	0.5-minute AAD-12 digestion	~0.17 µg
6	1-minute AAD-12 digestion	~0.17 µg
7	2- minute AAD-12 digestion	~0.17 µg
8	4-minute AAD-12 digestion	~0.17 µg
9	8-minute AAD-12 digestion	~0.17 µg
10	16-minute AAD-12 digestion	~0.17 µg
11	10% Neutralized AAD-12	~0.017 µg

Figure 42. Simulated Gastric Fluid Analysis of AAD-12 Protein (Western Blot)

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

(i) Acute Oral Toxicity

An acute oral toxicity study with the *P. fluorescens*-derived AAD-12 protein was conducted in mice administered 2000 mg AAD-12/kg body weight after adjustment for purity (5666 mg/kg of test substance at 35.3% purity) (Wiescinski & Golden 2008). The study was conducted following OECD Guideline 423 and used a total of 10 mice (5 male and 5 female). All animals survived and no clinical signs were observed during the study. All animals gained weight by study termination on day 15. There were no treatment-related gross pathological observations. Therefore, the acute oral LD_{50} and no observed effect level (NOEL) of AAD-12 in male and female mice was greater than 2000 mg/kg based on fact that no mortality was observed and there were no observable effects (adverse or non-adverse effects) with the AAD-12-treated animals. AAD-12 protein displays very low acute toxicity potential.

3.3 b Assessment of Toxicity Potential of PAT

(i) Amino Acid Sequence Comparison to Known Toxins

The PAT protein does not share any amino acid sequence similarity with known toxins that would present any safety concerns. Amino acid homologies with the PAT protein sequence were evaluated using BLASTp search algorithm against the GenBank non-redundant protein sequences (up to date as of February 23, 2012). By their annotations, the majority of proteins returned by BLASTp with statistically significant alignments are phosphinothricin acetyltransferase, other acetyltransferases, and hypothetical proteins without assigned function. None of these proteins is associated with known protein toxins that are harmful to humans and animals (Guttikonda 2012).

(ii) Lability in Simulated Gastric Fluid and Heat Lability

The PAT protein is rapidly degraded in simulated gastric fluid (EPA 1997, Herouet et al 2005, OECD 1999) and is readily denatured by heat (EPA 1997, OECD 1999). Numerous glufosinate tolerant products including those in canola, soybean, corn and cotton have been reviewed by USDA with no concerns identified.

There is no evidence available indicating that the PAT protein is toxic to either humans or animals. In acute toxicity studies mice gavaged with high levels of PAT protein showed no treatment-related significant toxic effects (EPA 1997, OECD 1999). The US EPA has concluded, after reviewing data on the acute toxicity and digestibility of the PAT protein, that there is a reasonable certainty that no harm will result from aggregate exposure to the USA population, including infants and children, to the PAT protein and the genetic material necessary for its introduction (EPA 1997). US EPA has consequently established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for PAT and the genetic material necessary for its production in all plants (40 CFR §174.522).

The overall safety assessment of PAT supports the conclusion that food and feed products containing DAS-8191Ø-7 cotton or derived from DAS-8191Ø-7 cotton are as safe as non-transgenic cotton currently on the market for human and animal consumption.

3.4 Potential Allergenicity of the Novel Protein

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

Song P, 2013a. Sequence Similarity Assessment of the AAD-12 Protein to Known Allergens by Bioinformatics AnalysisStudy ID 130068, Dow AgroSciences, LLC, Indianapolis, IN

Song P, 2013b. Sequence Similarity Assessment of the PAT Protein to Known Allergens by Bioinformatics AnalysisStudy ID 130069, Dow AgroSciences, LLC, Indianapolis, IN

Korjagin VA, 2001a. In vitro simulated gastric fluid digestibility study of microbially derived Cry1Ac (synpro). Study ID 010026, Dow AgroSciences LLC, Indianapolis, IN

3.4 a Assessment of Allergenicity Potential of AAD-12 Protein

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

(i) Amino Acid Sequence Comparison to Known Allergens

The step-wise, weight-of-evidence approach (Codex Alimentarius Commission 2009) was used to assess the allergenic potential of the AAD-12 protein. The AAD-12 protein does not share meaningful amino acid sequence similarities with known allergens. No significant sequence homology was identified when the AAD-12 protein sequence was compared with known allergens in the FARRP (Food Allergy Research and Resource Program) version 13.00 allergen database (Released in February, 2013), using the search criteria of either a match of eight or more contiguous identical amino acids, or >35% identity over 80 or longer amino acid residues (Song 2013a).

(ii) Heat Lability

The thermal stability of the *P. fluorescens*-derived AAD-12 protein was evaluated by heating protein solutions for 30 min at 50, 70 and 95 °C; and 20 min in an autoclave (120 °C at ~117 kPa (~17 PSI)) in a phosphate-based buffer. The AAD-12 protein activity was measured by a modified enzyme assay based on the procedure described in Fukumori and Hausinger (Fukumori & Hausinger 1993). In the presence of Fe(II), the AAD-12 protein catalyzes the conversion of dichlorphenoxyacetate to 2,4-dichlorophenol and glyoxylate concomitant with the decomposition of α-ketoglutarate to form succinate and carbon dioxide. The resulting phenol is measured with an AAPPC assay or the Emerson reaction (Emerson 1943). As shown in Table 11, all heating conditions eliminated the enzymatic activity of the AAD-12 protein (Schafer 2008).

Table 11. **Summary of AAD-12 Enzyme Activity Results**

Treatment Temperature	Time (min)	% Enzymatic Activity
4 °C	NA	100.0%
50 °C	30	0.0%
70 °C	30	0.0%
95 °C	30	0.0%
120 °C (autoclave)	20	0.0%

Note: Relative activity of the 4 °C treatment is designated as 100%. The results are averaged from enzymatic assay absorbance readings of serial dilutions of the AAD-12. % Enzymatic Activity = (OD heat treatment - blank) / (OD 4 °C - blank) x 100.

In summary, a step-wise weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the AAD-12 amino acid sequence. AAD-12 protein is rapidly degraded in simulated gastric fluid and is readily denatured by heat. There is no evidence of acute toxicity of the AAD-12 protein in mice at a dose of 2000 mg/kg body weight of AAD-12. These results indicate that DAS-8191Ø-7 cotton AAD-12 is unlikely to cause allergenic or adverse effects in humans or animals.

3.4 b Assessment of Allergenicity Potential of PAT Protein

(i) Amino Acid Sequence Comparison to Known Allergens

The PAT protein has no biologically meaningful sequence similarities to known allergens using a sequence evaluation program based on that formulated by the joint FAO/WHO Expert Consultation (2001) and by the Codex Alimentarius (Codex Alimentarius Commission 2001). This search looks for a match of at least eight contiguous amino acids or greater than 35% identity over 80 or longer amino-acid stretches using an allergen database (FARRP 2013) and no such matches were found (Song 2013b).

In summary, a step-wise weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the PAT protein. Bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence. PAT protein is rapidly degraded in simulated gastric fluid and is readily denatured by heat. There is no evidence of acute toxicity of the PAT protein in mice.

3.5 Toxicity of novel herbicide metabolites in DAS-8191Ø -7 cotton

The nature and safety of the metabolites resulting from the herbicide interaction with novel proteins expressed in DAS-81910-7 have already been assessed as part of previous applications (for AAD-12/2,4-D, see application A1046 (event DAS-68416-4)) or, in the case of PAT/glufosinate-ammonium, have significant history of safe use. Therefore, spraying of DAS-81910-7 cotton with 2,4-D or glufosinate-ammonium is not expected to result in the production of metabolites that are not also found in crops sprayed with the same herbicides and already used in the food supply.

3.6 Compositional Analysis

Fast B J, Johnson T Y, (2012). Nutrient composition of a transformed soybean cultivar expressing Cry1Ac, Cry1F, and PAT: event DAS-81419-2. Indianapolis, IN, Dow AgroSciences LLC.

3.6 a Selection of the Comparators for DAS-8191Ø-7 Cotton

Proper selection of comparator (control) plants is important to ensure the accurate assessment of the impact of transgene insertion on various characteristics of DAS-8191Ø-7 cotton. The control plants should have a genetic background similar to that of DAS-8191Ø -7 cotton but lack the transgenic insert. In all cases, a genotypically similar non-transgenic near-isogenic control was used. Additionally, the results of compositional analysis were compared with concurrently grown non-transgenic cotton varieties and published literature ranges for non-transgenic cotton (Appendix 6).

3.6 b DAS-8191Ø-7 Cottonseed Composition

Data from the composition analysis was statistically analyzed for each sub-experiment comparing unsprayed DAS-8191Ø-7 cotton to the appropriate unsprayed non-transgenic near-isogenic control and non-transgenic reference lines in sub-experiment one and cottonseed from the herbicide-treated and unsprayed DAS-8191Ø-7 plants in sub-experiment two.

Composition assessment of DAS-8191Ø-7 cotton was conducted in which key nutrient and anti-nutrient levels of unsprayed DAS-8191Ø-7 cottonseed were compared with the appropriate non-transgenic near-isogenic control and non-transgenic reference lines and between cottonseed from herbicide-treated and unsprayed DAS-8191Ø-7 cottonseed. The assessment was conducted using the principles and analytes in the OECD consensus document for cotton composition (OECD 2009a), in which samples were analyzed for proximates, fiber, minerals, amino acids, fatty acids, vitamins, and anti-nutrients. Levels of the analytes in DAS-8191Ø-7 cottonseed were compared with: 1) corresponding levels in cottonseed from a non-transgenic, near-isogenic control, grown concurrently, under the same field conditions and 2) natural ranges generated from an evaluation of commercial non-transgenic cotton reference lines grown concurrently, under the same field conditions and 3) from data published in the scientific literature for non-transgenic cotton. Comparison with data published in the literature places any potential differences between the assessed crop and its comparators in the context of the documented variations in the concentrations of crop nutrients and anti-nutrients.

Of the 73 cotton analytes assayed, 59 analytes had sufficient quantitative data for inclusion in the combined site statistical analysis. Although a limited number of statistically significant differences occurred between DAS-8191Ø-7 cotton and the control, these differences were not biologically relevant because the results were within ranges found for non-transgenic cotton reference lines included in this

study and/or within available literature ranges for non-transgenic cotton. Based on these results, it is concluded that DAS-8191Ø-7 cotton is compositionally equivalent to non-transgenic cotton.

(i) Field Study Design

Field trials were conducted at eight US sites (one site each in Tallassee, Alabama; Sycamore, Georgia; Washington, Louisiana; Stoneville, Mississippi; Fisk, Missouri; Mebane, North Carolina; Groom, Texas; and East Bernard, Texas) in 2012 to produce samples of DAS-8191Ø-7 cotton (unsprayed and sprayed with 2,4-D plus glufosinate-ammonium), near isogenic non-transgenic control, and non-transgenic reference line cottonseed for nutrient composition analysis. Because non-transgenic cotton plants (including the control and reference lines) are sensitive to 2,4-D, two sub-experiments were conducted at each site to spatially separate the non-transgenic entries from the entries where 2,4-D was applied. Sub-experiment one was unsprayed and contained the control, reference varieties and one entry of DAS-8191Ø-7 cotton. Sub-experiment two contained two entries of DAS-8191Ø-7 cotton; one unsprayed and one sprayed with 2,4-D plus glufosinate-ammonium.

The entries were arranged in a randomized complete block design within each sub-experiment, and the two sub-experiments were separated by 100 ft (30 m) to prevent control and reference entries in sub-experiment one from potentially being injured by 2,4-D drift from the applications in sub-experiment two. Both sub-experiment one and two were present at all field testing sites. Randomization of entries within blocks was unique at each field testing site. Test, control, and reference variety cotton was planted at a seeding rate of approximately 100 seeds per 25 ft (7.62 m) of row (one seed per 3 in or 7.6 cm). Four replicate plots of each entry were established at each site for each sub-experiment, with each plot consisting of four rows that were 25 ft (7.62 m) long with a row spacing of approximately 30 in (76 cm). Each four row plot was bordered by two rows of a non-transgenic cotton cultivar.

Herbicides were applied in a spray volume of approximately 20 gallons per acre (187 L/ha), and all herbicide applications included 2% v/v ammonium sulfate. The 2,4-D (GF-2654) and glufosinate-ammonium (Ignite 280 SL) were applied in a tank mixture as two broadcast applications to one entry of DAS-8191Ø-7 cotton in sub-experiment two. Application timings were at the 3 node and 6 node growth stages. The target application rates for both application timings were 1120 g ae/ha (2,4-D) and 596 g ai/ha (glufosinate-ammonium). Actual application rates ranged from 1108 – 1142 g ae/ha (2,4-D) and 589 – 622 g ai/ha (glufosinate-ammonium) for the 3 node application and 1061 – 1141 g ae/ha (2,4-D) and 564 – 621 g ai/ha (glufosinate-ammonium) for the 6 node application.

(ii) Compositional Analysis

Samples of acid delinted cottonseed were analysed at Covance Laboratories Inc. for 73 cotton analytes (Table 12). Methods for analyte compositional analysis are described in Appendix 5. Data from the composition analysis was statistically analysed for each sub-experiment comparing unsprayed DAS-8191Ø-7 cotton to the appropriate unsprayed non-transgenic near-isogenic control and non-transgenic

reference lines in sub-experiment one and cottonseed from the herbicide-treated and unsprayed DAS-8191Ø-7 plants in sub-experiment two. The results were then placed into context with reported scientific literature values (Appendix 6).

3.6 c Statistical Analysis

Analysis of variance was conducted for each sub-experiment separately across field testing sites for composition data using a mixed model (SAS Institute Inc. 2009). Entry was considered a fixed effect and location, block within location, and location-by-entry were designated as random effects. Paired contrasts were conducted using t-tests, and the significance of overall treatment effects was estimated using an F-test. Significant differences were declared at the 95% confidence level ($\alpha = 0.05$).

Of the 73 analytes evaluated in this study, 59 produced sufficient data (>50% of data points above the limited of quantitation (LOQ)) for inclusion in the combined site statistical analysis. Therefore, 59 comparisons were to be made in each sub-experiment in this study [Note that inclusion of analytes in the statistical analysis where a predominance of the data are less than the LOQ violates the assumptions of ANOVA due to non-normal data distributions and artificially reduced variance estimates.].

In the 59 comparisons in each sub-experiment that were included in the statistical analysis, the probability of declaring one or more false differences based on unadjusted P-values was very high due to a multiplicity effect in each sub-experiment. Multiplicity occurs when a large number of comparisons are made in a single study to look for unexpected effects. As a result, under these conditions, the probability of falsely declaring differences based on comparison-wise P-values is very high at 95.151% ($1-0.95^{59}$) ($1-0.95^{\text{number of comparisons}}$).

One method that has been used to handle multiplicity is to adjust P-values to control the experiment-wise error rate. However, the power for detecting specific effects can be reduced significantly when many comparisons are made, such as in a study like this. An alternative approach with much greater power is to adjust P-values to control the probability that each declared difference is significant (Curran-Everett 2000). This can be accomplished using a False Discovery Rate (FDR) control procedure (Benjamini & Hochberg 1995), which is a commonly used approach in studies examining transgenic crops (Coll et al 2008, Herman et al 2010, Herman et al 2007, Huls et al 2008, Jacobs et al 2008, Stein et al 2009).

Table 12 Cottonseed Composition Analytes

Proximates and Fiber (9)		
Protein	Moisture	NDF
Fat	Carbohydrates	Total Dietary Fiber
Ash	ADF	Crude Fiber
Minerals (12)		

Calcium	Manganese[b]	Selenium
Copper	Molybdenum	Sodium
Iron	Phosphorus	Sulfur
Magnesium	Potassium	Zinc
Amino Acids (18)		
Alanine	Histidine	Proline
Arginine	Isoleucine	Serine
Aspartic Acid	Leucine	Threonine
Cystine	Lysine	Tryptophan
Glutamic Acid	Methionine	Tyrosine
Glycine	Phenylalanine	Valine
Fatty Acids (22)		
8:0 Caprylic[a]	16:1 Palmitoleic[b]	20:0 Arachidic
10:0 Capric[a]	17:0 Heptadecanoic[a]	20:1 Eicosenoic[a]
12:0 Lauric[a]	17:1 Heptadecenoic[a]	20:2 Eicosadienoic[a]
14:0 Myristic[b]	18:0 Stearic	20:3 Eicosatrienoic[a]
14:1 Myristoleic[a]	18:1 Oleic[b]	20:4 Arachidonic[a]
15:0 Pentadecanoic[a]	18:2 Linoleic[b]	22:0 Behenic
15:1 Pentadecenoic[a]	18:3 Linolenic	
16:0 Palmitic	18:3 γ-Linolenic[a]	
Vitamins (7)		
Vitamin A (Beta Carotene) [a]	Vitamin B3 (Niacin)	α-tocopherol (Vitamin E)
Vitamin B1 (Thiamine HCl)	Vitamin B6 (Pyridoxine HCl)	
Vitamin B2 (Riboflavin)	Vitamin B9 (Folic Acid)	
Anti-Nutrients (5)		
Dihydrosterculic Acid	Sterculic Acid	Total Gossypol[b]
Malvalic Acid[b]	Free Gossypol	

[a] Analytes excluded from combined site and analysis due to more than 50% of samples < LOQ
[b] Analytes with statistically significant adjusted FDR differences in the combined site analysis.

Therefore, the P-values from the composition analysis were each adjusted using the FDR method to improve discrimination of true differences among treatments from random effects (false positives). In this study differences were considered significant if the FDR-adjusted P-value was less than 0.05.

3.6 d Composition Analysis Results

Combined summary and statistical analysis of composition data from the non-transgenic near-isogenic control and DAS-8191Ø-7 cotton is found in Table 13 through Table 18 and Figure 43 through Figure 48) for both sub-experiment one and two. For each analyte and entry, the least-square mean, standard error, and minimum and maximum sample value are reported. Also, for comparison, the minimum and maximum values for the reference lines and literature ranges are reported. Arithmetic means from each

field site are plotted in figures and literature ranges are shaded (literature ranges reported as not detectable or <LOD are plotted as zeros).

The following sections discuss results first for sub-experiment one (unsprayed DAS-8191Ø-7, control, and reference lines), followed by a discussion of results for sub-experiment two (sprayed and unsprayed DAS-8191Ø-7). Sub-experiment one was used to evaluate if the composition of unsprayed DAS-8191Ø-7 cotton is equivalent to reference and control lines, and sub-experiment two was used to evaluate if applying 2,4-D plus glufosinate-ammonium impacts the composition of DAS-8191Ø-7 cotton.

(i) Sub-experiment One - Proximate and Fibre Analysis of Seed

Results from the combined site analysis of the proximate and fiber composition for the control and DAS-8191Ø-7 cottonseed are provided in Table 13 and Figure 43. Nine analytes were analyzed including ash, carbohydrates, crude fat, protein, moisture, acid detergent fiber (ADF), crude fiber, neutral detergent fiber (NDF) and dietary fiber. No significant FDR-adjusted P-values were observed for DAS-8191Ø-7 unsprayed for all nine analytes. In addition, mean results for all nine DAS-8191Ø-7 unsprayed proximate and fiber analytes fell within the reference variety ranges and literature ranges. Based on these data, statistical analyses found no differences between the levels of nutrient components in cottonseed from DAS-8191Ø-7 and the non-transgenic control, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 13. Combined Site Analysis Results for Proximates & Fiber in control, DAS-8191Ø-7, and Reference Variety Cottonseed and Literature Ranges

Analytical Component (Units)	Overall Trtmt Effect (Pr > F)	Control	DAS-8191Ø-7 Unsprayed	Reference Variety Range	Overall Trtmt Effect (Pr > F)	DAS-8191Ø-7 Unsprayed	DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium	Literature Range
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
Ash (% Dry Weight)	**0.047**	4.29 ± 0.11 3.67 - 5.14	4.17 ± 0.11 3.62 - 4.76 (**0.047**, 0.185)	3.53 - 5.21	0.411	4.31 ± 0.13 3.69 - 5.08	4.28 ± 0.13 3.79 - 5.02 (0.411, 0.945)	3.7 - 5.342
Carbohydrates (% Dry Weight)	**0.007**	47.5 ± 0.9 44.5 - 53.6	49.1 ± 0.9 44.7 - 57.9 (**0.007**, 0.050)	42.3 - 54.2	0.485	50.2 ± 1.1 46.9 - 56.6	50.5 ± 1.1 45.7 - 58.6 (0.485, 0.945)	39.0 - 53.62
Crude Fat (% Dry Weight)	0.368	21.7 ± 0.7 18.3 - 25.2	21.3 ± 0.7 15.6 - 26.2 (0.368, 0.642)	15.8 - 27.9	0.319	20.9 ± 0.9 17.3 - 25.9	20.5 ± 0.9 15.1 - 26.6 (0.319, 0.945)	14.4 - 27.292
Protein (% Dry Weight)	**0.008**	26.4 ± 0.9 22.3 - 31.5	25.4 ± 0.9 22.2 - 31.1 (**0.008**, 0.055)	21.5 - 32.3	0.669	24.6 ± 1.0 19.2 - 29.7	24.7 ± 1.0 19.2 - 29.7 (0.669, 0.945)	12 - 32.97
Moisture (% Fresh Weight)	0.663	8.2 ± 0.2 6.64 - 9.74	8.0 ± 0.2 6.56 - 9.39 (0.663, 0.815)	6.37 - 10.2	0.985	8.2 ± 0.2 6.95 - 9.20	8.2 ± 0.2 6.92 - 9.13 (0.985, 0.998)	2.25 - 15.9
ADF (% Dry Weight)	0.381	25.9 ± 0.5 23.0 - 28.6	25.3 ± 0.5 21.3 - 28.6 (0.381, 0.642)	20.4 - 29.4	0.405	26.0 ± 0.5 22.1 - 29.8	25.7 ± 0.5 22.4 - 30.0 (0.405, 0.945)	19.74 - 40.5
Crude Fiber (% Dry Weight)	0.677	18.1 ± 0.3 16.0 - 21.3	17.9 ± 0.3 14.8 - 22.7 (0.677, 0.815)	15.1 - 23.5	0.060	18.6 ± 0.3 13.7 - 23.6	17.8 ± 0.3 15.6 - 19.8 (0.060, 0.945)	13.45 - 23.10
NDF (% Dry Weight)	0.667	34.0 ± 0.6 30.7 - 40.3	33.8 ± 0.6 28.3 - 39.7 (0.667, 0.815)	27.2 - 38.2	0.557	35.2 ± 0.6 30.6 - 39.3	34.8 ± 0.6 31.3 - 39.3 (0.557, 0.945)	25.56 - 53.6
Dietary Fiber (% Dry Weight)	0.283	44.8 ± 1.0 40.3 - 53.5	45.7 ± 1.0 40.7 - 56.5 (0.283, 0.591)	37.6 - 51.3	0.309	46.6 ± 1.1 40.0 - 54.0	47.2 ± 1.1 41.7 - 54.1 (0.309, 0.945)	33.69 - 47.55

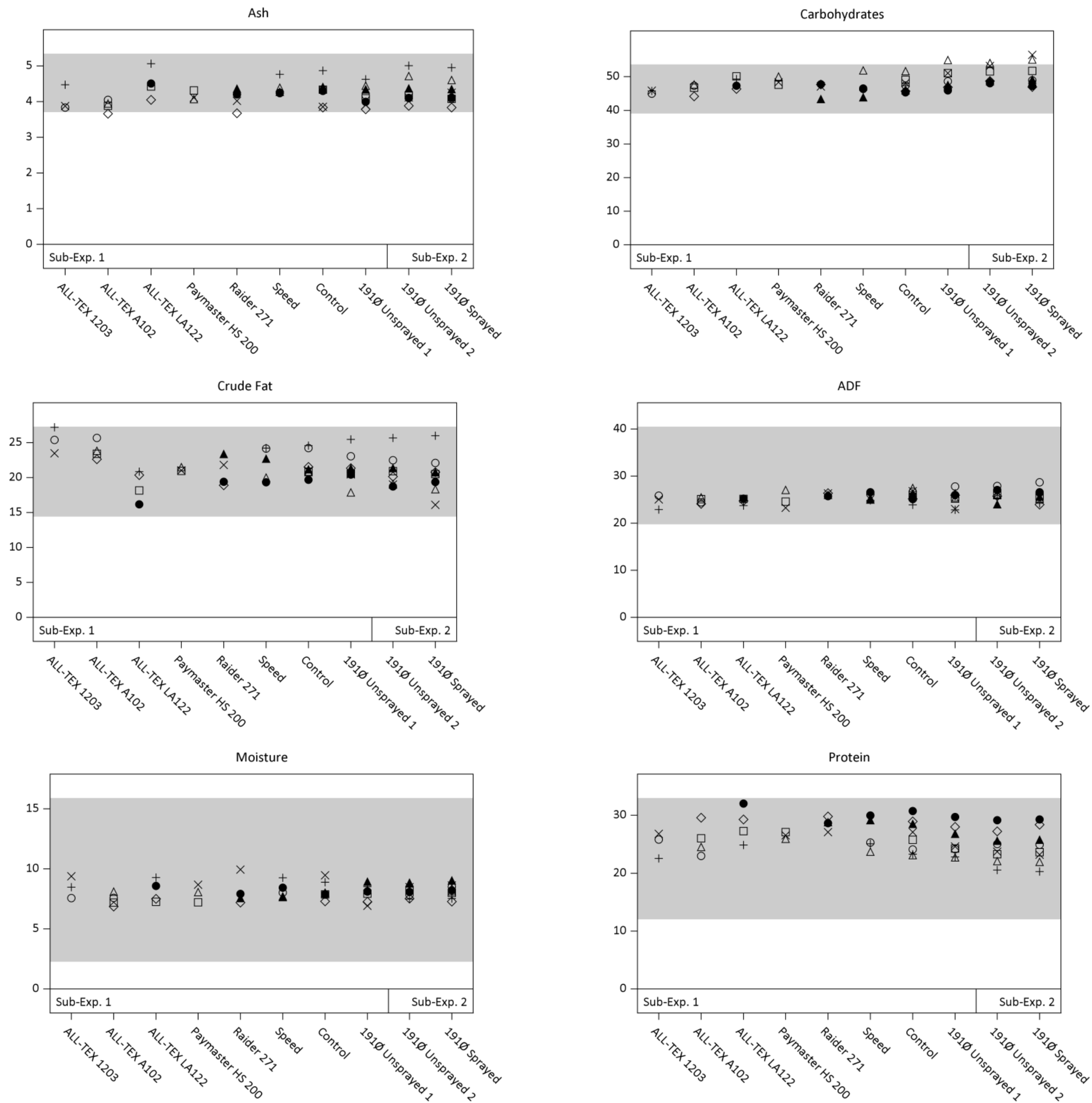


Figure 43. **Proximates & Fiber in Control, DAS-8191Ø-7 and Reference Variety Cottonseed**

Y axis: Moisture = % fresh weight, all others = % dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

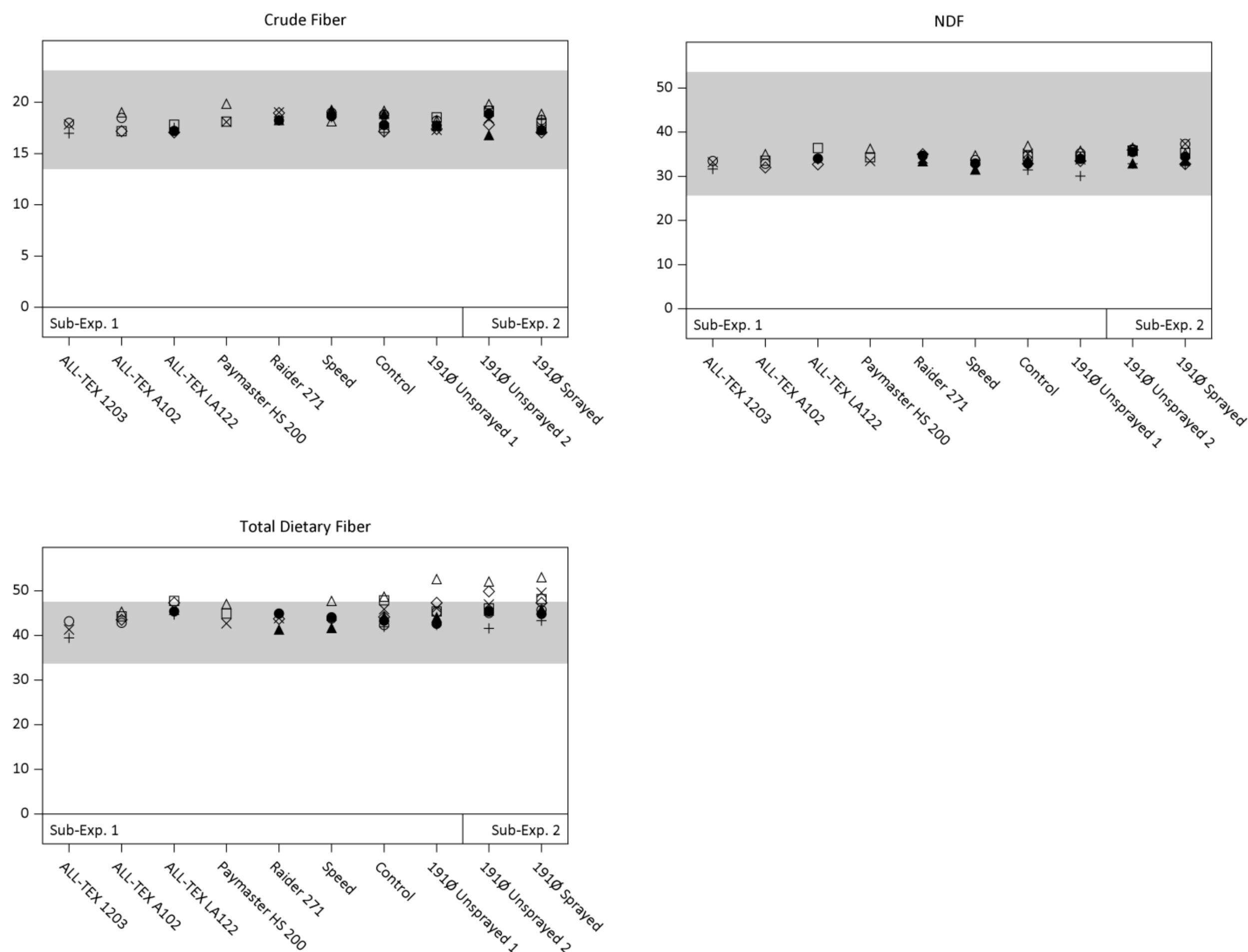


Figure 43 (Cnt) Proximates & Fiber in Control, DAS-8191Ø-7 and Reference Variety Cottonseed

Y axis: Moisture = % fresh weight, all others = % dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed.191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

(ii) Sub-experiment One - Mineral Analysis of Seed

Results from the combined site analysis of the mineral composition for control and DAS-8191Ø-7 cottonseed are provided in Table 14 and Figure 44. Twelve analytes were measured including calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium, sulfur and zinc. Mean results for all minerals tested in DAS-8191Ø-7 unsprayed fell within the reference variety ranges and literature ranges where available (no values were reported in the literature for selenium and sulfur).

No significant FDR-adjusted P-values were observed for DAS-8191Ø-7 unsprayed except for manganese. Manganese showed no significant differences for FDR-adjusted P-values among the eight individual field sites. Significant FDR-adjusted P-values were only identified in the combined site analysis. The relative magnitude of the difference between the mean values for DAS-8191Ø-7 unsprayed and the non-transgenic control for the combined site analysis was a decrease of 15.0%, which was less than the variability observed for the control sample for manganese (range 1.17 – 2.36, a relative difference of 101.7%). The observed differences in manganese between DAS-8191Ø-7 and the non-transgenic control were not considered to be meaningful from a food and safety or nutritional perspective because they were small, less than the variability seen in the control, statistically insignificant when compared at each individual field site and the mean DAS-891Ø-7 value for manganese was within both reference variety and literature ranges.

For the remaining mineral analytes, mean results for DAS-8191Ø-7 unsprayed fell within the reference variety ranges and literature ranges. Statistical analyses found no differences between the levels of nutrient components in cottonseed from DAS-8191Ø-7 and the non-transgenic control, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 14. Combined Site Analysis Results for Minerals in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (mg/100 g Dry Weight)	**Overall Trtmt Effect (Pr > F)**	**Control**	**DAS-8191Ø-7 Unsprayed**	**Reference Variety Range**	**Overall Trtmt Effect (Pr > F)**	**DAS-8191Ø-7 Unsprayed**	**DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium**	**Literature Range**
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
Calcium	**0.011**	124 ± 7 88.1 - 164	133 ± 7 98.0 - 172 (**0.011**, 0.059)	78.9 - 204	0.960	136 ± 7 113 - 178	136 ± 7 114 - 178 (0.960, 0.998)	100 - 330
Copper	**0.021**	0.90 ± 0.07 0.525 - 1.37	0.86 ± 0.07 0.532 - 1.30 (**0.021**, 0.096)	0.466 - 1.44	0.944	0.86 ± 0.08 0.446 - 1.32	0.86 ± 0.08 0.426 - 1.41 (0.944, 0.998)	0.313 - 2.457
Iron	0.546	4.20 ± 0.24 3.24 - 6.26	4.25 ± 0.24 3.45 - 5.76 (0.546, 0.747)	3.43 - 6.45	0.353	4.25 ± 0.21 3.28 - 5.71	4.30 ± 0.21 3.43 - 5.57 (0.353, 0.945)	3.671 - 31.838
Magnesium	0.420	387 ± 18 291 - 487	384 ± 18 282 - 488 (0.420, 0.679)	285 - 470	0.278	391 ± 19 250 - 494	388 ± 19 283 - 498 (0.278, 0.945)	340 - 493.12
Manganese	**0.002**	1.59 ± 0.10 1.17 - 2.36	1.35 ± 0.10 0.968 - 2.20 (**0.002**, **0.021**)	0.983 - 2.28	0.789	1.31 ± 0.08 0.985 - 1.93	1.31 ± 0.08 1.01 - 2.04 (0.789, 0.990)	1.069 - 2.216
Molybdenum	0.460	0.039 ± 0.012 0.00412 - 0.107	0.036 ± 0.012 0.00427 - 0.106 (0.460, 0.702)	0.00326 - 0.122	0.331	0.036 ± 0.011 0.00557 - 0.0980	0.034 ± 0.011 0.00539 - 0.0999 (0.331, 0.945)	NR
Phosphorus	0.079	652 ± 42 491 - 924	633 ± 42 469 - 921 (0.079, 0.259)	460 - 901	0.378	655 ± 45 412 - 936	649 ± 45 472 - 933 (0.378, 0.945)	482.54 - 991.57

Table 14. Combined Site Analysis Results for Minerals in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (mg/100 g Dry Weight)	Overall Trtmt Effect (Pr > F)	Control	DAS-8191Ø-7 Unsprayed	Reference Variety Range	Overall Trtmt Effect (Pr > F)	DAS-8191Ø-7 Unsprayed	DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium	Literature Range
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
Potassium	0.106	1078 ± 23 958 - 1230	1055 ± 23 937 - 1190 (0.106, 0.331)	938 - 1290	0.613	1074 ± 23 939 - 1200	1070 ± 23 934 - 1190 (0.613, 0.945)	960 - 1448.35
Selenium (ppb Dry Weight)	0.379	110 ± 30 <LOQ - 382	122 ± 30 <LOQ - 378 (0.379, 0.642)	<LOQ - 676	0.670	194 ± 95 <LOQ - 2010	180 ± 95 <LOQ - 1070 (0.670, 0.945)	NR
Sodium	0.249	123 ± 8 77.5 - 178	111 ± 8 64.7 - 188 (0.249, 0.577)	73.9 - 192	0.187	128 ± 9 78.7 - 207	136 ± 9 75.3 - 192 (0.187, 0.945)	5.4 - 740
Sulfur	0.541	495 ± 67 305 - 952	535 ± 67 311 - 1850 (0.541, 0.747)	331 - 847	0.734	520 ± 74 279 - 1430	499 ± 74 303 - 1180 (0.734, 0.945)	NR
Zinc	0.290	3.36 ± 0.13 2.63 - 3.93	3.47 ± 0.13 2.47 - 5.29 (0.290, 0.591)	2.77 - 4.26	0.821	3.51 ± 0.21 2.33 - 5.53	3.48 ± 0.21 2.23 - 5.94 (0.821, 0.998)	2.70 - 5.95

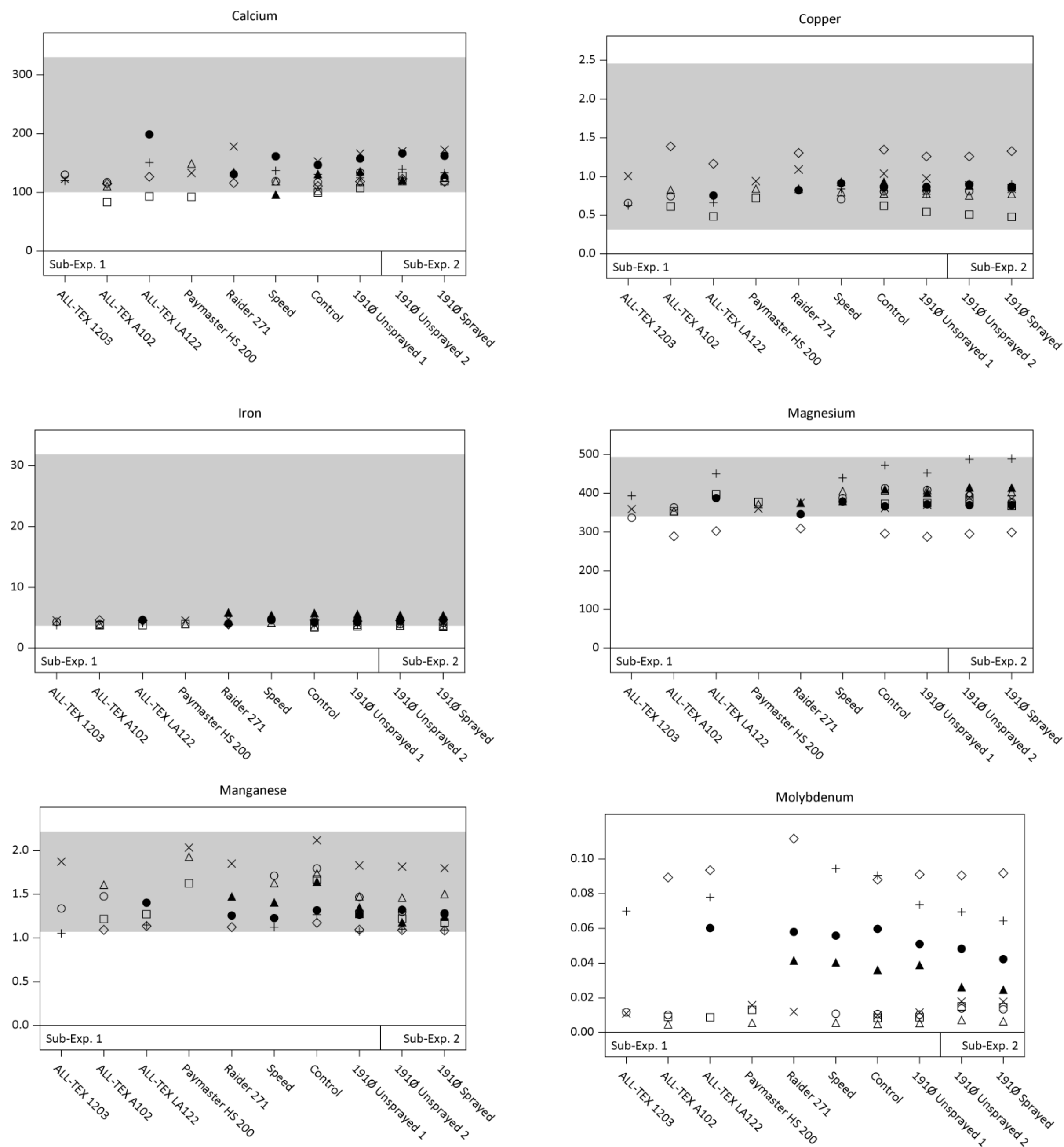


Figure 44. Minerals in Control, DAS-8191Ø-7 and Reference Variety CottonseedY axis: selenium = ppb dry weight, all others = mg/100 g dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range (where available).

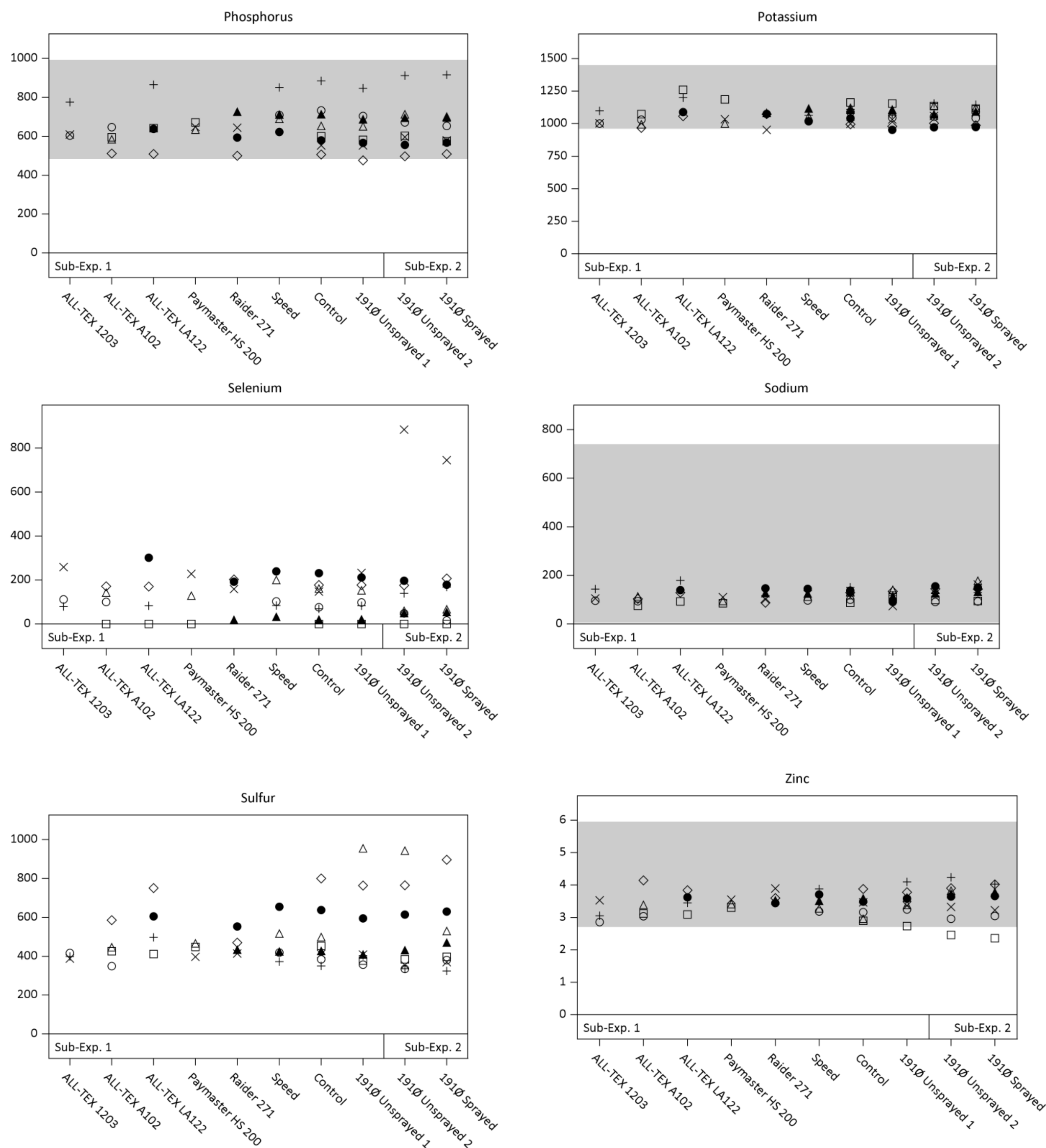


Figure 44 (Cnt) Minerals in control, DAS-8191Ø-7, and reference variety cottonseed

Y axis: selenium = ppb dry weight, all others = mg/100 g dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range (where available).

(iii) Sub-experiment One - Amino Acid Analysis of Seed

Results from the combined site analysis of the amino acid composition for control and DAS 8191Ø 7 cottonseed are provided in Table 15 and Figure 45. Eighteen analytes were measured including alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. Mean results for all eighteen of the amino acids tested in DAS-8191Ø-7 unsprayed fell within the reference variety ranges and/or literature ranges.

For all amino acid analytes measured, no significant FDR-adjusted P-values were observed for DAS-8191Ø-7 unsprayed. In addition, mean results for all eighteen DAS-8191Ø-7 unsprayed analytes fell within the reference variety ranges and literature ranges. Based on these data, statistical analyses found no differences between the levels of nutrient components in cottonseed from DAS-8191Ø-7 and the non-transgenic control, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 15. Combined Site Analysis Results for Amino Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (Units)	**Overall Trtmt Effect (Pr > F)**	**Control**	**DAS-8191Ø-7 Unsprayed**	**Reference Variety Range**	**Overall Trtmt Effect (Pr > F)**	**DAS-8191Ø-7 Unsprayed**	**DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium**	**Literature Range**
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				Sub-Experiment 2 (2,4-D Applied)			
Alanine	0.705	4.44 ± 0.03 4.29 - 4.598	4.45 ± 0.03 4.22 - 4.741 (0.705, 0.815)	4.18 - 4.656	0.549	4.48 ± 0.05 4.265 - 4.734	4.49 ± 0.05 4.25 - 4.746 (0.549, 0.945)	4.08 - 5.30
Arginine	**0.020**	12.64 ± 0.16 11.87 - 13.73	12.49 ± 0.16 11.50 - 13.67 (**0.020**, 0.096)	11.57 - 13.67	0.677	12.41 ± 0.20 11.27 - 13.47	12.39 ± 0.20 11.31 - 13.77 (0.677, 0.945)	10.83 - 15.18
Aspartic Acid	0.349	10.09 ± 0.15 9.67 - 11.03	10.25 ± 0.15 9.75 - 12.03 (0.349, 0.642)	9.59 - 11.42	0.182	10.32 ± 0.22 9.69 - 11.74	10.56 ± 0.22 9.70 - 12.33 (0.182, 0.945)	9.00 - 12.37
Cystine	0.145	1.785 ± 0.040 1.563 - 2.126	1.833 ± 0.040 1.529 - 2.214 (0.145, 0.427)	1.593 - 2.339	0.701	1.799 ± 0.029 1.559 - 2.235	1.786 ± 0.029 1.542 - 2.165 (0.701, 0.945)	1.53 - 2.35
Glutamic Acid	0.701	20.11 ± 0.13 19.17 - 20.73	20.07 ± 0.13 18.58 - 20.72 (0.701, 0.815)	19.44 - 21.23	0.437	19.92 ± 0.20 18.91 - 20.69	19.83 ± 0.20 18.33 - 20.65 (0.437, 0.945)	20.24 - 22.90
Glycine	0.949	4.42 ± 0.04 4.14 - 4.61	4.41 ± 0.04 4.081 - 4.546 (0.949, 0.969)	4.10 - 4.594	0.382	4.45 ± 0.05 4.053 - 4.633	4.44 ± 0.05 4.070 - 4.738 (0.382, 0.945)	4.29 - 5.72
Histidine	0.267	2.841 ± 0.016 2.695 - 3.048	2.868 ± 0.016 2.604 - 3.001 (0.267, 0.583)	2.651 - 3.077	0.652	2.840 ± 0.019 2.685 - 2.999	2.829 ± 0.019 2.606 - 2.978 (0.652, 0.945)	2.91 - 3.88
Isoleucine	0.746	3.62 ± 0.02 3.284 - 3.790	3.61 ± 0.02 3.391 - 3.799 (0.746, 0.847)	3.180 - 3.817	0.737	3.63 ± 0.03 3.268 - 3.880	3.62 ± 0.03 3.329 - 3.876 (0.737, 0.945)	3.10 - 4.46
Leucine	0.702	6.30 ± 0.02 6.15 - 6.49	6.31 ± 0.02 6.18 - 6.45 (0.702, 0.815)	6.04 - 6.54	0.153	6.34 ± 0.04 6.15 - 6.60	6.32 ± 0.04 6.14 - 6.60 (0.153, 0.945)	6.03 - 8.11

Table 15. Combined Site Analysis Results for Amino Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (% Total Amino Acid)	**Overall Trtmt Effect (Pr > F)**	**Control**	**DAS-8191Ø-7 Unsprayed**	**Reference Variety Range**	**Overall Trtmt Effect (Pr > F)**	**DAS-8191Ø-7 Unsprayed**	**DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium**	**Literature Range**
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				Sub-Experiment 2 (2,4-D Applied)			
Lysine	0.254	4.69 ± 0.06 4.36 - 5.09	4.73 ± 0.06 4.44 - 5.107 (0.254, 0.577)	4.27 - 5.03	0.727	4.72 ± 0.07 4.40 - 5.209	4.74 ± 0.07 4.39 - 5.149 (0.727, 0.945)	4.62 - 6.60
Methionine	0.464	1.640 ± 0.023 1.406 - 1.801	1.625 ± 0.023 1.460 - 1.774 (0.464, 0.702)	1.339 - 1.785	0.728	1.651 ± 0.033 1.434 - 1.988	1.645 ± 0.033 1.449 - 1.908 (0.728, 0.945)	1.27 - 2.28
Phenylalanine	0.504	5.70 ± 0.06 5.49 - 6.12	5.68 ± 0.06 5.201 - 6.01 (0.504, 0.726)	5.44 - 6.02	0.137	5.68 ± 0.06 5.35 - 5.88	5.63 ± 0.06 5.223 - 5.97 (0.137, 0.945)	5.44 - 7.23
Proline	0.983	4.04 ± 0.01 3.908 - 4.116	4.04 ± 0.01 3.895 - 4.187 (0.983, 0.983)	3.780 - 4.192	0.897	4.04 ± 0.02 3.903 - 4.200	4.05 ± 0.02 3.92 - 4.160 (0.897, 0.998)	3.81 - 5.30
Serine	0.878	4.63 ± 0.04 4.352 - 4.88	4.63 ± 0.04 4.32 - 4.875 (0.878, 0.925)	4.26 - 5.05	0.897	4.63 ± 0.03 4.354 - 4.915	4.63 ± 0.03 4.291 - 4.928 (0.897, 0.998)	4.15 - 5.87
Threonine	0.849	3.54 ± 0.03 3.312 - 3.705	3.54 ± 0.03 3.291 - 3.684 (0.849, 0.911)	3.186 - 3.756	0.705	3.57 ± 0.04 3.261 - 3.800	3.58 ± 0.04 3.338 - 3.799 (0.705, 0.945)	2.67 - 4.26
Tryptophan	0.557	1.433 ± 0.019 1.240 - 1.592	1.422 ± 0.019 1.267 - 1.651 (0.557, 0.747)	1.295 - 1.677	0.927	1.422 ± 0.018 1.254 - 1.595	1.420 ± 0.018 1.199 - 1.652 (0.927, 0.998)	0.91 - 1.40
Tyrosine	0.155	3.335 ± 0.015 3.201 - 3.457	3.313 ± 0.015 3.180 - 3.422 (0.155, 0.436)	3.190 - 3.459	0.507	3.331 ± 0.020 3.192 - 3.490	3.320 ± 0.020 3.157 - 3.468 (0.507, 0.945)	2.63 - 3.46
Valine	0.426	4.75 ± 0.02 4.43 - 4.96	4.74 ± 0.02 4.50 - 4.92 (0.426, 0.679)	4.36 - 5.02	0.610	4.76 ± 0.04 4.36 - 5.023	4.74 ± 0.04 4.45 - 5.059 (0.610, 0.945)	4.49 - 6.24

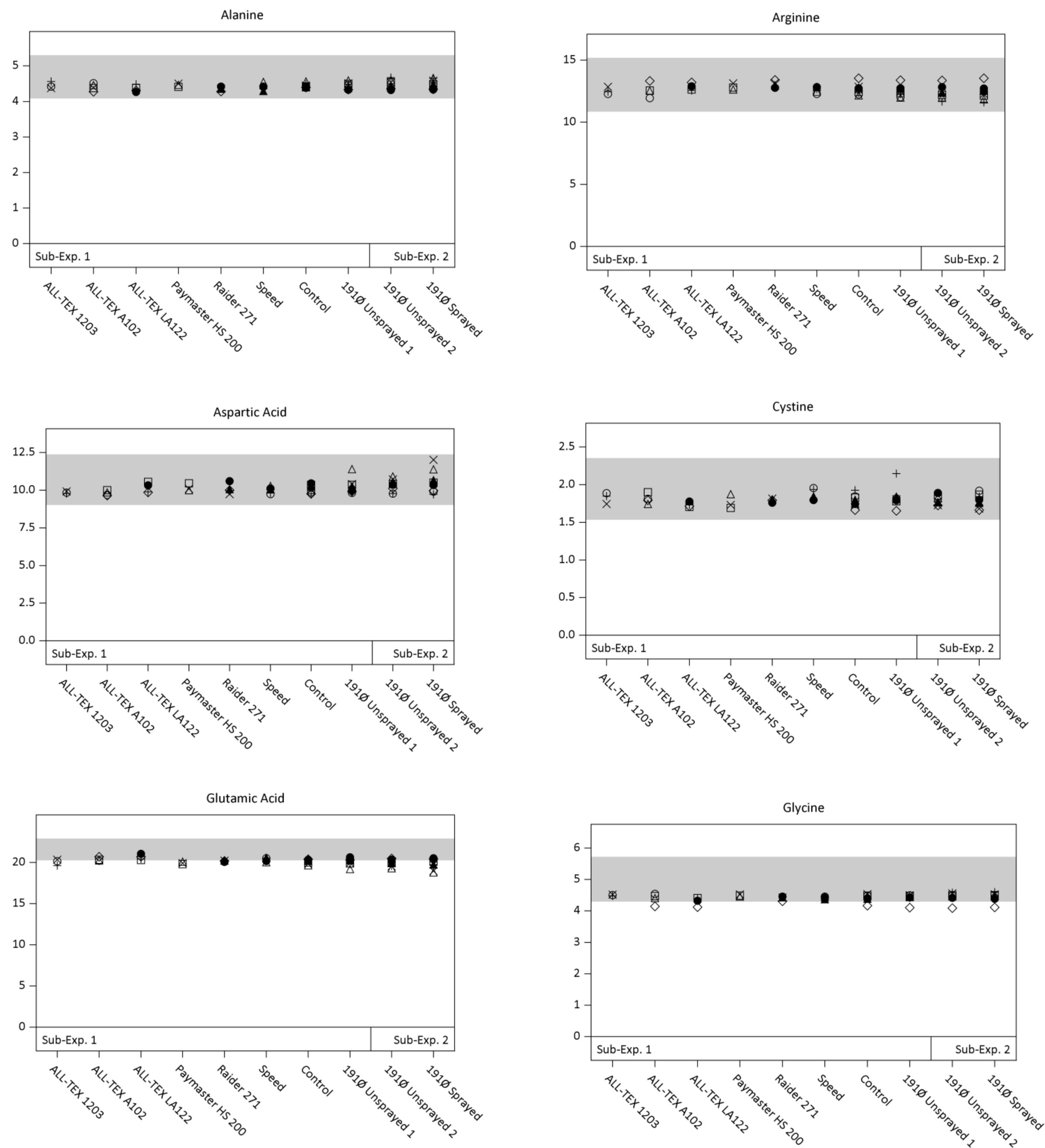


Figure 45. **Percent Total Amino Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed**Y axis: % Total Amino Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

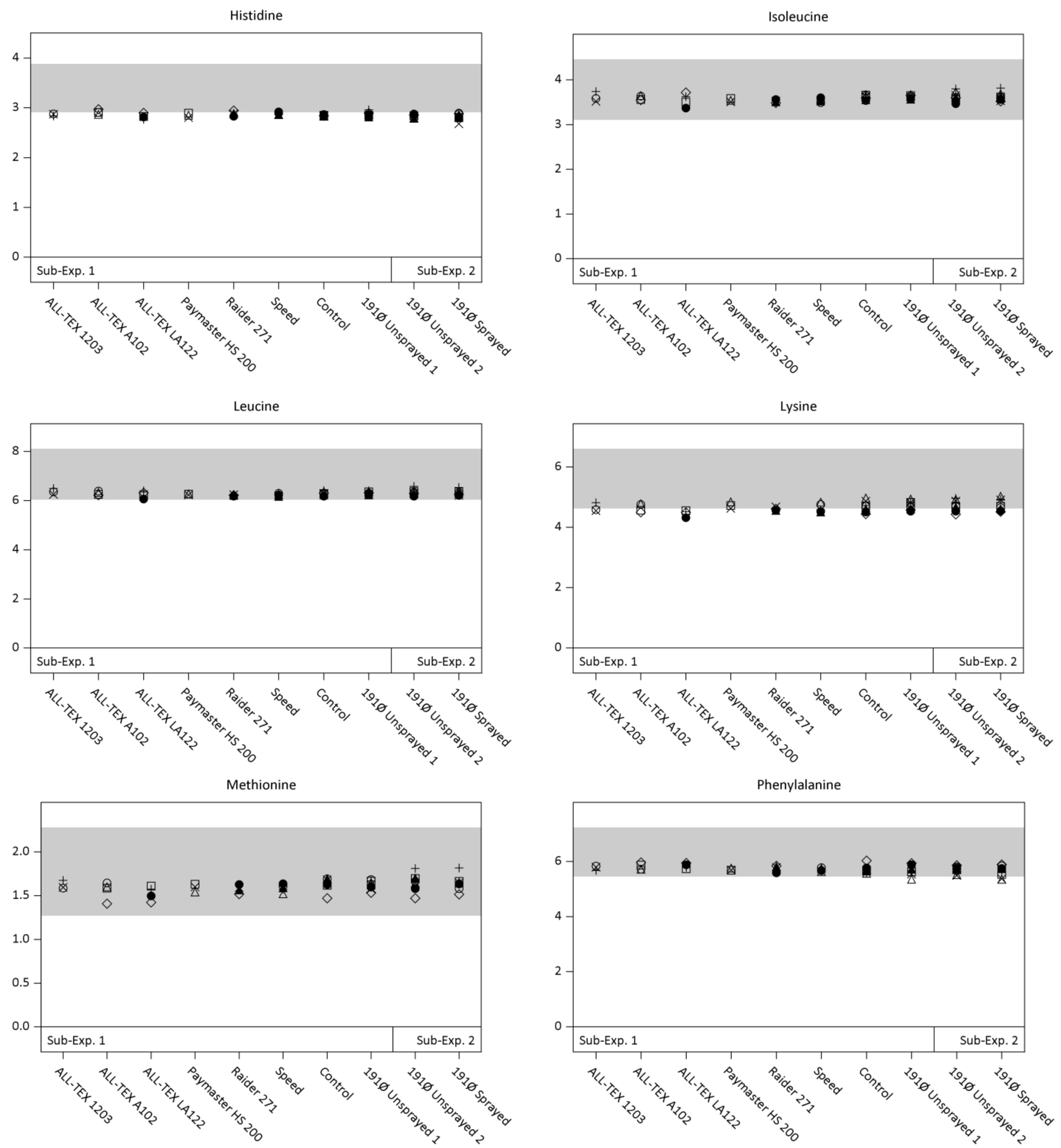


Figure 45. Percent Total Amino Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed

Y axis: % Total Amino Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment

2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range

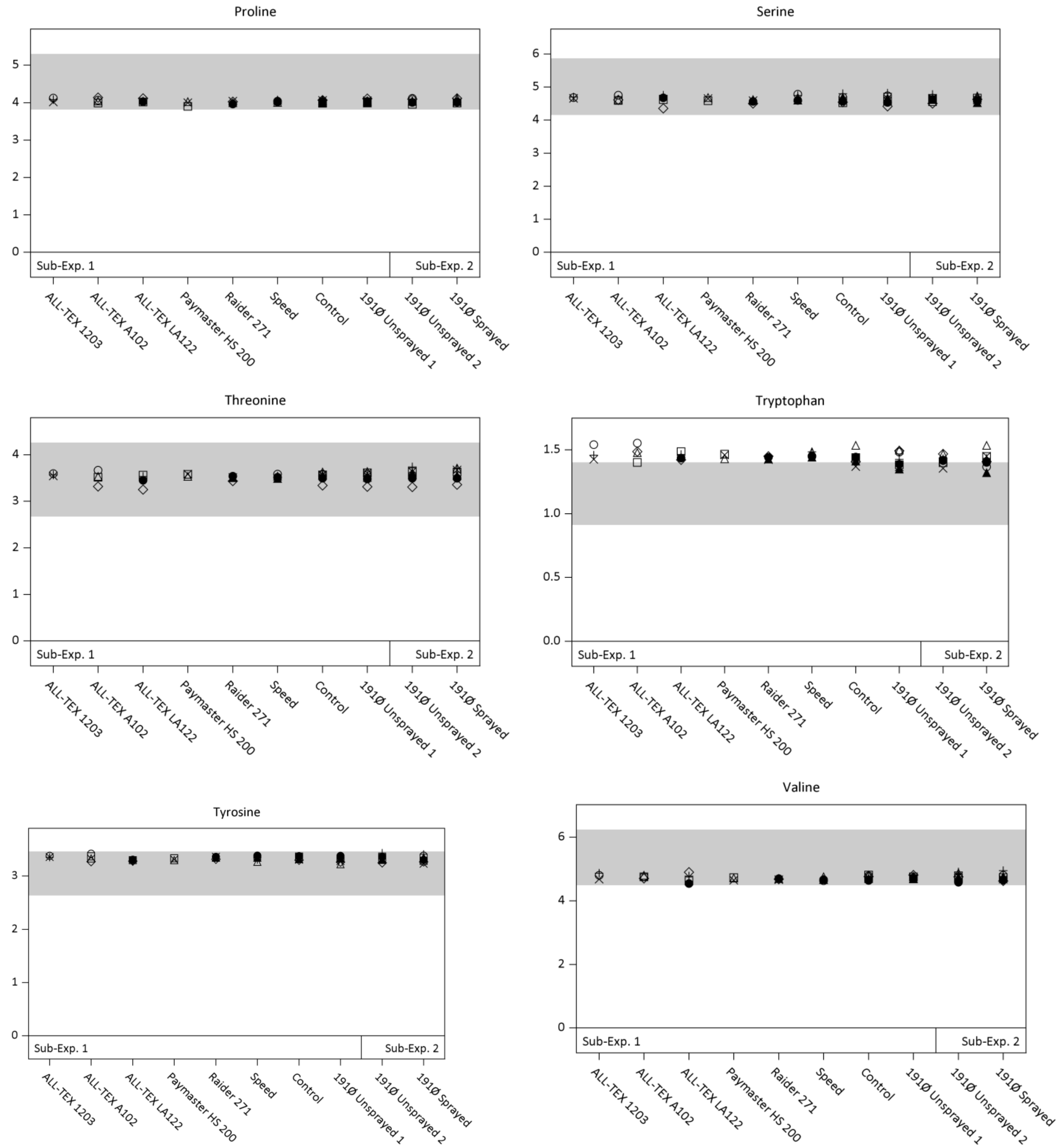


Figure 45. Percent Total Amino Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed

Y axis: % Total Amino Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range

(iv) Sub-experiment One - Fatty Acid Analysis of Seed

Results from the combined site analysis of the fatty acid composition (22 analytes) for control and DAS-8191Ø-7 cottonseed are provided in Table 16 and Figure 46 of the 22 fatty acids analysed (Table 16), thirteen were excluded from the analysis because the majority of the results were less than the LOQ Table 18 and Table 16). Mean results in DAS-8191Ø-7 unsprayed for the remaining nine fatty acids including 14:0 myristic, 16:0 palmitic, 16:1 palmitoleic, 18:1 oleic, 18:2 linoleic, 18:0 stearic, 18:3 linolenic, 20:0 arachidic, and 22:0 behenic, fell within the reference variety ranges and/or literature ranges.

The FDR-adjusted P-values for 14:0 myristic, 16:1 palmitoleic, 18:1 oleic, and 18:2 linoleic were significant. Statistical differences for 16:1 palmitoleic and 18:1 oleic were not consistently observed among individual sites, in which 16:1 palmitoleic decreased at one of the eight sites by a mean difference of -2.3%, while 18:1 oleic acid decreased at one of the eight sites by a mean difference of -5.3%. In addition, the relative magnitudes of the differences between the mean values for DAS-8191Ø-7 unsprayed and the non-transgenic control for the combined site analysis were a decrease of 6.9% for 16:1 palmitoleic and a decrease of 5.9% for 18:1 oleic acid. This was less than the variability observed for the control sample for 16:1 palmitoleic (range .4105 – 0.639, a relative difference of 55.7%) and 18:1 oleic (range 13.74 – 17.16 a relative difference of 24.9%). The observed differences in 16:1 palmitoleic and 18:1 oleic between DAS-8191Ø-7 and the non-transgenic control were not considered to be meaningful from a food and safety or nutritional perspective because they were small, not consistently reproducible across the individual sites and the mean DAS-891Ø-7 values for 16:1 palmitoleic and 18:1 oleic were within both reference variety and literature ranges.

For 14:0 myristic and 18:2 linoleic, no significant differences for FDR-adjusted P-values were observed among the individual sites. Significant FDR-adjusted P-values were only identified in the combined site analysis. The relative magnitudes of the differences between the mean values for DAS-8191Ø-7 unsprayed and the non-transgenic control for the combined site analysis were a decrease of 8.75% for 14:0 myristic and an increase of 2.05% for 18:2 linoleic. This was less than the variability observed for the control sample for 14:0 myristic (range 0.545 – 0.907, a relative difference of 66.4%) and 18:2 linoleic (range 54.4 – 61.1, a relative difference of 12.3%). The observed differences in 14:0 myristic and 18:2 linoleic between DAS-8191Ø-7 and the non-transgenic control were not considered to be meaningful from a food and safety or nutritional perspective because they were small and the mean DAS-891Ø-7 values for 14:0 myristic and 18:2 linoleic were within both reference variety and literature ranges.

For 16:0 palmitic, 18:0 stearic, 18:3 linolenic, 20:0 arachidic, and 22:0 behenic, mean results for DAS-8191Ø-7 unsprayed fell within the reference variety ranges and literature ranges. Statistical analyses found no FDR-adjusted differences between the levels of nutrient components in cottonseed from DAS-8191Ø-7 and the non-transgenic control, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 16. **Combined Site Analysis Results for Fatty Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges**

Analytical Component (% Total Fatty Acid)	**Overall Trtmt Effect (Pr > F)**	**Control**	**DAS-8191Ø-7 Unsprayed**	**Reference Variety Range**	**Overall Trtmt Effect (Pr > F)**	**DAS-8191Ø-7 Unsprayed**	**DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium**	**Literature Range**
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
8:0 Caprylic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
10:0 Capric	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
12:0 Lauric	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
14:0 Myristic	**<0.001**	0.720 ± 0.041 0.545 - 0.907	0.657 ± 0.041 0.489 - 0.872 (**<0.001**, **0.002**)	0.4324 - 1.046	0.541	0.651 ± 0.041 0.508 - 0.871	0.648 ± 0.041 0.506 - 0.866 (0.541, 0.945)	0.455 - 2.40
14:1 Myristoleic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
15:0 Pentadecanoic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	0.050 - 0.481
15:1 Pentadecenoic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
16:0 Palmitic	**0.027**	22.55 ± 0.58 20.37 - 25.67	22.26 ± 0.58 20.04 - 26.12 (**0.027**, 0.114)	18.76 - 26.07	0.216	22.26 ± 0.66 19.71 - 26.14	22.16 ± 0.66 19.53 - 25.99 (0.216, 0.945)	15.11 - 28.10
16:1 Palmitoleic	**<0.001**	0.494 ± 0.022 0.4105 - 0.639	0.460 ± 0.022 0.3917 - 0.588 (**<0.001**, **0.003**)	0.3787 - 0.636	0.203	0.454 ± 0.023 0.3776 - 0.587	0.451 ± 0.023 0.3728 - 0.592 (0.203, 0.945)	0.464 - 1.190

Table 16. Combined Site Analysis Results for Fatty Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (% Total Fatty Acid)	**Overall Trtmt Effect (Pr > F)**	**Control**	**DAS-8191Ø-7 Unsprayed**	**Reference Variety Range**	**Overall Trtmt Effect (Pr > F)**	**DAS-8191Ø-7 Unsprayed**	**DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium**	**Literature Range**
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
17:0 Heptadecanoic	NA	NA <LOQ - 0.0990	NA <LOQ - 0.1046	<LOQ - 0.1085	NA	NA <LOQ - 0.1044	NA <LOQ - 0.1030	0.092 - 0.119
17:1 Heptadecenoic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
18:0 Stearic	0.373	2.311 ± 0.068 1.951 - 2.685	2.334 ± 0.068 1.943 - 2.645 (0.373, 0.642)	1.801 - 2.962	0.124	2.341 ± 0.073 1.959 - 2.628	2.313 ± 0.073 1.875 - 2.612 (0.124, 0.945)	0.20 - 3.11
18:1 Oleic	**<0.001**	14.84 ± 0.35 13.74 - 17.16	13.95 ± 0.35 12.76 - 16.50 (**<0.001**, **0.001**)	12.93 - 17.09	0.698	13.82 ± 0.34 12.54 - 16.50	13.80 ± 0.34 12.64 - 16.08 (0.698, 0.945)	12.8 - 25.3
18:2 Linoleic	**<0.001**	58.5 ± 0.8 54.4 - 61.1	59.7 ± 0.8 54.9 - 62.5 (**<0.001**, **0.003**)	52.36 - 63.9	0.247	59.9 ± 0.9 54.45 - 62.9	60.0 ± 0.9 54.7 - 62.91 (0.247, 0.945)	46.00 - 59.4
18:3 Linolenic	0.249	0.2032 ± 0.0083 0.1733 - 0.2412	0.2117 ± 0.0083 0.1782 - 0.2991 (0.249, 0.577)	0.1460 - 0.2567	0.617	0.2126 ± 0.0090 0.1829 - 0.2761	0.2150 ± 0.0090 0.1828 - 0.2688 (0.617, 0.945)	0.11 - 0.35
18:3 γ-Linolenic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	0.097 - 0.232
20:0 Arachidic	0.489	0.2509 ± 0.0105 0.2088 - 0.2949	0.2492 ± 0.0105 0.2029 - 0.3063 (0.489, 0.722)	0.1855 - 0.3242	0.660	0.2530 ± 0.0111 0.2010 - 0.3113	0.2521 ± 0.0111 0.2067 - 0.2991 (0.660, 0.945)	0.186 - 0.414
20:1 Eicosenoic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	0.095 - 0.098

Table 16. Combined Site Analysis Results for Fatty Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (% Total Fatty Acid)	Overall Trtmt Effect (Pr > F)	Control	DAS-8191Ø-7 Unsprayed	Reference Variety Range	Overall Trtmt Effect (Pr > F)	DAS-8191Ø-7 Unsprayed	DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium	Literature Range
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
20:2 Eicosadienoic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
20:3 Eicosatrienoic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
20:4 Arachidonic	NA	NA <LOQ	NA <LOQ	<LOQ	NA	NA <LOQ	NA <LOQ	NR
22:0 Behenic	0.181	0.1373 ± 0.0060 0.1119 - 0.1692	0.1341 ± 0.0060 0.1057 - 0.1693 (0.181, 0.464)	0.1035 - 0.1749	0.373	0.1358 ± 0.0071 0.1101 - 0.1757	0.1386 ± 0.0071 0.1092 - 0.1726 (0.373, 0.945)	0.104 - 0.295

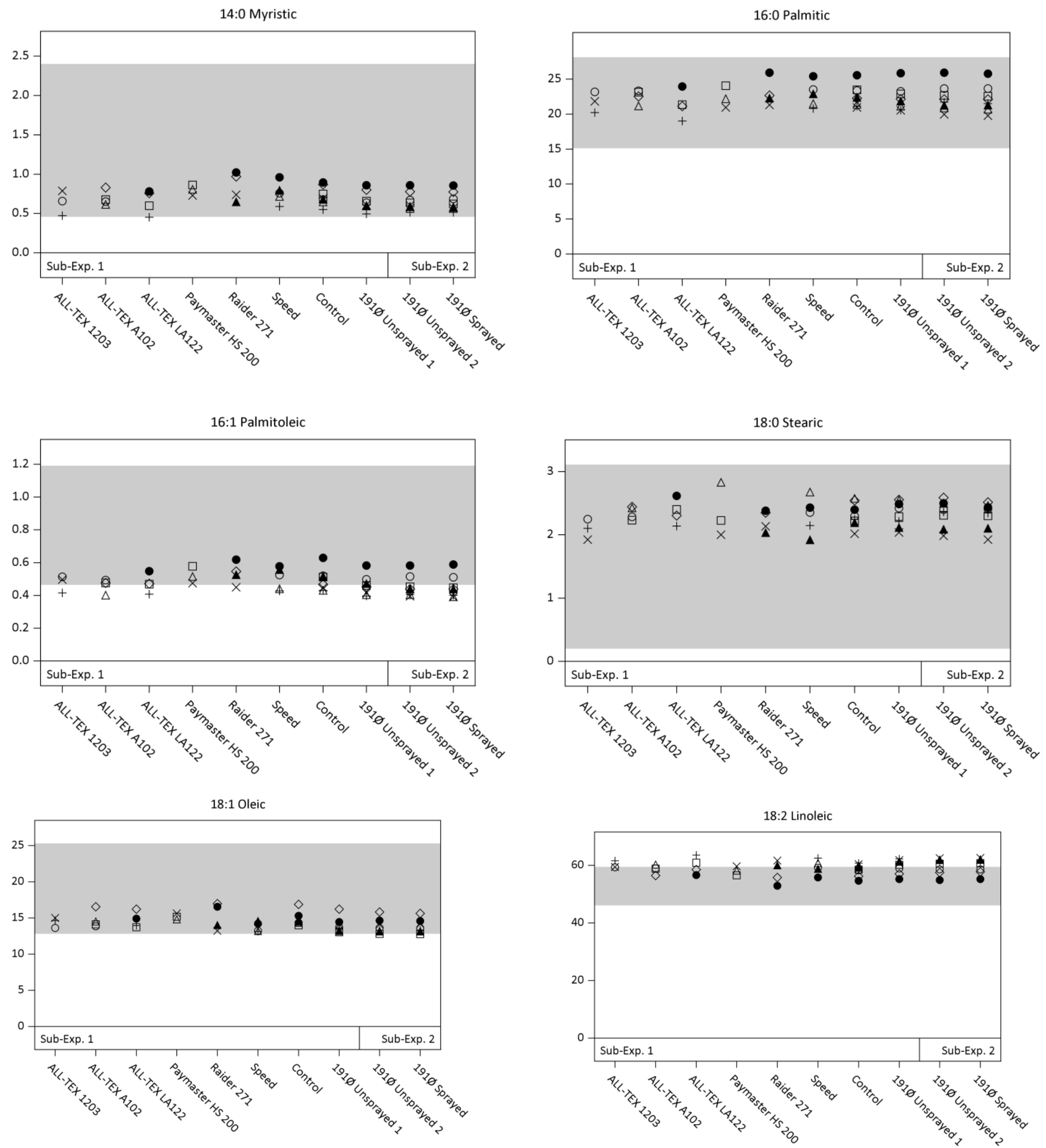


Figure 46. **Percent Total Fatty Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed** Y axis: % Total Fatty Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

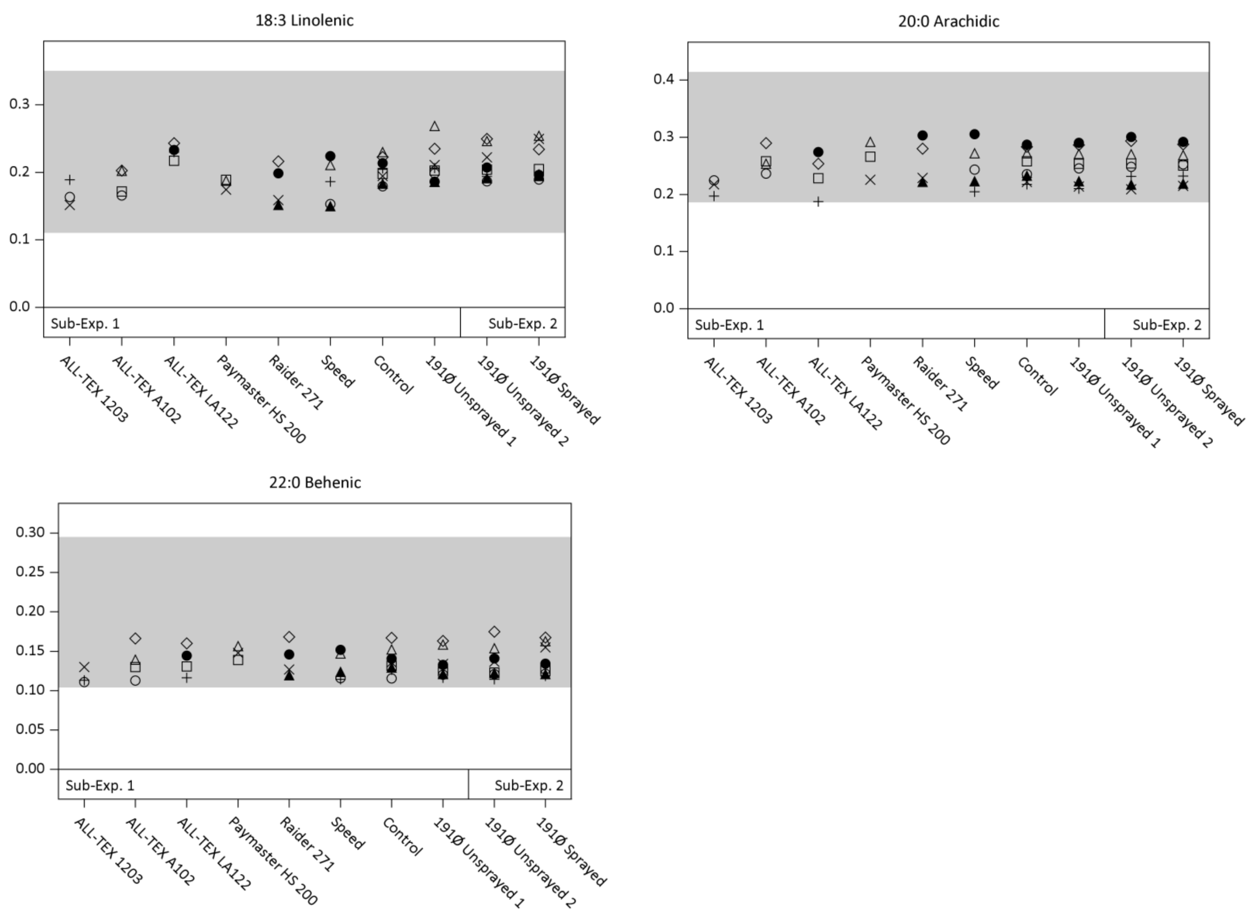


Figure 46. Percent Total Fatty Acids in Control, DAS-8191Ø-7 and Reference Variety Cottonseed

Y axis: % Total Fatty Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

(v) Sub-Experiment One - Vitamin Analysis of Seed

Results from the combined site analysis of the vitamin composition from control and DAS-8191Ø-7 cottonseed are provided in and Figure 47. Seven vitamin analytes were measured including vitamin E, A, B1, B2, B3, B6, and B9 (Table 17). Vitamin A (beta carotene) was excluded from the analysis because the majority of the results were less than the LOQ. Mean results in DAS-8191Ø-7 unsprayed for the remaining six vitamins fell within the reference variety ranges and the literature ranges (only available for vitamin E). No significant FDR-adjusted P-values were observed for DAS-8191Ø-7 unsprayed in the six vitamins that were included in the statistical analysis, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 17. **Combined Site Analysis Results for Vitamins in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges**

Analytical Component (mg/kg Dry Weight)	Overall Trtmt Effect (Pr > F)	Control	DAS-8191Ø-7 Unsprayed	Reference Variety Range	Overall Trtmt Effect (Pr > F)	DAS-8191Ø-7 Unsprayed	DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium	Literature Range
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
Alpha Tocopherol (Vitamin E)	0.612	90 ± 11 30.3 - 134	88 ± 11 26.7 - 136 (0.612, 0.803)	31.1 - 151	0.360	90 ± 10 20.4 - 135	89 ± 10 27.6 - 129 (0.360, 0.945)	70.825 - 197.243
Vitamin A (Beta Carotene)	NA	NA <LOQ - 0.264	NA <LOQ - 0.323	<LOQ - 0.267	NA	NA <LOQ - 0.249	NA <LOQ - 0.238	NR
Vitamin B1 (Thiamine HCl)	0.953	10.3 ± 0.4 7.52 - 12.6	10.3 ± 0.4 8.28 - 13.2 (0.953, 0.969)	5.54 - 14.7	0.912	10.4 ± 0.5 6.14 - 13.4	10.4 ± 0.5 7.42 - 15.4 (0.912, 0.998)	NR
Vitamin B2 (Riboflavin)	0.813	6.2 ± 0.5 3.52 - 9.95	6.3 ± 0.5 3.82 - 9.82 (0.813, 0.888)	3.44 - 9.52	0.998	6.6 ± 0.5 3.69 - 12.5	6.6 ± 0.5 3.97 - 10.0 (0.998, 0.998)	NR
Vitamin B3 (Niacin)	0.315	27.8 ± 1.2 21.7 - 35.6	28.5 ± 1.2 22.7 - 35.3 (0.315, 0.620)	20.4 - 36.8	0.905	28.2 ± 1.1 21.3 - 37.7	28.1 ± 1.1 23.5 - 34.3 (0.905, 0.998)	NR
Vitamin B6 (Pyridoxine HCl)	0.176	3.83 ± 0.09 3.15 - 4.49	3.72 ± 0.09 3.10 - 4.54 (0.176, 0.464)	2.84 - 5.12	0.535	3.70 ± 0.13 3.18 - 4.72	3.77 ± 0.13 3.08 - 5.28 (0.535, 0.945)	NR
Vitamin B9 (Folic Acid)	0.806	1.66 ± 0.07 1.14 - 2.38	1.67 ± 0.07 1.17 - 2.26 (0.806, 0.888)	1.10 - 2.40	0.160	1.56 ± 0.09 0.872 - 2.31	1.64 ± 0.09 1.15 - 2.29 (0.160, 0.945)	NR

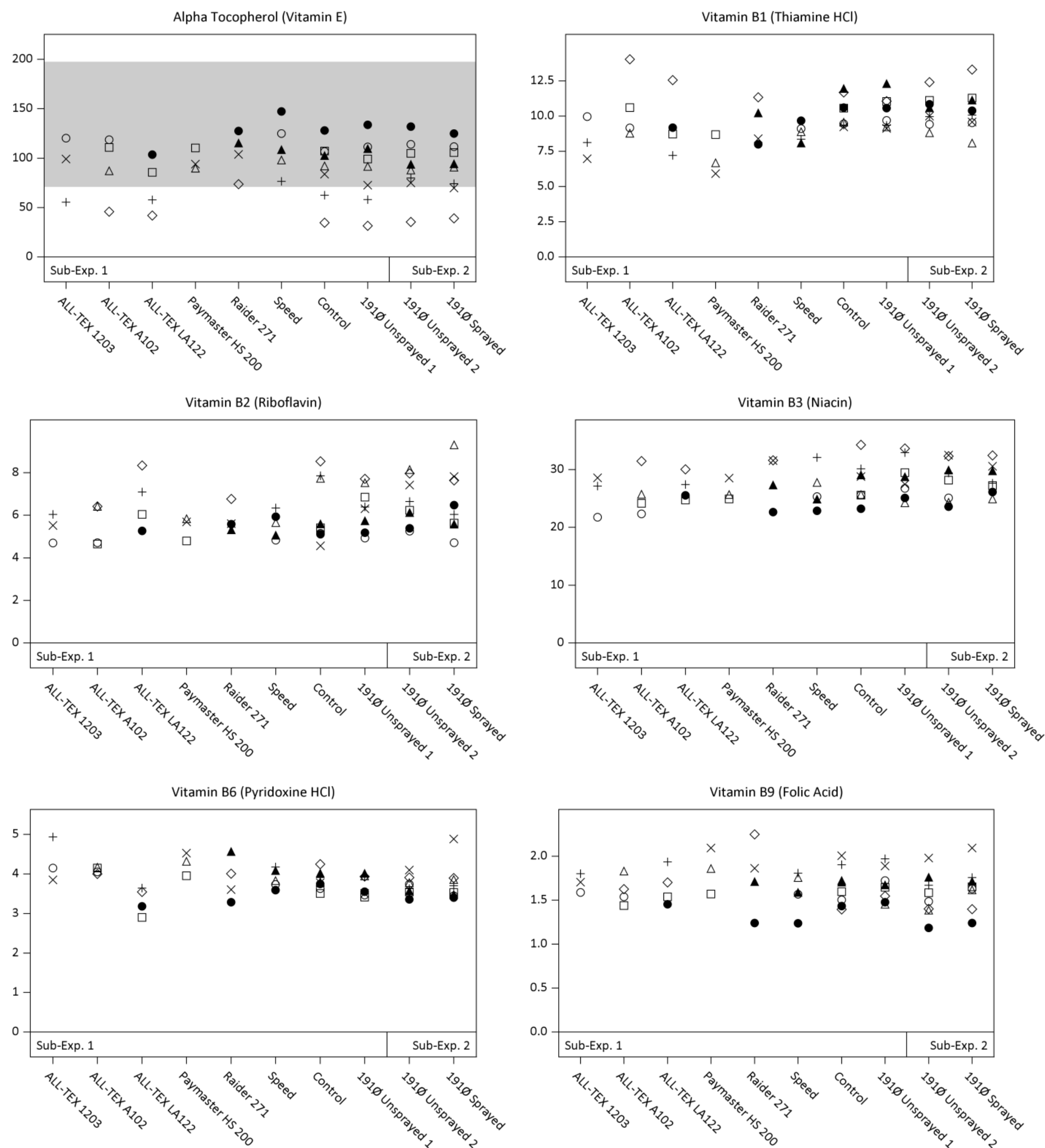


Figure 47. Vitamins in Control, DAS-8191Ø-7, and Reference Variety Cottonseed

Y axis: mg/kg Dry Weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

(vi) Sub-Experiment One - Anti-Nutrient Analysis of Seed

Results from the combined site analysis of the five anti-nutrient analytes including dihydrosterculic acid, malvalic acid, sterculic acid, free gossypol and total gossypol, from control and DAS-8191Ø-7 cottonseed are provided in Table 18 and Figure 48. Mean results in DAS-8191Ø-7 unsprayed for the five anti-nutrients analyzed fell within the reference variety ranges and the literature ranges.

The FDR-adjusted P-values were significant in DAS-8191Ø-7 unsprayed for malvalic acid and total gossypol. For both analytes, no significant differences for FDR-adjusted P-values were observed among the individual sites. Significant FDR-adjusted P-values were only identified in the combined site analysis. The relative magnitudes of the differences between the mean values for DAS-8191Ø-7 unsprayed and the non-transgenic control for the combined site analysis were an increase of 10.1% for malvalic acid and a decrease of 12% for total gossypol. This was less than the variability observed for the control sample for both malvalic acid (range 0.403 – .645, a relative difference of 60.0%) and total gossypol (range 0.829 – 1.44, a relative difference of 73.7%). The observed differences in malvalic acid and total gossypol between DAS-8191Ø-7 and the non-transgenic control were not considered to be meaningful from a food and safety or nutritional perspective because they were small, less than the variability seen in the control, statistically insignificant when compared at each individual field site and the mean DAS-891Ø-7 values for malvalic acid and total gossypol were within both reference variety and literature ranges.

For dihydrosterculic acid, sterculic acid and free gossypol mean results for DAS-8191Ø-7 unsprayed fell within the reference variety ranges and literature ranges. Statistical analyses found no FDR-adjusted differences between the levels of nutrient components in cottonseed from DAS-8191Ø-7 and the non-transgenic control, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 18. Combined Site Analysis Results for Anti-Nutrients in Control, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (Units)	**Overall Trtmt Effect (Pr > F)**	**Control**	**DAS-8191Ø-7 Unsprayed**	**Reference Variety Range**	**Overall Trtmt Effect (Pr > F)**	**DAS-8191Ø-7 Unsprayed**	**DAS-8191Ø-7 Sprayed w/ 2,4-D + Glufosinate-ammonium**	**Literature Range**
		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)	Min - Max
	Sub-Experiment 1 (No 2,4-D Applied)				**Sub-Experiment 2 (2,4-D Applied)**			
Dihydrosterculic Acid (% Total Fatty Acid)	0.074	0.205 ± 0.007 0.150 - 0.247	0.214 ± 0.007 0.174 - 0.261 (0.074, 0.259)	0.153 - 0.325	0.578	0.216 ± 0.006 0.175 - 0.269	0.214 ± 0.006 0.181 - 0.254 (0.578, 0.945)	0.075 - 0.310
Malvalic Acid (% Total Fatty Acid)	**0.003**	0.524 ± 0.023 0.403 - 0.645	0.577 ± 0.023 0.426 - 0.762 (**0.003**, **0.029**)	0.402 - 0.854	0.174	0.591 ± 0.026 0.474 - 0.722	0.612 ± 0.026 0.450 - 0.806 (0.174, 0.945)	0.17 - 0.759
Sterculic Acid (% Total Fatty Acid)	0.077	0.275 ± 0.011 0.173 - 0.447	0.297 ± 0.011 0.209 - 0.358 (0.077, 0.259)	0.196 - 0.440	0.964	0.301 ± 0.011 0.215 - 0.366	0.301 ± 0.011 0.196 - 0.391 (0.964, 0.998)	0.13 - 0.56
Free Gossypol (% Dry Weight)	**0.010**	0.96 ± 0.06 0.593 - 1.36	0.83 ± 0.06 0.556 - 1.17 (**0.010**, 0.059)	0.492 - 1.28	0.624	0.82 ± 0.06 0.479 - 1.20	0.81 ± 0.06 0.498 - 1.13 (0.624, 0.945)	0.454 - 1.399
Total Gossypol (% Dry Weight)	**0.001**	1.08 ± 0.05 0.829 - 1.44	0.95 ± 0.05 0.719 - 1.19 (**0.001**, **0.010**)	0.551 - 1.41	0.867	0.92 ± 0.05 0.519 - 1.31	0.93 ± 0.05 0.624 - 1.32 (0.867, 0.998)	0.547 - 1.522

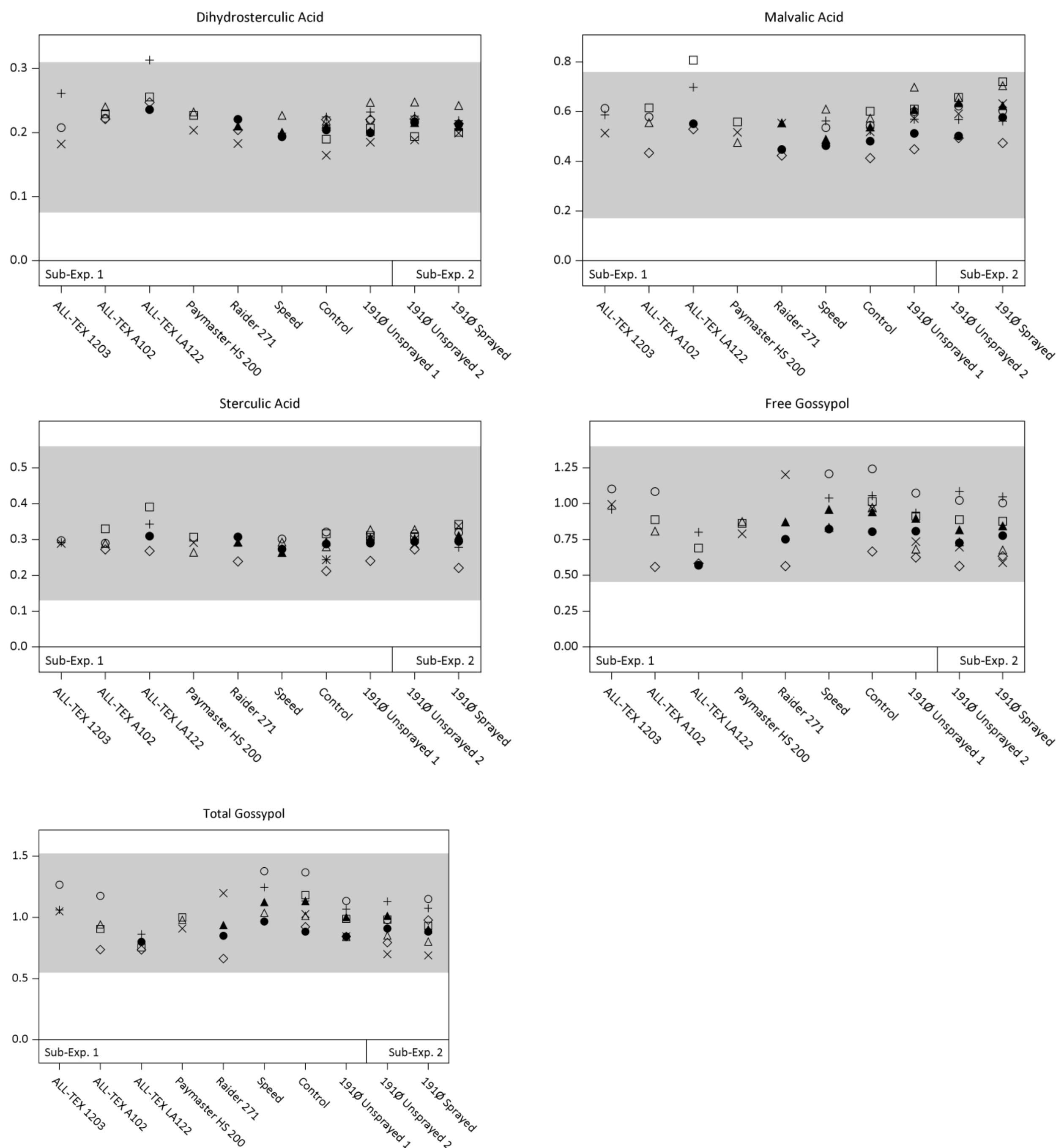


Figure 48. Anti-Nutrients in Control, DAS-8191Ø-7, and Reference Variety CottonseedY axis: see for specific values. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D + glufosinate-ammonium in sub-experiment 2. Symbols for each location shown: open circle = AL, × = GA, + = LA, open triangle = MO, open square = MS, open diamond = NC, filled circle = TX1, filled triangle = TX2. The shaded band represents the literature range.

(vii) Sub-Experiment Two - Composition Results

The comparisons between sprayed and unsprayed entries of DAS-8191Ø-7 cotton in sub-experiment two indicated no statistically significant differences for all 59 analytes included in the combined site analysis (Table 13 - Table 18 and Figure 43 -Figure 48). In addition, comparisons of unsprayed cotton in sub-experiment two with unsprayed cottons in sub-experiment one indicated no statistically significant differences for all 59 analytes. Based on these comparisons, it is concluded that sprayed DAS-8191Ø-7 cottonseed in sub-experiment two is compositionally equivalent to unsprayed DAS-8191Ø-7 cotton in sub-experiment one and indicates that spraying DAS-8191Ø-7 with 2,4-D plus glufosinate-ammonium has no significant effect on cottonseed composition. Based on these results, it is concluded that DAS-8191Ø-7 (unsprayed or sprayed with 2,4-D plus glufosinate-ammonium) cottonseed is compositionally equivalent to non-transgenic cottonseed.

3.6 e Composition Summary

Field trials were conducted at eight U.S. field sites in 2012 to produce cottonseed samples of DAS-8191Ø-7 cotton (unsprayed and sprayed with 2,4-D plus glufosinate-ammonium), non-transgenic control and non-transgenic cotton lines for nutrient composition analysis. Cottonseed samples were analyzed for 73 analytes including proximates, fiber, minerals, amino acids, fatty acids, vitamins, and anti-nutrients, which included analytes that are deemed important for the assessment of new cotton varieties for use in food and feed (OECD 2009a). Of the 73 analytes tested, 14 were excluded from the combined site analysis because more than 50% of the results for those analytes were less than the limit of quantitation (<LOQ) (Table 12).

Statistical analyses found no FDR-adjusted P-value differences in cottonseed from DAS-8191Ø-7 and the non-transgenic control for proximate, fiber, amino acid or vitamin analytes. In addition, mean values for these analytes all fell within reference variety ranges and / or literature ranges supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

For mineral, fatty acid and anti-nutrient composition analysis, all but seven analytes showed no statistical differences in FDR-adjusted P-values in combined site analysis of DAS-8191Ø-7 unsprayed cotton and the non-transgenic control. The mean values for mineral, fatty acid and anti-nutrient analytes with no statistical difference also fell within reference variety ranges and / or literature ranges.

Seven analytes including manganese, 14:0 myristic, 16:1 palmitoleic, 18:1 oleic, 18:2 linoleic, malvalic acid and total gossypol all showed statistical differences in FDR-adjusted P-values between the control and DAS-8191Ø-7 unsprayed and the non-transgenic control. However, the observed differences between the seven analytes in DAS-8191Ø-7 and the non-transgenic control were not considered to be meaningful from a food and safety or nutritional perspective because the differences between DAS-8191Ø-7 and the non-transgenic control were small, were less than the variability seen in the non-

transgenic control and the mean values for the seven analytes were all within reference variety and / or literature ranges.

Additionally, no statistically significant differences were detected between DAS-8191Ø-7 unsprayed and DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate-ammonium, which indicates that spraying DAS-8191Ø-7 with 2,4-D plus glufosinate-ammonium has no significant effect on cottonseed composition. Based on these results, it is concluded that DAS-8191Ø-7 (unsprayed or sprayed with 2,4-D plus glufosinate-ammonium) cottonseed is compositionally equivalent to non-transgenic cottonseed.

3.6 f Conclusions

Composition assessment of DAS-8191Ø-7 cotton was conducted in which key nutrient and anti-nutrient levels of unsprayed DAS-8191Ø-7 cottonseed were compared with the appropriate non-transgenic near-isogenic control and non-transgenic reference lines and between cottonseed from herbicide-treated and unsprayed DAS-8191Ø-7 cottonseed. The assessment was conducted using the principles and analytes in the OECD consensus document for cotton composition, in which samples were analyzed for proximates, fiber, minerals, amino acids, fatty acids, vitamins, and anti-nutrients.

Of the 73 analytes evaluated in this study, 59 produced sufficient data (>50% of data points above the limited of quantitation (LOQ)) for inclusion in the combined site statistical analysis. Therefore, 59 comparisons were made in each sub-experiment in this study. Of the 59 analytes included in the combined site analysis, 52 showed no statistical differences in FDR-adjusted P-values between the non-transgenic control and DAS-8191Ø-7 cottonseed, mean results fell within the reference variety ranges and / or the literature ranges, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Statistically significant differences ($P<0.05$) in FDR-adjusted P-values between the non-transgenic control and DAS-8191Ø-7 cottonseed were detected in seven analytes including manganese, 14:0 myristic, 16:1 palmitoleic, 18:1 oleic, 18:2 linoleic, malvalic acid and total gossypol. Mean analyte values that were observed to be significantly different from those of the non-transgenic control were generally shown to be of small relative magnitudes and mean results fell within the reference variety ranges and / or the literature ranges, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Additionally, no statistically significant differences were detected between DAS-8191Ø-7 unsprayed and DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate, which indicates that spraying DAS-8191Ø-7 with 2,4-D plus glufosinate-ammonium has no significant effect on cottonseed composition.

Based on the results of this composition analysis, it is concluded that cottonseed from DAS-8191Ø-7 is compositionally equivalent to non-transgenic cotton and therefore the food and feed safety and nutritional quality of this product is comparable to that of commercially cultivated cotton.

4. NUTRITIONAL IMPACT

Fast BJ, Johnson TY. 2013. Nutrient Composition of a Cotton Cultivar Containing Aryloxyalkanoate Dioxygenase-12 (AAD-12) and Phosphinothricin Acetyltransferase (PAT): Event DAS-8191Ø-7Study ID 120040.01, Dow AgroSciences LLC, Indianapolis IN
Fleming CR, 2013. Global Dietary and Livestock Assessment of PAT and AAD-12 Proteins for DAS Cotton Cultivar Based on Event DAS 8191Ø-7Study ID: 130093 Dow AgroSciences, LLC., Indianapolis, IN
Hill RC, 2013. Protein Expression of a Transformed Cotton Line Containing Aryloxyalkanoate Dioxygenase (AAD-12) and Phosphinothricin Acetyltransferase (PAT) - Event DAS-81910-7Study ID 120040.2, Dow AgroSciences LLC., Indianapolis IN
Schafer BW, 2010. Summary of the Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12Study ID 101047, Dow AgroSciences LLC, Indianapolis, IN
Wiescinski CM, Golden RM, 2008. AAD-12: Acute Oral Toxicity Study in Crl:CD1(ICR) MiceStudy ID 081037, The Dow Chemical Company, Midland, MI

4.1 Human Dietary Risk Assessment

4.1 a AAD-12 Protein Human Dietary Risk Assessment

Expression levels of AAD-12 in plant tissues of DAS-8191Ø-7 cotton were used in addition to conservative human dietary consumption data for cotton to estimate dietary exposure to the AAD-12 protein from DAS-8191Ø-7 cotton. Furthermore, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment for the AAD-12 protein in DAS-8191Ø-7 cotton supports the conclusion that there is negligible risk to human health (Fleming 2013).

The field expression of AAD-12 protein in DAS-8191Ø-7cotton has been measured using an AAD-12 specific enzyme linked immunosorbent assay (ELISA) in several plant tissues of cotton (Hill 2013). AAD-12 field expression data is available for trials conducted at six test sites located within the major cotton-producing regions of the U.S representing diverse agronomic practices and environmental conditions (Table 8).

For the human diet, only the cottonseed of cotton plants is useful for food. Cottonseed is not directly consumed by humans because the majority of commercial cotton varieties contain the anti-nutrients gossypol and cyclopropenoid fatty acids. For the human diet, cottonseed is used in food applications in which the seeds are mainly used to obtain refined edible oil, which contains undetectable amounts of protein (OECD 2009a, Reeves III & Weihrauch 1979); therefore, oil produced from DAS-8191Ø-7 will contain extremely low levels of AAD-12 protein.

The full range of applicable AAD-12 expression values in DAS-8191Ø-7 cottonseed was 6.75 to 27.77 ng/mg tissue (Table 8) however, because cottonseed and cottonseed oil are blended commodities,

exposure to the maximum AAD-12 expression level is highly unlikely. Instead an average AAD-12 expression value is most appropriate for determining human dietary risk assessment analyses. In DAS-8191Ø-7 cottonseed, the average value of AAD-12 protein was 18.75 ng AAD-12 protein/mg tissue on a dry weight basis (Table 8).

This assessment assumes that 100% of consumed cotton products are derived from DAS-8191Ø-7 cottonseed, which is a conservative estimate for exposure to the AAD-12 protein from DAS-8191Ø-7 cotton. The actual dietary exposure of the protein from DAS-8191Ø-7 cotton will be lower because: 1) there may be protein degradation during transport and storage; 2) cottonseed containing AAD-12 will be mixed with non- AAD-12 cottonseed; 3) human consumption of cotton products is primarily in food forms which are cooked and heat is known to denature the AAD-12 protein and 4) the majority of consumer dietary exposure to cotton is *via* edible cottonseed oil, which according to USDA National Nutrient Database for Standard Reference, Release 25 (USDA 2012) does not contain any protein. Therefore, no human dietary exposure to the AAD-12 protein is expected from consumption of oils derived from DAS-8191Ø-7 cotton based on USDA Nutrient Database.

A conservative short term intake (STI) consumption of cotton was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint, the No Observed Adverse Effect Level (NOAEL) of 2000 mg/kg (Wiescinski & Golden 2008). Acute risk assessments are typically not required for substances with acute NOAEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki et al 2005). Nevertheless, to place the AAD-12 protein exposure estimate in context, a comparison of the exposure information to the lower limit NOAEL has been made to provide a margin of exposure (MOE) (MOE = NOAEL / Exposure). The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold.

For a US assessment, the MOE values were calculated using the DEEM dietary exposure model program, DEEM-FCID version 3.15 (EPA 2013). The only cotton commodity included in the dietary model in DEEM is cottonseed oil. There is no protein in cottonseed oil; therefore, estimated exposure to AAD-12 based on this model is 0 mg/kg bw/day (Table 19).

The US assessment is incongruent with an assessment performed based on the WHO maximum global consumption data for acute exposures, which indicated that the US was the top global consumer for both cottonseed and cottonseed oil. The 97.5th percentile exposure estimates from WHO for cottonseed were 0.05 g/kg bw/day, indicating very low consumption levels. The likely source of this exposure was the variety of FDA-approved food additives derived from cottonseed [see 21 CFR 172.894 (FDA 2012)]; these additives may be minor enough components of foods to not be included in the DEEM model.

When the WHO “SO 691 Cottonseed” acute consumption information is coupled to the protein average field expression level for cottonseed, the following potential acute exposures are calculated (reported in Table 19).

For AAD-12 protein at 18.75 ppm, exposure *via* cottonseed is estimated as:

- 0.000938 mg protein/kg-bw/day, for general population (i.e. adults)
- 0.000938 mg protein/kg-bw/day, for children of 6 years or younger

These exposure estimates are significantly below the acute oral toxicity endpoint (NOAEL) of 2000 mg/kg and support a conclusion of negligible risk to humans based on such low potential AAD-12 protein consumption from DAS-8191Ø-7 cottonseed.

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold. MOEs based on the WHO consumption information are reported in Table 19. The calculated MOE values for the AAD-12 protein in cottonseed are very large (>2,000,000). For chemicals, MOEs greater than 100 are typically considered acceptable; thus all MOEs for AAD-12 protein indicate negligible concern for adverse effects from DAS-8191Ø-7 cotton based on the available safety threshold information.

These very small levels of exposure to humans to DAS-8191Ø-7 AAD-12 in cottonseed, along with the data above, support the conclusion that there is negligible risk to human health when DAS-8191Ø-7 AAD-12 is present in their diets.

Table 19. Human Dietary Margins of Exposure for AAD-12 Protein in Cotton

Populations	Food Intake (g/kg-bw)	Exposure (mg AAD-12 /kg-bw/day)	NOAEL (mg/kg-bw)	MOE
		US DEEM 97.5th		
U.S. Population	0	0	>2000	NA
		WHO 97.5th (United States)[a]		
General Population	0.05	0.000938	>2000	>2132196
Children <6 year	0.05	0.000938	>2000	>2132196

[a] Based on WHO 97.5th percentile consumption of cottonseed under commodity SO 691.

4.1 a PAT Protein Human Dietary Risk Assessment

Expression levels of PAT protein in DAS-8191Ø-7 cotton were used with conservative human dietary consumption data for cotton to estimate human dietary exposure to the PAT protein from DAS-8191Ø-7 cotton. In addition, the relevance of the exposure estimate was placed into context based on known mammalian toxicity information. A dietary exposure assessment for the PAT protein in DAS-8191Ø-7 cotton supports the conclusion that there is negligible risk to human health.

The field expression of PAT protein in DAS-8191Ø-7cotton has been measured using a PAT specific enzyme-linked immunosorbent assay (ELISA) in several plant tissues of cotton (see section 3.2 c(ii)). PAT field expression data is available for trials conducted at six test sites located within the major cotton-producing regions of the U.S representing diverse agronomic practices and environmental conditions (Table 10).

For the human diet, only the cottonseed of cotton plants is useful for food. Cottonseed is not directly consumed by humans because the majority of commercial cotton varieties contain the anti-nutrients gossypol and cyclopropenoid fatty acids. For the human diet, cottonseed is used in food applications in which the seeds are mainly used to obtain refined edible oil, which contains undetectable amounts of protein (OECD 2009a, Reeves III & Weihrauch 1979); therefore, oil produced from DAS-8191Ø-7 will contain extremely low levels of PAT protein.

The full range of applicable PAT expression values in DAS-8191Ø-7 cottonseed was 2.37 to 5.71 ng/mg tissue (Table 10) however, because cottonseed and cottonseed oil are a blended commodities, exposure to the maximum PAT expression level is highly unlikely. Instead an average PAT expression value is most appropriate for determining human dietary risk assessment analyses. In DAS-8191Ø-7 cottonseed, the average value of PAT protein was 3.85 ng PAT protein/mg tissue on a dry weight basis (Table 10).

This assessment assumes that 100% of consumed cotton products are derived from DAS-8191Ø-7 cottonseed, which is a conservative estimate for exposure to the PAT protein from DAS-8191Ø-7 cotton. The actual dietary exposure of the protein from DAS-8191Ø-7 cotton will be lower because: 1) there may be protein degradation during transport and storage, 2) cottonseed containing PAT will be mixed with non-PAT cottonseed, 3) human consumption of cotton products is primarily in food forms which are cooked and heat is known to denature the PAT protein and 4) the majority of consumer dietary exposure to cotton is *via* edible cottonseed oil, which according to USDA National Nutrient Database for Standard Reference, Release 25 (USDA 2012) does not contain protein. Therefore, no human dietary exposure to the PAT protein is expected from consumption of oils derived from DAS-8191Ø-7 cotton based on USDA National Nutrient Database.

A conservative short term intake (STI) consumption of cotton was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint, the No Observed Adverse Effect Level (NOAEL) of 5000 mg/kg (OECD 1999). Acute risk assessments are typically not

required for substances with acute NOAEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki et al 2005). Nevertheless, to place the PAT protein exposure estimate in context, a comparison of the exposure information to the lower limit NOAEL has been made to provide a margin of exposure (MOE) (MOE = NOAEL / Exposure). The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold.

For a US assessment, the MOE values were calculated using the DEEM dietary exposure model program DEEM-FCID version 3.15 (EPA 2013). The only cotton commodity included in the dietary model in DEEM is cottonseed oil. There is no protein in cottonseed oil; therefore, estimated exposure to PAT based on this model is 0 mg/kg bw/day (Table 20).

The US assessment is incongruent with an assessment performed on the WHO maximum global consumption data for acute exposures, which indicated that the US was the top global consumer of both cottonseed and cottonseed oil. The 97.5th percentile exposure estimates from WHO for cottonseed were 0.05 g/kg bw/day, indicating very low consumption levels. The likely source of this exposure was the variety of FDA-approved food additives derived from cottonseed [see 21 CFR 172.894 (FDA 2012)]; these additives may be minor enough components of foods to not be included in the DEEM model.

When the WHO "SO 691 Cottonseed" acute consumption information is coupled to the protein average field expression level for cottonseed, the following potential acute exposures are calculated (reported in Table 20).

For PAT protein at 3.85 ng, exposure *via* cottonseed is estimated as:

- 0.000193 mg protein/kg-bw/day, for general population (*i.e.* adults)
- 0.000193 mg protein/kg-bw/day, for children of 6 years or younger

These exposure estimates are significantly below the acute oral toxicity endpoint (NOAEL) of 5000 mg/kg (OECD 1999) and support a conclusion of negligible risk to humans based on such low potential PAT protein consumption from DAS-8191Ø-7 cottonseed.

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold. MOEs based on the WHO consumption information are reported in Table 20. The calculated MOE values for the PAT protein in cottonseed are very large (>2,000,000). For chemicals, MOEs greater than 100 are typically considered acceptable; thus all MOEs for PAT protein indicate negligible concern for adverse effects from DAS-8191Ø-7 cotton based on the available safety threshold information.

These very small levels of exposure to humans to DAS-8191Ø-7 PAT in cottonseed, along with the data above, support the conclusion that there is negligible risk to human health when DAS-8191Ø-7 PAT is present in their diets.

Table 20. Human Dietary Margins of Exposure for PAT Protein in Cotton

Populations	Food Intake (g/kg-bw)	Exposure (mg PAT /kg-bw/day)	NOAEL (mg/kg-bw)	MOE
US DEEM 97.5th				
U.S. Population	0	0	>5000	NA
WHO 97.5th (United States)[a]				
General Population	0.05	0.000193	>5000	>25906736
Children <6 year	0.05	0.000193	>5000	>25906736

[a] Based on WHO 97.5th percentile consumption of cottonseed under commodity SO 691

4.2 Livestock Dietary Risk Assessment

4.2 a AAD-12 Protein Livestock Dietary Risk Assessment

Expression levels of the AAD-12 protein in DAS-8191Ø-7 cotton were used with conservative (i.e. protective) livestock dietary assumptions for cotton commodities to estimate livestock dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margin of exposure (MOE) values for the AAD-12 protein in DAS-8191Ø-7 cotton, indicating negligible concern for adverse effects from acute dietary exposure (Fleming 2013).

The cotton commodity forms that are considered potential animal feeds are derived primarily from the cottonseed, which can be fed whole or processed into meal and hulls; cotton gin by-products are also sometimes used as a supplemental cattle feed. The US typically uses highest average field trial (HAFT) value for animal feed inputs but uses average residue values for blended commodities like grain and meal. Because the various feed commodities derived from cottonseed vary in protein content, conversion factors were calculated based on crude protein content to convert measured AAD-12 protein concentrations in whole cottonseed to concentrations in the processed commodities. The crude protein content of whole cottonseed is 21.8% - 34.2% and cotton meal can contain 41.7% - 48.9% crude protein depending on the process used (OECD 2009a). Hence a conservative concentration value of 2.2X (48.9 / 21.8) is used for determining AAD-12 protein levels in cotton meal based on AAD-12 cottonseed protein expression levels. Cotton hulls contain 4.2% - 6.2% crude protein (OECD 2009a), therefore the ratio of 6.2/21.8 is used conservatively to estimate the 0.28X reduction of protein in hulls relative to the whole seed.

Cotton gin by-products (also called cotton gin trash) consist of the plant residues remaining from ginning cotton and can contain several parts of the cotton plant including stems, leaves, seed, burr, and lint. Gin by-products are not a high value feed commodity and so are not processed for removal of the anti-nutrient, gossypol; as such, they are only a relevant feed commodity for ruminants. A conservative estimate of the expression of AAD-12 in cotton gin by-products was obtained by taking the highest expression level detected in the portions of the plant typically found in gin by-products (seed, leaf, boll and whole plant). For this assessment, the highest expression for AAD-12 was found in the leaf at 51.26 ng/mg dry tissue (Table 21).

These expression level input values are conservative estimates for exposure to the transgenic proteins from cotton; actual dietary exposure of the protein will be lower due to degradation of the protein before consumption and to mixing with traditional cotton. Degradation of the protein will occur during transport and storage. Additionally heat applied during preparation of cotton derived foods and feeds may lessen exposure to AAD-12; when heated AAD-12 is functionally unstable (Schafer 2010). Furthermore, this assessment has assumed that 100% of cotton consumed is derived from DAS-8191Ø-7 cotton; cotton is a blended commodity and actual cotton-derived foods and feeds will contain cotton from a mixture of sources, further reducing exposure from that estimated in this assessment.

Table 21. Concentration Levels of AAD-12 Protein Applicable for Livestock Exposure

Test		**AAD-12 ng/mg Tissue Dry Weight[a,b]**			
Matrix	**Description**	**Overall Mean**	**Std. Dev. (n=200)**	**Min/Max Range**	**HAFT**
Seed (Maturity)	DAS-8191Ø-7	**18.75**	4.81	6.75 – 27.77	25.81
Leaf (1st Open Boll)	DAS-8191Ø-7	**51.26**	19.63	17.38 – 89.23	78.17
Bolls (Peak Bloom)	DAS-8191Ø-7	17.17	7.91	4.36 – 33.39	29.88
Whole Plant (Maturity)	DAS-8191Ø-7	16.42	12.18	ND – 46.98	33.63
		Food and Feed Estimates			
Cottonseed Meal	Estimated as 2.2X Seed	**41.25**			
Cottonseed Hull	Estimated as 0.28X Seed	**5.25**			
Cotton Gin By-products	Estimated as highest detected expression (Leaf)	**51.26**			

[a] Bolded values used as input in livestock dietary calculations, when appropriate.
[b] AAD-12 ng/mg Tissue Dry Weight from measured protein expression in field trials.

The presence of AAD-12 protein in cotton is not anticipated to have an impact for feed ration formulation, because nutrient composition analyses have shown that DAS-8191Ø-7 cottonseed (with the PAT and AAD-12 protein) is substantially equivalent to non transgenic cottonseed (Fast & Johnson 2013). Therefore, a dietary exposure estimate for AAD-12 protein in livestock diets based on traditional use of the unmodified feeds is provided here by coupling field expression information for the proteins in DAS-8191Ø-7 cotton with livestock dietary consumption assumptions for feed commodities derived from cotton.

A US livestock assessment was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (EPA 2008a). All the components relevant for livestock assessments can be found in Table 21. This US assessment includes several cotton commodity forms as potential animal feeds: seed, meal, hulls and gin by-products. The US MRBD guidance is used to construct a maximum cotton feed contribution for swine, poultry and cattle. No guidance on sheep diets is offered in the MRBD guidance, so all possible commodities were included as a conservative approach.

Variations in livestock feed diets elsewhere in the world could result in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for AAD-12 in cotton. US EPA currently assumes the following for reference animals for dietary assessments based on the balance of Complex Carbohydrates (CC), Protein Concentrate (PC) and Roughage (R) within the feeds for animals in finishing or feedlots (EPA 2008b):

Beef: Finishing or feedlot beef (body weight at slaughter, **544 kg,** daily feed intake of **9.1 kg** dry matter feed). Feedlot rations in the finishing stage consist of high amounts of grain or grain supplements (80% CC), forages (15% R), and protein sources (5% PC) in last 120 to 180 days (4 to 6 months) before slaughter at **16 to18 months of age.**

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

Poultry: Chicken: Laying hen (body weight, **1.9 kg**, average daily intake of
52 grams or **0.052 kg of feed**). Laying hens are usually slaughtered **after 18 months**. A daily ration includes grain or grain supplement (75% CC) and protein source (25% PC). Alternate poultry would be frying and rotisserie chickens weighing 3 to 4 lb, with an average life span of 38 to 42 days. The broiler diet contains 85% CC and 15% PC.

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

Sheep: Ram/Ewe (body weight **85 kg**, average daily intake of **2 kg of feed**). Sheep are not a typical US reference animal and therefore are not discussed in detail in the MRBD guidance; however, they have been included in this assessment as the reported consumption of cotton for sheep as a percentage of the diet is higher than other livestock (OECD 2009b). Standard body weights and food intakes for the US are offered by OECD (OECD 2009b), however no suggested ratio of CC, PC, and R components in the daily ration is available for sheep. For this assessment, all cotton commodities were conservatively included at the percentage indicated by OECD.

Table 22. US Intake Animal Dietary Burdens for Livestock from AAD-12

			Dietary Contribution (%)						**Animal Dietary Burden (ppm)**				
Feed-stuff[a]	Type	Dry Matter (%)[b]	Beef	Dairy	Poultry	Pig	Sheep[c]	AAD-12 (ppm)[d]	Beef	Dairy	Poultry	Pig	Sheep[c]
								US					
Cottonseed	PC[e]	88	Nu[f]	*Meal used*	Nu	Nu	25	**18.75**	-	-	-	-	4.69
Cottonseed meal	PC[e]	89	5	10	20	15	15	**41.25**	2.06	4.13	8.25	6.19	6.19
Cottonseed hulls	R[e]	90	*10*	Nu	Nu	Nu	15	**5.25**	0.53	-	-	-	0.79
Cotton gin by-products	R[e]	90	*5*	Nu	Nu	Nu	Nu	**51.26**	2.56	-	-	-	-
								Total	**5.15**	**4.13**	**8.25**	**6.19**	**11.67**

[a] US dietary contributions determined based on the Maximum Reasonably Balanced Diet (MRBD) method
[b] Protein concentration data in Table 24 is expressed on a dry weight basis, so the correction for % dry matter used in determining chemical residue exposures was not applied here.
[c] Recommended ratios of Roughage, Carbohydrate Concentrate and Protein Concentrate are not given for use of the MRBD method in sheep, so for the US sheep diet, all potential constituents of the diet were included at the maximum percentage.
[d] Note Expression Inputs for Feedstuff values are found in Table 21.
[e] Roughage (R), Protein Concentrate (PC)
[f] Nu = Not Used

The resulting US intake dietary burden for animal feeds is totalled in Table 22. Because only cotton feeds are considered, the remaining nutritional balance of the diets is assumed to be comprised of unmodified feeds. Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal and results are found in Table 23. The highest estimated exposed US animal is the sheep with 0.27 mg AAD-12/kg-bw. Lower estimates for beef cattle, dairy cattle, swine and poultry were ≤0.23

mg/kg-bw (Table 23**.** Livestock Daily Dose Estimates of AAD-12 Protein from Cotton Animal Feeds). When these values are compared to the acute mammalian NOAEL of >2000 mg/kg-bw (see section 3.3 b), there is an high margin of safety for livestock because the MOEs are large (>7200). This dietary exposure assessment for AAD-12 in DAS-8191Ø-7 cotton supports the conclusion that there is negligible risk to animal health.

Table 23. Livestock Daily Dose Estimates of AAD-12 Protein from Cotton Animal Feeds

	Cattle		**Swine**	**Poultry**	**Sheep**
	Beef	**Dairy**	**Finishing**	**Laying Hen**	**Ram/Ewe**
		US ASSESSMENT			
Body Weight (kg)	544	612	113	1.9	85
Daily Maximum Feed (kg Dry Matter (DM))	9.1	24	3.1	0.052	2
Maximum AAD-12 Intake (mg/kg feed)[a]	5.15	4.13	8.25	6.19	11.67
Maximum Intake (mg/kg-bw)[b]	0.09	0.16	0.23	0.17	0.27
MOE vs. Mammalian NOAEL	>23216	>12349	>8837	>11806	>7284

[a] Derived in previous table
[b] Maximum intake (mg/kg-bw) values were rounded in the table and thus, if used as listed, will result in slightly different MOE values.

4.2 b PAT Livestock Dietary Risk Assessment

Expression levels of the PAT protein in DAS-8191Ø-7 cotton were used with conservative livestock dietary consumption assumptions for cotton commodities to estimate livestock dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the PAT protein in DAS-8191Ø-7 cotton, indicating negligible concern for adverse effects from acute dietary exposure.

The cotton commodity forms that are considered potential animal feeds are derived primarily from the cottonseed, which can be fed whole or processed into meal and hulls; cotton gin by-products are also sometimes used as a supplemental cattle feed. The US typically uses the highest average field trial (HAFT) value for animal feed inputs but uses average residue values for blended commodities like grain and meal. Because the various feed commodities derived from cottonseed vary in protein content, conversion factors were calculated based on crude protein content to convert measured PAT protein concentrations in whole cottonseed to concentrations in the processed commodities. The crude protein content of whole cottonseed is 21.8% - 34.2% and cotton meal can contain 41.7% - 48.9% crude protein

depending on the process used (OECD 2009a). Hence a conservative concentration value of 2.2X (48.9 / 21.8) is used for determining protein levels in cotton meal based on cottonseed protein expression levels. Cotton hulls contain 4.2% - 6.2% crude protein (OECD 2009a), therefore the ratio of 6.2/21.8 is used conservatively to estimate the 0.28X reduction of protein in hulls relative to the whole seed.

Cotton gin by-products (also called cotton gin trash) consist of the plant residues remaining from ginning cotton and can contain several parts of the cotton plant including stems, leaves, seed, burr, and lint. Gin by-products are not a high value feed commodity and so are not processed for removal of the anti-nutrient, gossypol; as such, they are only a relevant feed commodity for ruminants. A conservative estimate of the expression of PAT in cotton gin by-products was obtained by taking the highest expression level detected in the portions of the plant typically found in gin by-products (seed, leaf, boll and whole plant). For this assessment, the highest expression for PAT was found in the leaf (Table 24) at 9.14 ng/mg dry tissue.

These expression level input values are conservative estimates for exposure to the transgenic proteins from cotton; actual dietary exposure and impact of the protein will be lower due to degradation of the protein before consumption and to mixing with traditional cotton. Degradation of the protein will occur during transport and storage. Additionally heat applied during preparation of cotton derived foods and feeds may lessen exposure to PAT protein; heat is known to denature PAT (EPA 1997). Furthermore, this assessment has assumed that 100% of cotton consumed is derived from DAS-8191Ø-7 cotton; cotton is a blended commodity and actual cotton-derived foods and feeds will contain cotton from a mixture of sources, further reducing exposure from that estimated in this assessment.

Table 24. Mean Concentration Levels of PAT Protein Applicable for Livestock Exposure

Test		**PAT ng/mg Tissue Dry Weight[a,b]**			
Matrix	**Description**	**Overall Mean**	**Std. Dev. (n=200)**	**Min/Max Range**	**HAFT**
Seed (Maturity)	DAS-8191Ø-7	**3.85**	0.79	2.37-5.71	4.79
Leaf (1st Open Boll)	DAS-8191Ø-7	**9.14**	3.92	4.11 – 18.56	16.13
Bolls (Peak Bloom)	DAS-8191Ø-7	3.16	1.11	1.62 – 6.27	5.13
Whole Plant (Maturity)	DAS-8191Ø-7	0.97	1.02	ND – 3.97	3.24
Food and Feed Estimates					
Cottonseed Meal	Estimated as 2.2X Seed	**8.47**			
Cottonseed Hull	Estimated as 0.28X Seed	**1.08**			
Cotton Gin By-products	Estimated as highest detected expression (Leaf)	**9.14**			

[a] Bolded values used as input in livestock dietary calculations, when appropriate.
[b] PAT ng/mg Tissue Dry Weight from measured protein expression in field trials.

The presence of PAT protein in cotton is not anticipated to have an impact for feed ration formulation, because nutrient composition analyses have shown that DAS-8191Ø-7 cottonseed (with the PAT and AAD-12 protein) is substantially equivalent to non-transgenic cottonseed. Therefore a dietary exposure estimate for PAT protein in livestock diets based on traditional use of the unmodified feeds is provided here by coupling field expression information for the proteins in DAS-8191Ø-7 cotton with livestock dietary consumption assumptions for feed commodities derived from cotton.

A US livestock assessment was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (EPA 2008a). All the components relevant for livestock assessments can be found in Table 24. This US assessment includes several cotton commodity forms as potential animal feeds: seed, meal, hulls and gin by-products. The US MRBD guidance is used to construct a maximum cotton feed contribution for swine, poultry and cattle. No guidance on sheep diets is offered in the MRBD guidance, so all possible commodities were included as a conservative approach.

Variations in livestock feed diets elsewhere in the world could result in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for PAT in cotton. See section 4.2 a for details on US EPA's current references for animal dietary assessments based on the balance of Complex Carbohydrates (CC), Protein Concentrate (PC) and Roughage (R) within the feeds for animals in finishing or feedlots.

The resulting US intake dietary burden for animal feeds is totalled in Table 25. Because only cotton feeds are considered, the remaining nutritional balance of the diets is assumed to be comprised of unmodified feeds. Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal and results are found in Table 26.

The highest estimated exposed US animal is the sheep with 0.06 mg PAT/kg-bw. Lower estimates for beef cattle, dairy cattle, swine and poultry were ≤0.05 mg/kg-bw (Table 26). When these values are compared to the acute mammalian NOAEL of >5000 mg/kg-bw, there is a significant margin of safety for livestock because the MOEs are very large (>88,000). This dietary exposure assessment for the PAT protein in DAS-8191Ø-7 cotton supports the conclusion that there is negligible risk to animal health.

Table 25. US Intake Animal Dietary Burdens for Livestock from PAT

			Dietary Contribution (%)						Animal Dietary Burden (ppm)				
Feed-stuff[a]	Type	Dry Matter (%)[b]	Beef	Dairy	Poultry	Pig	Sheep[c]	PAT (ppm)[d]	Beef	Dairy	Poultry	Pig	Sheep[c]
US													
Cottonseed	PC[e]	88	Nu[f]	*Meal used*	Nu	Nu	25	**3.85**	-	-	-	-	0.96
Cottonseed meal	PC[e]	89	5	10	20	15	15	**8.47**	0.42	0.85	1.69	1.27	1.27
Cottonseed hulls	R[e]	90	*10*	Nu	Nu	Nu	15	**1.08**	0.11	-	-	-	0.16
Cotton gin by-products	R[e]	90	*5*	Nu	Nu	Nu	Nu	**9.14**	0.46	-	-	-	-
								Total	**0.99**	**0.85**	**1.69**	**1.27**	**2.39**

[a] US dietary contributions determined based on the Maximum Reasonably Balanced Diet (MRBD) method
[b] Protein concentration data in Table 24 is expressed on a dry weight basis, so the correction for % dry matter used in determining chemical residue exposures was not applied here.
[c] Recommended ratios of Roughage (R), Carbohydrate Concentrate (CC) and Protein Concentrate (PC) are not given for use of the MRBD method in sheep, so for the US sheep diet, all potential constituents of the diet were included at the maximum percentage.
[d] Note Expression Inputs for Feedstuff values are found in Table 24.
[e] Roughage (R), Protein Concentrate (PC)
[f] Nu = Not Used

Table 26. Livestock Daily Dose Estimates of PAT Protein from Cotton Animals Feeds

	Cattle		Swine	Poultry	Sheep
	Beef	**Dairy**	**Finishing**	**Laying Hen**	**Ram/Ewe**
US ASSESSMENT					
Body Weight (kg)	544	612	113	1.9	85
Daily Maximum Feed (kg Dry Matter (DM))	9.1	24	3.1	0.052	2
Maximum PAT Intake (mg/kg feed)[a]	0.99	0.85	1.69	1.27	2.39
Maximum Intake (mg/kg-bw)[b]	0.02	0.03	0.05	0.03	0.06
MOE vs. Mammalian NOAEL	>301920	>150000	>107845	>143852	>88912

[a] Derived in previous table
[b] Maximum intake (mg/kg-bw) values were rounded in the table and thus, if used as listed, will result in slightly different MOE values.

4.3 DAS-8191Ø-7 Cotton is "As Safe As" Non-transgenic Cotton

The primary use of cotton in the USA is for fibres which are mainly used in the manufacturing of a large number of textiles. Only the cottonseed is processed into four major food and feed products: oil, meal, hulls and linters. Only cottonseed oil and linters (fibre) from cottonseed are used in food applications in which the seeds are mainly used to obtain edible oil and used as livestock feed. Due to the harsh conditions used in cottonseed processing, cottonseed oil and linters contain undetectable or negligible amounts of protein, therefore, oil and other products produced from DAS-8191Ø-7 cotton will contain extremely low levels of AAD-12 and PAT protein.

The *aad-12* and *pat* expression cassettes introduced into DAS-8191Ø-7 cotton are the same as those introduced into DAS-68416-4 soybean which has previously been previously approved by FSANZ (A1046,(FSANZ 2011). In addition, the *aad-12* and *pat* expression cassettes are the same as those introduced into DAS-444Ø6-6 soybean which is approved by FSANZ (A1073, (FSANZ 2013) and currently under US FDA consultation (BNF000133).

As with DAS-68416-4 soybean and DAS-444Ø6-6 soybean, the data and information presented in this petition support the conclusion that food and feed derived from DAS-8191Ø-7 cotton is as safe and nutritious as those derived from non-transgenic cotton. The conclusion was based on 1) detailed molecular characterization of DAS-8191Ø-7 cotton, 2) safety assessment of the introduced AAD-12 and PAT proteins and 3) nutrient composition analysis of DAS-8191Ø-7 cotton.

The *aad-12* and *pat* genes were introduced into DAS-8191Ø-7 cotton using *Agrobacterium* mediated transformation. Molecular characterization by Southern blot analyses of DAS-8191Ø-7 cotton confirmed that a single, intact DNA insert containing the *aad-12* and *pat* gene expression cassettes was stably integrated into the cotton genome. Southern blot analyses also confirmed the absence of the plasmid backbone DNA in DAS-8191Ø-7 cotton. The integrity of the inserted DNA was demonstrated in five different breeding generations. Data from segregating generations confirmed the predicted Mendelian inheritance pattern. These data confirmed the stability of DAS-8191Ø-7 cotton during traditional breeding procedures.

The microbial-derived AAD-12 and PAT proteins have been extensively assessed to establish the safety of the proteins. DAS-8191Ø-7 cotton-derived AAD-12 and PAT proteins were determined to be biochemically equivalent to the corresponding proteins from microbial-derived expression host organisms. A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the AAD-12 and PAT proteins. Bioinformatic analyses revealed no meaningful homologies with known or putative allergens or toxins for the AAD-12 or PAT amino acid sequences. Both proteins hydrolyzed rapidly in simulated gastric fluid. There was no evidence of acute toxicity in mice at a dose of 2000 mg/kg body weight of AAD-12 protein and 5000 mg/kg body weight of PAT protein.

AAD-12 and PAT protein expression levels in DAS-8191Ø-7 cotton were measured using a protein-specific enzyme-linked immunosorbent assay (ELISA). Protein expression was analyzed in multiple tissues collected throughout the growing season from DAS-8191Ø-7 cotton plants untreated and treated with 2,4-D plus glufosinate-ammonium. Glycosylation analysis revealed no detectable covalently linked carbohydrates in either AAD-12 or PAT proteins expressed in DAS-8191Ø-7 cotton plants. The low level expression of these proteins presents a low exposure risk to humans and animals, and the results of the overall safety assessment of AAD-12 and PAT indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

A composition assessment was conducted in which levels of key nutrients and anti-nutrients of DAS-8191Ø-7 cottonseed were compared with the appropriate non-transgenic near-isogenic control and non-transgenic reference lines. Samples were analysed for proximates, fiber, minerals, amino acids, fatty acids, vitamins, and anti-nutrients. Fifty-nine cotton analytes were assayed and the analyses conclude that DAS-8191Ø-7 cotton is compositionally equivalent to non-transgenic cotton.

The information and data presented in this submission demonstrate that DAS-8191Ø-7 cotton is compositionally equivalent to commercially cultivated non-transgenic cotton with the exception of its tolerances to 2,4-D and glufosinate-ammonium herbicides. Because DAS-8191Ø-7 cotton is compositionally equivalent to non-transgenic cotton, no significant impact is expected on human or animal health via commodity food and feed cotton products.

5. APPENDICES

Appendix 1. Methods for Molecular Characterization of DAS-8191Ø-7 Cotton

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

Appendix 3. Methods and Results for Characterization of PAT Protein

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

Appendix 5. Methods for Compositional Analysis

Appendix 6. Literature Ranges for Compositional Analysis

Appendix 1. Methods for Molecular Characterization of DAS-8191Ø-7 Cotton

DAS-8191Ø-7 cotton Material

Transgenic cotton seeds from five distinct generations of cotton containing event DAS-8191Ø-7 were planted in the greenhouse. After at least one week of growth for emerged seedlings, leaf punches were taken from each plant and were tested for PAT protein expression using a Lateral Flow Strip (LFS) test according to the manufacturer's instructions (Envirologix Inc.). Each plant was given a "+" or "-" for the presence or absence of the PAT protein.

Control Cotton Material

Seeds from the non-transgenic cotton variety Coker 310 were planted in the greenhouse. The Coker 310 seeds had a genetic background representative of the transgenic seeds but did not contain the *aad-12* or *pat* genes.

Reference Materials

DNA of the plasmid pDAB4468 was added to samples of the non-transgenic control genomic DNA at a ratio approximately equivalent to 1 copy of the transgene per cotton genome with a cotton genome size of ~2.2×10^9 bp (Arumuganathan & Earle 1991) and used as the positive control for the Southern hybridization. DIG-labelled DNA Molecular Weight Marker II and DIG-labelled DNA Molecular Weight Marker VII (Roche Diagnostics), each containing a mixture of DNA fragments with different sizes, served as size standards for agarose gel electrophoresis and Southern blot analysis.

DNA Probe Preparation

DNA probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labelled nucleotide, [DIG]-dUTP, into fragments generated by primers specific to genetic elements and backbone regions from plasmid pDAB4468. Generation of DNA probes by PCR synthesis was carried out using a PCR DIG Probe Synthesis Kit (Roche Diagnostics). Labeled probes were purified from agarose gels and were quantified by a PicoGreen reagent (Invitrogen).

Sample Collection and DNA Extraction

Leaf samples were collected from greenhouse-grown plants for genomic DNA extraction. Genomic DNA was extracted following a modified CTAB method. Briefly, leaf samples were ground individually in liquid nitrogen, and then CTAB extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 2% PVP-10, 2% β-ME) was added to samples at a ratio of about 5:1 plus over 10 µL each of RNase-A (Qiagen) and proteinase K (Qiagen). After approximately 2 hours of incubation at ~65 °C with gentle shaking, samples were centrifuged and the supernatants were extracted with equal volume of chloroform : octanol = 24:1 three times. DNA was precipitated by mixing the supernatants with approximately 0.7 volume of isopropanol. The precipitated DNA was rinsed with 70% ethanol, air-dried, then dissolved in appropriate volume of 1× TE buffer (pH 8.0). The resultant genomic DNA was further purified with a Genomic-tip 500/G following the manufacturer's instructions (Qiagen). The DNA was

quantified with a PicoGreen reagent (Invitrogen), and was visualized on an agarose gel to check for genomic DNA quality.

DNA Digestion and Electrophoretic Separation of the DNA Fragments

Genomic DNA extracted from the cotton leaf tissue was digested with restriction enzymes by combining approximately 10 μg of genomic DNA with approximately 5-10 units of the selected restriction enzyme per μg of DNA in the corresponding reaction buffer. Each sample was incubated at 37°C overnight for digestion. 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 at 35-65 V for 18-22 hr to achieve fragment separation. The gels were stained with ethidium bromide and the DNA was visualized under UV light. A photographic record was made for each stained gel.

Southern Transfer

DNA fragments in the agarose gels were depurinated, denatured, neutralized *in situ*, and transferred to nylon membranes in 10× SSC buffer using a wicking system. After transfer to the membrane, the DNA was fixed to the membrane by crosslinking through UV treatment.

Hybridization

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-labelled DNA molecular weight marker II and VII were used to determine the hybridizing fragment size on the Southern blots.

Detection

DIG-labelled probes bound to the nylon membranes after stringent wash were incubated with Alkaline Phosphatase (AP)-conjugated anti-Digoxigenin antibody for ~1 hr at room temperature. The anti-DIG antibody specifically bound to the probes was then visualized using CDP-Star Chemiluminescent Nucleic Acid Detection System (Roche Diagnostics). Blots were exposed to chemiluminescent film to detect the hybridizing fragments and to visualize the molecular weight markers. The images were then scanned and stored. The number and size of all the detected band were documented for each digest and probe combination.

Once the data were recorded, membranes were rinsed with milli-Q water and then stripped of the bound probe in a solution containing 0.2 M NaOH and 1.0% SDS. The alkali-based stripping procedure successfully removes the labelled probes from the membranes, allowing them to be re-hybridized with a different DNA probe.

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

Materials & Methods for Characterization of AAD-12 Protein

DAS-8191Ø-7 transgenic cotton material

The AAD-12 protein was extracted from the leaf tissue of greenhouse grown transgenic cotton event DAS-8191Ø-7 (T_3 generation see Figure 2). Prior to harvest, the AAD-12 protein expression was verified by lateral flow strip testing and the leaf tissue from DAS-8191Ø-7 cotton was harvested, frozen, lyophilized, ground, and stored at -80 °C. Test material used for characterization of DAS-8191Ø-7 cotton AAD-12 are listed in Table 27.

Control cotton material

The control cotton line had a genetic background representative of DAS-8191Ø-7 cotton plants, but did not contain *aad-12*. Seeds from this isogenic non-transgenic cotton line were planted and the resulting leaf tissue was harvested and processed under the same conditions as DAS-8191Ø-7 cotton.

AAD-12 Reference material

Recombinant AAD-12 protein was produced and purified from the microbe *Pseudomonas fluorescens* and had a molecular weight of 32 kDa. The commercially available reference substances used in this study are listed in Table 28.

Table 27. Test Material for AAD-12 Characterization

Test Substance	Source	Lot Number	Assay	Reference
Cotton AAD-12	Cotton DAS-8191Ø-7	TSN304178	SDS-PAGE, Glycosylation, MALDI-TOF, MALDI-TOF/TOF MS/MS, Activity Assay	NA
Control Cotton Line	isogenic / non-transgenic cotton	TSN304177	SDS-PAGE, Western	NA
Cotton crude leaf extract	Cotton DAS-8191Ø-7	NA	SDS-PAGE, Western	NA
Microbe-derived AAD-12	*Pseudomonas fluorescens*	TSN030732-003 (466-028B)	SDS-PAGE, Western, Glycosylation, Activity Assay	
Microbe-derived AAD-12	*Pseudomonas fluorescens*	TSN030732-002 (466-028A)	MALDI-TOF, MALDI-TOF/TOF MS/MS or ESI-LC/MS	

Table 28. Commercially available reference substances for AAD-12 Characterization

Reference Substance	Product Name	Lot Number	Assay	Reference
Mass Spectrometry Mass Standards Kit	Mass Standards Kit for Calibration of AB SCIEX TOF/TOF Instruments	A1068	Protein sequence analysis	AB SCIEX
Soybean Trypsin Inhibitor (STI)	A component of the GelCode glycoprotein staining kit	NH175044	Glycosylation assay	ThermoFisher
Horseradish Peroxidase (HRP)	A component of the GelCode glycoprotein staining kit	ND171686	Glycosylation assay	ThermoFisher
Bovine Serum Albumin (BSA)	Pre-diluted BSA protein assay standard set	NH175569	SDS-PAGE & Glycosylation assay	ThermoFisher
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	1167391	SDS-PAGE, western blot & Glycosylation assay	Invitrogen: Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 10, and 3.5 kDa

SDS-PAGE and western blot analysis of crude cotton leaf extracts

SDS-PAGE and western blot analyses of the crude protein extracts prepared from the transgenic DAS-8191Ø-7 and non-transgenic-cotton leaf were performed with Bio-Rad Criterion gels fitted in a Criterion Gel chamber with XT MES running buffer (Bio-Rad). Extracts were prepared by bead-grinding (Geno-grinder) ~40 mg of the ground cotton leaf tissue for 3 minutes in a micro-centrifuge tube containing ~1.0 mL PBST supplemented with 5 mM EDTA, 23 mM β-mercaptoethanol, and protease inhibitors. Supernatants were isolated by subjecting the homogenate to centrifugation at ~20,000 × g (4 °C), and 100 µL of each extract was mixed with 100 µL of 2× Laemmli sample buffer (Bio-Rad) containing freshly added β -mercaptoethanol (Bio-Rad) and heated for ~10 minutes at ~95 °C. After a brief centrifugation (2 min at 20,000 × g), 30 µL of each supernatant was loaded directly on the gel. The reference standards, microbe-derived AAD-12 and BSA (ThermoScientific), were diluted to an appropriate concentration and combined with freshly prepared Laemmli sample buffer containing 5% β -mercaptoethanol. The electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes.

After separation, the gel was cut in half and one half was stained with ThermoScientific GelCode Blue protein stain and then scanned with a densitometer (GE Healthcare) to obtain a permanent record of the gel. The remaining half of the gel was electro-blotted to a nitrocellulose membrane (Bio-Rad) with a Criterion transfer cell (Bio-Rad) for 60 minutes under a constant voltage of 100 V. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (α-AAD-12 PAb, DAS 1197-167-2, 4.3 mg/mL) for 60 minutes (1:5000 dilution) at room temperature. A 1:10,000 dilution of conjugated goat anti-rabbit

IgG (H+L) with horseradish peroxidase (ThermoScientific) was used as the secondary antibody. GE Healthcare ECL chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to detection film (ThermoScientific) for various time points and subsequently developed with an All-Pro 100 Plus film developer.

Protein purification of AAD-12 from DAS-8191Ø-7 cotton plant tissue for structural analyses
An AAD-12 immunoaffinity resin was prepared by mixing 50 µL of a slurry of Protein A/G resin (ThermoScientific) with 200 µg α-AAD-12 monoclonal antibody and incubating overnight at 4 °C. The resin was washed with PBST and the bound IgG irreversibly coupled to the resin by incubating in 200 µL PBST containing 1.8 mM DSS for 60 minutes at room temperature with mixing. Cross-linking was terminated by incubating for 5 minutes following the addition of 500 µL of 1.0 M Tris-HCl pH 8.0. The resin was then washed extensively with PBST and used immediately for immune-capture of the AAD-12 protein.

To generate a cotton leaf extract suitable for AAD-12 isolation, 5-10 grams of ground, lyophilized DAS-8191Ø-7 cotton leaf tissue was suspended by stirring in cold extraction buffer (50 mM HEPES pH 7.8, 300 mM NaCl, 10 mM EDTA) at a ratio of ~15 mL of buffer per gram of dry tissue. A protease inhibitor tablet (Roche) and 10% (w/w) PVPP were added to the suspended tissue. The mixture was stirred at 4 °C for 30 minutes to fully hydrate and extract soluble proteins. The mixture was then filtered through a single layer of pre-wetted miracloth (Calbiochem) and the extract then further clarified by centrifugation at 38,000 × g for 30 minutes. The resulting primary supernatant was removed and mixed with an equal volume of extraction buffer supplemented with 2 M Urea and 0.2% Tween-20 for a final concentration of 1 M and 0.1% Tween-20 respectively. To isolate AAD-12, typically 15 mL of the leaf extract was incubated with 50 µL of freshly prepared AAD-12 capture resin. Binding reactions were incubated in batch at 4 °C for 3 hours to overnight. The resin was collected by centrifugation and then successively washed twice with 1.0 mL extraction buffer containing 1 M urea + 0.1% Tween-20 and then washed one time each with 1.0 mL extraction buffer with 0.5 M Urea + 0.05% Tween-20 followed by 1.0 mL extraction buffer alone. Lastly, the resin was washed once with either1.0 mL of 50 mM HEPES pH 7.8, 0.1 mM EDTA or 1.0 mL of PBST. The resin containing captured AAD-12 was either stored at -80 ºC or analysed immediately.

Detection of post-translational glycosylation
Glycosylation analysis was used to determine whether DAS-8191Ø-7 cotton AAD-12 was post-translationally modified with covalently bound carbohydrate moieties. The resin containing the immunoaffinity-captured, cotton-derived AAD-12 protein was mixed with 50 – 100 µL Laemmli sample buffer (Bio-Rad) lacking β -mercaptoethanol and the sample was then incubated at ~95 °C for 10 minutes to solubilize AAD-12. The microbe-derived AAD-12, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were similarly diluted with 2X SDS-PAGE sample buffer to the approximate concentration of the purified cotton AAD-12 protein. As with AAD-12, all control proteins were also incubated for 10 minutes at ~95 °C. All samples were then centrifuged at 20,000 × g for 2

minutes to obtain a clarified supernatant. The resulting supernatants were applied directly to duplicate Mini-Protean TGX gels (Bio-Rad) and electrophoresed at 150 V for ~50 minutes.

After electrophoresis, one gel was stained with GelCode Blue stain (ThermoScientific) for total protein according to the manufacturers' protocol. After the staining was complete, the gel was scanned with a densitometer to obtain a permanent visual record of the gel. The second gel was stained with a GelCode Glycoprotein Staining Kit (ThermoScientific) according to the manufacturers' protocol to visualize the glycoproteins. The procedure for glycoprotein staining is briefly described as follows: After electrophoresis, the gel was fixed in 50% methanol for 30 minutes and rinsed with 3% acetic acid. This was followed by an incubation period with the oxidation solution from the staining kit for 15 minutes. The gel was once again rinsed with 3% acetic acid and incubated with GelCode glycoprotein staining reagent for 30 minutes. Finally, the gel was immersed in the reduction solution for 5 minutes, and rinsed with 3% acetic acid. Glycoproteins were visualized as magenta bands on a light pink background. After the glycoprotein staining was complete, the gel was scanned with a GE Healthcare densitometer to obtain a permanent visual record of the gel.

Mass spectrometry peptide mass fingerprinting and sequence analysis of plant and microbe-derived AAD-12 protein

The immunoaffinity purified plant-derived AAD-12 protein was subjected to in-gel digestion by trypsin, chymotrypsin, and Asp-N followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF MS-MS. Prior to each enzyme digestion, purified AAD-12 protein was resolved on a SDS-PAGE gel and bands corresponding to AAD-12 protein (one band was used per digest containing approximately 100 ng per lane) were excised with a sterile scalpel and processed as follows (a half lane section of the standard protein of the gel was processed alongside with the protein sample, in a separate tube, using identical procedure). Gel pieces were crushed with a sterile micro-pestle in a siliconised microcentrifuge tube, and destained as follows: 0.4 mL of 50% ACN and 0.4 mL of ammonium bicarbonate buffer were added, the tube was sealed and shaken at room temperature for 30 min in a Thermomixer R at 1100 rpm; the tube was centrifuged to settle the gel pieces, and the supernatant was removed with a pipette tip and discarded; the destaining procedure was repeated 2 times.

The proteins were reduced and alkylated in-gel as follows: (1) 0.2 mL of DTT solution was added to gel pieces, and the tube was incubated at room temperature for 30 min in a Thermomixer R; (2) the tube was centrifuged, and the DTT solution was removed by a pipette tip; (3) the gel pieces were washed with 0.5 mL of 25 mM ammonium bicarbonate buffer, the tube was centrifuged, and the buffer was removed; (4) 0.2 mL of IAA solution was added to the gel pieces, and the tube was incubated in darkness at ambient temperature for 1 hour; (5) the gel pieces were washed twice with 0.5 mL of 25 mM ammonium bicarbonate buffer (the tube was centrifuged, and the buffer was removed after each wash). After the destaining/ reduction/ alkylation procedures, the gel pieces were shrunk in neat acetonitrile and then dried in a centrifugal evaporator for 30 min.

The dried gel pieces were re-hydrated with a trypsin solution (25 μg in 500 μL of 25 mM ammonium bicarbonate buffer, pH 7.8; prepared fresh), chymotrypsin solution (25 μg in 500 μL of 1 mM HCl; prepared fresh), or Asp-N solution (2 μg in 50 μL of 50 mM sodium phosphate buffer, pH 8.0; prepared fresh) and incubated in an incubator at 37 ºC for approximately 16 hours (overnight). Afterwards, the peptides were extracted from the gel slices sequentially with 0.4 mL of 50% ACN/ 0.1% TFA, then 0.4 mL of 50% ACN/ 5% FA, and finally 0.4 mL of 75% ACN/ 5% FA (30 min per extraction in a Thermomixer R at room temperature, shaking at 1100 rpm). The extracts for each sample were combined and dried in a centrifugal evaporator.

Dried peptides were reconstituted in 30 μL of 0.1% TFA in water and were purified for MALDI MS analysis using C18 Zip-Tips (Millipore), according to the manufacturer's procedure. Purified peptides were eluted directly onto MALDI plate sequentially, with aqueous 10%, 25%, 50%, and 75% ACN (buffered with 0.1% TFA). The ZipTip C18 fractions were mixed with 4 µL of CHCA matrix (10 mg/mL CHCA in 50% ACN supplemented with 0.1% TFA), and 1 µL of the sample-matrix mixture was deposited on the MALDI target and allowed to air dry.

The sample preparations were analysed directly by MALDI-TOF mass spectrometry. All mass spectra were acquired on an AB Sciex 4800 MALDI-TOF/TOF mass spectrometer. Mass calibration was performed with a Mass Standards Kit for Calibration of AB SCIEX TOF/TOF Instruments, consisting of the calibration mixture (theoretical monoisotopic $[M+H]^+$ *m/z* values used): des-Arg^1-Bradykinin, *m/z* 904.4681; Angiotensin I, *m/z* 1296.6853; Glu^1-Fibrinopeptide B, *m/z* 1570.6774; ACTH (fragment 1-17), *m/z* 2093.0867; ACTH (fragment 18-39), *m/z* 2465.1989; ACTH (fragment 7-38) m/z 3657.9294. The plate wide calibration model was used for MS calibration.

The peptide fragments of the cotton-derived ADD-12 protein (including the N- and C-termini) were analysed and compared with the sequence of the microbe-derived protein.

Activity assay of the AAD-12 protein derived from DAS-8191Ø-7 cotton plant tissue

To prepare a cotton-derived AAD-12 fraction suitable for enzymatic analysis, a primary leaf extract was prepared as previously described. Following the initial centrifugation at 38,000 × g, powdered ammonium sulfate was added to the primary supernatant to achieve a final concentration of 30%. The mixture was incubated with stirring at 4 °C for 1 hour. The sample was subjected to centrifugation at 38,000 × g for 30 minutes at 4 °C. The 30% ammonium sulfate pellet was discarded and additional ammonium sulfate was added to the 30% supernatant to achieve a final concentration of 55%. The sample was then incubated overnight at 4 °C with stirring. The sample was again subjected to centrifugation at 38,000 × g for 30 minutes at 4 °C. The resulting supernatant was discarded and the pellet containing the bulk of the AAD-12 was dissolved in 50 mM HEPES pH 7.8, 1 mM EDTA. The 30 – 50% ammonium sulfate protein fraction was divided into 850 µL aliquots, snap frozen in liquid nitrogen and stored at -80 °C.

A colorimetric assay was used to assess the activity of both the cotton leaf- and microbe-derived enzymes. Assays were performed in 1.5 mL Eppendorf tubes at room temperature with a total assay volume of 150 µL as previously described (Fukumori & Hausinger 1993). Typical assays contained 100 mM HEPES pH 7.0, 1 mM $Fe(II)(SO_4)_2(NH_4)_2$, 1 mM sodium ascorbate, S-Dichloroprop (in DMSO), and 0.05 µM AAD-12. All reactions were initiated by addition of α-ketoglutarate to a final concentration of 2 mM.

Prior to assay, the AAD-12 protein was serially diluted in 50 mM HEPES pH 7.0, 1 mM EDTA to match the concentration of the cotton-derived enzyme (0.0865 µM). After the appropriate incubation time, assays were terminated by addition of 10 µL 100 mM sodium EDTA followed by 15 µL of borate buffer pH ~10 (0.309 g boric acid + 0.373 g KCl + 4.4 mL 1 N KOH). Phenol products were detected by the addition of 2 µL 2 % 4-aminoantipyrine and 2 µL 8% potassium ferricyanide. Following centrifugation for 30 seconds at 10,000 × g, 150 µL of the supernatant was transferred to a 96-well plate and the absorbance at 510 nm was recorded on a SpectraMax® M2 microplate reader. Authentic product standard curves were run in parallel with 2,4 dichlorophenol in the range of 0 – 125 µM added in place of substrate. Control reactions contained all reagents except for substrates. Blanks lacking enzyme were analysed to account for the contaminating phenols in the substrate formulation which were found to be negligible (not shown). Kinetic experiments were performed as described with S-dichloroprop varied over a concentration range from 0 – 125 µM.

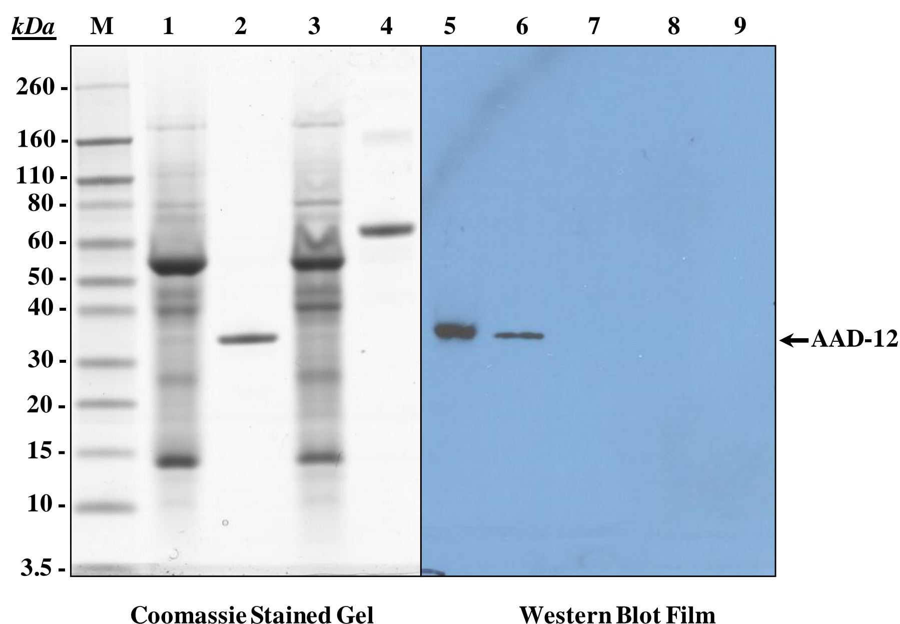
Results for Characterization of AAD-12 Protein

SDS-PAGE and western blot analysis of crude cotton leaf protein extracts

SDS-PAGE and western analysis was conducted to confirm the identity DAS-8191Ø-7 cotton-derived AAD-12 and to show molecular weight and immunoreactive equivalence between the microbe-derived and DAS-8191Ø-7 cotton-derived AAD-12 proteins. Both the microbe-derived AAD-12 protein and the transgenic DAS-8191Ø-7 cotton leaf extract contained a positive signal at the expected molecular weight of 32 kDa by polyclonal antibody western blot analysis (Figure 49). Importantly, the non-transgenic cotton extracts and the BSA control samples did not contain any immunoreactive protein bands, as expected. The results demonstrated that the anti-AAD-12 antibodies recognized the DAS-8191Ø-7 cotton AAD-12 protein and the identical migration pattern for both transgenic cotton and microbe-derived AAD-12 strongly suggests that DAS-8191Ø-7 cotton AAD-12 is not fragmented, glycosylated or otherwise post-translationally modified which would add or subtract from the overall protein molecular weight. Both SDS-PAGE and western analysis results demonstrate the microbe-derived AAD-12 and DAS-8191Ø-7 cotton derived proteins to be equivalent in both molecular weight and immunoreactive.

Purification results of DAS-8191Ø -7 cotton AAD-12

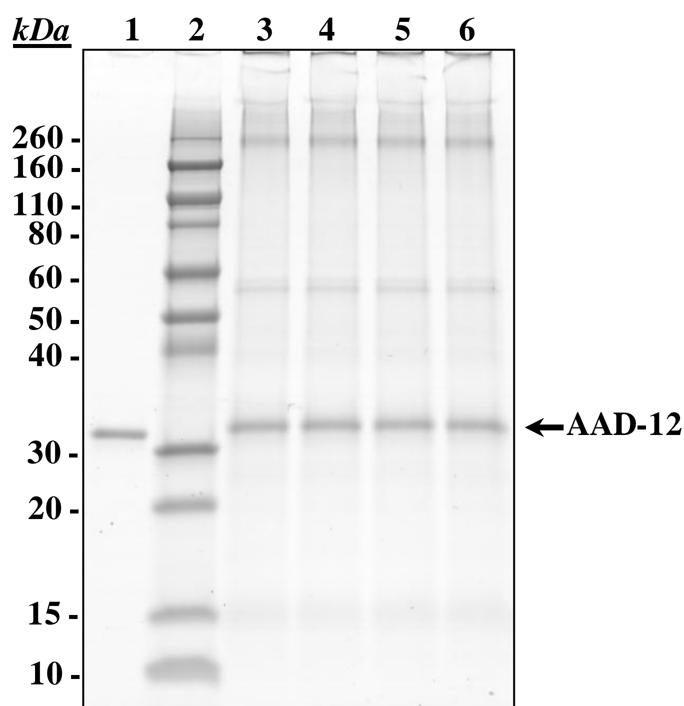
To conduct additional equivalency analysis of DAS-8191Ø-7 cotton AAD-12 to that of microbe-derived AAD-12, AAD-12 immunoaffinity precipitation was conducted on an aqueous extract of 5 – 10 grams of ground DAS-8191Ø-7 cotton lyophilized transgenic leaf tissue. Proteins bound to the anti-AAD-12 monoclonal antibody column were examined by SDS-PAGE along with the microbe-derived AAD-12. For the microbe-derived AAD-12, a single major protein band of 32 kDa was visualized following Coomassie staining of the SDS-PAGE gel and as expected, the corresponding DAS-8191Ø-7 cotton-derived AAD-12 protein was identical in size to the microbe-derived AAD-12 protein (Figure 50).



Lane	Sample	Amount Loaded
M	Novex prestained MW markers	10.0 µL
1	DAS-8191Ø-7 cotton crude leaf extract	30.0 µL
2	Microbe-derived AAD-12	1.00 µg
3	Control cotton (non-transgenic) leaf extract	30.0 µL
4	BSA protein standard	1.00 µg
5	DAS-8191Ø-7 cotton crude leaf extract	30.0 µL
6	Microbe-derived AAD-12 standard	1.30 ng
7	Blank	-
8	Control cotton (non-transgenic) leaf extract	30.0 µL
9	BSA protein standard	1.25 ng

Figure 49. SDS-PAGE and Western Blot Analysis of Microbe- & DAS-8191Ø-7 Cotton-Derived AAD-12 Proteins

Predictably, the plant purified fractions contained a minor amount of impurities in addition to the AAD-12 protein. The co-eluted proteins were likely retained on the resin by weak interactions with the column matrix or antibody leaching off of the column under the elution conditions. Other researchers have also reported the non-specific adsorption of proteins, peptides, and amino acids on activated agarose immunoadsorbents (Holroyde et al 1976, Kennedy & Barnes 1983, Williams et al 2006) as well as antibody leaching from the column (Goldberg et al 1991).



Coomassie Stained Gel

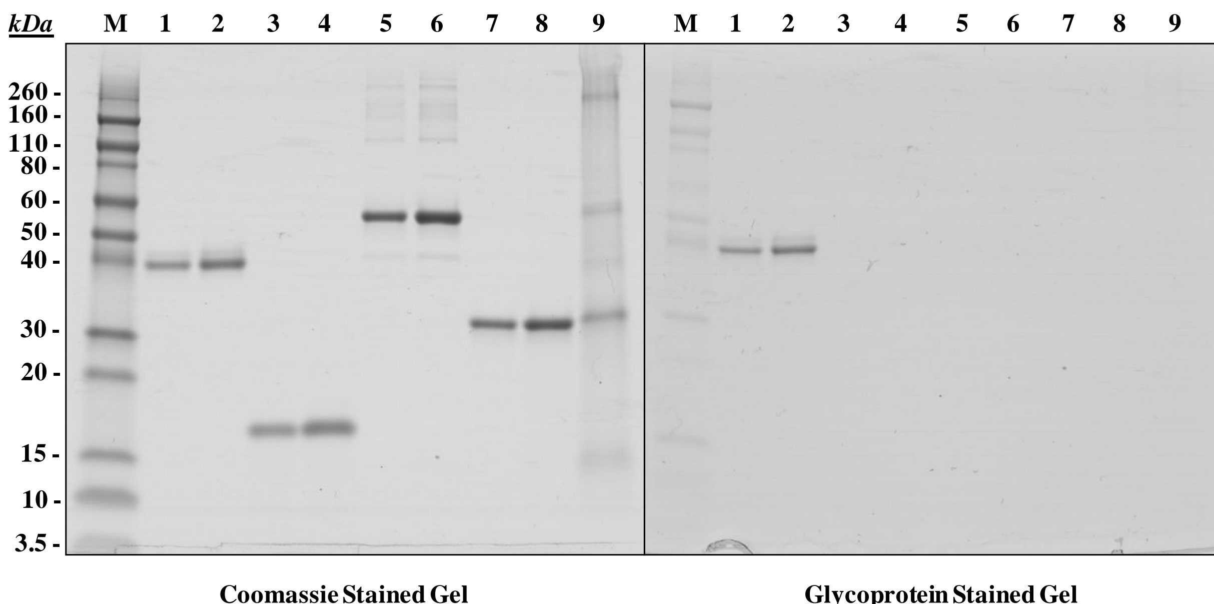
Lane	Sample	Amount Loaded
1	Microbe-derived AAD-12 Standard	0.34 µg
2	Novex prestained MW markers	10 µL
3	DAS-8191Ø-7 purified AAD-12	25 µL
4	DAS-8191Ø-7 purified AAD-12	25 µL
5	DAS-8191Ø-7 purified AAD-12	25 µL
6	DAS-8191Ø-7 purified AAD-12	25 µL

Figure 50. SDS-PAGE Analysis of Immunoaffinity Purified DAS-8191Ø-7 Cotton Derived AAD-12

Glycosylation detection of DAS-8191Ø-7 cotton AAD-12 protein

Detection of carbohydrates, possibly covalently linked to immunoaffinity-purified DAS-8191Ø-7 cotton-derived AAD-12 protein, was assessed using the GelCode Glycoprotein Staining Kit from ThermoScientific. The purified DAS-8191Ø-7 cotton AAD-12 was electrophoresed simultaneously with a set of control and reference protein standards.

As seen in the Coomassie stained gel in Figure 51, the glycoprotein, horseradish peroxidase, was loaded as a positive control indicator for glycosylation and two non-glycoproteins, soybean trypsin inhibitor and bovine serum albumin, were employed as negative controls. As expected, the glycoprotein stained gel shows a positive signal from the glycosylated horseradish peroxidase positive control, while the non-glycosylated negative controls, soybean trypsin inhibitor and bovine serum albumin, show no signal. Importantly, as with the negative control samples, neither the microbe-derived or DAS-8191Ø-7 purified AAD-12 exhibited detectable glycosylation following glycoprotein staining. These results confirm that microbe-derived and DAS-8191Ø-7 cotton derived AAD-12 contains no detectable covalently linked carbohydrates.



Lane	Sample	Amount Loaded
M	Novex prestained MW markers	10 µL
1	Horseradish peroxidase (+ control)	0.5 µg
2	Horseradish peroxidase (+ control)	1.0 µg
3	Soybean trypsin inhibitor (- control)	0.5 µg
4	Soybean trypsin inhibitor (- control)	1.0 µg
5	Bovine serum albumin (- control)	0.5 µg
6	Bovine serum albumin (- control)	1.0 µg
7	Microbe-derived AAD-12	0.5 µg
8	Microbe-derived AAD-12	1.0 µg
9	DAS-8191Ø-7 immunopurified AAD-12	20 µL

Figure 51. Glycoprotein Analysis of Purified DAS-8191Ø-7 Cotton AAD-12

Coomassie (left) and glycoprotein (right) stained SDS-PAGE gels containing control proteins and microbe-derived and cotton derived AAD-12 protein.

Results of MALDI-TOF MS and MALDI-TOF MS/MS amino acid sequence analysis of DAS-8191Ø-7 cotton-derived AAD-12 protein

To confirm the amino acid sequence of the purified DAS-8191Ø-7 cotton-derived AAD-12 protein, the purified protein was resolved by SDS-PAGE (Figure 50) and the respective bands were excised and subjected to in-gel digestion with trypsin, chymotrypsin, or Asp-N. The masses of the digested DAS-8191Ø-7 cotton AAD-12 protein were compared with those deduced from expected masses of trypsin, chymotrypsin, and ASP-N cleavage sites in AAD-12 using Protein Analysis Worksheet (PAWS) freeware from Proteometric LLC (Figure 52 - Figure 54). The masses of the DAS-8191Ø-7 cotton detected peptides were compared with the expected in silico masses to confirm the identity of the purified AAD-12 protein.

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 52. *In Silico* Trypsin Cleavage of DAS-8191Ø-7 Cotton AAD-12

Theoretical cleavage of the AAD-12 protein with trypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC. Theoretical trypsin digest peptides are indicated by the continuum of upper (black) or lower (red) case letters.

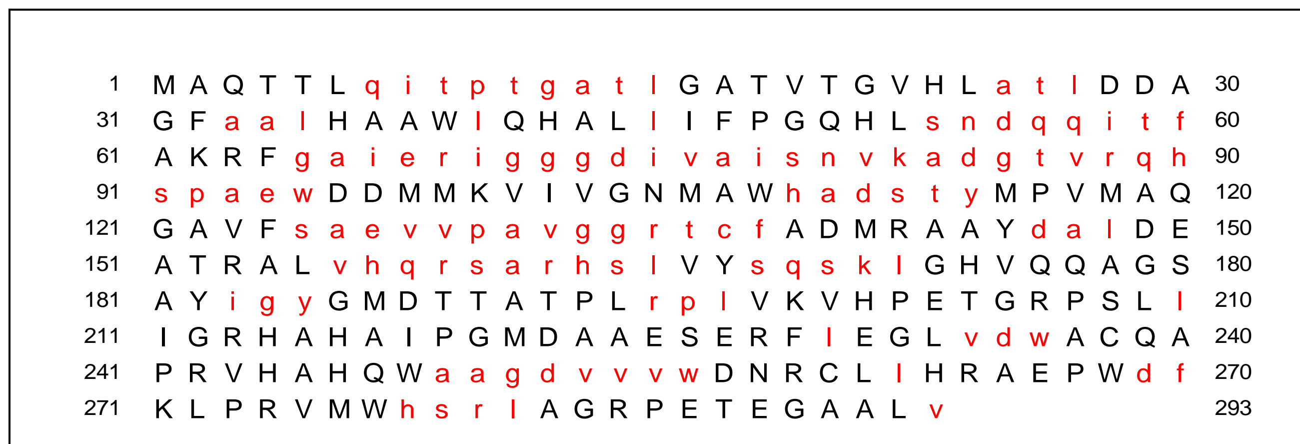


Figure 53. ***In Silico*** **Chymotrypsin Cleavage of DAS-8191Ø-7 Cotton AAD-12**

Theoretical cleavage of the AAD-12 protein with chymotrypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC. Theoretical trypsin digest peptides are indicated by the continuum of upper (black) or lower (red) case letters.

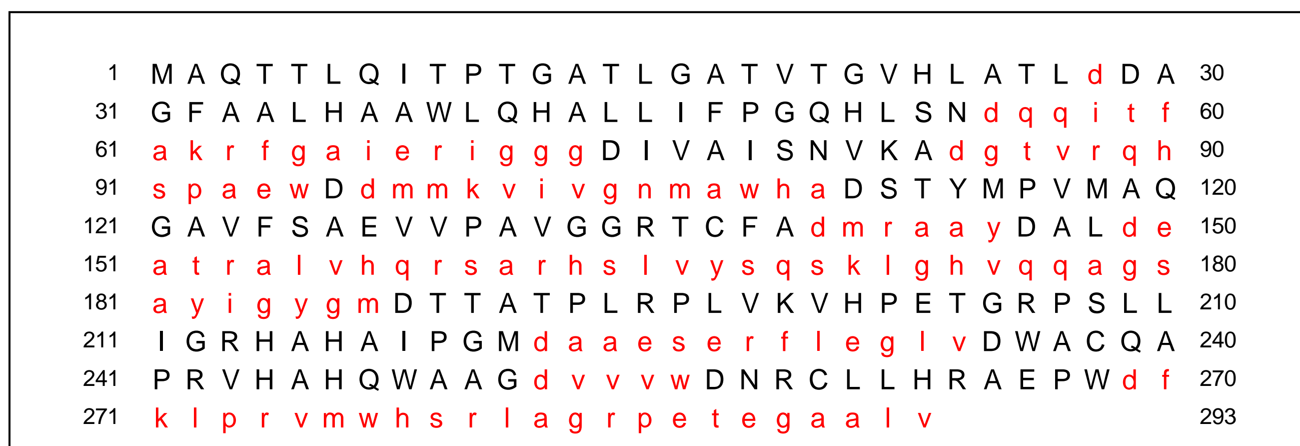


Figure 54. ***In Silico*** **Asp-N Cleavage of DAS-8191Ø-7 Cotton AAD-12**

Theoretical cleavage of the AAD-12 protein with Asp-N generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC. Theoretical trypsin digest peptides are indicated by the continuum of upper (black) or lower (red) case letters

Figure **55** is a comprehensive peptide coverage map illustrating the peptides identified and sequence verified for each endoproteinase (trypsin, chymotrypsin, and Asp-N) digest. Observed sequence coverage for trypsin, chymotrypsin and Asp-N was 61.4%, 49.1%, and 36.5%, respectively. For DAS-8191Ø-7 cotton-derived AAD-12, overall sequence coverage analysis was excellent at 88.1%. Peptide sequence that was missed did not contain sequence motifs that are typically required for glycosylation

(N-X-S or N-X-T where X is any amino acid) (Hamby & Hirst 2008). The theoretical and observed amino acid digest (and molecular weights) of the cotton-derived AAD-12 protein is also described in the attached report (Clement 2013).

The amino acid residues at the N-and C-termini of the cotton-derived AAD-12 protein were measured and compared with the previously determined sequence of the microbe-derived protein. The protein sequences were measured by MALDI-TOF, MALDI-TOF/TOF MS/MS or ESI-LC/MS. Asp-N digestion revealed that both the microbe- and DAS-8191Ø-7 cotton-derived AAD-12 N-termini were identical with the N-terminal amino acid of the cotton-derived AAD-12 containing an acetylated alanine (A) (Table 29).

Table 29. Summary of N-terminal Sequences of AAD-12 Derived Proteins

Expected	M^1	A^2 Q T T L Q I T P T G A T L G...
DAS-8191Ø-7	*N-acetyl-*	A^2 Q T T L Q I T P T G A T L G...
Microbe-derived		A^2 Q T T L Q I T P T G A T L G...

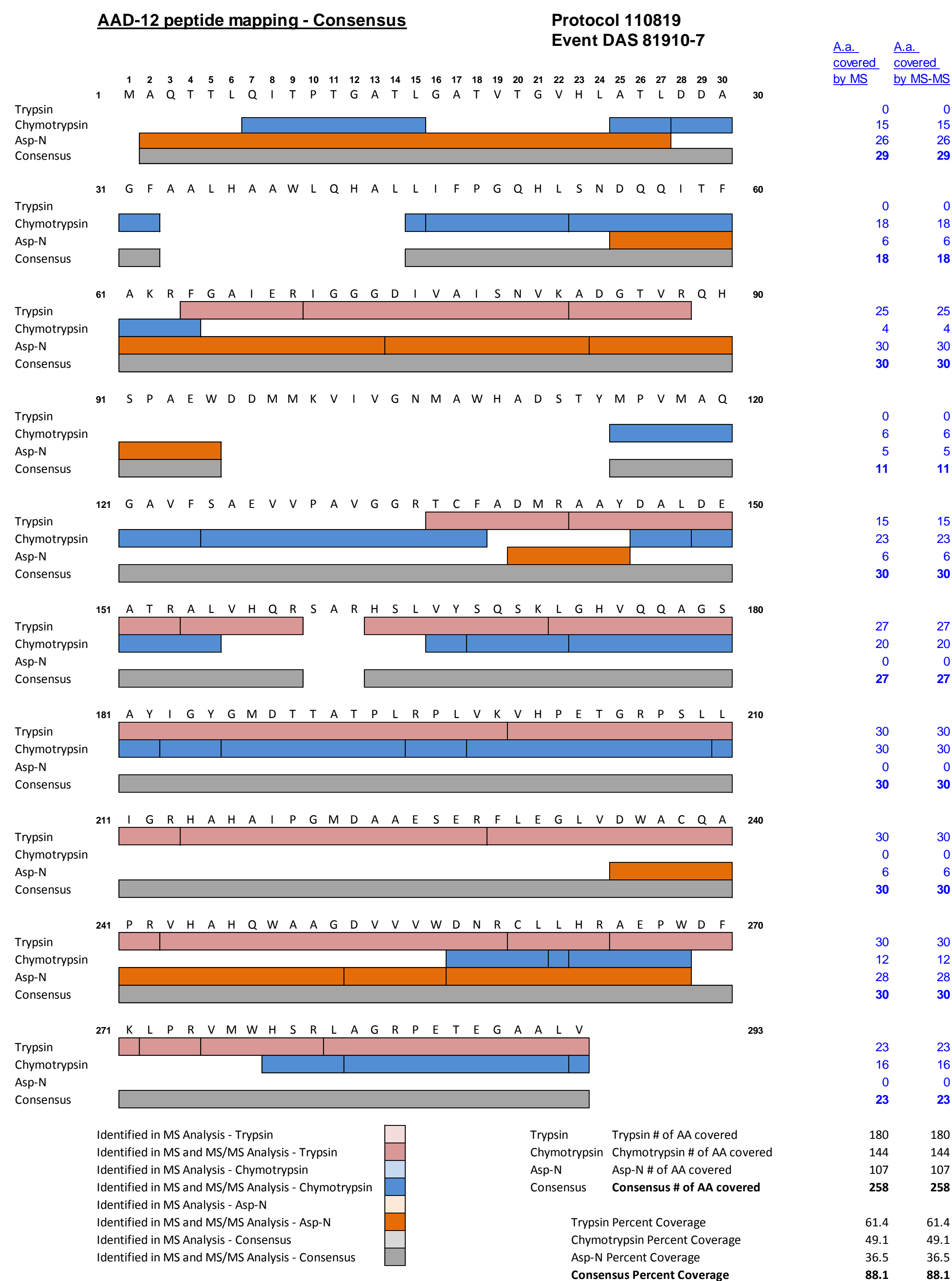


Figure 55. Comprehensive MS and MS/MS Sequence Coverage Map for Trypsin, Chymotrypsin and Asp-N Digest of DAS-8191Ø-7 Cotton-Derived AAD-12

Based on the *aad-12* DNA sequence, the N-terminal DAS-8191Ø-7 cotton and microbe-derived AAD-12 protein sequences are expected to contain a methionine residue (Table 29). This result indicated that the N-terminal methionine of the microbe-derived and DAS-8191Ø-7 cotton proteins had been removed. In addition, in DAS-8191Ø-7 the second amino acid alanine was acetylated. This result is encountered frequently with eukaryotic (plant) expressed proteins since approximately 80-90% of the N-terminal protein residues are modified in such a way (Polevoda & Sherman 2003, Wellner et al 1990). This result determined that during or after AAD-12 translation in cotton and *P. fluorescens*, the N-terminal methionine is cleaved by a methionine aminopeptidase (MAP). MAPs cleave methionyl residues rapidly when the second residue on the protein is small, such as Gly, Ala, Ser, Cys, Thr, Pro, and Val (Walsh 2005).

Also, it has been shown that proteins with serine and alanine at the N-termini are frequently acetylated (Polevoda & Sherman 2003). The two cotranslational 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 & Sherman 2003). However, examples demonstrating that N-terminal protein acetylation result in biological significance are rare (Polevoda & Sherman 2003).

The C-terminal sequence of the DAS-8191Ø-7 cotton- and microbe-derived AAD-12 proteins were determined essentially as described above for AAD-12 N-terminal sequence. The resulting amino acid C-terminal sequences were compared to the expected translated *aad-12* DNA sequence (Table 30). The results indicated that the measured DAS-8191Ø-7 cotton- and microbe-derived AAD-12 C-terminal sequences were identical to the expected protein sequence, and both the cotton- and microbe-derived AAD-12 proteins were indistinguishable and unaltered at the C-terminus.

Table 30. Summary of C-terminal Sequences of AAD-12 Derived Proteins

Expected	R^{284} P E T E G A A L V^{293}
DAS-8191Ø-7	R^{284} P E T E G A A L V^{293}
Microbe-derived	R^{284} P E T E G A A L V^{293}

Results of the activity assay of the AAD-12 protein derived from DAS-8191Ø-7 cotton plant tissue
To evaluate AAD-12 enzymatic activity, kinetic assays were performed with both microbe-derived and DAS-8191Ø-7 cotton-derived AAD-12. Assays were performed for 6 minutes at 28 °C with 0.05 μM enzyme. AAD-12 isolated from both *P. flourescens* and DAS-8191Ø-7 cotton displayed the expected hyperbolic activity profiles when evaluated over a range of S-dichloroprop substrate concentrations (Figure 56). Michaelis-Menten curve fitting revealed apparent K_m values of 41.2 ± 4.8 μM and 29.8 ± 1.0 μM for microbe-derived and cotton-derived AAD-12 respectively. For these assays, the apparent V_{max} values for microbe-derived and cotton-derived AAD-12 were also similar at 6.8 ± 0.3 and 5.0 ± 0.1 μM/min respectively. The relative agreement in the measured kinetic parameters for both microbe- and DAS-8191Ø-7 cotton-derived AAD-12 indicates that the enzymes are functionally equivalent.

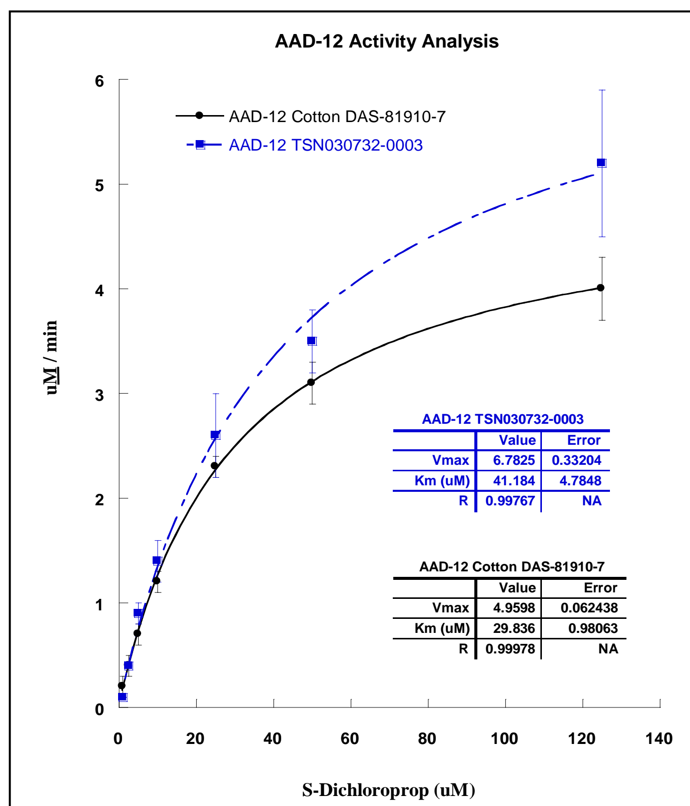


Figure 56. Kinetic Analysis of Microbe and DAS-8191Ø-7 Cotton Derived AAD-12 Proteins with S-Dichloroprop Substrate
The Michaelis-Menten plots of microbe-derived AAD-12 (black circles) and cotton-derived PAT (blue squares) using L-Glufosinate-ammonium as a substrate for PAT. The average of three independent experiments is shown and error bars indicate the standard deviation of the analyses.

Conclusions

This study demonstrated that the biochemical identity and biological function of *P. fluorescens*-produced AAD-12 protein was equivalent to the protein purified from the leaf tissue of DAS-8191Ø-7 cotton. Both the DAS-8191Ø-7 cotton and microbe-derived AAD-12 proteins had an apparent molecular weight of 32 kDa and were immunoreactive on western blots using an AAD-12 specific polyclonal antibody. The observation that DAS-8191Ø-7 cotton and microbe-derived AAD-12 co-migrate on both SDS-PAGE and western blots strongly indicates a lack of posttranslational modifications apart from the N-terminal acetylation. Moreover, it was experimentally determined that both DAS-8191Ø-7 cotton and microbe-derived AAD-12 are not glycosylated as empirically determined by MALDI-MS and glycoprotein stained gels. DAS-8191Ø-7 cotton - and *P. fluorescens*-derived AAD-12 were equally active using S-dichloroprop as a substrate and displayed similar kinetic parameters, indicating that the proteins are enzymatically equivalent. Finally, the amino acid sequence of cotton-derived AAD-12 was directly confirmed using enzymatic peptide mass fingerprinting by MALDI-TOF MS and verified by MS/MS analysis. Collectively, these data support the conclusion that the AAD-12 protein produced by *P. fluorescens* and transgenic cotton are biochemically and functionally equivalent.

Appendix 3. Methods and Results for Characterization of PAT Protein

Materials & Methods for Characterization of PAT Protein

DAS-8191Ø-7 transgenic Cotton material

The PAT protein was extracted from the leaf tissue of greenhouse grown transgenic cotton event DAS-8191Ø-7 (T_3 generation see Figure 2). Prior to harvest, the PAT protein expression was verified by western blot and the leaf tissue from DAS-8191Ø-7 cotton was harvested, frozen, lyophilized, ground, and stored at -80 °C. Test material used for characterization of DAS-8191Ø-7 cotton PAT are listed in Table 31.

Control Cotton material

The control cotton line had a genetic background representative of DAS-8191Ø-7 cotton plants, but did not contain the *pat* gene. Seeds of the non-transgenic cotton line were planted, grown, tested, harvested, and processed under the same conditions as DAS-8191Ø-7 cotton.

Microbe-Derived PAT

Recombinant microbe-derived PAT protein was produced and purified from *Pseudomonas fluorescens* and has an apparent molecular weight of ~20 kDa.

Reference materials

The commercially available reference substances used in this study are listed in Table 32.

Table 31. Test Material for PAT Characterization

Test Substance	Source	Lot Number	Assay	Reference
Cotton PAT	Cotton DAS-8191Ø-7	TSN304178	SDS-PAGE, Western blot, Glycosylation, MALDI-TOF, MALDI-TOF/TOF MS/MS, Activity Assay	NA
Control Cotton Line	isogenic / non-transgenic cotton	TSN304177	SDS-PAGE, Western blot, Glycosylation, MALDI-TOF, MALDI-TOF/TOF MS/MS, Activity Assay	NA
Cotton crude leaf extract	Cotton DAS-8191Ø-7	NA	SDS-PAGE, Western	NA
Microbe-derived PAT	*Pseudomonas fluorescens*	TSN303589 (ENBK 132436-001)	SDS-PAGE, Western, Glycosylation, MALDI-TOF, MALDI-TOF/TOF MS/MS, Activity Assay	

Table 32. Commercially Available Reference Substances for PAT Characterization

Reference Substance	Product Name	Lot Number	Assay	Reference
Mass Spectrometry Mass Standards Kit	Mass Standards Kit for Calibration of AB SCIEX TOF/TOF Instruments	A1068	Protein sequence analysis	AB SCIEX
Soybean Trypsin Inhibitor (STI)	A component of the GelCode glycoprotein staining kit	NH175044	Glycosylation assay	ThermoFisher
Horseradish Peroxidase (HRP)	A component of the GelCode glycoprotein staining kit	ND171686	Glycosylation assay	ThermoFisher
Bovine Serum Albumin (BSA)	Pre-diluted BSA protein assay standard set	NH175569	SDS-PAGE, Glycosylation assay	ThermoFisher
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	1167391	SDS-PAGE, western blot, Glycosylation assay	Invitrogen: Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 10, and 3.5 kDa

SDS-PAGE and western blot analysis of crude cotton leaf extracts

SDS-PAGE and western blot analysis of the crude protein extracts prepared from the transgenic DAS-8191Ø-7 cotton tissue and non-transgenic isoline tissue were performed with Bio-Rad Criterion gels fitted in a Criterion Gel chamber with XT MES running buffer (Bio-Rad). Extracts were prepared by Geno-grinding the ground cotton tissue at 40 mg/mL in PBST buffer containing 5 mM EDTA, 0.16% (v/v) β-mercaptoethanol, and 1× protease inhibitor cocktail with 2 steel beads for 3 minutes at 1,500 strokes/min in a 2 mL micro-centrifuge tube. The supernatants were clarified by centrifuging the samples at ~20,000 × g (4 °C) for 5 minutes, and 100 µL of each extract was mixed with 100 µL of 2× Laemmli sample buffer (Bio-Rad) containing freshly added β-mercaptoethanol (Bio-Rad) and heated for 5 minutes at ~95 °C. After a brief centrifugation (2 min at 20,000 × g, 4 °C), 20 µL of each supernatant was loaded directly on the gel. The reference standards, microbe-derived PAT, and control standard, BSA (Thermo Scientific), were diluted to an appropriate concentration and combined with Laemmli sample buffer containing β-mercaptoethanol. The electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes.

After separation, the gel was cut in half and one half was stained with Thermo Scientific GelCode Blue protein stain and scanned with a densitometer (GE Healthcare) to obtain a permanent record of the gel. The remaining half of the gel was electro-blotted to a nitrocellulose membrane (Bio-Rad) using a Criterion trans-blot electrophoretic transfer cell (Bio-Rad) with transfer buffer containing 20% methanol, 10% Tris/glycine buffer under a constant voltage of 100 V for 60 minutes. After transfer, the membrane

was probed with a PAT specific polyclonal rabbit antibody for 60 minutes (1:40,000 dilution) at room temperature. A 1:80,000 dilution of conjugated goat anti-rabbit IgG (H+L) with horseradish peroxidase (Thermo) was used as the secondary antibody. ThermoPierce ECL chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to detection film (Thermo Scientific) for various time points and subsequently developed with an All-Pro 100 Plus film developer.

Protein purification of PAT from DAS-8191Ø-7 cotton plant tissue for structural analyses

The PAT protein was extracted from the ground cotton tissue using 50 mM HEPES (pH 7.8), 300 mM NaCl, 10 mM EDTA (Extraction buffer) by weighing and transferring ground leaf tissue into a chilled glass beaker and adding Extraction buffer to 20 mL per 1 gram of tissue. The tissue was mixed briefly and PVPP (insoluble) was added to the mixture at 10% (w/w). The mixture was stirred for 20 min and the solution was filtered through 1 layer of pre-wetted miracloth (Calbiochem) and clarified by centrifugation at ~30,000 × g, 4 °C for 30 minutes. The supernatant was removed and combined with an equal volume of Extraction buffer supplemented with 2 M urea, 0.2% Tween-20 to yield a final extract containing 1 M urea and 0.1% Tween-20.

The PAT protein was purified from the supernatant by immuno-precipitation using polyclonal antibodies cross-linked to Protein A/G Agarose resin (Thermo Scientific) at 4 mg of antibody per milliliter of resin. For each 15 mL of clarified supernatant, 50 µL (200 µg of antibody) of coupled resin was added and allowed to incubate on a rotating mixer for 3 hours at 4 °C. The resin was recovered by centrifugation at ~3,500 × g for 5 minutes at 4 °C and the resin was transferred to a 1.5 mL microcentrifuge tube using 1 mL of wash buffer. The resin was then washed sequentially for a total of 5 washes using different buffers (*vide infra*). Each wash was accomplished by adding buffer to the tube containing resin, rotating the tube on a mixer for ~5 min at 4 °C, centrifuging the sample at ~500 x g for 1 min, and discarding the supernatant. The resin was first washed twice with 0.9 mL of Extraction buffer supplemented with 1 M urea and 0.1% Tween-20, once with 0.9 mL of Extraction buffer supplemented with 0.5 M urea and 0.05% Tween-20, once with Extraction buffer, and once with 50 mM HEPES (pH 7.8), 0.1 mM EDTA. After the final wash, spin, and decant, the resin was centrifuged for 1 min at 500 × g at 4 °C to pellet the resin and the supernatant was discarded. Finally, the resin was centrifuged for 10 sec at 20,000 × g at 4 °C to pellet the resin and supernatant was again discarded. The immuno-purified protein was stored bound to the resin at -20 °C until used for SDS-PAGE followed by mass spectrometry analysis and SDS-PAGE followed by glycosylation analysis.

Detection of post-translational glycosylation

For detection of potential post-translational glycosylation, cotton-derived PAT protein was immuno-purified as described above. The resin with bound PAT protein from a single immuno-precipitation preparation was combined with 60 µL of 2× Laemmli sample buffer (no β-mercaptoethanol added) and was heated at 95 °C for 5 minutes. Protein standards and controls including microbe-derived PAT, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase proteins were diluted with

2× Laemmli sample buffer (Bio-Rad) to the approximate concentration of the purified cotton-derived PAT protein and heated at 95 °C for 5 minutes. All samples were centrifuged at 20,000 × g for 1 minute prior to loading on SDS-PAGE gel.

The resulting sample supernatants were applied directly to a Bio-Rad Mini-Protean TGX gel and were electrophoresed at 150 V for ~50 minutes. Two identical gels were run in parallel and after electrophoresis, one gel was stained with GelCode Blue stain (Thermo Scientific) for detection of total protein according to the manufacturers' protocol. After the staining was complete, the gel was scanned with a densitometer to obtain a permanent visual record of the gel. The second gel was stained with a GelCode Glycoprotein Staining Kit (Thermo Scientific) according to the manufacturers' protocol to visualize the glycoproteins.

The procedure for glycoprotein staining is briefly described as follows: After electrophoresis, the gel was fixed in 50% methanol for 30 minutes and rinsed with 3% acetic acid. This was followed by an incubation period with the oxidation solution from the staining kit for 15 minutes. The gel was once again rinsed with 3% acetic acid and incubated with GelCode glycoprotein staining reagent for 30 minutes. Finally, the gel was immersed in the reduction solution for 5 minutes, and rinsed with 3% acetic acid. The glycoproteins were visualized as magenta bands on a light pink background. After the glycoprotein staining was complete, the gel was scanned with a GE Healthcare densitometer to obtain a permanent visual record of the gel.

Mass spectrometry peptide mass fingerprinting and sequence analysis of cotton event DAS-8191Ø-7- and microbe-derived PAT proteins

Immunoaffinity purified plant-derived PAT protein was subjected to in-gel digestion by trypsin and Asp-N followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF MS-MS. For each enzyme digestion, bands resolved on a SDS-PAGE gel at ~20 kDa corresponding to PAT protein (two bands were used per digest containing approximately 100 ng per lane, resulting in 200 ng total protein per digest reaction) were excised with a sterile scalpel and processed as follows (a half lane section of the standard protein of the gel was processed alongside with the protein sample, in a separate tube, using identical procedure). Gel pieces were crushed with a sterile micro-pestle in a siliconized microcentrifuge tube, and destained as follows: 0.4 mL of 50% ACN and 0.4 mL of ammonium bicarbonate buffer were added, the tube was sealed and shaken at room temperature for 30 min in a Thermomixer R at 1100 rpm; the tube was centrifuged to settle the gel pieces, and the supernatant was removed with a pipette tip and discarded; the destaining procedure was repeated 2 times.

The proteins were reduced in-gel as follows: (1) 0.2 mL of DTT solution was added to the gel pieces, and the tube was incubated at room temperature for 30 min in a Thermomixer R; (2) the tube was centrifuged, and the DTT solution was removed by a pipette tip; (3) the gel pieces were washed twice with 0.5 mL of 25 mM ammonium bicarbonate buffer, the tube was centrifuged, and the buffer was

removed. After the destaining/ reduction procedures, the gel pieces were shrunk in neat acetonitrile and then dried in a centrifugal evaporator for 30 min.

The dried gel pieces were re-hydrated with a trypsin solution (25 μg in 500 μL of 25 mM ammonium bicarbonate buffer, pH 7.8; prepared fresh) or Asp-N solution (2 μg in 50 μL of 50 mM sodium phosphate buffer, pH 8.0; prepared fresh) and incubated in an incubator at 37 ºC for approximately 16 hours (overnight). Afterwards, the peptides were extracted from the gel slices sequentially with 0.4 mL of 50% ACN/ 0.1% TFA, then 0.4 mL of 50% ACN/ 5% FA, and finally 0.4 mL of 75% ACN/ 5% FA (30 min per extraction in a Thermomixer R at room temperature, shaking at 1100 rpm). The extracts for each sample were combined and dried in a centrifugal evaporator.

Dried peptides were reconstituted in 30 μL of 0.1% TFA in water and were purified for MALDI MS analysis using C18 Zip-Tips (Millipore), according to the manufacturer's procedure. Purified peptides were eluted directly onto MALDI plate sequentially, with aqueous 10%, 25%, 50%, and 75% ACN (buffered with 0.1% TFA). The ZipTip C18 fractions were mixed with 4 μL of CHCA matrix (10 mg/mL CHCA in 50% ACN supplemented with 0.1% TFA), and 1 μL of the sample-matrix mixture was deposited on the MALDI target and allowed to air dry. The sample preparations were analysed directly by MALDI-TOF mass spectrometry. All mass spectra were acquired on an AB Sciex 4800 MALDI-TOF/TOF mass spectrometer. Mass calibration was performed with an Mass Standards Kit for Calibration of AB SCIEX TOF/TOF Instruments, consisting of the calibration mixture (theoretical monoisotopic $[M+H]^+$ *m/z* values used): des-Arg[1]-Bradykinin, *m/z* 904.4681; Angiotensin I, *m/z* 1296.6853; Glu[1]-Fibrinopeptide B, *m/z* 1570.6774; ACTH (fragment 1-17), *m/z* 2093.0867; ACTH (fragment 18-39), *m/z* 2465.1989; ACTH (fragment 7-38) m/z 3657.9294. The plate wide calibration model was used for MS calibration. The peptide fragments of the cotton-derived PAT protein (including the N- and C-termini) were analysed and compared with the sequence of the microbe-derived protein.

<u>Protein purification of PAT from DAS-8191Ø-7 cotton plant tissue for functional analyses</u>

The PAT protein was extracted with 350 mL of 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, supplemented with 1% PVPP (polyvinylpolypyrrolidone, insoluble) and a protease inhibitor cocktail by blending ground cotton leaf tissue (15 g) using a stick blender (~1 g of tissue per 20 mL of buffer). The blended material was strained through 2 layers of pre-wetted Miracloth (Calbiochem), and clarified by centrifuging at ~35,000 × g, 4 °C for 45 minutes to remove all particulates and PVPP.

The supernatant was transferred to a pre-chilled beaker and solution was stirred at 4 °C. Ammonium sulfate was slowly added to the extract over a 1 hr time-course until 40% ammonium sulfate saturation was achieved. The solution was stirred for an additional 2 hrs following final addition of salt. The solution was centrifuged at 35,000 × g, 4 °C for 60 minutes to remove precipitated proteins.

The supernatant, containing PAT, was collected and returned to the chilled beaker at 4 °C with stirring. Ammonium sulfate was added to the extract over 1 hr until a concentration of 70% saturation was reached. The solution was allowed to equilibrate overnight. The precipitated protein material was pelleted by centrifuging the mixture at ~35,000 × g, 4 °C for 60 minutes and the supernatant was discarded. The pellet containing PAT was resuspended in 45 mL of chilled HIC Buffer A (HIC Buffer A = 50 mM Tris (pH 7.5), 5% glycerol with 1.25 M ammonium sulfate; HIC Buffer B = 50 mM Tris (pH 7.5), 5% glycerol). The resuspension was centrifuged at ~35,000 × g, 4 °C for 60 minutes to remove any non-dissolved particulates prior to chromatography.

The clarified supernatant was loaded onto a 10 mL Phenyl HP HiTrap column (two 5 mL columns connected in serial, GE Healthcare) equilibrated in HIC Buffer A. The column was washed with 15 column volumes (CV) of HIC Buffer A, followed by 5 CV of 5% HIC Buffer B. The bound proteins were eluted with a 50 CV linear elution gradient to 100% HIC Buffer B. Fractions (3.5 mL volume) were collected starting at 10% HIC Buffer B conditions and odd fractions were assayed for PAT content by Lateral Flow Strip Assay (Envirologix, Inc) and by the PAT Activity Assay.

Fractions containing the PAT protein were pooled and concentrated using a 10 kDa MWCO filter device (Amicon) from 122.5 mL to 4.2 mL. The salt content of the concentrated sample was adjusted to match the initial chromatographic conditions (100% HIC Buffer A) and was loaded onto a 9.4 mL Phenyl HP HiScreen column (two 4.7 mL columns connected in serial, GE Healthcare) equilibrated in HIC Buffer A. The column was washed with 6 column volumes (CV) of HIC Buffer A, and the bound proteins were eluted with a 20 CV linear elution gradient to 100% HIC Buffer B. Fractions (2.0 mL volume) were collected starting at 0% HIC Buffer B conditions and odd fractions were assayed for PAT content by Lateral Flow Strip Assay (Envirologix, Inc) and by the PAT activity assay.

Fractions containing the PAT protein were pooled and concentrated using a 10 kDa MWCO filter device (Amicon) from 46 mL to 3.0 mL. The concentrated sample was then applied to a 120 mL HiLoad 16/600 Superdex 200 column (GE Healthcare) equilibrated with 50 mM Tris (pH 7.5), 150 mM NaCl, and 5% glycerol. One-milliliter fractions were collected and assayed for PAT content by Lateral Flow Strip (Envirologix, Inc) and by the PAT Activity Assay. Fractions containing the PAT protein were pooled and concentrated using a 10 kDa MWCO filter device (Amicon) from 8 mL to 0.8 mL. The final purification product was aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

Activity assay of the PAT protein derived from DAS-8191Ø-7 cotton plant tissue
Cotton-derived PAT was purified using traditional purification procedures as described above. The activity of both the cotton- and microbe-derived enzymes was verified using an established spectrophotometric assay with minor modifications (De Block et al 1987, Mahan et al 2006).

Assays were performed in 96-well plates at room temperature in a total volume of 150 µL (a 150 µL total volume results in a 0.446 cm path length). Assays contained 50 mM HEPES (pH 7.0), 1 mM EDTA, 1 mM

5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 500 µM of acetyl-CoA, 25 nM PAT, and varied levels of DL-glufosinate-ammonium (25 µM –2 mM). Reactions were initiated by the addition of acetyl-CoA and the absorbance at 412 nm was recorded every 30 seconds for a total of 5 minutes on a Molecular Devices SpectraMax® M2 plate reader. Separate positive control reactions were assayed using microbe-derived PAT at the same concentration as the plant-derived protein (25 nM). Negative control reactions were assayed in the absence of DL-glufosinate-ammonium to monitor non-specific acetyl-CoA consumption. All reactions were run in triplicate.

PAT activity was quantified by measuring the liberation of the free CoA sulfhydryl group which forms concomitantly with transfer of the acetyl group to glufosinate-ammonium. The reaction of the sulfyhydryl group of free CoA with DTNB yields a molar equivalent of the chromophore 5-thio-2-nitrobenzoic (ε = 14,150 M^{-1} cm^{-1} at 412 nm). The initial rates were determined from the raw data, and were corrected for non-specific acetyl-CoA consumption (based upon control reactions lacking glufosinate-ammonium) and converted to µM/min. A racemic mixture of glufosinate was used for the assays, therefore the final values are presented as a function of L-glufosinate, the active enantiomer. These converted initial rates were plotted against L-glufosinate concentrations and subsequently fitted to the Michaelis–Menten equation allowing for the extrapolation of K_m and V_{max}. The data were analysed using KaleidaGraph software (v.4.03).

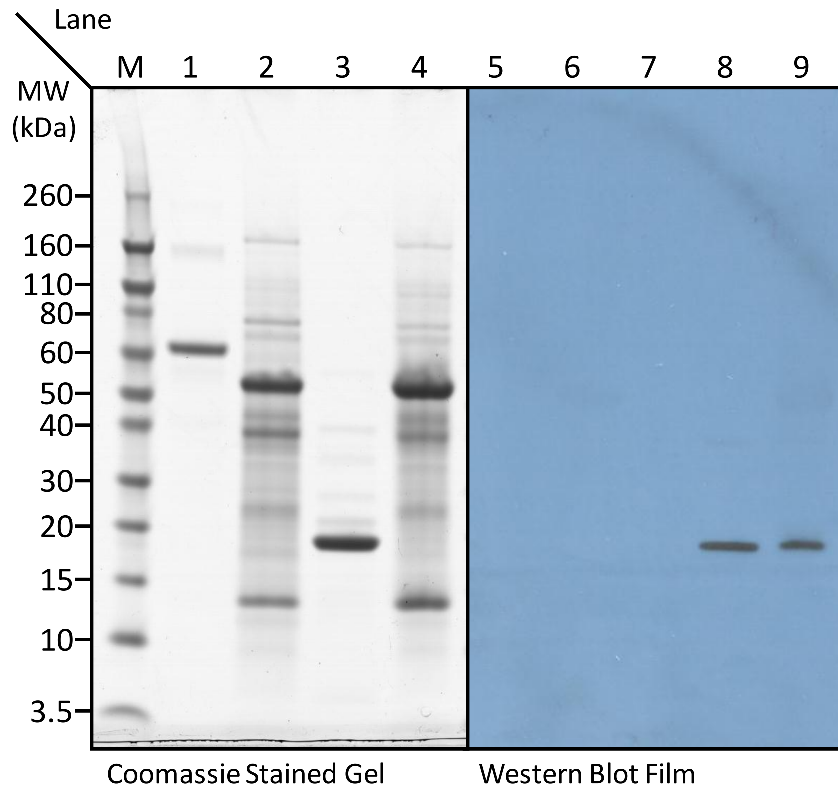
Results for Characterization of PAT Protein

SDS-PAGE and western blot analysis of cotton leaf protein extracts

SDS-PAGE and western analysis was conducted to confirm the identity DAS-8191Ø-7 cotton-derived PAT and to show molecular weight and immunoreactive equivalence between the microbe-derived and DAS-8191Ø-7 cotton derived PAT proteins. For the microbe-derived PAT protein, the major protein band was approximately ~20 kDa (Figure 57) as visualized on Coomassie stained SDS-PAGE gels. As expected, the corresponding DAS-8191Ø-7 cotton-derived PAT protein was visualized by immunospecific polyclonal antibodies at an identical size to the microbe-expressed protein. In the PAT western blot analysis, no immunoreactive proteins, consistent with the PAT protein, were observed in the control isoline extract or the BSA lanes (Figure 57). The results demonstrated that the anti-PAT antibodies recognized the DAS-8191Ø-7 cotton PAT protein and the identical migration pattern for both transgenic cotton- and microbe-derived PAT strongly suggests that the PAT protein expressed in cotton is not post-translationally glycosylated (note: the PAT enzyme does not contain N-glycosylation sites (Herouet et al 2005)) or processed in such a manner which would have added to or subtracted from the overall protein molecular weight. Both SDS-PAGE and western analysis results demonstrate the microbe-derived PAT and DAS-8191Ø-7 cotton derived proteins to be equivalent in both molecular weight and immunoreactive.

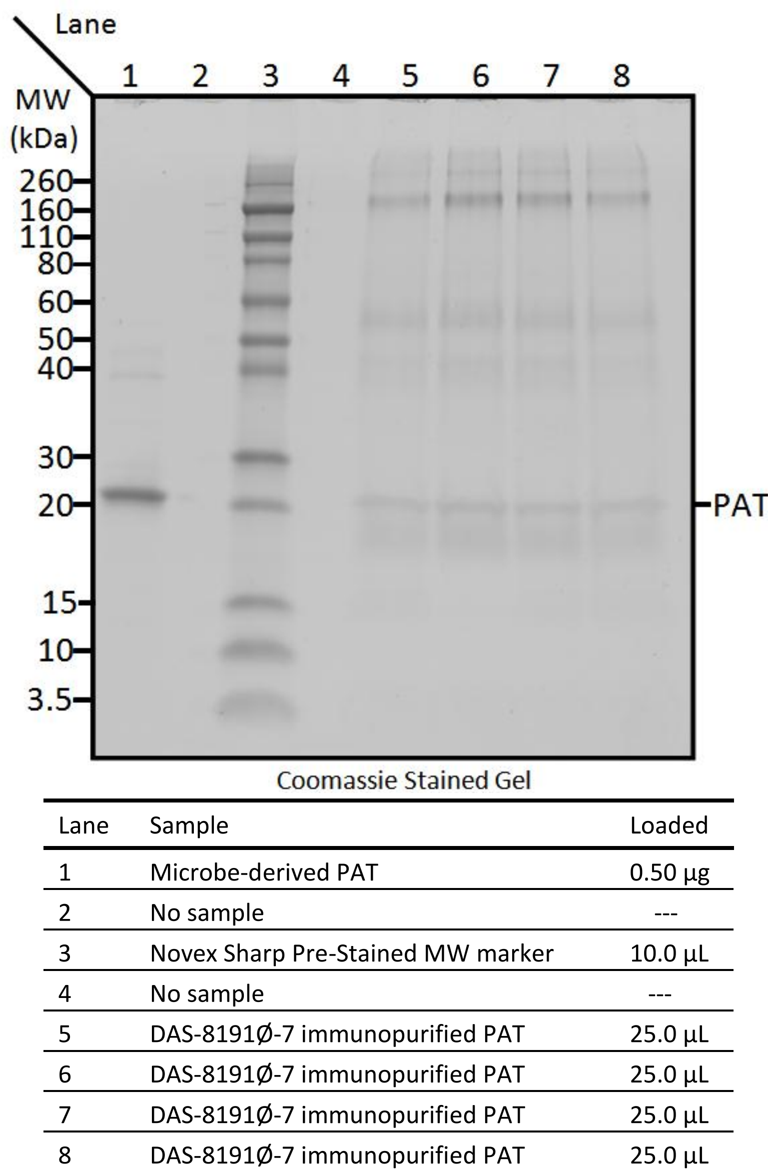
Purification results of DAS-8191Ø-7 cotton PAT for structural analysis

To conduct additional equivalency analysis of DAS-8191Ø-7 cotton-derived PAT to that of microbe-derived PAT, immuno-precipitation was conducted on an aqueous extract of ~6 grams of ground DAS-8191Ø-7 cotton leaf tissue to further purify the DAS-8191Ø-7 cotton-derived PAT protein. The total protein bound to the polyclonal antibody resin was examined by SDS-PAGE analysis which demonstrated that DAS-8191Ø-7 cotton-derived PAT protein from the tissue extract with an approximate molecular weight of ~20 kDa was captured during the immuno-precipitation procedure (Figure 58). Following immuno-precipitation, the DAS-8191Ø-7 cotton-derived PAT was then compared with the microbe-derived protein. For the microbe-derived PAT protein, the major protein band was approximately 20 kDa (Figure 58) as visualized on Coomassie stained SDS-PAGE gels.



Lane	Sample	Amount Loaded
M	Novex Sharp Pre-Stained MW marker	10.0 µL
1	Bovine Serum Albumin (BSA)	1.00 µg
2	Control cotton (non-transgenic) leaf extract	20.0 µL
3	Microbe-derived PAT	1.00 µg
4	DAS-8191Ø-7 cotton extract	20.0 µL
5	Bovine Serum Albumin	1.25 ng
6	Control cotton (non-transgenic) leaf extract	20.0 µL
7	No sample	---
8	Microbe-derived PAT	1.30 ng
9	DAS-8191Ø-7 cotton crude leaf extract	20.0 µL

Figure 57. SDS-PAGE and Western Blot Analysis of Microbe- and DAS-8191Ø-7 Cotton-Derived PAT Proteins



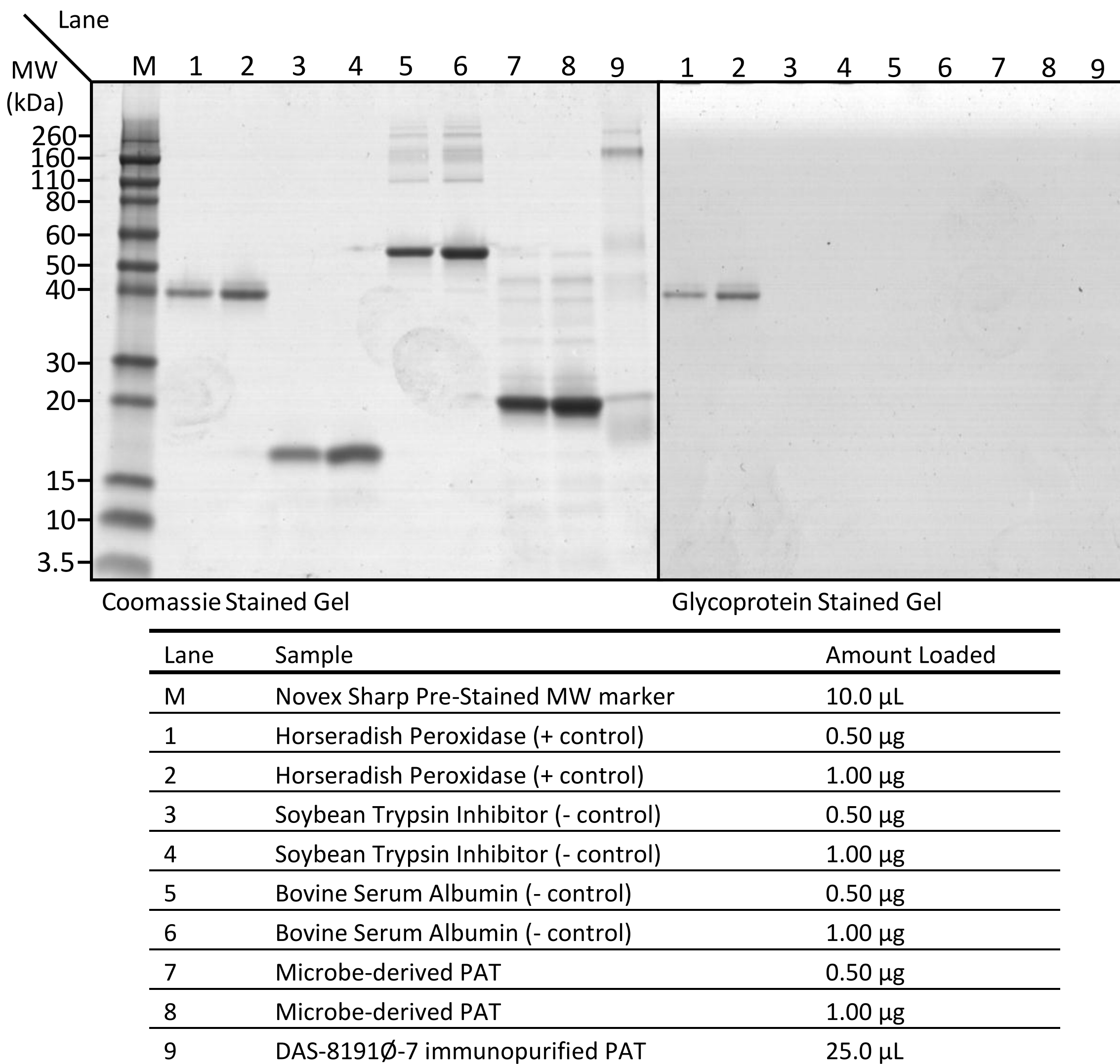
Lane	Sample	Loaded
1	Microbe-derived PAT	0.50 µg
2	No sample	---
3	Novex Sharp Pre-Stained MW marker	10.0 µL
4	No sample	---
5	DAS-8191Ø-7 immunopurified PAT	25.0 µL
6	DAS-8191Ø-7 immunopurified PAT	25.0 µL
7	DAS-8191Ø-7 immunopurified PAT	25.0 µL
8	DAS-8191Ø-7 immunopurified PAT	25.0 µL

Figure 58. SDS-PAGE Analysis of the Immunoaffinity Purified DAS-8191Ø-7 Cotton Derived PAT

As expected, the corresponding cotton-derived PAT protein was visualized by immunospecific polyclonal antibodies at an identical size to the microbe-expressed protein (Figure 58). The plant-derived immuno-precipitation samples contained a minor amount of impurities in addition to the full-length PAT protein. The co-bound proteins were likely retained to the resin by weak interactions with the matrix or antibody released during sample preparation for SDS-PAGE analysis. Other researchers have also reported the non-specific adsorption of proteins, peptides, and amino acids on activated agarose immuno-adsorbents (Holroyde et al 1976, Kennedy & Barnes 1983, Williams et al 2006) as well as antibody leaching from the resin (Goldberg et al 1991).

Results of detection of glycosylation of PAT protein

Detection of carbohydrates, possibly covalently linked to DAS-8191Ø-7 cotton-derived PAT protein, was assessed by resolving immuno-precipitated PAT using SDS-PAGE followed by visualization of the gel with a stain that specifically detects glycosylated proteins. The immunoaffinity-purified DAS-8191Ø-7 cotton PAT protein was electrophoresed simultaneously with a set of control and reference protein standards. A glycoprotein, horseradish peroxidase, was loaded as a positive control indicator for glycosylation, and non-glycoproteins including microbe-derived PAT, soybean trypsin inhibitor, and bovine serum albumin, were employed as negative controls.



Lane	Sample	Amount Loaded
M	Novex Sharp Pre-Stained MW marker	10.0 µL
1	Horseradish Peroxidase (+ control)	0.50 µg
2	Horseradish Peroxidase (+ control)	1.00 µg
3	Soybean Trypsin Inhibitor (- control)	0.50 µg
4	Soybean Trypsin Inhibitor (- control)	1.00 µg
5	Bovine Serum Albumin (- control)	0.50 µg
6	Bovine Serum Albumin (- control)	1.00 µg
7	Microbe-derived PAT	0.50 µg
8	Microbe-derived PAT	1.00 µg
9	DAS-8191Ø-7 immunopurified PAT	25.0 µL

Figure 59. Glycoprotein Analysis of Immunopurified DAS-8191Ø-7 Derived PAT

SDS-PAGE gels containing microbe- and cotton-derived PAT protein and standards visualized for total protein and glycoprotein stains.

As expected, the glycoprotein stained gel shows a positive signal from the glycosylated horseradish peroxidase positive control, while the non-glycosylated negative controls, soybean trypsin inhibitor and bovine serum albumin, shows no signal. As with the negative control sample, both the microbial-derived and DAS-8191Ø-7 cotton purified PAT samples were not detected on the glycoprotein stained gel. This was anticipated as the PAT protein does not contain any sites predicted for *N*-glycosylation, the most common form of glycosylation found on proteins (Herouet et al 2005). The results showed that both the DAS-8191Ø-7 cotton and microbe-derived PAT proteins had no detectable covalently linked carbohydrates (Figure 59).

Results of MALDI-TOF MS and MALDI-TOF MS/MS tryptic and Asp-N peptide sequencing of DAS-8191Ø-7 cotton-derived PAT protein

The PAT protein derived from DAS-8191Ø-7 cotton tissue was separated by SDS-PAGE (Figure 58) and the respective bands were excised and subjected to in-gel digestion by trypsin and Asp-N endoproteases. The resulting peptide mixture was analysed by MALDI-TOF MS and sequence verified by MS/MS to determine the peptide sequences. The masses of the detected peptides were compared with the expected masses based on trypsin or Asp-N cleavage sites in the sequence of the cotton-derived PAT protein.

Figure 60 and Figure 61 illustrate the theoretical cleavage of the PAT protein generated *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The theoretical and observed amino acid digest (and molecular weights) of the cotton-derived PAT peptides is also described in. The PAT protein, once denatured, is readily digested by endoproteases to yield numerous peptides that are able to be detected using mass spectrometry.

```
1    M S P E R r p v e i r p a t a a d m a a v c d i v n h y i e   30
31   t s t v n f r T E P Q T P Q E W I D D L E R l q d r Y P W L   60
61   V A E V E G V V A G I A Y A G P W K a r N A Y D W T V E S T   90
91   V Y V S H R h q r L G L G S T L Y T H L L K s m e a q g f k   120
121  S V V A V I G L P N D P S V R l h e a l g y t a r G T L R a   150
151  a g y k H G G W H D V G F W Q R d f e l p a p p r p v r p v   180
181  t q i                                                         183
```

Figure 60. *In Silico* Trypsin Cleavage of DAS-8191Ø-7 Cotton PAT

Theoretical cleavage of the PAT protein with trypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC. Trypsin endoprotease specifically hydrolyses protein and peptide bonds C-terminally of lysine and arginine. Theoretical trypsin digest peptides are indicated by the continuum of upper (black) or lower (red) case letters.

```
1    M S P E R R P V E I R P A T A A d m a a v c D I V N H Y I E   30
31   T S T V N F R T E P Q T P Q E W I d D L E R L Q d r y p w l   60
61   v a e v e g v v a g i a y a g p w k a r n a y D W T V E S T   90
91   V Y V S H R H Q R L G L G S T L Y T H L L K S M E A Q G F K   120
121  S V V A V I G L P N d p s v r l h e a l g y t a r g t l r a   150
151  a g y k h g g w h D V G F W Q R d f e l p a p p r p v r p v   180
181  t q i                                                         183
```

Figure 61. *In Silico* Asp-N Cleavage of DAS-8191Ø-7 Cotton PAT

Theoretical cleavage of the PAT protein with Asp-N generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC. Asp-N endoprotease specifically hydrolyses protein and peptide bonds N-terminally of aspartic acid. Theoretical Asp-N digest peptides are indicated by the continuum of upper (black) or lower (red) case letters**.**

In the endoprotease digests of the transgenic cotton-derived PAT protein, the peptide sequence coverage from peptide mass fingerprint (PMF) data was extensive at 91.3%. Of the 91.3% sequence coverage from PMF data, all peptide sequences were confirmed by tandem mass spectrometry sequencing. The detected peptide fragments covered nearly the entire protein sequence with only a few peptide fragments undetected (**Figure 62**). This analysis confirmed the DAS-8191Ø-7 cotton-derived protein amino acid sequence matched that of the microbe-derived PAT protein at the N- and C-terminus as well as a major portion of the internal sequence.

Table 33. Summary of N-terminal Sequences of PAT Derived Proteins

Expected	$M^1 S^2$ P E R R P V E I R P A T A A
DAS-8191Ø-7	S^2 P E R R P V E I R P A T A A
Microbe-derived	S^2 P E R R P V E I R P A T A A

Table 34. Summary of C-Terminal Sequences of PAT Derived Proteins

Expected	P^{173} P R P V R P V T Q I^{183}
DAS-8191Ø-7	P^{173} P R P V R P V T Q I^{183}
Microbe-derived	P^{173} P R P V R P V T Q I^{183}

Based on the described results, one post-translational modification was observed for the DAS-8191Ø-7 cotton-derived PAT protein which included the removal of the N-terminal methionine. The excision of Met at position 1 is a common modification found in plant-expressed proteins (Li & Chang 1995). No other post-translational modifications were observed. Collectively, the mass spectrometry data, glycosylation staining, and SDS-PAGE western blot analyses all provide evidence for the lack of glycosylation of both the DAS-8191Ø-7 cotton-derived PAT and the microbe-derived PAT proteins. The results of these analyses indicate that the amino acid sequence of the DAS-8191Ø-7 cotton-derived PAT protein was equivalent to the *P. fluorescens*-expressed protein previously characterized.

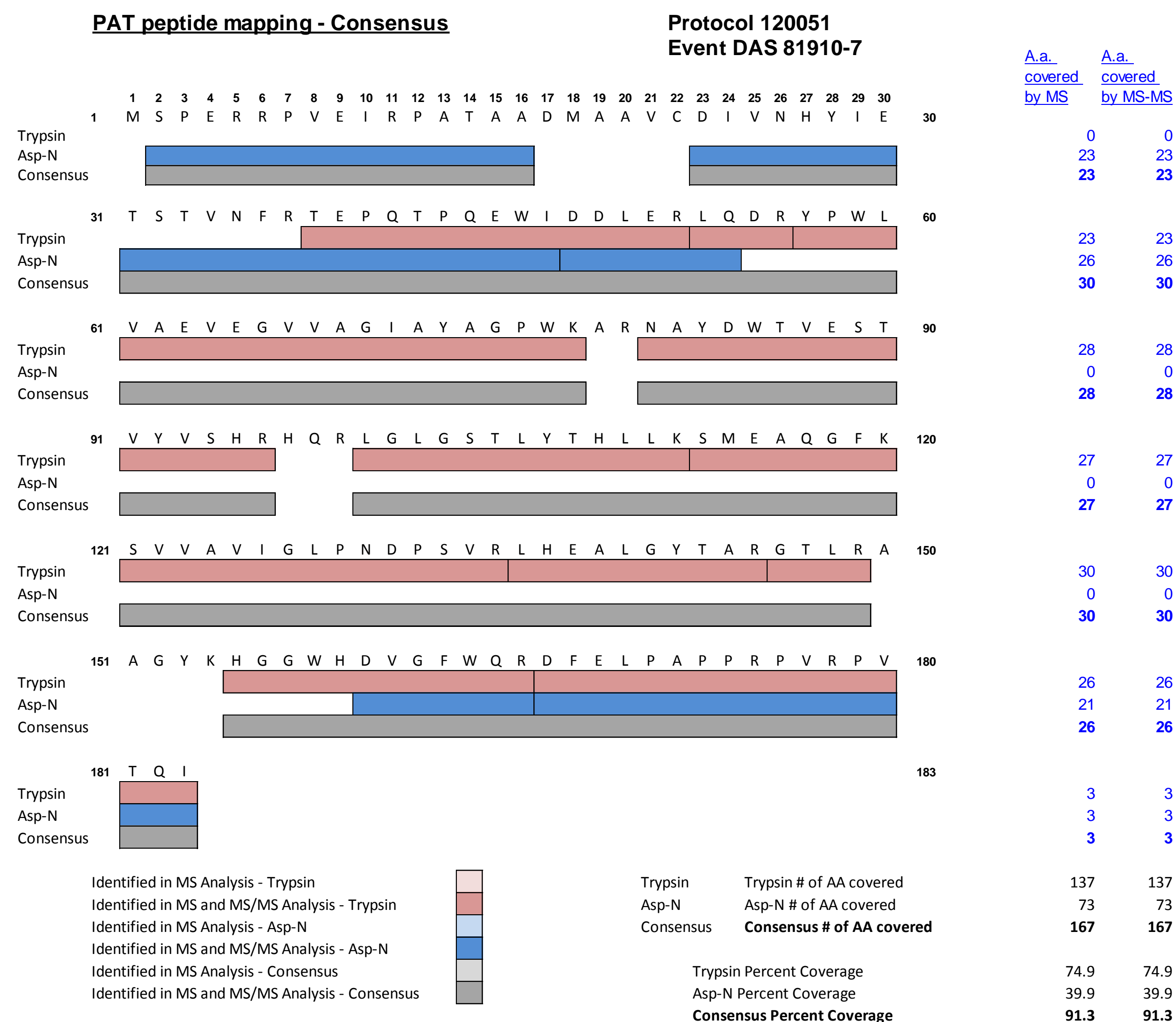


Figure 62. Comprehensive MS and MS/MS Sequence Coverage Map for Trypsin and Asp-N digests of DAS-8191Ø-7 Cotton-Derived PAT

Sequence coverage was 91.3% with PMF data and 91.3% by tandem MS.

Results of the enrichment and activity assay of the PAT protein derived from DAS-8191Ø-7 cotton plant tissue

Microbe and DAS-8191Ø-7 cotton-derived PAT proteins were assayed using an established spectrophotometric assay with minor modifications (De Block et al 1987, Mahan et al 2006). Both the DAS-8191Ø-7 cotton and microbe-derived PAT displayed hyperbolic kinetic plots when evaluated over a range of glufosinate-ammonium concentrations (Figure 9). Michaelis-Menten curve fitting revealed that microbe and DAS-8191Ø-7 cotton-derived PAT have similar V_{max} values of 9.30 ± 0.10 and 7.19 ± 0.06 µM/min, respectively. The K_m value for the microbe-derived PAT (150 ± 3 µM) was comparable to that of the cotton-derived enzyme (117 ± 4 µM). Both enzyme preparations also displayed similar k_{cat} values of

6.20 s^{-1} for microbe-derived and 4.80 s^{-1} for cotton-derived PAT. The calculated catalytic efficiencies (k_{cat}/K_m) were nearly identical at 4.13 x 10^4 and 4.11 x 10^4 $M^{-1} s^{-1}$ for both the microbe- and DAS-8191Ø-7 cotton-derived enzymes, respectively. These results demonstrate that PAT derived from DAS-8191Ø-7 cotton leaf displays similar kinetic parameters to PAT purified from *P. fluorescens* and are therefore functionally equivalent.

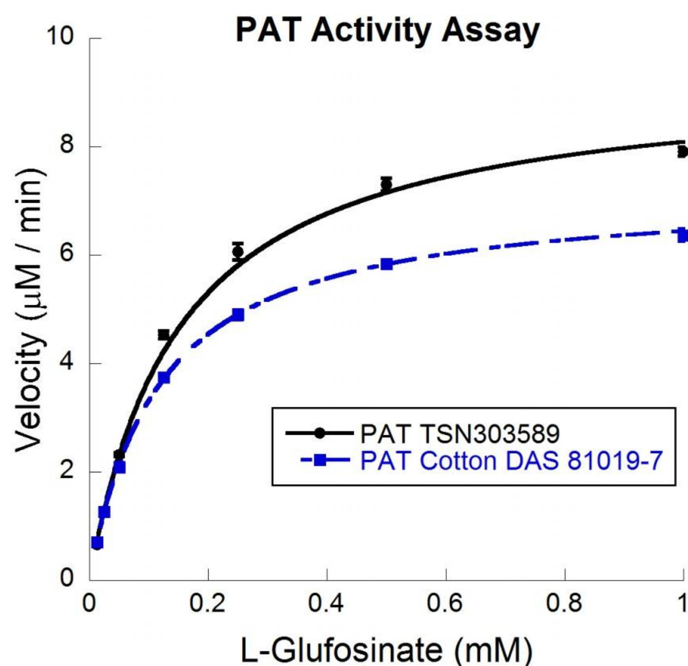


Figure 63. Kinetic Analysis of Microbe- and DAS-8191Ø-7 Cotton-Derived PAT Proteins
The Michaelis-Menten plots of microbe-derived PAT (black circles) and cotton-derived PAT (blue squares) using L-Glufosinate as a substrate for PAT. The average of three independent experiments is shown and error bars indicate the standard deviation of the analyses.

Conclusions

It was demonstrated that the biochemical identity and biological function of the microbe-derived PAT protein was equivalent to the protein purified from leaf tissue of cotton event DAS-8191Ø-7. Both the cotton- and microbe-derived PAT proteins had an apparent molecular weight of ~20 kDa and were immunoreactive to a PAT protein-specific polyclonal antibody in western blot assays. Greater than 90% of the amino acid sequences were confirmed by enzymatic peptide mass fingerprinting by MALDI-TOF MS and verified by MS/MS. The lack of glycosylation of the cotton-derived PAT protein provided additional support that the PAT protein produced by *P. fluorescens* and transgenic cotton were equivalent. Enzymatic activity assays using glufosinate-ammonium as a substrate were performed to

evaluate the kinetic parameters of both enzymes. These results demonstrate that PAT derived from DAS-8191Ø-7 cotton leaf displays similar kinetic parameters to PAT purified from *Pseudomonas fluorescens*. Collectively, these biochemical tests indicate that the plant- and microbe-derived PAT proteins are biochemically and biologically equivalent and therefore the microbe-derived protein is acceptable for use in regulatory studies.

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

Experimental Design

Cotton samples were collected from a field study conducted in the US in 2012 that included six (6) field sites; Alabama, Georgia, Louisiana, Missouri, North Carolina, and Texas. Each site consisted of one plot of each treatment per block, with 4 blocks per location. Plots were arranged in a randomized complete block (RCB) design, with a unique randomization at each site. Plot size was 4 rows by 25 feet with row spacing of approximately 30-40 inches. Each cotton plot was bordered by 2 rows of non-transgenic cotton of similar maturity. Blocks were separated from each other and outside border rows by an alley of at least 10 ft of bare soil or a non-sexually compatible non-crop buffer (e.g., turf grass). At each location, all blocks were used for collection of samples for protein expression analysis. With the exception of seed, expression samples were collected from rows 1 and 4 of each four row plot. Seed expression samples were collected from rows 2 and 3 (the centre two rows) of each four row plot. Herbicide treatments were designed to replicate maximum label rates.

2,4-D plus Glufosinate-Ammonium Treatment:

2,4-D (GF-2654) plus glufosinate-ammonium (Ignite 280 SL) as a tank mixture was applied as two broadcast applications to DAS-8191Ø-7 cotton. Individual applications were at approximately 3 node and 6 node stages. Individual target application rates were 1.0 lb ae/A for GF-2654, or 1120 g ae/ha. Individual target application rates were 0.53 lb ae/A for Ignite 280 SL, or 596 g ai/ha.

Tissue Sampling and Processing

Tissue Sampling

A total of ten tissue samples were collected for AAD-12 and PAT protein expression analysis. Details of each tissue type are as described:

a. *Leaf (4-leaf, first white bloom, and first open boll growth stages)*

One leaf sample per plot (representing 10-14 leaves collected from separate plants) was collected for each test and control entry. Each leaf was collected from a different plant in the plot. Each leaf sample was the youngest set of fully expanded leaves.

b. *Squares (first white bloom growth stage)*

One square sample per plot (representing 10-14 squares) was collected for each test and control entry. Each square was collected from a different plant in the plot consisting of a flower bud and bracts, but not the stem.

c. *Pollen (early bloom growth stage)*

One pollen sample per plot (representing a volume of 0.2-0.5 mL of pollen) was collected from each plot for each test and control entry. Each pollen sample was collected across the plants within each plot from white flowers just starting to open.

d. *Flower (peak bloom growth stage)*
One flower sample per plot (representing 14-18 flowers) was collected for each test and control entry. Each flower consisted of white flowers from a different plant in the plot.

e. *Bolls (peak bloom)*
One boll sample per plot (representing 10-14 bolls) was collected for each test and control entry. Each boll was collected from a different plant in the plot. Boll samples were either open or closed on the first position of fruiting branches.

f. *Root (Maturity)*
One root sample (representing 1-2 plants) per plot was collected for each test and control entry at the maturity stage by cutting a circle around the base of the plant. The root ball was removed and cleaned.

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

h. *Seed (Maturity)*
One individual sample was collected from each plot of each test and control entry. Each sample contained approximately 500 grams of grain.

Tissue Processing
Samples were shipped to Dow AgroSciences laboratories and maintained frozen until use. Samples of cotton tissues were prepared for expression analysis by coarse grinding, lyophilizing and/or fine-grinding with a Geno/Grinder (Certiprep, Metuchen, NJ).

Determination of AAD-12 Protein in Cotton Tissue Samples
ELISA method DAS 120999 was used to determine AAD-12 protein concentration in cotton tissue samples (Theoharis 2013).

AAD-12 Protein Extraction and Analysis
The AAD-12 protein was extracted from cotton tissues except grain and pollen with a phosphate buffered saline solution containing 0.05% (v/v) Tween-20 (PBST), 0.75% ovalbumin (OVA) and 1.0% polyvinylpyrrolidone (PVP). For grain and pollen, the protein was extracted with a phosphate buffered saline solution containing 0.30% (v/v) Tween-20 (PBST), 0.75% OVA and 1.0% PVP.

The plant tissue and grain extracts were centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer if necessary, and analysed using an AAD-12 ELISA kit. Briefly, an aliquot of the diluted sample and a horseradish peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate were incubated in the wells of a microtiter plate coated with an immobilized anti-AAD-12 polyclonal antibody. These antibodies bind with AAD-12 protein in the wells and form a "sandwich" with AAD-12 protein bound between soluble and the immobilized antibodies. The unbound samples and excess conjugate were then removed from the plate by washing with PBST. Subsequent addition of an enzyme substrate generated a coloured product. The reaction was stopped by adding a dilute acid solution.

Since the AAD-12 was bound in the antibody sandwich, the level of colour development, determined by measuring the absorbance of the solution, was related to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower colour development). The absorbance at 450 nm with a background subtraction at 650 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the AAD-12 concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analysed in duplicate wells with the average concentration of the duplicate wells being reported.

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

Tissue	**AAD-12 (ng/mg)**	
	LOD	LOQ
Bolls	0.5	1
Flower		
Leaf		
Pollen		
Root		
Seed		
Squares		
Whole Plant		

Determination of PAT Protein in Cotton Tissue Samples

ELISA method GRM07.26 was used to determine PAT protein concentration in cotton tissue samples.

PAT Protein Extraction and Analysis

The PAT protein was extracted from cotton tissues with a phosphate buffered saline solution with 0.05% Tween-20 (PBST) and 1.0% polyvinylpyrrolidone (PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted with PBST/1.0% PVP if necessary, and analysed using a PAT ELISA kit.

Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT monoclonal antibody and anti-PAT polyclonal antibodies coated in the wells of a 96-well plate in a sandwich ELISA format. At the end of the incubation period, the unbound reagents were removed from the plate by washing. Subsequent addition of an enzyme substrate generated a coloured product. The reaction was stopped by adding a dilute acid solution.

Since PAT was bound in the antibody sandwich, the level of colour development, determined by measuring the absorbance of the solution, was related to the concentration of PAT in the sample (*i.e.*, lower residue concentrations result in lower colour development). The absorbance at 450 nm with a background subtraction at 650 nm was measured using a Molecular Devices Spectra Max M2 plate reader or a Grifols Triturus Automated Immunoassay Analyzer. A calibration curve was generated and the PAT concentration in unknown samples was calculated from the polynomial regression equation using Soft-MAX Pro™ or Triturus Version 4.01B software which was compatible with the plate reader. Samples were analysed in duplicate wells with the average concentration of the duplicate wells being reported.

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

Tissue	**PAT (ng/mg)**	
	LOD	LOQ
Bolls	0.025	0.06
Flower		
Leaf		
Pollen		
Root		
Seed		
Squares		
Whole Plant		

Appendix 5. Methods for Compositional Analysis

Samples of DAS-8191Ø-7 (unsprayed and sprayed with 2,4-D plus glufosinate-ammonium), near isogenic non-transgenic control, and reference variety cottonseed were analysed at Covance Laboratories Inc. for 73 composition analytes. The following methods were used by Covance Laboratories to determine Proximates & Fibre (9), Minerals (12), Amino Acids (18), Fatty Acids (22), Vitamins (7) and Anti-Nutrients (5). See section 3.6 b for composition analysis study design and results.

<u>Proximates and Fibre</u>

- **Protein**

The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulphuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25.

- **Fat -** by Soxhlet Extraction (FSOX)

The samples were weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the samples to remove the fat. The extract was then evaporated, dried, and weighed.

- **Ash**

All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash.

- **Moisture**

The samples were dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture.

- **Carbohydrates**

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

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

- **Acid Detergent Fibre** (ADF)

Sample aliquots were weighed into pre-weighed filter bags. The fats and pigments were then removed by an acetone wash. Due to the high fat content of the samples, this was followed by an additional 12-hour acetone soak with no agitation. The filter bags were placed in an ANKOM2000 Fibre analyzer

where the protein, carbohydrate, and ash content were dissolved by boiling acidic detergent solution. After drying, the bags were reweighed and the acid detergent fibre was determined gravimetrically.

- **Neutral Detergent Fibre** (NDF)

Sample aliquots were weighed into pre-weighed filter bags. The fats and pigments were then removed by an acetone wash. Due to the high fat content of the samples, this was followed by an additional 12-hour acetone soak with no agitation. The filter bags were placed in an ANKOM2000 Fibre analyzer where the protein, carbohydrate, and ash content were dissolved by a boiling detergent solution at a neutral pH. Hemicellulose, cellulose, lignin and insoluble protein fraction were left in the filter bag and determined gravimetrically.

- **Total Dietary Fibre**

Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each of the samples to precipitate the soluble fibre. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fibre in the samples was calculated using protein and ash values and the weighed residue fractions.

- **Crude Fibre**

Crude fibre was quantitated as the loss on ignition of dried residue remaining after digestion of the samples with 1.25% sulphuric acid and 1.25% sodium hydroxide solutions under specific conditions.

Minerals

Mineral Analysis (12 Total): ICP Emission Spectrometry
The samples were dried, precharred, and ashed overnight in a muffle furnace set to maintain 500°C. The ashed samples were re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown samples, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions.

Mineral Reference Standards:

Manufacturer	Mineral	Lot No.	Concentration (µg/ml)
Inorganic Ventures	Calcium	F2-MEB453071MCA, F2-MEB453073	200, 1000
Inorganic Ventures	Copper	F2-MEB453071MCA, F2-MEB453072MCA	2.00, 10.0
Inorganic Ventures	Iron	F2-MEB453071MCA, F2-MEB453074	10.0, 50.0
Inorganic Ventures	Magnesium	F2-MEB453071MCA, F2-MEB453072MCA	50.0, 250
Inorganic Ventures	Manganese	F2-MEB453071MCA, F2-MEB453072MCA	2.00, 10.0
Inorganic Ventures	Phosphorus	F2-MEB453071MCA, F2-MEB453073	200, 1000
Inorganic Ventures	Potassium	F2-MEB453071MCA, F2-MEB453073	200, 1000
Inorganic Ventures	Sodium	F2-MEB453071MCA, F2-MEB453073	200, 1000
Inorganic Ventures	Zinc	F2-MEB453071MCA, F2-MEB453072MCA	10.0, 50.0

Molybdenum & Sulfur: ICP-Mass Spectrometry
The samples were wet-ashed with nitric acid using microwave digestion. Using inductively coupled plasma mass spectrometry, the amount of each element was determined by comparing the counts generated by the unknowns to those generated by standard solutions of known concentrations.

Molybdenum and Sulfur Reference Standards:

Manufacturer	Mineral	Lot No.	Concentration (µg/ml)
Inorganic Ventures	Molybdenum	F2-MEB421115MCA	1.000
Inorganic Ventures	Sulfur	E2-S01119	100

Selenium by Inductively Coupled Plasma-Mass Spectrometry (SEICPMS)
The samples were closed-vessel microwave digested with nitric acid (HNO3) and water. After digestion, the solutions were brought to a final volume with water. To normalize the organic contribution between samples and standards, a dilution was prepared for analysis that contained methanol. The selenium concentration was determined with Se78 using an inductively coupled plasma-mass spectrometer (ICP-MS) with a dynamic reaction cell (DRC) by comparing the counts generated by standard solutions.

Selenium Reference Standard:

Manufacturer	Mineral	Lot No.	Concentration (mg/L)
SPEX CertiPrep.	Selenium	18-29SE	1000

Amino Acid Composition (TAALC/TRPLC)

Samples Analysed (18 total)

Alanine (total)
Arginine (total)
Aspartic acid (including asparagines) (total)
Cystine (including cysteine) (total)
Glutamic acid (including glutamine) (total)
Glycine (total)
Histidine (total)
Isoleucine (total)
Leucine (total)
Lysine (total)
Methionine (total)
Phenylalanine (total)
Proline (total)
Serine (total)
Threonine (total)
Tryptophan (total)
Tyrosine
Valine (total)

The samples were hydrolysed in 6N hydrochloric acid for approximately 24 hours at approximately 106-118ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine were converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan was hydrolysed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours.

The samples were analysed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection.

Amino Acid Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
L-Alanine	Sigma-Aldrich	BCBC5470	99.8
L-Arginine Monohydrochloride	Sigma-Aldrich	1361811	100
L-Aspartic Acid	Sigma-Aldrich	BCBB9274	100.6
L-Cystine	Sigma-Aldrich	1451329	100
L-Glutamic Acid	Sigma-Aldrich	1423805	100.2
Glycine	Sigma-Aldrich	1119375	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	BCBB1348	99.9
L-Isoleucine	Sigma-Aldrich	1423806	100
L-Leucine	Sigma-Aldrich	BCBC6907	99.9
L-Lysine Monohydrochloride	Sigma-Aldrich	1362380	100.2
L-Methionine	Sigma-Aldrich	1423807	99.9
L-Phenylalanine	Sigma-Aldrich	BCBC5774	100
L-Proline	Sigma-Aldrich	1414414	99.7
L-Serine	Sigma-Aldrich	1336081	99.9

L-Threonine	Sigma-Aldrich	1402329	100
L-Tryptophan	Sigma-Aldrich	BCBC1685	>99
		BCBB1284	99.8
L-Tyrosine	Sigma-Aldrich	BCBC2417	100
L-Valine	Sigma-Aldrich	1352709	100

Fatty Acids (FAPM)

The lipid was extracted and saponified with 0.5N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analysed by gas chromatography using external standards for quantitation. Fatty acid results were converted to their triglyceride equivalents.

Fatty Acids Analysed and Reference Standards (22 AA total)

Manufacturer	Lot No.	Component	Weight (%)		Purity (%)
			JY-10	MA7-W	
Nu-Chek Prep GLC Reference Standard Covance 1 Covance 2	JY-10 MA7-W	Methyl Octanoate (8:0 Caprylic)	3.0	1.25	99.7
		Methyl Decanoate (10:0 Capric)	3.25	1.25	99.6
		Methyl Laurate (12:0 Lauric)	3.25	1.25	99.8
		Methyl Myristate (14:0 Myristic)	3.25	1.25	99.8
		Methyl Myristoleate (14:1 Myristoleic)	1.0	1.25	99.5
		Methyl Pentadecanoate (15:0 Pentadecanoic)	1.0	1.25	99.6
		Methyl Pentadecenoate (15:1 Pentadecenoic)	1.0	1.25	99.4
		Methyl Palmitate (16:0 Palmitic)	10.0	15.75	99.8
		Methyl Palmitoleate (16:1 Palmitoleic)	3.0	1.25	99.7
		Methyl Heptadecanoate (17:0 Heptadecanoic))	1.0	1.25	99.6
		Methyl 10-Heptadecenoate (17:1 Heptadecenoic)	1.0	1.25	99.5
		Methyl Stearate (18:0 Stearic)	7.0	14.00	99.8
		Methyl Oleate (18:1 Oleic)	10.0	15.75	99.8
		Methyl Linoleate (18:2 Linoleic)	10.0	15.75	99.8
		Methyl Gamma Linolenate (18:3 γ-Linolenic)	1.0	1.25	99.4
		Methyl Linolenate (18:3 Linolenic)	3.0	1.25	99.5
		Methyl Arachidate (20:0 Arachidic)	2.0	1.25	99.8
		Methyl 11-Eicosenoate (20:1 Eicosenoic)	2.0	1.25	99.6
		Methyl 11-14 Eicosadienoate (20:2 Eicosadienoic)	1.0	1.25	99.5
		Methyl 11-14-17 Eicosatrienoate (20:3 Eicosatrienoic)	1.0	1.25	99.5
		Methyl Arachidonate (20:4 Arachidonic)	1.0	1.25	99.4
		Methyl Behenate (22:0 Behenic)	1.0	1.25	99.8

Vitamins

- **Vitamin A (Beta Carotene)**

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

Vitamin A Reference Standard:

Component	Manufacturer	Lot No.	Purity (%)
Beta-Carotene	Sigma-Aldrich	091M1417V	98.4 (Lambda Maximum 450 to 451 nm in hexane)

- **Vitamin B1 (Thiamine HCl)**

The samples were subjected to acid hydrolysis to denature matrix and free bound thiamine analogs. The treated sample was brought to volume, filtered and injected onto a reversed phase column using a high-performance liquid chromatography system with a post-column derivatization reaction coil and detected via a fluorescence detector. As thiamine monophosphate is not completely reacted, thiamine and thiamine monophosphate are both quantitated separately. Final results are the sum of the two components converted to thiamine hydrochloride form.

Vitamin B1 Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Thiamine monophosphate chloride dihydrate	Sigma-Aldrich	BCBF5554V	99.3
Thiamine hydrochloride	USP	P0K366	99.7

- **Vitamin B2** (Riboflavin)

The samples were hydrolysed with dilute hydrochloric acid and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the samples, using the bacteria *Lactobacillus rhamnosus*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically.

Vitamin B2 Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Riboflavin	USP	N1J079	99.7

- **Vitamin B3 (Niacin)**

The samples were hydrolysed with sulphuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the samples, using the bacteria *Lactobacillus plantarum,* with the growth response of a niacin standard. This response was measured turbidimetrically.

Vitamin B3 Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Niacin	USP	J0J235	99.8

- **Vitamin B6 (Pyridoxine Hydrochloride)**

The samples were hydrolysed with dilute sulphuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the samples, using the yeast *Saccharomyces cerevisiae,* with the growth response of a pyridoxine standard. The response was measured turbidimetrically. Results were reported as pyridoxine hydrochloride.

Vitamin B6 Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Pyridoxine hydrochloride	USP	Q0G409	99.8

- **Vitamin B9 (Folic Acid)**

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

Vitamin B9 Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Folic Acid	USP	Q0G151	98.9

- **α-Tocopherol (Vitamin E)**

The samples were saponified to break down any fat and release vitamin E. The saponified mixtures were extracted with ethyl ether and then quantitated by high- performance liquid chromatography using a silica column. *Note: Alpha tocopherol is part of a mixed standard which also includes beta, delta, and gamma isomers. The reference standard material for those isomers may contain small amounts of alpha tocopherol. All reference standards that contributed to the alpha tocopherol concentration are listed below.*

α-Tocopherol Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Alpha Tocopherol	USP	O0K291	98.5
D-gamma-Tocopherol	Acros Organics	A0083534	99.3
(+)-δ-Tocopherol	Sigma-Aldrich	090M1916V	92.0

Anti-Nutrients

Sterculic Acid, Malvalic Acid, Dihydrosterculic Acid

The total lipid fraction was extracted from the samples using chloroform and methanol. A portion of the lipid fraction was then saponified with a mild alkaline hydrolysis. The free fatty acids were extracted with ethyl ether and hexane. The free fatty acids were then converted to their phenacyl derivatives with 2-bromoacetophenone. The derivatized extracts were injected on a high-performance liquid chromatography system equipped with an ultraviolet detector. The relative percent of total fatty acids for each peak was calculated from peak areas.

Sterculic, Malvalic and Dihydrosterculic Acid Reference Standards:

Manufacturer	Lot No.	Component	Weight (%)	Purity (%)
Nu-Chek Prep	N-22A-A17-Q	Behenic acid	NA	>99
Nu-Chek Prep	N-24A-A28-Q	Lignoceric acid	NA	>99
Matreya LLC	22990	Dihydrosterculic acid	NA	>98
Nu-Chek Prep GLC Reference Standard PSA 1	AU19-V	Methyl Myristate	2.2	99.6
		Methyl Pentadecanoate	0.7	99.5
		Methyl Palmitate	47.0	99.5
		Methyl Palmitoleate	0.5	99.4
		Methyl Heptadecanoate	2.0	99.6
		Methyl Stearate	36.0	99.7
		Methyl Oleate	9.0	99.5
		Methyl Linoleate	1.0	99.4
		Methyl Linolenate	0.5	99.5
		Methyl Nonadecanoate	0.6	99.4
		Methyl Arachidate	0.5	99.6

NA=Not applicable

Gossypol, Free
The samples were extracted with an aqueous acetone solution and filtered. Duplicate aliquots were made and the active aliquot was reacted with aniline with heat applied in a water bath. Active and inactive aliquots were brought to volume with an aqueous isopropyl alcohol solution and read on a spectrophotometer at 440 nm. The absorbance difference was then compared to a linear curve calculated from standards that were aliquoted, reacted, and read in the same fashion as the samples.

Gossypol, Free & Total Reference Standard:

Component	Manufacturer	Lot No.	Purity (%)
Gossypol from cotton seeds	Sigma-Aldrich	041M4117V	99.64

Gossypol, Total (GOSS)
Total gossypol defines gossypol and gossypol derivatives, both free and bound, in cottonseed products that are capable of reacting with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol under the conditions of the method. Gossypol, gossypol analogs, and gossypol derivatives having an available aldehyde moiety were measured by the method (see references above).

Appendix 6. Literature Ranges for Compositional Analysis

Composition Literature Ranges for Non-Transgenic Cottonseed

	Analyte	Units	ILSI 2010		Literature		Literature Citations	
			Min	Max	Min	Max	Min	Max
Proximates & Fibre (9 Total)	Ash	% DW	3.761	5.342	3.7	5.29	(Nida et al 1996)	(Hamilton et al 2004)
	Carbohydrates	% DW	39.0	53.6	41.0	53.62	(Nida et al 1996)	(Hamilton et al 2004)
	Fat	% DW	17.201	27.292	14.4	25.5	(Bertrand et al 2005)	(Nida et al 1996)
	Protein	% DW	21.48	32.97	12	32	(Kohel et al 1985)	(Kohel et al 1985)
	Moisture	% FW	2.3	9.9	2.25	15.9	(Hamilton et al., 2004)	(Berberich et al 1996)
	ADF	% DW	19.74	38.95	21.10	40.5	(Hamilton et al., 2004)	(Bertrand et al., 2005)
	Crude Fibre	% DW	13.86	23.10	13.45	19.31	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	NDF	% DW	25.56	51.87	32.92	53.6	(Hamilton et al., 2004)	(Bertrand et al., 2005)
	Total Dietary Fibre	% DW	33.69	47.55	NR	NR	NR	NR
Minerals (12 Total)	Calcium	mg/100g dry wt.	103.23	325.81	100	330	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Copper	mg/100g dry wt.	0.313	2.457	0.333	1.114	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Iron	mg/100g dry wt.	3.671	31.838	3.927	7.215	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Magnesium	mg/100g dry wt.	347.09	493.12	340	470	(Belyea et al 1989)	(Hamilton et al., 2004)
	Manganese	mg/100g dry wt.	1.069	2.196	1.106	2.216	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Molybdenum	mg/100g dry wt.	NR	NR	NR	NR	NR	NR
	Phosphorus	mg/100g dry wt.	482.54	991.57	560	860	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Potassium	mg/100g dry wt.	983.45	1448.35	960	1240	(Belyea et al 1989)	(Hamilton et al., 2004)

	Selenium	ppb_DW	NR	NR	NR	NR	NR	NR
	Sodium	mg/100g dry wt.	11.183	735.477	5.4	740	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Sulfur	mg/100g dry wt.	NR	NR	NR	NR	NR	NR
	Zinc	mg/100g dry wt.	2.70	5.95	2.89	4.862	(Belyea et al., 1989)	(Hamilton et al., 2004)
Amino Acids (AA) (18 Total)	Alanine	% total amino acid	4.08	4.51	4.15	5.30	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Arginine	% total amino acid	10.85	12.77	10.83	15.18	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Aspartic Acid	% total amino acid	9.00	10.60	9.63	12.37	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Cystine	% total amino acid	1.53	2.35	1.60	2.32	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Glutamic Acid	% total amino acid	20.61	22.90	20.24	21.61	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Glycine	% total amino acid	4.29	4.68	4.44	5.72	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Histidine	% total amino acid	2.91	3.22	3.00	3.88	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Isoleucine	% total amino acid	3.10	3.71	3.10	4.46	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Leucine	% total amino acid	6.03	6.65	6.27	8.11	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Lysine	% total amino acid	4.62	5.46	4.85	6.60	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Methionine	% total amino acid	1.27	2.16	1.46	2.28	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Phenylalanine	% total amino acid	5.44	6.04	5.51	7.23	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Proline	% total amino acid	3.81	4.49	3.93	5.30	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Serine	% total amino acid	4.15	5.31	4.16	5.87	(Hamilton et al., 2004)	(Hamilton et al., 2004)

(Continued) Composition Literature Ranges for Non-Transgenic Cottonseed

	Analyte	Units	ILSI 2010		Literature		Literature Citations	
			Min	Max	Min	Max	Min	Max
AA (Cont.)	Threonine	% total amino acid	2.67	3.59	3.26	4.26	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Tryptophan	% total amino acid	0.91	1.31	0.94	1.40	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Tyrosine	% total amino acid	2.63	2.93	2.65	3.46	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Valine	% total amino acid	4.49	5.31	4.72	6.24	(Hamilton et al., 2004)	(Hamilton et al., 2004)
Fatty Acids (22 Total)	8:0 Caprylic	% total fatty acid	ND	ND	NR	NR	NR	NR
	10:0 Capric	% total fatty acid	ND	ND	NR	NR	NR	NR
	12:0 Lauric	% total fatty acid	ND	ND	NR	NR	NR	NR
	14:0 Myristic	% total fatty acid	0.455	2.400	0.55	2.40	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	14:1 Myristoleic	% total fatty acid	ND	ND	NR	NR	NR	NR
	15:0 Pentadecanoic	% total fatty acid	0.103	0.481	0.050	0.17	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	15:1 Pentadecenoic	% total fatty acid	ND	ND	NR	NR	NR	NR
	16:0 Palmitic	% total fatty acid	15.11	27.90	21.23	28.10	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	16:1 Palmitoleic	% total fatty acid	0.464	1.190	0.57	0.57	(Bertrand et al., 2005)	(Bertrand et al., 2005)
	17:0 Heptadecanoic	% total fatty acid	0.092	0.119	NR	NR	NR	NR
	17:1 Heptadecenoic	% total fatty acid	ND	ND	NR	NR	NR	NR
	18:0 Stearic	% total fatty acid	0.20	3.11	1.99	3.11	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	18:1 Oleic	% total fatty acid	12.8	25.3	12.90	20.10	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	18:2 Linoleic	% total fatty acid	46.0	59.4	46.00	57.10	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	18:3 Linolenic	% total fatty acid	0.11	0.35	0.18	0.18	(Bertrand et al.,	(Bertrand et al.,

							2005)	2005)
	18:3 gamma Linolenic	% total fatty acid	0.097	0.232	NR	NR	NR	NR
	20:0 Arachidic	% total fatty acid	0.186	0.414	0.24	0.34	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	20:1 Eicosenoic	% total fatty acid	0.095	0.098	NR	NR	NR	NR
	20:2 Eicosadienoic	% total fatty acid	ND	ND	NR	NR	NR	NR
	20:3 Eicosatrienoic	% total fatty acid	ND	ND	NR	NR	NR	NR
	20:4 Arachidonic	% total fatty acid	ND	ND	NR	NR	NR	NR
	22:0 Behenic	% total fatty acid	0.104	0.295	0.12	0.24	(Hamilton et al., 2004)	(Hamilton et al., 2004)
Vitamins (7 Total)	Alpha Tocopherol (Vitamin E)	mg/kg DW	70.825	197.243	NR	NR	NR	NR
	Vitamin A (Beta Carotene)	mg/kg DW	NR	NR	NR	NR	NR	NR
	Vitamin B1 (Thiamine HCl)	mg/kg DW	NR	NR	NR	NR	NR	NR
	Vitamin B2 (Riboflavin)	mg/kg DW	NR	NR	NR	NR	NR	NR
	Vitamin B3 (Niacin)	mg/kg DW	NR	NR	NR	NR	NR	NR
	Vitamin B6 (Pyridoxine HCl)	mg/kg DW	NR	NR	NR	NR	NR	NR
	Vitamin B9 (Folic Acid)	mg/kg DW	NR	NR	NR	NR	NR	NR
Anti-Nutrients (5 Total)	Dihydrosterculic Acid	% total fatty acid	0.075	0.310	0.12	0.24	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Malvalic Acid	% total fatty acid	0.229	0.759	0.17	0.61	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Sterculic Acid	% total fatty acid	0.190	0.556	0.13	0.56	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Free Gossypol	mg/kg DW	0.454	1.399	0.53	1.20	(Hamilton et al., 2004)	(Hamilton et al., 2004)
	Total Gossypol	% DW	0.547	1.522	0.55	1.46	(Bertrand et al., 2005)	(Nida et al 1996)

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