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Dow AgroSciences

Petition for Determination of Nonregulated Status for Herbicide Tolerant DAS-8191Ø-7 Cotton

OECD Unique Identifier: DAS-8191Ø-7

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

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Release of Information

Dow AgroSciences LLC (DAS) is submitting the information in this petition for deregulation to USDA-APHIS as part of the regulatory process. By submitting this information, DAS does not authorize its release to any third party. If USDA receives a Freedom of Information Act request pursuant to 5 U.S.C., § 552 and 7 CFR Part 1 regarding this information, DAS expects that USDA will provide DAS with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds (*e.g.*, responsiveness, confidentiality, and/or competitive concerns). DAS understands that a copy of this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Except in accordance with the foregoing, DAS does not authorize the release, publication or other distribution of this information (including website posting) without prior notice and consent from DAS.

Certification

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination and that it includes all relevant data and information known to the petitioner, unfavorable as well as favorable, associated with DAS-8191Ø-7 cotton.

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Summary

Dow AgroSciences LLC (herein referred to as "DAS") is submitting a Petition for Determination of Nonregulated Status for DAS-8191Ø-7 cotton. DAS requests from USDA Animal and Plant Health Inspection Service (APHIS) that cotton transformation event DAS-8191Ø-7 and any cotton lines derived from crosses between DAS-8191Ø-7 cotton and conventional cotton or biotechnology-derived cotton granted nonregulated status by APHIS, no longer be considered regulated articles under 7 CFR Part 340.

DAS has developed transgenic cotton plants containing *aad-12* and *pat,* which confer tolerance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate. DAS-8191Ø-7 cotton will provide growers with greater flexibility in selection of herbicides for the improved control of economically important weeds; allow an increased application window for effective weed control; and provide an effective weed resistance management solution to the growing 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.

In addition to AAD-12, DAS-8191Ø-7 cotton plants have been genetically modified to express the phosphinothricin acetyltransferase (PAT) protein. The PAT enzyme acetylates the primary amino group of the herbicide phosphinothricin, rendering the herbicide inactive. The *pat* gene expressing the PAT protein was derived from *Streptomyces viridochromogenes* and provides tolerance to the herbicide glufosinate in DAS-8191Ø-7 cotton plants. The *pat* gene has been extensively reviewed by regulatory authorities in over eleven countries and has a long history of safe use, being used in over eight crop species representing over twenty-two biotechnology plant events.

The *aad-12* and *pat* expression cassettes introduced into DAS-8191Ø-7 cotton are the same as those introduced into DAS-68416-4 soybean, currently under review by USDA-APHIS (USDA petition number 09-349-01p). In addition, the *aad-12* and *pat* expression cassettes are the same as those introduced into DAS-444Ø6-6 soybean, also currently under USDA-APHIS review (USDA petition number 11-234-01p).

Plant Pest Assessment of DAS-8191Ø-7 Cotton

Data and information presented in this petition to USDA-APHIS confirm the lack of plant pest potential of DAS-8191Ø-7 cotton compared to non-transgenic cotton. The data demonstrate that DAS-8191Ø-7 cotton is agronomically, phenotypically, and compositionally comparable to non-transgenic cotton, with the exception of the presence of the *aad-12* and *pat* genes. DAS-8191Ø-7 cotton is unlikely to pose an increased plant pest risk, weediness potential or an adverse environmental impact compared to non-transgenic cotton. These conclusions are based on the outcome of extensive data and evaluation including:

- ***Cotton is a familiar crop*** that does not possess any of the attributes commonly associated with weeds and has a long history of safe use.

- Detailed ***molecular characterization*** of the inserted DNA demonstrated a single, intact copy of the T-DNA insert within the cotton genome.

- Detailed ***biochemical characterization*** of the expressed AAD-12 and PAT proteins confirmed they are unlikely to be allergens or toxins. In addition, PAT has been commercially available in crops including cotton, corn, rice, canola, soybean and several other crops previously reviewed by USDA.

- ***Compositional assessment*** of cottonseed confirmed the DAS-8191Ø-7 cotton is compositionally equivalent to non-transgenic cotton.

- ***Phenotypic, agronomic and ecological characterization*** assessments of DAS-8191Ø-7 cotton demonstrated no increased plant pest or weediness potential compared to non-transgenic cotton.

- Assessment on the potential impact on ***non-target and endangered species*** indicated that DAS-8191Ø-7 cotton is unlikely to have adverse effects on these organisms compared to non-transgenic cotton.

- ***Current agronomic management practices*** for cotton conclude that DAS-8191Ø-7 cotton is not likely to impact cotton agronomic practices or land use, with the exception of 2,4-D application on DAS-8191Ø-7 cotton varieties.

Cotton is a Familiar Crop

U.S. cotton is grown across 17 states in the southern region of the country. Cotton is the world's most widely grown fiber crop, accounting for over 35% of the total world fiber use. The United States is the top exporter of raw cotton, and third in world production behind China and India. In 2012, 9.4 million acres of cotton were harvested in the US; with production value estimated at just under $6 billion.

Cotton is a slow-growing plant that competes poorly with weeds. The commercial cotton species grown in the U.S. do not exhibit weedy characteristics, do not outcross to weedy relatives and are not invasive. Cotton is not listed as a weed and is not listed on the list of noxious weeds by USDA (7 CFR Part 360). Volunteers that may appear in subsequent rotational crops are easily controlled through traditional tillage practices or the use of appropriate herbicides.

Although feral populations of cultivated variants of cotton exist in the U.S., these populations do not occur in cotton growing areas. In addition, DAS-8191Ø-7 cotton would not be expected to confer a selective advantage or result in a plant pest or weediness potential if crossing with feral populations were to occur. Such unlikely outcomes could be controlled by current agronomic practices.

Molecular Characterization 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 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 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 both non-sprayed and sprayed with 2,4-D plus glufosinate. 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 DAS-8191Ø-7 cotton is unlikely to cause allergenic or toxic effects in humans or animals.

Compositional Assessment of DAS-8191Ø-7 Cotton
A compositional 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 analyzed 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.

Phenotypic, Agronomic and Ecological Characterization of DAS-8191Ø-7 Cotton
Evaluations of nine phenotypic, agronomic and ecological characteristics (early population, seedling vigor, flower initiation, nodes above white flower, plant height, percent open bolls, lint yield, disease incidence and insect damage) were conducted to investigate the equivalency of DAS-8191Ø-7 (non-sprayed and sprayed with 2,4-D plus glufosinate) cotton to non-transgenic cotton. Phenotypic, agronomic and ecological analysis of DAS-8191Ø-7

cotton revealed no statistically significant differences between non-sprayed DAS-8191Ø-7 cotton and the isoline (control). It was also determined that the phenotypic, agronomic and ecological characteristics of DAS-8191Ø-7 cotton sprayed with 2,4-D plus glufosinate are equivalent to non-sprayed DAS-8191Ø-7 cotton. In addition, germination and dormancy studies conducted under warm and cool conditions did not differ significantly from the non-transgenic cotton, near isogenic comparator.

The data support the conclusion that DAS-8191Ø-7 cotton is substantially equivalent to non-transgenic cotton with the exception of the introduced and expression of *aad-12* and *pat* genes and therefore no more likely to pose a plant pest risk or have a significant environmental impact compared to non-transgenic cotton.

Assessment on the Potential Impact on Non-Target and Endangered Species
A review of potential environmental impacts indicates that DAS-8191Ø-7 cotton is unlikely to have adverse effects on non-target organisms including threatened or endangered species under normal agricultural practices. This conclusion is based on several lines of evidence.

- The *aad-12* gene and expressed protein are present in nature in the soil bacterium *Delftia acidovorans*. The *pat* gene and the expressed protein are present in other crops grown in the United States with no effects on non-target organisms or endangered species.
- AAD-12 and PAT are not potential food allergens or toxins.
- DAS-8191Ø-7 cotton has been shown to be substantially equivalent to non-transgenic cotton based on the compositional analysis of cottonseed.
- Observations made during field testing of DAS-8191Ø-7 cotton revealed no effects on invertebrate populations.
- Agronomic characteristics were found to be equivalent to non-transgenic cotton.

Impact on Current Agronomic Management Practices
Because DAS-8191Ø-7 cotton is agronomically and compositionally similar to conventional cotton, no significant impact is expected on current crop production practices, non-target or endangered species, crop rotation, volunteer management, or commodity food and feed cotton products. The availability of DAS-8191Ø-7 cotton is expected to have a beneficial impact on weed control practices by providing growers with an advanced tool to address their weed control needs. The availability of DAS-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.

Conclusion
Information collected during field trials and laboratory analyses presented herein conclude that DAS-8191Ø-7 cotton is not likely to be a plant pest or result in weediness potential. DAS therefore requests a determination from USDA-APHIS that herbicide tolerant DAS-8191Ø-7 cotton and all progeny derived from crosses between DAS-8191Ø-7 cotton and non-transgenic cotton or biotechnology derived cotton be granted nonregulated status under 7 CFR Part 340.

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List of Acronyms and Scientific Terms

Symbol or Abbreviation	Definition
°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
ACCase	Acetyl CoA carboxylase
ADF	Acid detergent fiber
Adj. P	False Discovery Rate Adjusted P-value
ae	Acid equivalent
ae/ha	Acid equivalent per hectare
ai	Active ingredient
ai/ha	Active ingredient per hectare
AL	Alabama
ALS	Acetolactate synthase
ANOVA	Analysis of variance
AOSA	Association of Official Seed Analysis
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, U.S. FDA
cm	centimeter
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)
ESA	Endangered Species Act
ESI-LC/MS	Electrospray ionization-liquid chromatography mass spectrometry

Symbol or Abbreviation	Definition
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
ft	feet
FWS	U.S Fish & Wildlife Service
g	gram
GA	Georgia
GS	Glutamine synthetase
ha	Hectare
IAA	Indole acetic acid
ILSI	International Life Sciences Institute
Kb	Kilobase pair
kDa	Kilodalton
kPa	Kilopascals
L	Liter
LA	Louisiana
LFS	Lateral Flow Strip
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	meter
M	Molar
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MARC	Metabolism Assessment Review Committee - U.S. EPA
MCPA	((4-chlro-2-methyphenoxy) acetic acid)
MO	Missouri
MOA	Mode of Action
MS	Mississippi
µg	microgram
µL	microliter
µM	micromolar
mg	Milligrams
min	minute
mL	Milliliter
mM	millimolar
MOE	Margin of exposure
MW	Molecular Weight

Symbol or Abbreviation	Definition
NA	Not Applicable
NC	North Caroline
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
PAT	Phosphinothricin *N*-acetyltransferase protein
PBN	US FDA Pre-market Biotechnology Notice
PCR	Polymerase chain reaction
pDAB4468	DNA vector carrying the *aad-12* and *pat* expression cassettes
ppb	Parts Per Billion
ppm	Parts Per Million
PPO	Protoporphyrinogen oxidase
PPT	Phosphinothricin
PSI	Pounds per Square Inch
RB7 MAR	Matrix attachment region (MAR) from *Nicotiana tabacum*
RCB	Randomized complete block
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard Error
sec	second
SGF	Simulated gastric fluid
SpecR	Spectinomycin Resistance
STI	Short Term Intake
T-DNA	Transfer DNA
TX	Texas
USDA	United States Department of Agriculture
V	volt
w/v	weight / volume
w/w	weight / weight
WHO	World Health Organization

1. Rationale for the Development of DAS-8191Ø-7 Cotton

1.1. Basis for the Request for Nonregulated Status

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility under the Plant Protection Act (7 U.S.C. 7701-7772) to prevent the introduction or dissemination of plant pests into or within the United States. The APHIS regulations at 7 CFR 340.6 provide that an applicant may petition APHIS to evaluate submitted data on the genetically engineered crop to determine that a regulated article does not present a plant pest risk and therefore should no longer be regulated.

Dow AgroSciences LLC (herein referred to as "DAS") is submitting data for herbicide tolerant DAS-8191Ø-7 cotton and requests a determination from APHIS that DAS-8191Ø-7 cotton no longer be considered regulated articles under 7 CFR 340.

1.2. Benefits of DAS-8191Ø-7 Cotton

DAS has developed transgenic cotton plants containing *aad-12* and *pat*, which confers tolerance to the herbicides 2,4-D and glufosinate. The unique identifier for these plants, in accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2004), is DAS-8191Ø-7.

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-D to herbicidally-inactive 2,4-dichlorophenol (DCP). The *pat* gene encodes the enzyme phosphinothricin acetyltransferase that inactivates glufosinate.

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 U.S. include both grass and broadleaf species—*Lolium rigidum* (Rigid ryegrass), *Lolium multiflorum* (Italian ryegrass), *Sorghum halepense* (Johnsongrass), *Amaranthus palmeri* (Palmer amaranth), *Amaranthus rudis* (Common waterhemp), *Ambrosia artemisiifolia* (Common ragweed), *Ambrosia trifida* (Giant ragweed), *Conyza canadensis* (Horseweed), and *Conyza bonariensis* (Hairy fleabane). Additionally, weeds that had previously not been an agronomic problem prior to the wide use of glyphosate tolerant crops are now becoming more prevalent and difficult to control in the context of glyphosate tolerant crops. According to a 2012 survey across 31 states in the U.S., 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 the South, 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 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 commercialized in combination with other herbicide tolerant (*e.g.* glyphosate) and insect resistance cotton events. 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 modes 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.

1.3. Submission to Other Regulatory Agencies

DAS-8191Ø-7 cotton falls within the scope of the FDA policy statement, published in the Federal Register on May 29, 1992, concerning regulation of products derived from new plant varieties, including those developed via biotechnology. DAS submitted a pre-market biotechnology notification (PBN) to FDA in June 2013, BNF 000142 with anticipated review completed in late 2014.

The regulation and use of herbicides on DAS-8191Ø-7 cotton is governed by the U.S. EPA under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), which prohibits the sale and distribution of any pesticide that is not registered by EPA. In order to register a pesticide or a new use of a previously registered pesticide EPA must find that it will not pose an unreasonable risk to humans or the environment. Once registered, it is a violation of FIFRA to use a pesticide in a manner inconsistent with its EPA-approved label. The use of herbicides on DAS-8191Ø-7 cotton will be consistent with either currently authorized herbicide uses on cotton or uses that are currently in review by U.S. EPA.

Pesticides produced *in planta*, known as plant-incorporated protectants (PIPs), are also subject to regulation by the U.S. EPA under FIFRA (*e.g.* Cry proteins). Since AAD-12 and PAT are neither a herbicide or PIP, AAD-12 and PAT fall outside the scope of EPA regulations.

DAS intends to submit dossiers beginning in 2013 to the regulatory authorities of trade partners for import clearance and production approval which may include Canada, Japan, Korea, European Union, Australia / New Zealand, South Africa, Brazil and Mexico.

2. The Biology of Cotton and DAS-8191Ø-7 Recipient Cotton Cultivar

2.1. Overview of Cotton Biology

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)*
- Centers 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)*

2.2. Characterization 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 (Smith *et al.*, 1999; Bowman *et al.*, 2006).

3. Method of Development of DAS-8191Ø-7 Cotton

DAS-8191Ø-7 cotton was generated by *Agrobacterium* mediated transformation of cotton tissues from Coker 310 variety using plasmid pDAB4468 (Figure 1). This section describes the plasmid vector (pDAB4468), the transformation method, the donor genes (*aad-12* and *pat*), and the regulatory elements used in the development of DAS-8191Ø-7 cotton. The transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. The T-DNA insert in pDAB4468 (Figure 2) contains two synthetic genes, *aad-12* from *Delftia acidovorans* and *pat* from *Streptomyces viridochromogenes.*

3.1. pDAB4468

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 1 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 2). 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 and RB7-MAR in pDAB4468 are described below in sections 3.4.1, 3.4.2, 3.4.3 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.

3.2. Description of the Transformation System

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 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 (1.5% w/v) to screen for putative transformants. Those glufosinate tolerant plants were sampled and analyzed 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 4). The major steps described in the development of DAS-8191Ø-7 cotton are described in Figure 3 with the breeding diagram for DAS-8191Ø-7 cotton in Figure 4.

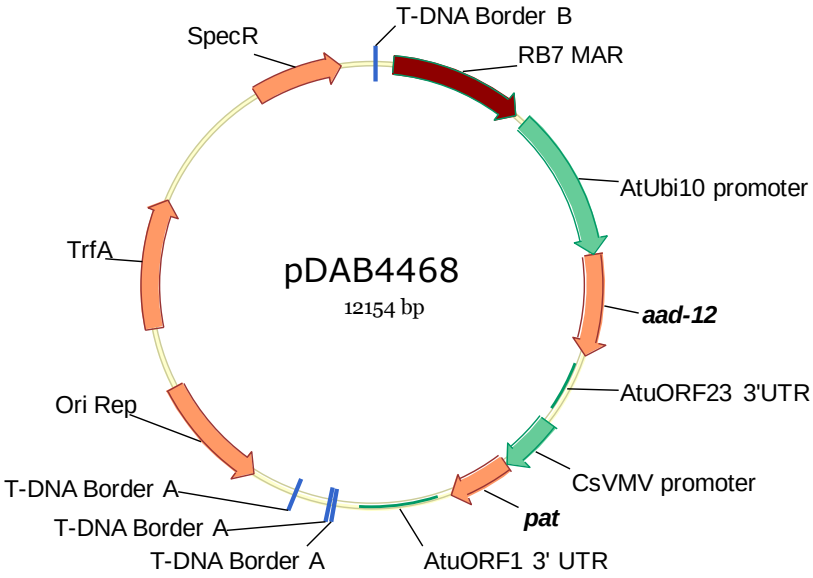


Figure 1. Plasmid map of pDAB4468

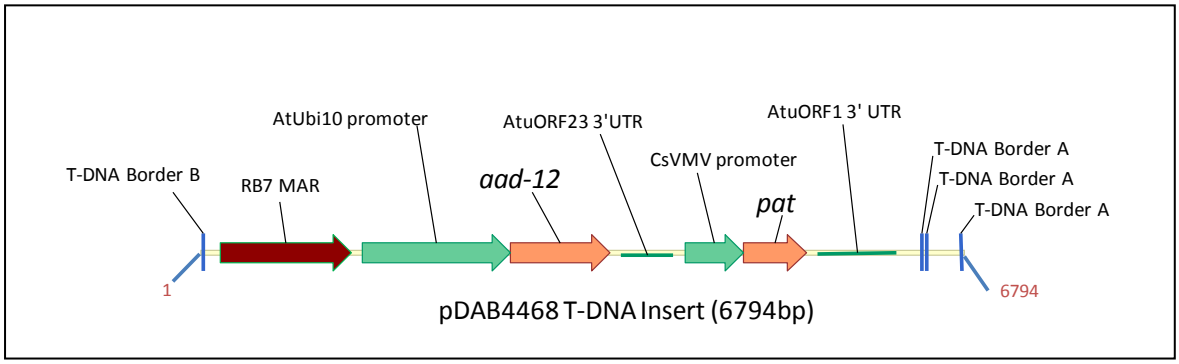


Figure 2. Diagram of T-DNA Region in Plasmid pDAB4468

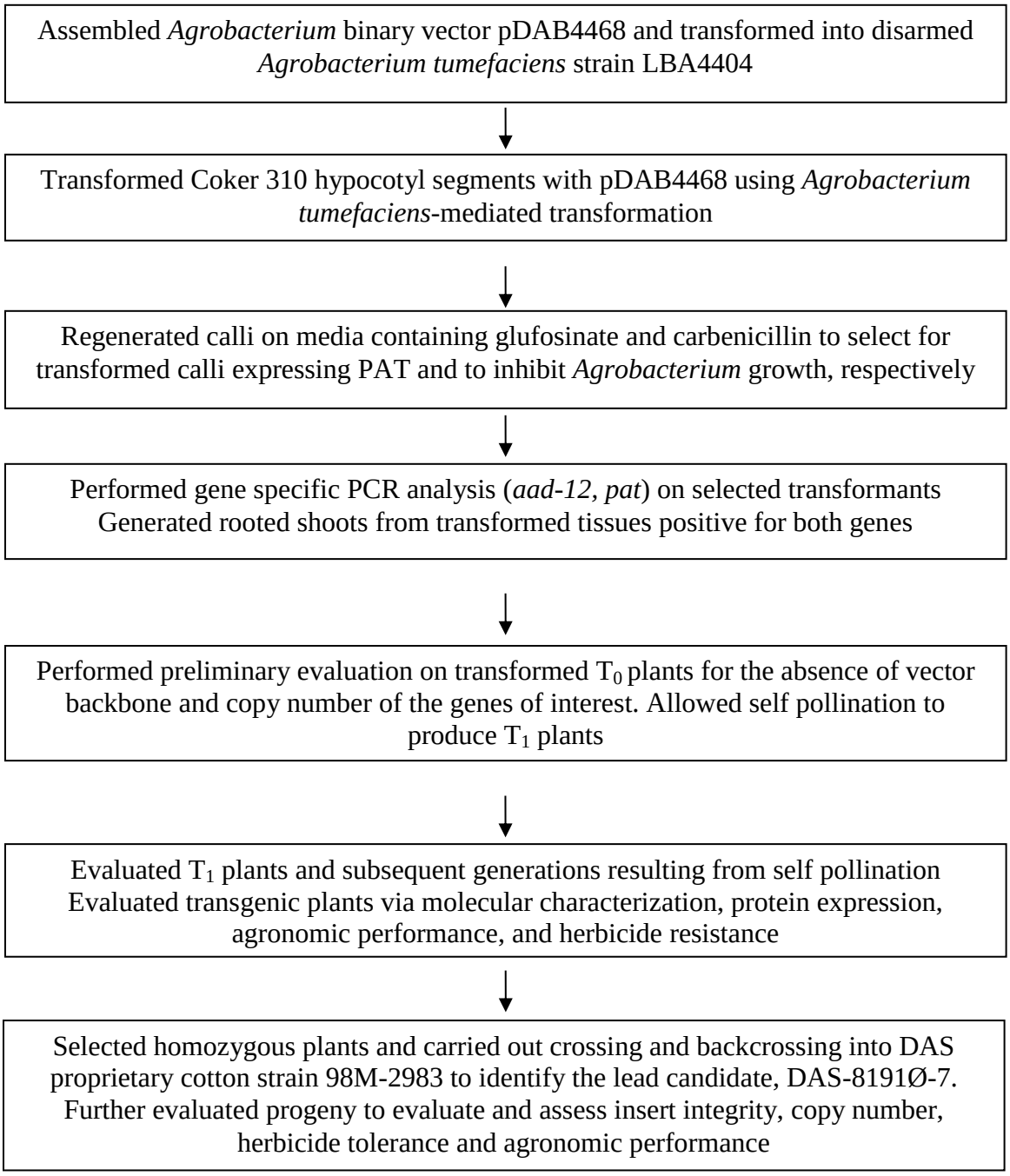
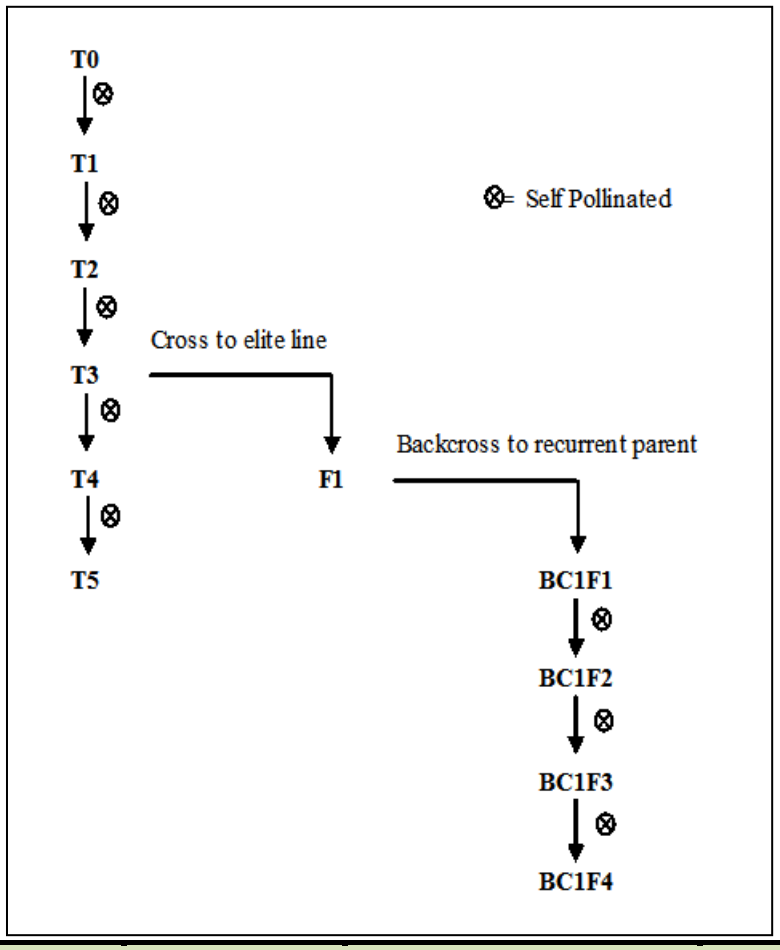


Figure 3. Schematic of the Development of DAS-8191Ø-7 Cotton



Analysis	Petition Section(s)	DAS-8191Ø-7 Cotton Generations	Control
Molecular Analysis	4, 4.2	T_2, T_3, T_4, T_5, BC_1F_2	Coker 310
Segregation Analysis (single generation)	4.4.1	BC_1F_2	None
Segregation Analysis (breeding generations)	4.4.2	T_1, BC_1F_2	None
Protein Characterization	5.1.3, 5.2.3	T_3	Coker 310
Protein Expression	5.1.4, 5.2.4	BC_1F_3	98M-2983
Composition	6.1	BC_1F_3	98M-2983
Agronomics	7.1	BC_1F_3	98M-2983
Germination & Dormancy	7.2.3	BC_1F_4	98M-2983 X Coker 310

Figure 4. Breeding Diagram of DAS-8191Ø-7 Cotton

3.3. 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 as a comparator (see Figure 4).

3.4. Donor Genes and Regulatory Sequences in DAS-8191Ø-7 Cotton

The transgenic insert in DAS-8191Ø-7 cotton contains two expression cassettes, *aad-12* and *pat* in addition to a matrix attachment region (MAR) of RB7 from *Nicotiana tobacum* (Figure 2). This section describes the details of each expression cassette including the source organism, history of safe use, the expressed traits and the genetic organization of each expression cassette. In addition, details of the RB7-MAR sequence are described.

3.4.1. *aad-12* expression cassette

The *aad-12* expression cassette consists of the AtUbi10 promoter, *aad-12* gene and AtuORF23 3′ UTR terminator (Figure 2, Table 1) and is identical to the *aad-12* expression cassette in DAS-68416-4 soybean and DAS-444Ø6-6 soybean (currently under USDA-APHIS review, petition numbers 09-349-01p, 11-234-01p 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-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.

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 (Von Graevenitz, 1985; Tamaoka *et al.*, 1987; Wen *et al.*, 1999). Strains of *D. acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Toms and Wood, 1970; Labuda *et al.*, 1992; Rao and Ravishankar, 2000; Shetty *et al.*, 2006).

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).

3.4.2. *pat* expression cassette

The *pat* expression cassette consists of the CsVMV promoter, *pat* gene and AtuORF1 3′ UTR terminator (Figure 2) and is identical to the *pat* expression cassette in DAS-68416-4 soybean and DAS-444Ø6-6 soybean (currently under USDA-APHIS review, petition numbers 09-349-01p, 11-234-01p respectively). 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.

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	Selectable marker. Synthetic, plant-optimized version of phosphinothricin *N*-acetyl transferase 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 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)

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 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
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

The presence of PAT protein in plants confers tolerance to glufosinate. 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 previously deregulated products (USDA, 1996; FDA, 1998, 2001; USDA, 2001; FDA, 2003, 2004a, 2004b, 2004c; USDA, 2004, 2005; FDA, 2011).

Expression of the *pat* gene is controlled by the cassava vein mosaic virus 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).

3.4.3. 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 (Han *et al.*, 1997; Abranches *et al.*, 2005; Verma *et al.*, 2005). It is hypothesized that MARs may act as a buffer to protect transgenes from neighboring chromosomal sequences that could destabilize their expression (Allen *et al.*, 1993; Allen *et al.*, 2000).

4. Genetic Characterization of DAS-8191Ø-7 Cotton

4.1. Overview of Genetic Characterization

Characterization of DAS-8191Ø-7 cotton was conducted by Southern blot 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.

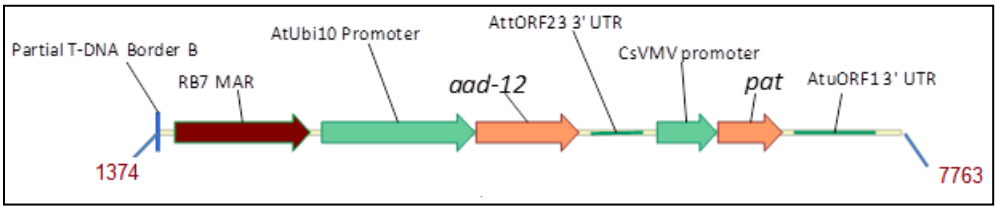


Figure 5. Diagram of Sequenced Transgene Insert in DAS-8191Ø-7 Cotton

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 characterize 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.*, 2010)
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 (Zambryski *et al.*, 1982; Wood *et al.*, 2001)
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 4). 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

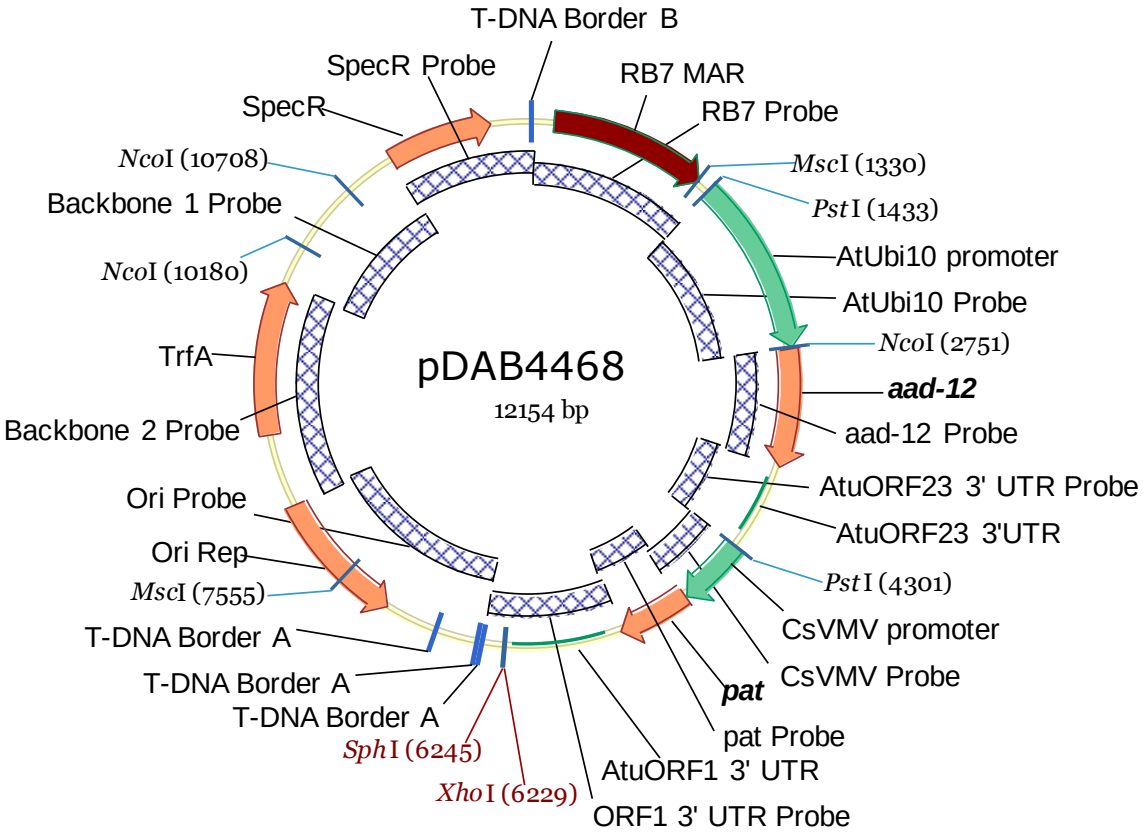


Figure 6. Probe Locations on pDAB4468 used in Southern Blot Analysis
The eleven probes described in Table 3 are shown as hashed boxes in the inner circle of the pDAB4468 plasmid map (outer circle).

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	

Probe	Restriction enzyme	Sample	Lane	Expected fragment sizes (bp)[1]	Observed fragment sizes (bp)[2]	Figure
	*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	Figure22
		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	

Probe	Restriction enzyme	Sample	Lane	Expected fragment sizes (bp)[1]	Observed fragment sizes (bp)[2]	Figure
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-labeled DNA Molecular Weight Marker fragments. Due to the incorporation of DIG molecules for visualization, the Marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

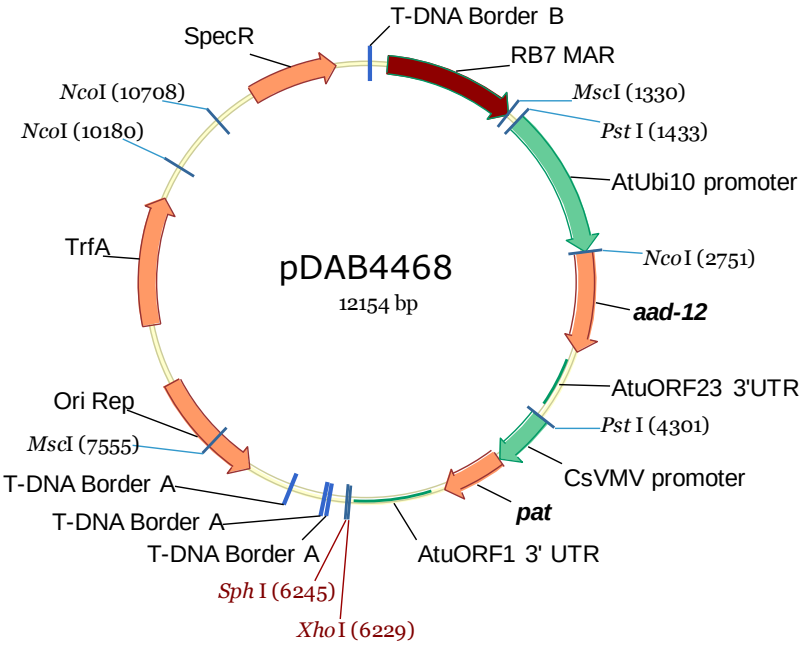
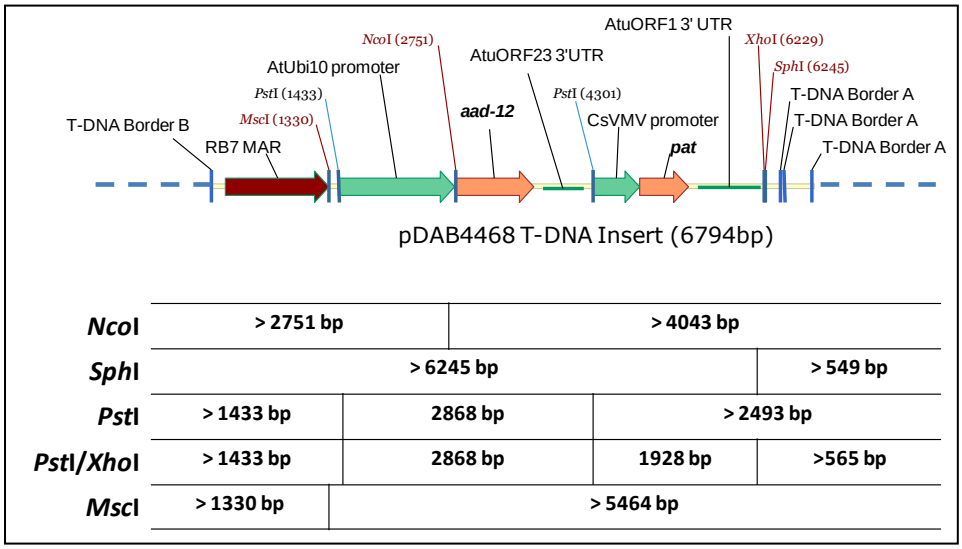


Figure 7. pDAB4468 Showing Location of Restriction Enzymes used for Southern Analysis



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.

4.1.1. Analysis of the Insert and Its Genetic Elements

4.1.1.1. 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 4). 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 8, 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 (Appendix 1). 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).

4.1.1.2. 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 4). 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, Figure22, 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, 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.

4.1.2. 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.

4.2. 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 4) 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.

4.3. Southern Blot Analysis Conclusions

Southern blot analysis confirms that DAS-8191Ø-7 cotton contains a single copy of the transgene insert from pDAB4468, gene expression cassettes *aad-12* and *pat*, and a RB7-MAR element (Section 4.1.1). No plasmid backbone sequences were detected in DAS-8191Ø-7 cotton (Section 4.1.2). 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 4.2).

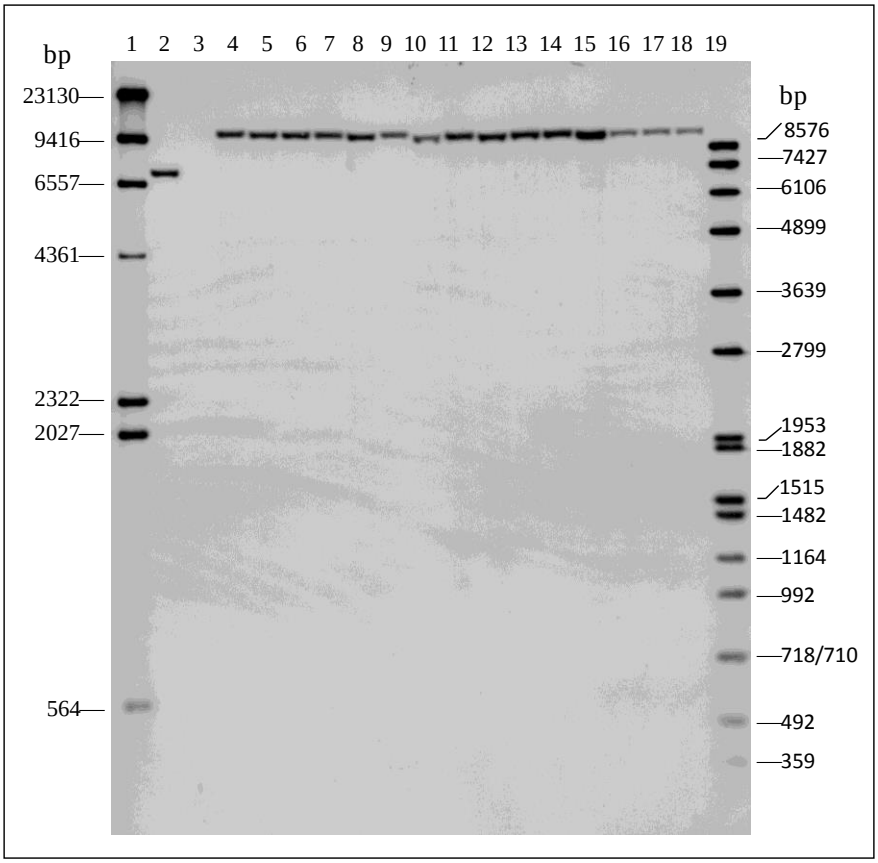


Figure 9. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Nco*I; *aad-12* Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

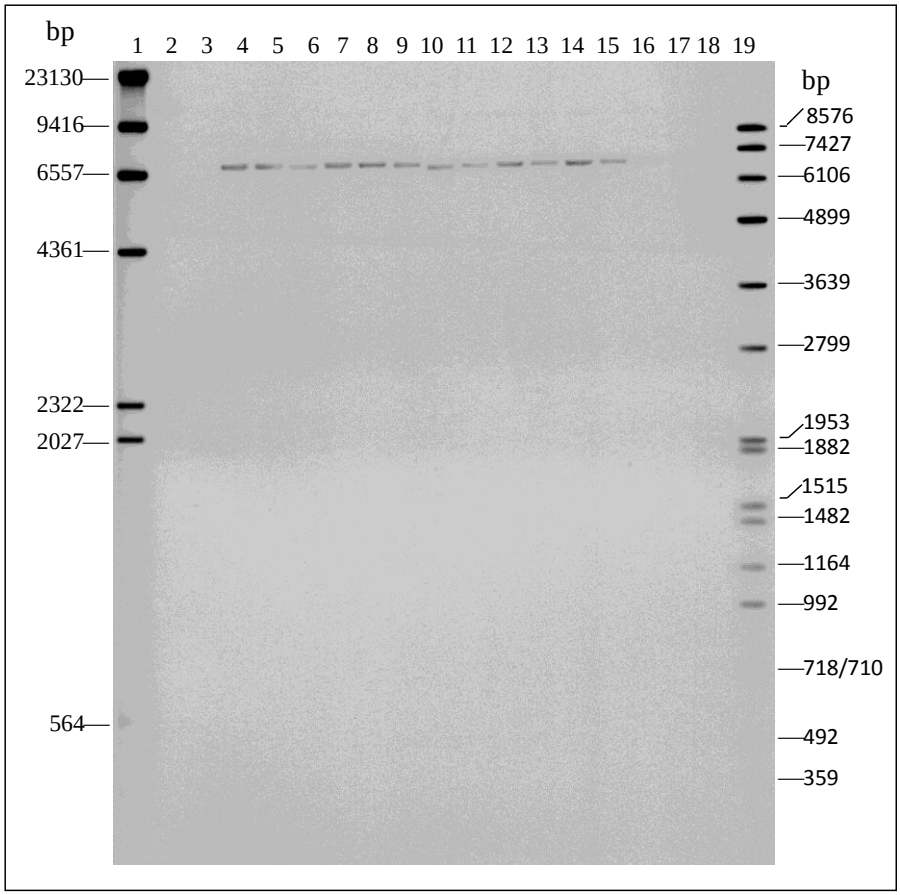


Figure 10. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; *aad-12* Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

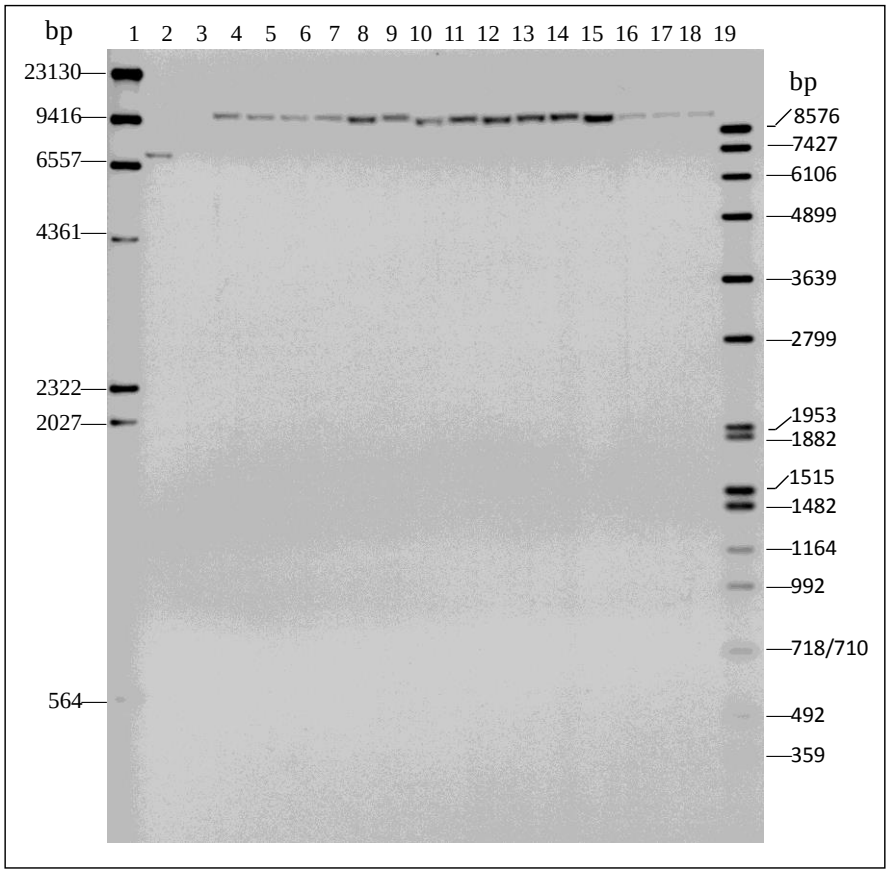


Figure 11. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Nco*I; *pat* Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

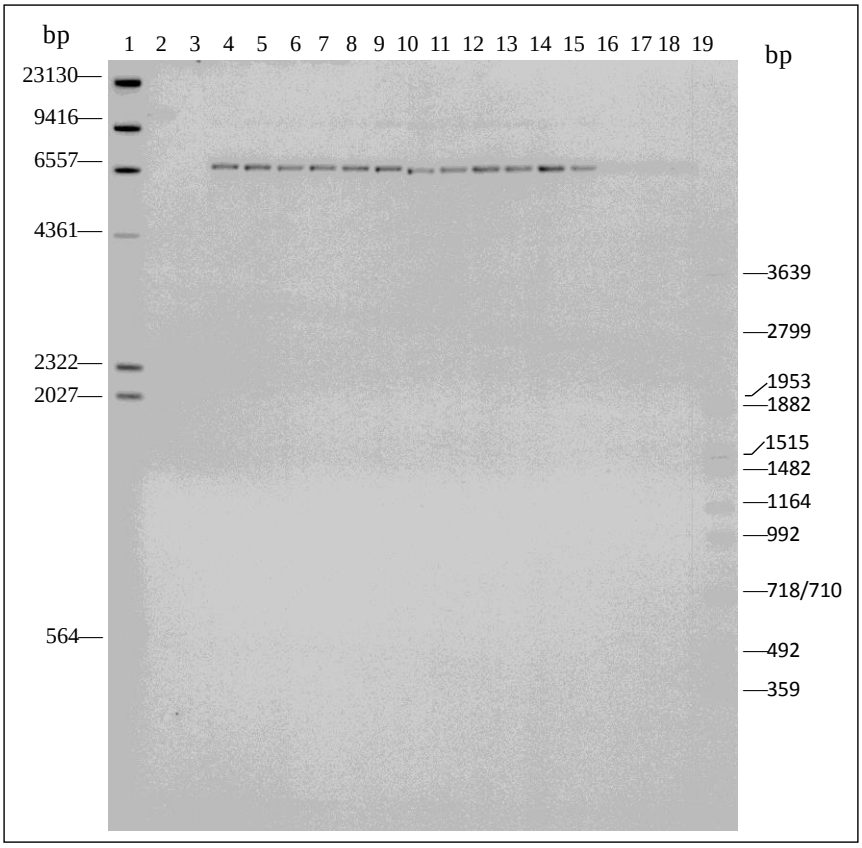


Figure 12. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; *pat* Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

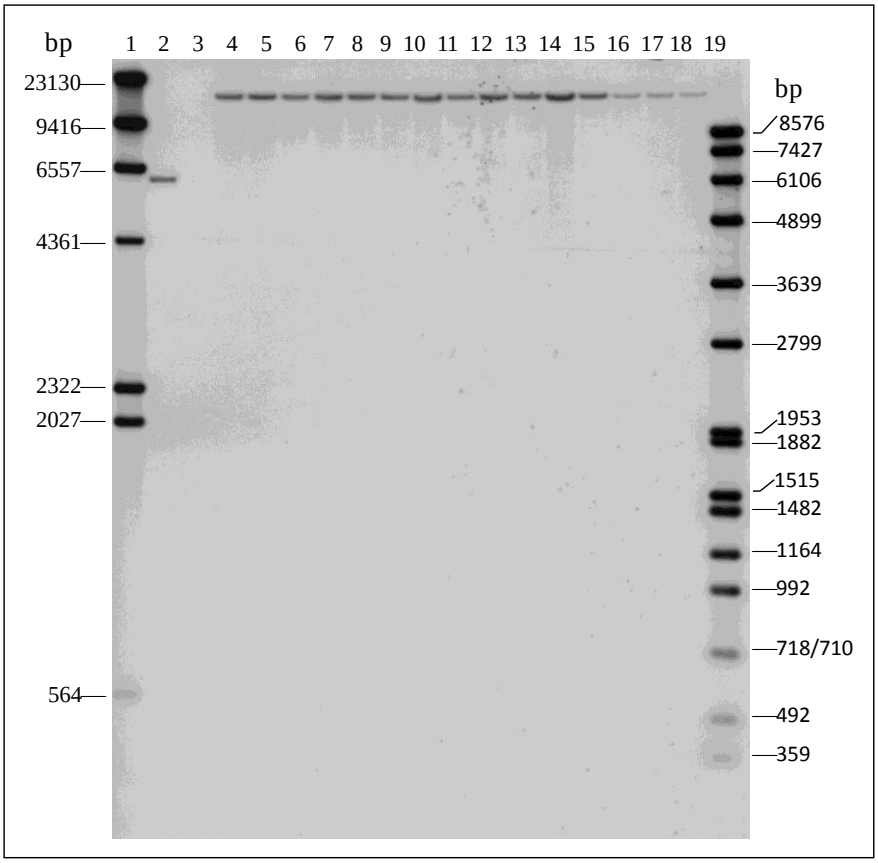


Figure 13. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Msc*I; AtUbi10 Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

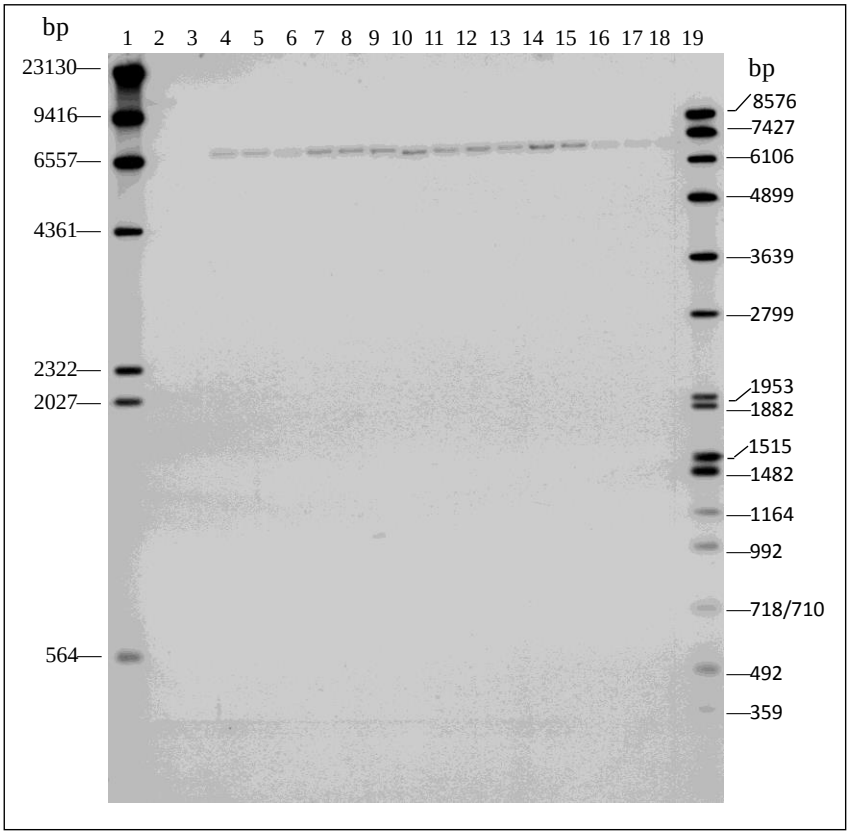


Figure 14. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; AtUbi10 Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

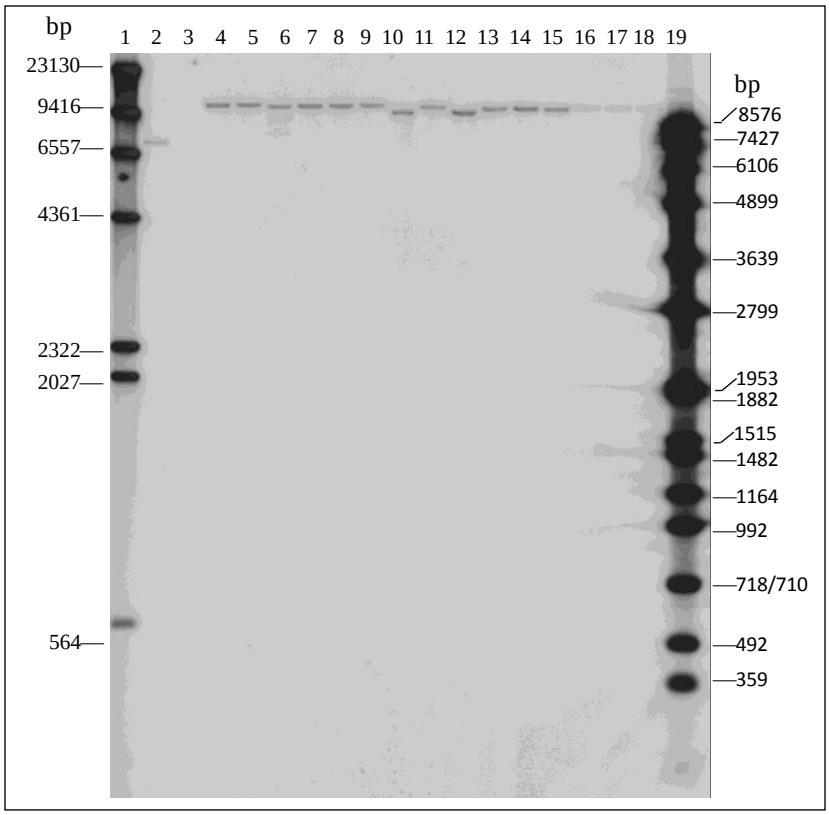


Figure 15. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Nco*I; AtuORF23 3′ UTR Probe

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-labeled 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		

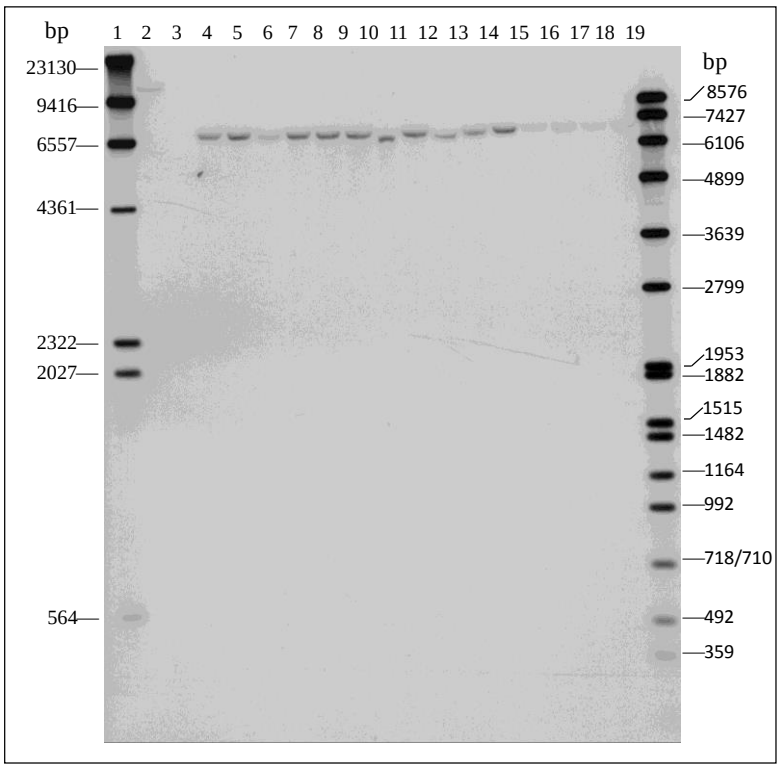


Figure 16. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; AtuORF23 3′ UTR Probe

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-labeled 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-T4-03
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-BC1F2-04
7	DAS-8191Ø-7-T3-02	17	DAS-8191Ø-7- BC1F2-05
8	DAS-8191Ø-7-T3-03	18	DAS-8191Ø-7- BC1F2-06
9	DAS-8191Ø-7-T3-04	19	DIG Molecular Weight Marker VII
10	DAS-8191Ø-7-T4-01		

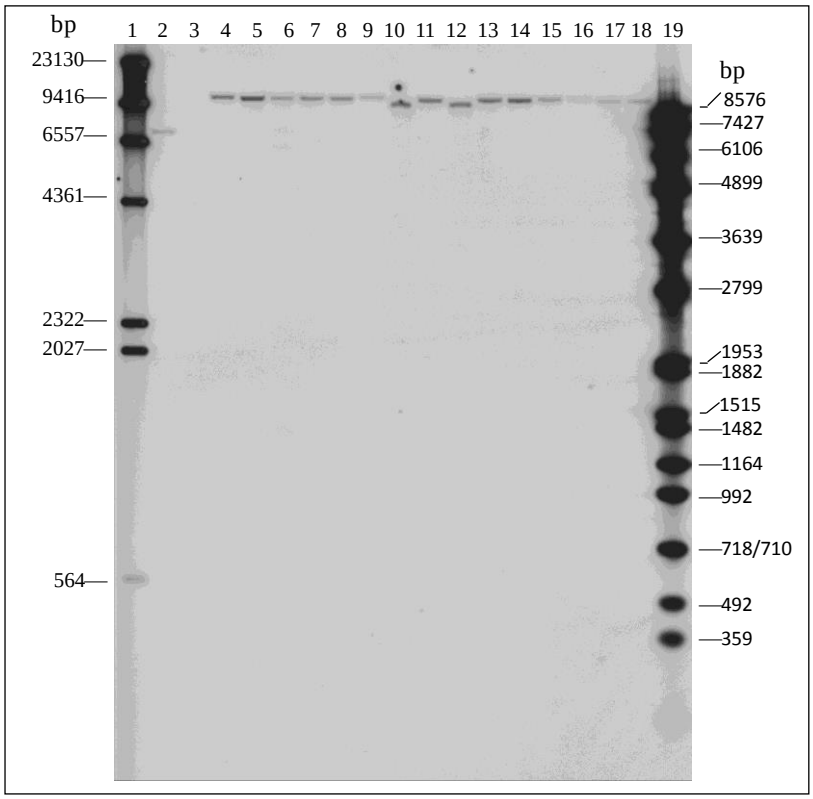


Figure 17. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Nco*I; CsVMV Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

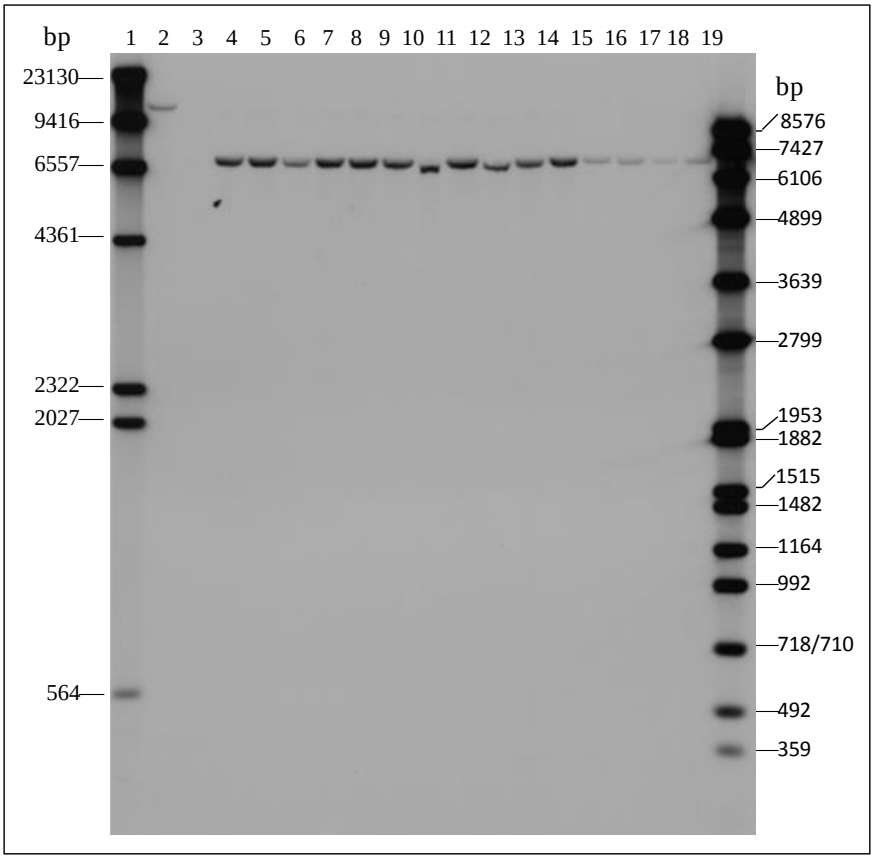


Figure 18. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; CsVMV Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

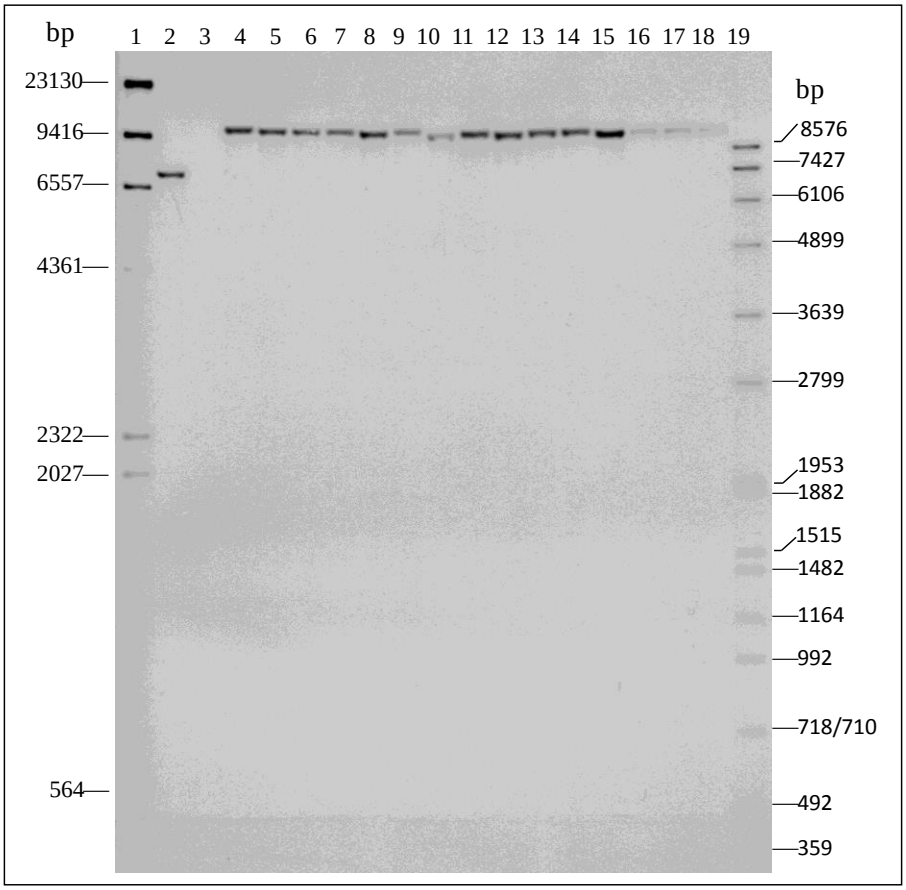


Figure 19. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Nco*I; AtuORF1 3′ UTR Probe

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-labeled 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		

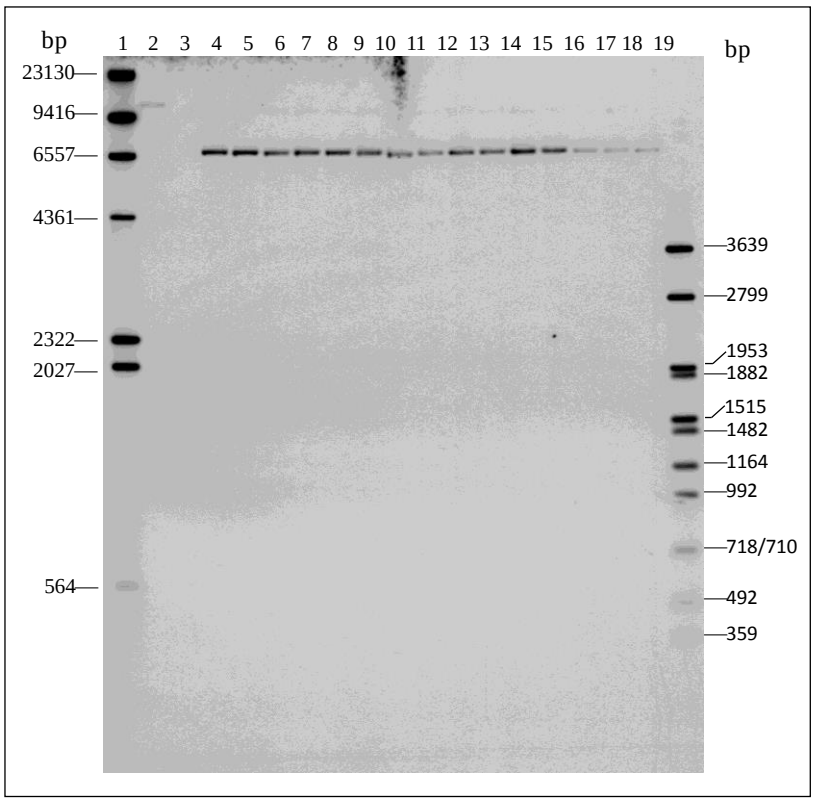


Figure 20. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; AtuORF1 3′ UTR Probe

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-labeled 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		

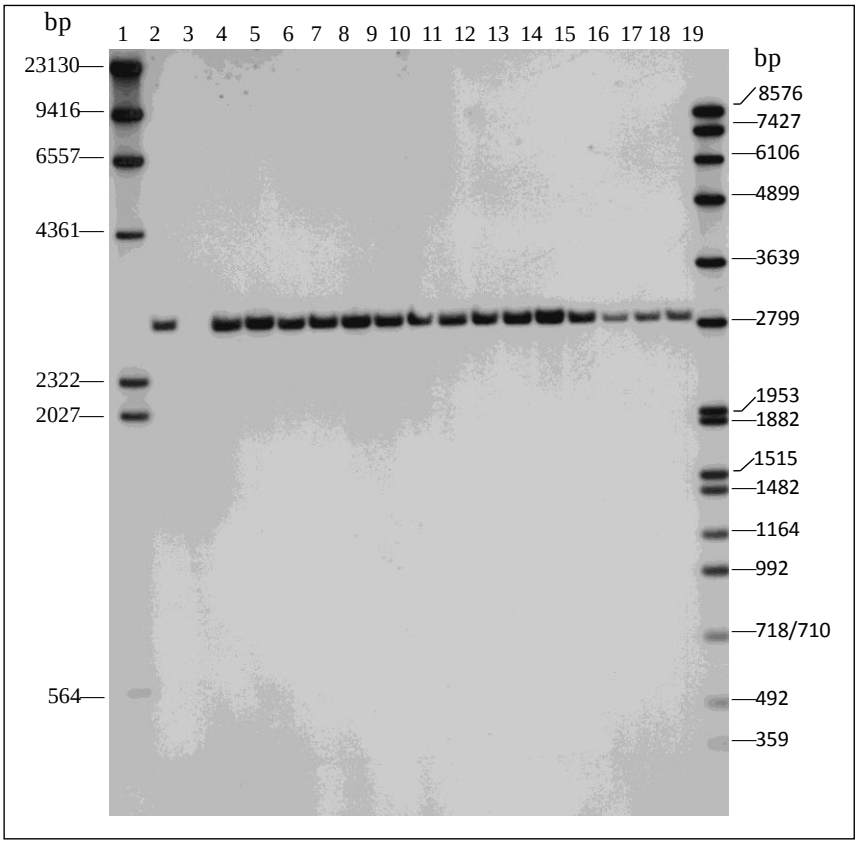


Figure 21. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I to Release *aad-12* Expression cassette; *aad-12* Probe

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-labeled 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		

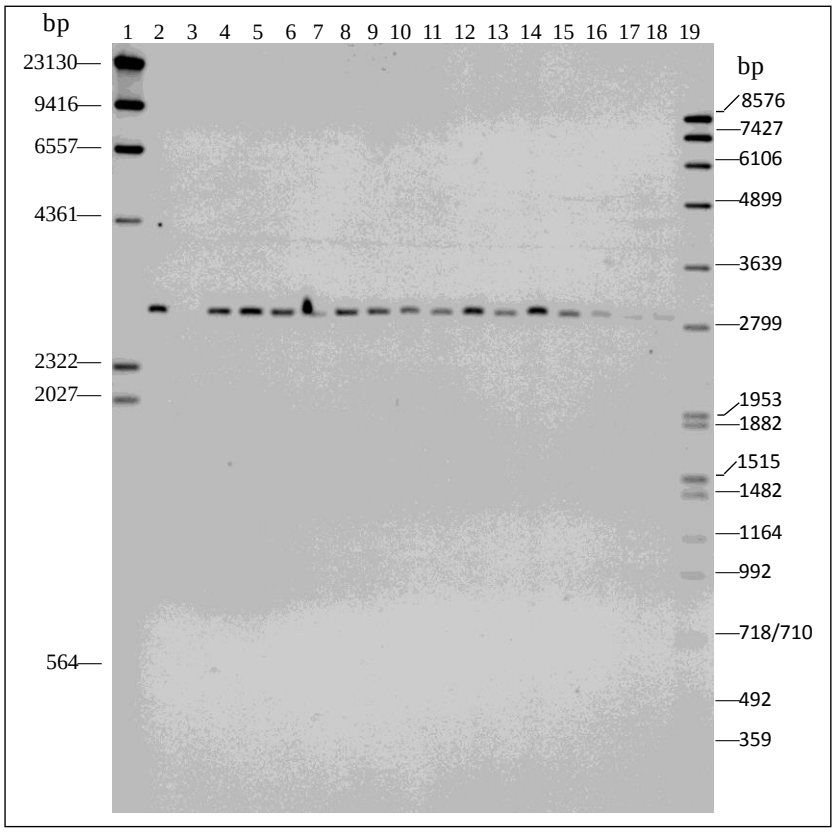


Figure22. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I to Release *aad-12* Expression Cassette; AtUbi10 Probe

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-labeled 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		

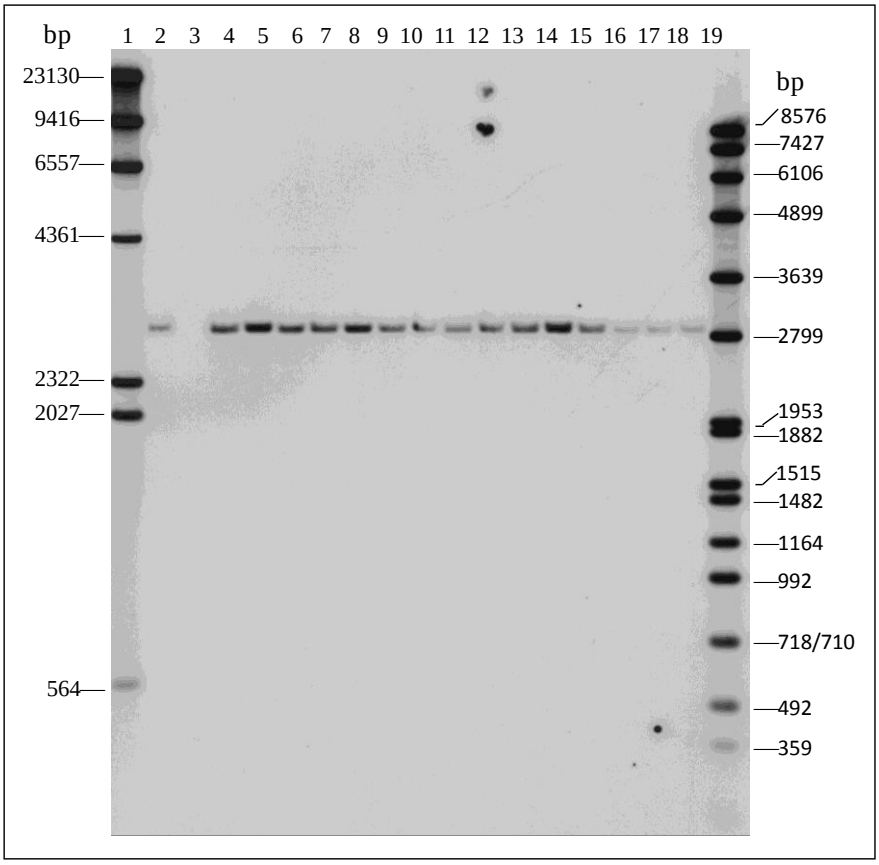


Figure 23. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I to Release *aad-12* Expression Cassette; AtuORF23 3′ UTR Probe

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-labeled 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		

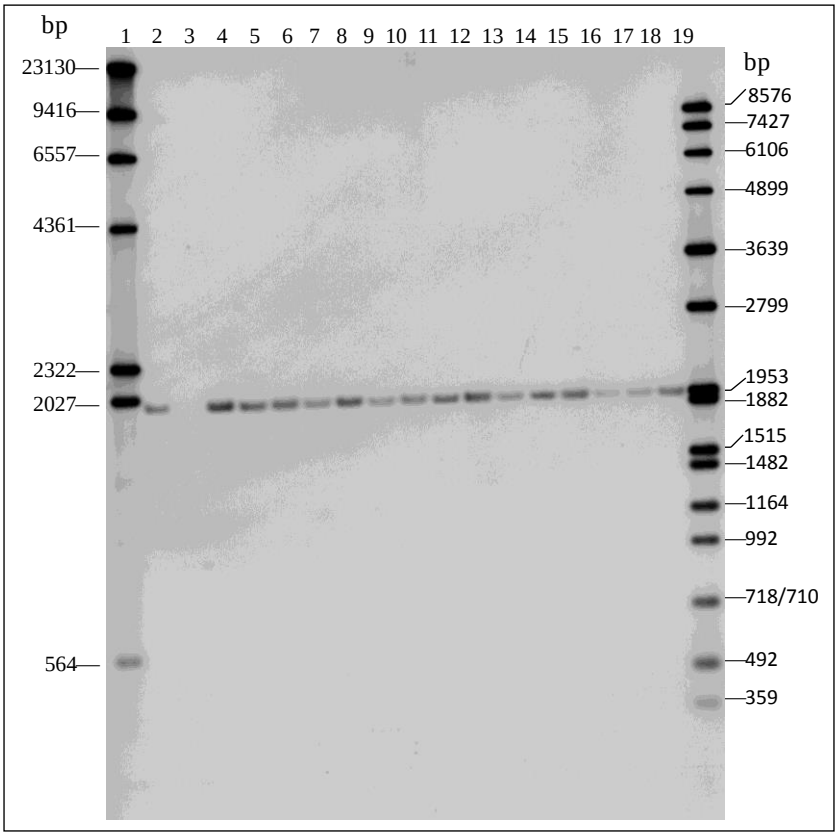


Figure 24. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I/*Xho*I to Release *pat* Expression Cassette; *pat* Probe

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-labeled 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		

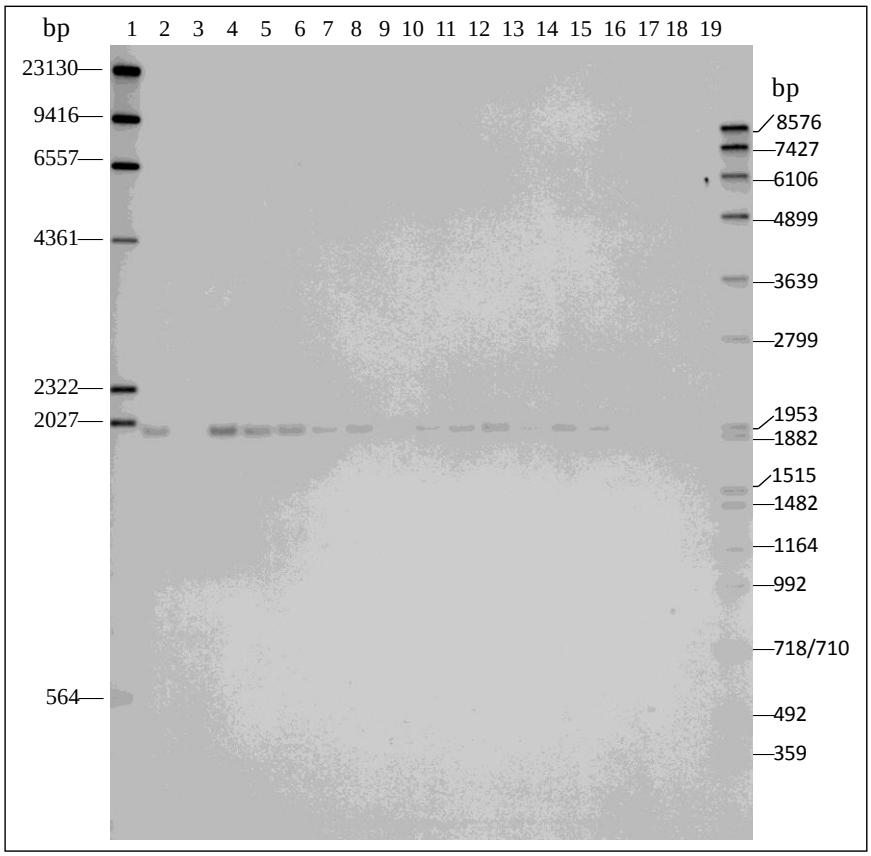


Figure 25. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I/*Xho*I to Release *pat* Expression Cassette; CsVMV Probe

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-labeled 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		

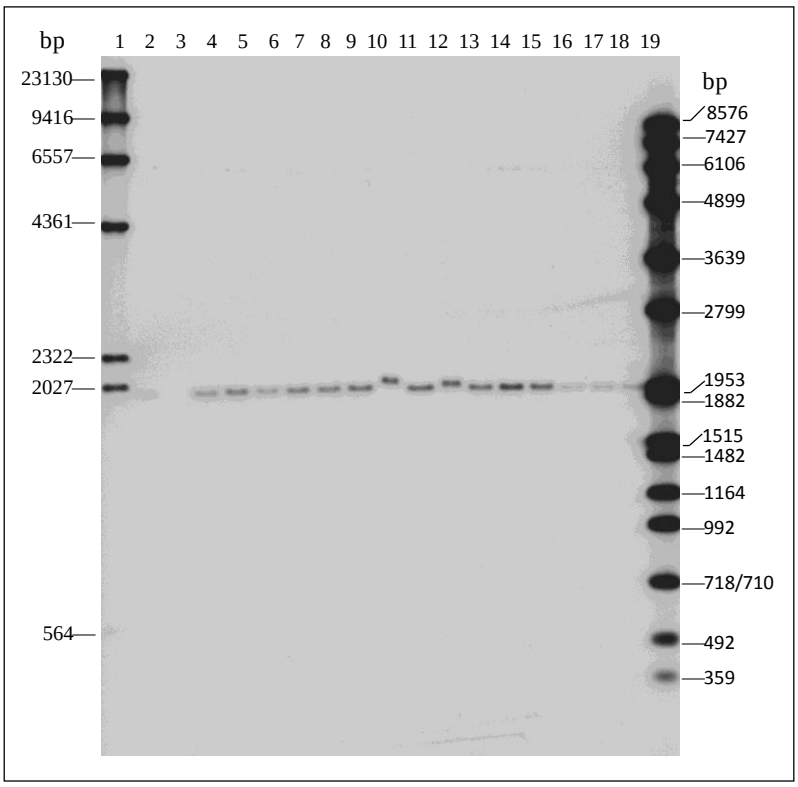


Figure 26. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I/*Xho*I to Release *pat* Expression Cassette; AtuORF1 3′ UTR Probe

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-labeled 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		

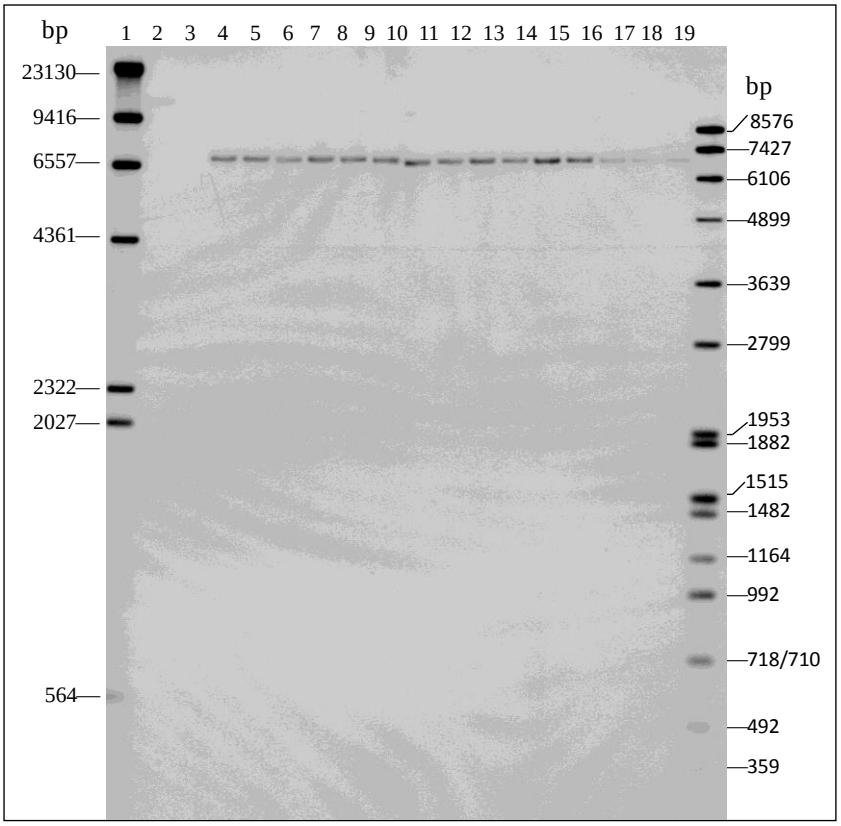


Figure 27. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Sph*I; RB7 Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

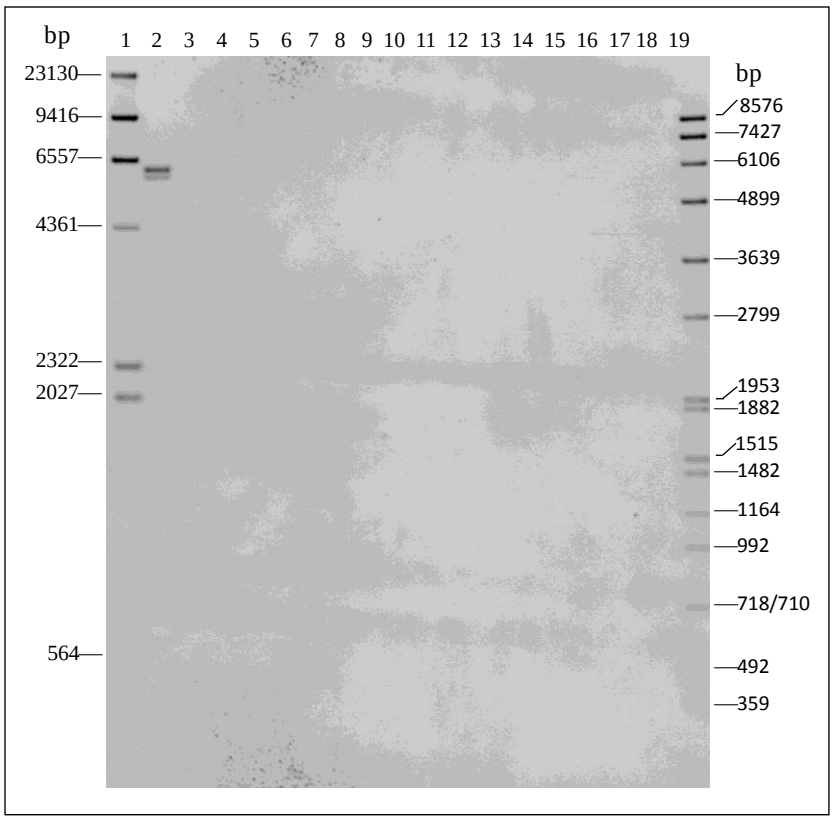


Figure 28. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Msc*I; *Ori* Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

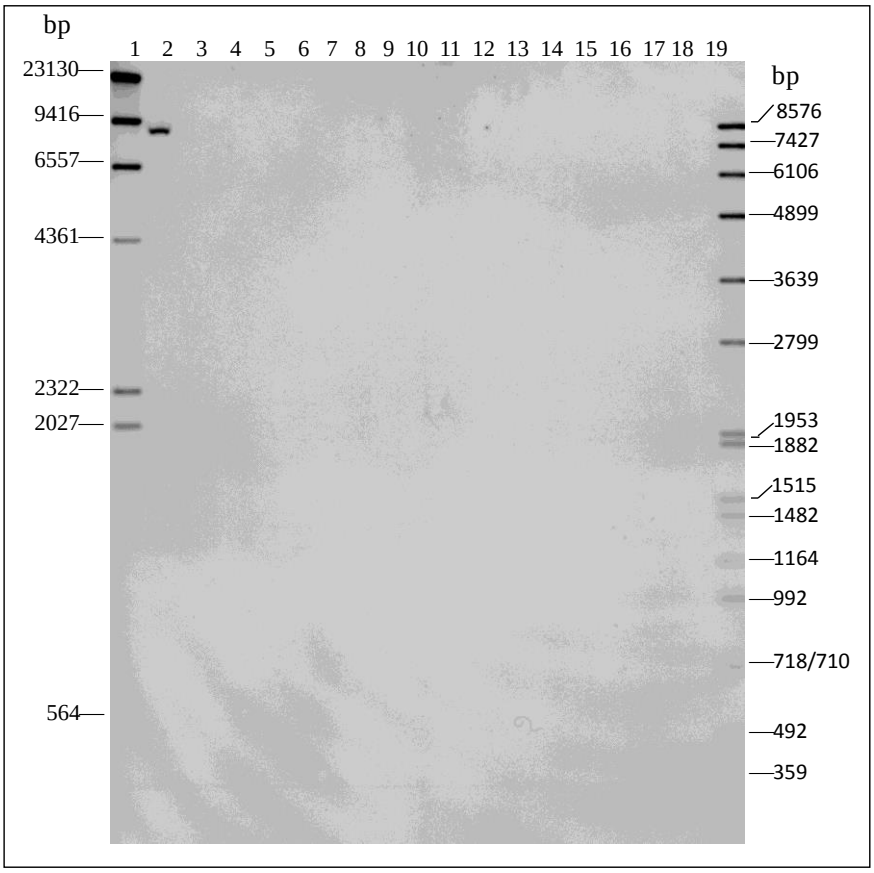


Figure 29. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I; Ori Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

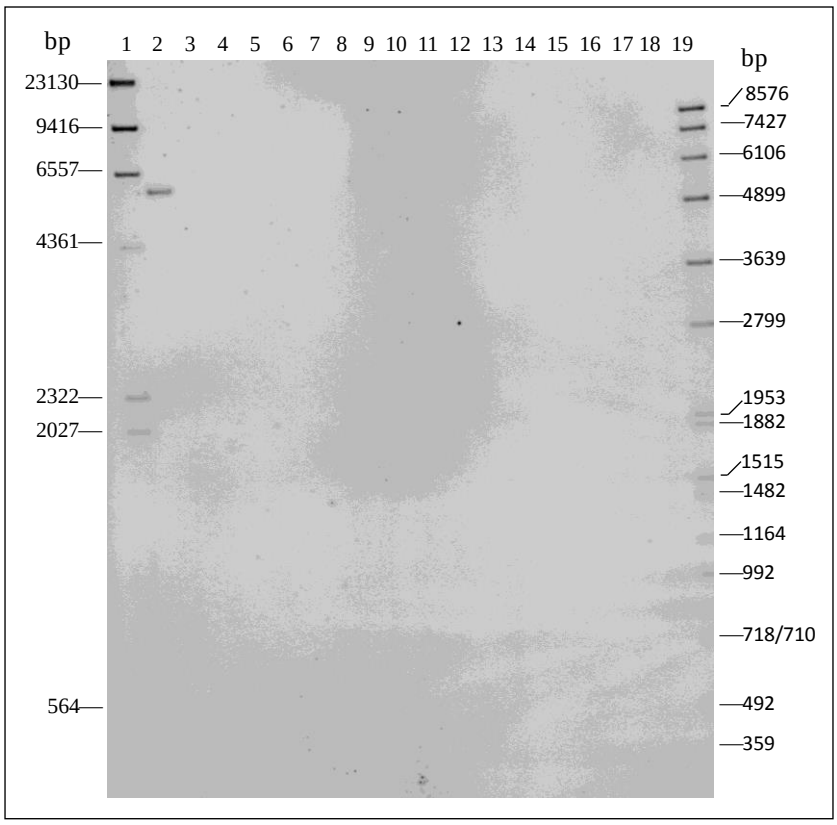


Figure 30. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Msc*I; Backbone2 Probe

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-labeled 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		

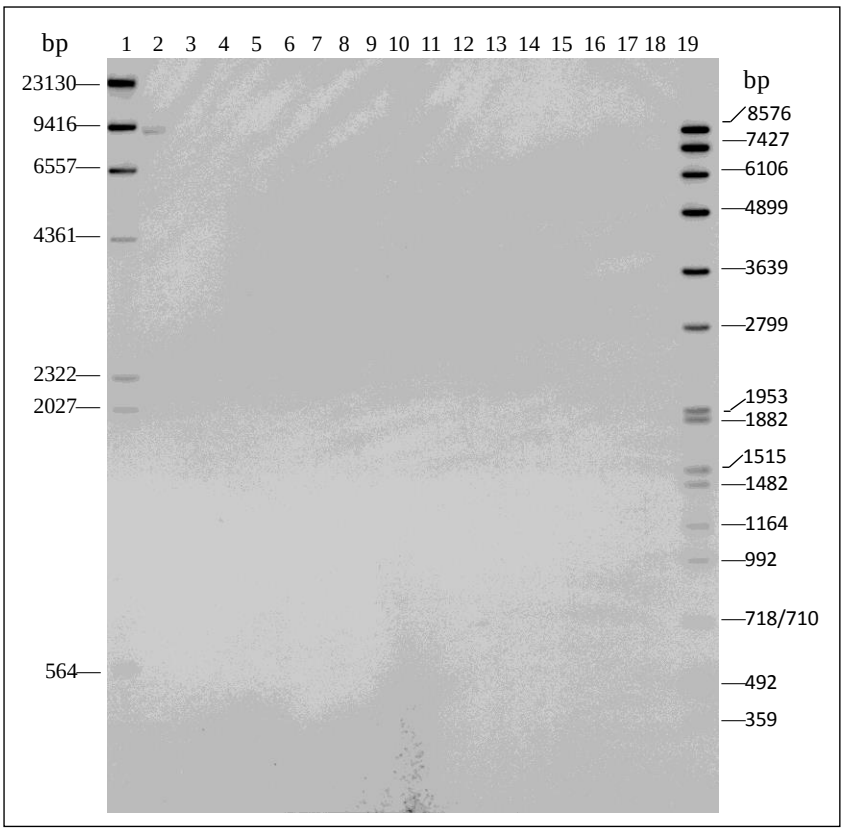


Figure 31. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I; Backbone2 Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

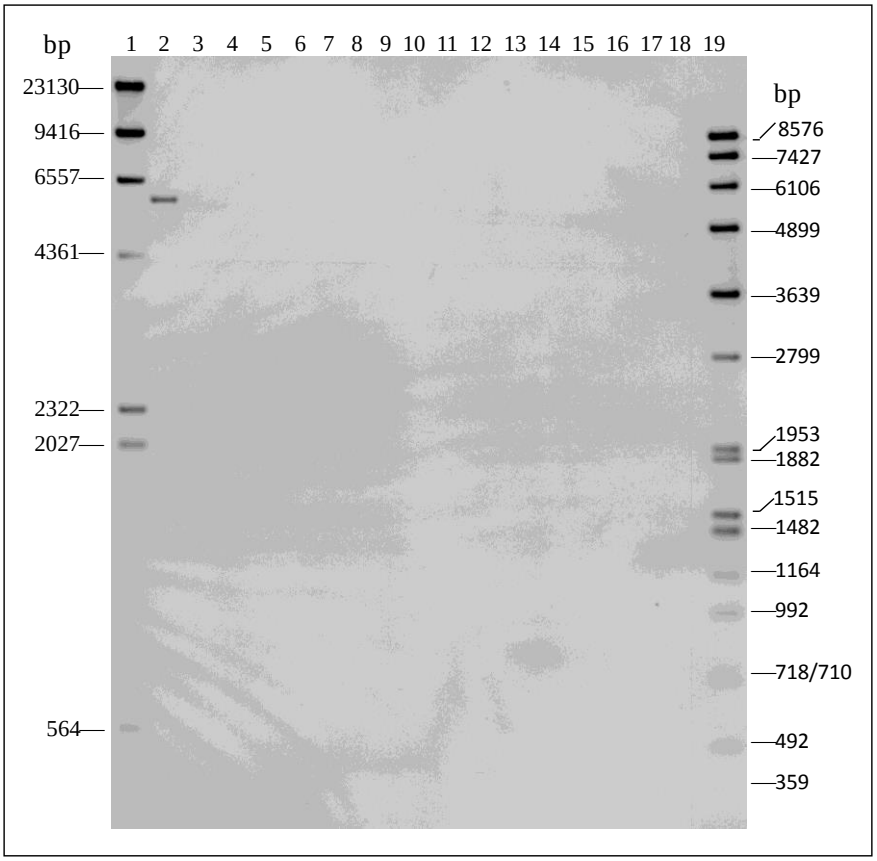


Figure 32. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Msc*I; Backbone1 Probe

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-labeled 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		

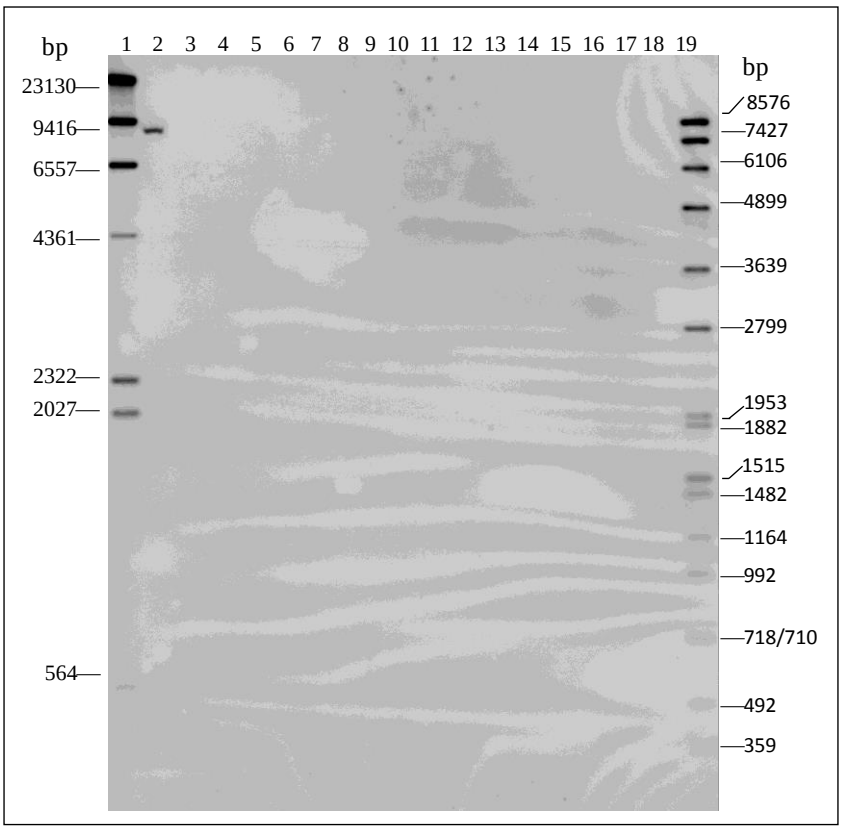


Figure 33. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I; Backbone1 Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

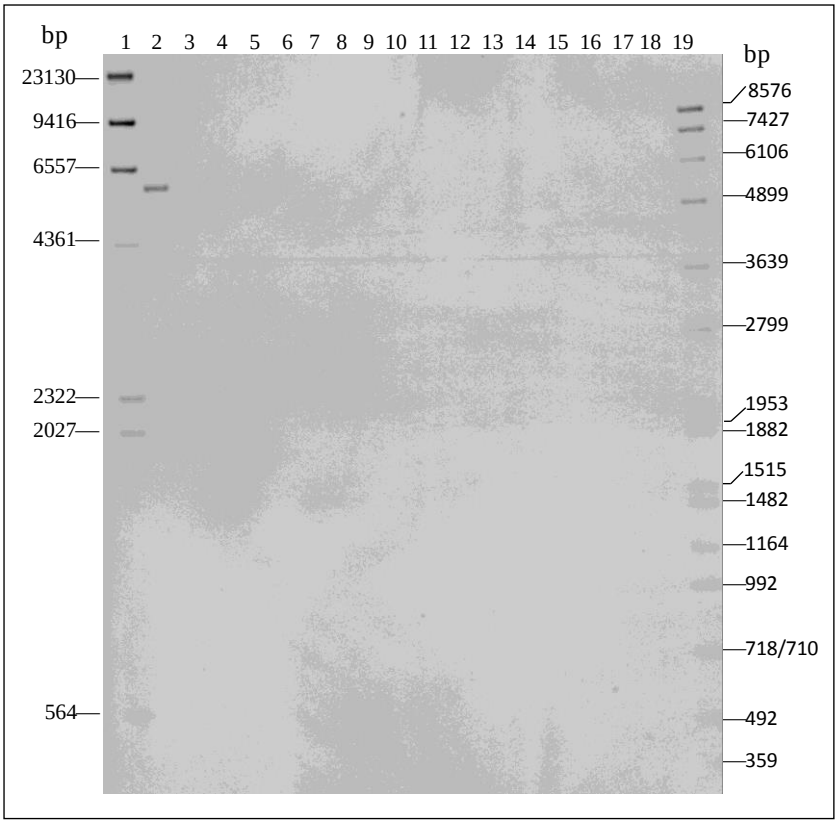


Figure 34. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Msc*I; *Spec*R Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

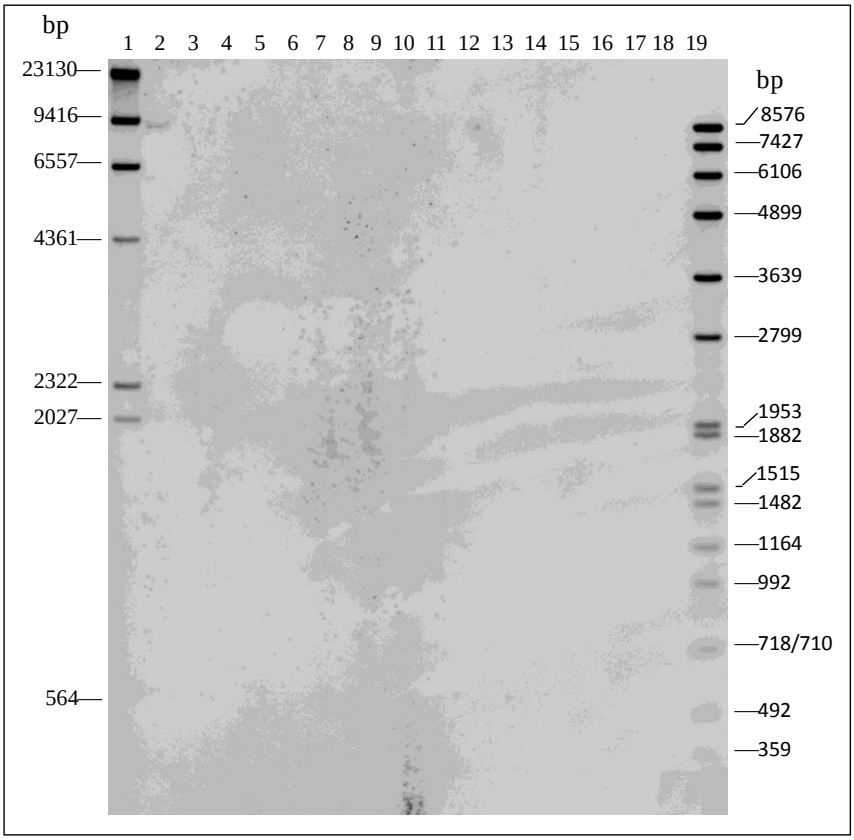


Figure 35. Southern Analysis of DAS-8191Ø-7 Cotton Digested with *Pst*I; *Spec*R Probe
Approximately 10 µg of genomic DNA was digested and loaded per lane. The positive control included plasmid pDAB4468 at approximately one copy of plasmid pDAB4468 per cotton genome in 10 µg of genomic DNA extracted from non-transgenic control Coker 310. Fragment sizes in bp of the DIG-labeled 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		

4.4. Segregation Analysis

4.4.1. 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 4).

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.

Table 5. Segregating Generation Results of BC_1F_2 Individual Plants

Tested Method	Total plants tested	Positive	Negative	Expected ratio	P-value [1]
AAD-12 LFS	136	104	32	3:1	0.6921
Event-Specific PCR	136	104	32	3:1	0.6921

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

4.4.2. 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 4). 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. Gene zygosity assays were conducted using TaqMan real time PCR with primers and probes specific to either *pat* or *aad-12* sequence to determine gene copy number of each assayed sample. DNA samples were extracted from 2571 BC_1F_2 and 196 T_1 plants and assayed for *aad-12* and *pat*, respectively. Quantitative analysis of the PCR results revealed the expected segregation ratio of 3:1 for plants containing the transgene insert versus plants that do not contain the transgene insert (segregated nulls) (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[1]
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

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

4.5. 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.

5. Characterization of the DAS-8191Ø-7 AAD-12 and PAT Proteins

As described in Section 3.4, DAS-8191Ø-7 cotton contains the *aad-12* and *pat* expression cassettes that, 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. These data were supplied to FDA for their evaluation in consultation BNF No. 000142 on the food and feed safety and compositional assessment of DAS-8191Ø-7 cotton.

5.1. AAD-12

5.1.1. Identity of the AAD-12 Protein

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 3.4.1)) 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 (currently under USDA-APHIS review, petition numbers 09-349-01p, 11-234-01p 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.

5.1.2. Mode of Action of the AAD-12 Protein

Expression of the AAD-12 protein in transgenic crops has been shown to provide tolerance to the herbicide 2,4-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).

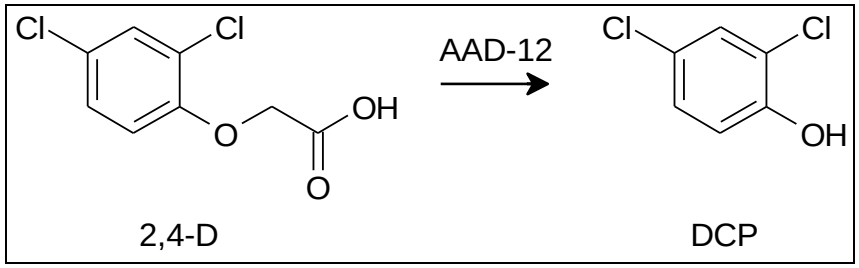


Figure 37. Degradation Reaction of 2,4-D Catalyzed by AAD-12

5.1.2.1. DCP

DCP (2,4-dichlorophenol) is a known primary degradate of 2,4-D in plants (Roberts, 1998). DCP has been observed as a degradate of 2,4-D in environmental matrices and is also observed in animal metabolism studies (Roberts, 1998; Barnekow *et al.*, 2001).

The U.S. EPA tolerance expression for 2,4-D does not include DCP in the plant residue definition, but DCP was at one point included in the livestock meat and milk tolerance expression. In 2004 the U.S. EPA's Health Effects Division, Metabolism Assessment Review Committee (MARC) recommended that DCP be deleted from the livestock tolerance expression for 2,4-D. The MARC committee stated DCP is "not of concern for either the tolerance expression or for risk assessment at the levels expected in livestock tissues and considering the likely lower toxicity of 2,4-DCP compared to 2,4-D" (EPA, 2003). This decision was included in the 2005 Registration Eligibility Decision document (EPA, 2005) and posted in the 2007 Federal Register (EPA, 2007). This action harmonizes U.S. tolerances with Codex, Japanese and European residue definitions which do not include DCP in any tolerance expression.

DAS will be submitting information to the U.S. EPA on the Nature of Residue and Magnitude of Residue resulting from the use of 2,4-D on cotton that expresses the AAD-12 protein. The U.S. EPA will evaluate this information as a component of their review of the use of 2,4-D on cotton that expresses the AAD-12 protein.

5.1.2.2. 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).

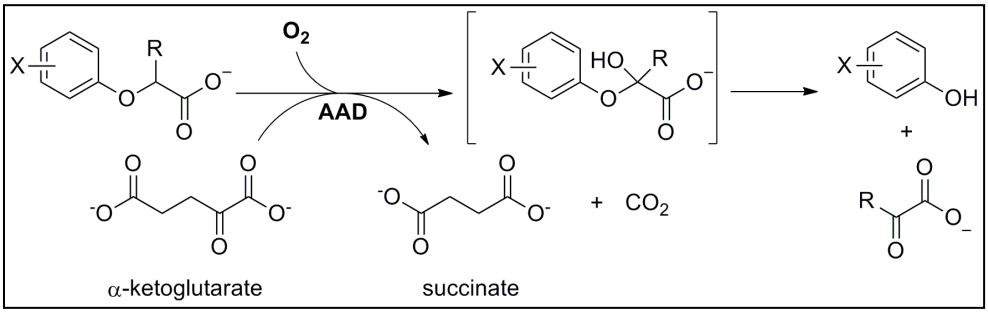


Figure 38. General Reaction Catalyzed by AAD-12 (R=H or CH_3)

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 (Griffin *et al.*, 2013).

5.1.3. Biochemical Characterization of the AAD-12 Protein

Large quantities of purified AAD-12 protein are required to perform safety assessment studies. Because it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans, 2004; 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. 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. 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 and 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 and Sherman, 2003). The C-terminal peptides from DAS-8191Ø-7 cotton and *P. fluorescens* were intact and empirically determined to be identical.

5.1.4. Expression of the AAD-12 Protein in Plant Tissues

A field expression study was conducted in the U.S. during 2012. Six sites (Alabama, Georgia, Louisiana, Missouri, North Carolina, and Texas) were planted with DAS-8191Ø-7 cotton and the non-transgenic control (98M-2983). The test sites represented regions of diverse agronomic practices and environmental conditions for cotton in North America. Two treatments of DAS-8191Ø-7 cotton (non-sprayed 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[1]	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

[1] 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. 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 non-sprayed treatments. No AAD-12 protein was detected in the control tissues.

Table 8. Summary of AAD-12 Protein Expression

Expression Tissue	Growth Stage	AAD-12 ng/mg Tissue Dry Weight[1,2]		
		Overall Mean	Std. Dev.[3] (n = 48)	Min/Max Range[4]
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[5]-46.98

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

5.1.5. Food and Feed Safety Assessment of AAD-12

The primary food source from cotton is refined cottonseed oil, which contains undetectable amounts of protein (Reeves III and Weihrauch, 1979). Therefore, assessing the allergenicity and toxicity to humans 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 food and feed safety assessment of AAD-12 expressed in DAS-8191Ø-7 cotton considers several factors including safety of the donor organism, history of safe use, allergenic potential, toxicity potential and dietary risk assessment based on consumption patterns. 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.

5.1.5.1. *Safety of Donor Organism and history of safe use*

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

Strains of *Delftia acidovorans* can be used to transform ferulic acid into vanillin and related flavor metabolites (Toms and Wood, 1970; Labuda *et al.*, 1992; Rao and Ravishankar, 2000; Shetty *et al.*, 2006).

5.1.5.2. Assessment of Allergenicity Potential

Studies were conducted to ascertain the potential allergenicity of AAD-12 and conclusions were as follows:

1) DAS-8191Ø-7 cotton AAD-12 originates from *Delftia acidovorans*, an organism that has not been reported as a source of allergens (5.1.5.1).

2) Bioinformatics analyses demonstrated that DAS-8191Ø-7 cotton AAD-12 does not share amino acid sequence similarities with known allergens and is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

3) *In vitro* digestive fate experiments conducted with AAD-12 demonstrate that the protein is rapidly digested in simulated gastric fluid (SGF).

4) AAD-12 is heat labile, in which enzyme activity is rapidly eliminated at temperatures as low as 50 °C in less than thirty minutes. Due to the harsh processing conditions of cottonseed oil and the lack of detectable proteins in processed cottonseed oil, AAD-12 is most likely absent in the oil and linters used in food products.

Taken together, these data conclude that DAS-8191Ø-7 cotton AAD-12 does not pose a significant allergenic risk.

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.

Lability in Simulated Gastric Fluid

The digestibility of the *P. fluorescens*-derived AAD-12 protein was tested *in vitro* using simulated gastric fluid (SGF). The AAD-12 protein was incubated in SGF (0.32% w/v pepsin at pH 1.2; (The United States Pharmacopeia, 1995) for various periods of time. The samples were then analyzed via SDS-PAGE and western blot analysis using a polyclonal antibody specific to AAD-12. The results demonstrated the AAD-12 protein was readily digested in less than thirty seconds in SGF, indicating that the proteins are unlikely to elicit allergenic reactions when consumed.

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 and Hausinger, 1993). In the presence of Fe(II), the AAD-12 protein catalyzes the conversion of dichlorphenoxyacetate to 2,4-dichlorophenol and glyoxylate concomitant with the decomposition of α-ketoglutarate to form succinate and carbon dioxide. The resulting phenol is measured with an AAPPC assay or the Emerson reaction (Emerson, 1943). All heating conditions eliminated the enzymatic activity of the AAD-12 protein.

5.1.5.3. Assessment of Toxicity Potential

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 (updated 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-like", and "putative alpha-ketoglutarate dependent dioxygenase". None of those proteins are associated with protein toxins that are harmful to humans or animals.

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). The study was conducted following OECD guideline 423 and used a total of 10 mice (5 male and 5 female) (OECD, 2001). There were no treatment-related gross pathological observations. Therefore, the acute oral LD_{50} and no observed effect level (NOEL) of AAD-12 in 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.

5.1.5.4. Human Dietary Risk Assessment for AAD-12

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 (Reeves III and Weihrauch, 1979; OECD, 2009); therefore, oil produced from DAS-8191Ø-7 will contain extremely low levels to no detectable levels of AAD-12 protein.

This assessment assumes that 100% of consumed cotton products are derived from DAS-8191Ø-7 cottonseed. This is a highly 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 ARS, 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.

Potential dietary risk to humans and livestock from the consumption of AAD-12 protein in food and feed derived from DAS-8191Ø-7 cotton was evaluated by determining margins of exposure (MOE). MOE is the ratio of the No Observable Effect Level (NOEL) of AAD-12 determined by a mouse acute oral toxicity study (2000 mg/kg body weight) to estimated dietary intake of AAD-12. To evaluate dietary risk for human food consumption, MOEs were determined for the U.S. population based on mean protein expression levels in cottonseed. For a U.S. 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.

The U.S. assessment is incongruent with an assessment performed based on the WHO maximum global consumption data for acute exposures, which indicated that the U.S. 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. When the WHO "SO 691 Cottonseed" acute consumption information is coupled to the protein average field expression level for cottonseed (Table 8), the dietary risk assessments revealed that the calculated MOE values for the AAD-12 protein in cottonseed for both adults and infants are very large with both great than 2,000,000. For chemicals, MOEs greater than 100 are typically considered acceptable. Based on the available safety threshold information, all MOEs for AAD-12 protein indicate negligible concern for adverse effects from DAS-8191Ø-7 cotton. Accordingly, there is negligible risk to human health when DAS-8191Ø-7 AAD-12 is present in the human diet.

5.1.5.5. Livestock Dietary Risk Assessment for AAD-12

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.

AAD-12 Livestock Dietary Risk Assessment Analysis

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 byproducts are also sometimes used as a supplemental cattle feed. Cotton gin byproducts (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 byproducts 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.

This assessment has assumed that 100% of cotton consumed is derived from DAS-8191Ø-7 cotton. Actual dietary exposure to AAD-12 protein will be lower because: 1) cotton is a blended commodity; cotton-derived food and feed will contain cotton from a mixture of sources, 2) degradation of the protein will occur during transport and storage, and 3) heat applied during preparation of cotton derived foods and feeds may lessen exposure to AAD-12, as AAD-12 is functionally unstable when heated (Section 5.1.5.2).

A U.S. livestock assessment was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (EPA, 2008). This U.S. assessment includes several cotton commodity forms as potential animal feeds: seed, meal, hulls and gin byproducts. The U.S. 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.

The highest estimated exposed U.S. animal is the sheep with 0.27 mg AAD-12/kg-bw. Lower estimates for beef cattle, dairy cattle, swine and poultry were $\leq$0.23 mg AAD-12/kg-bw. When these values are compared to the acute mammalian NOAEL of >2000 mg/kg-bw (see section 5.1.5.3), there is a 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 when AAD-12 is present in their diets.

5.1.6. 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.

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 AAD-12 is unlikely to cause allergenic or adverse effects in humans or animals.

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. The overall safety assessment of the AAD-12 protein supports the conclusion that DAS-8191Ø-7 cotton is as safe as non-transgenic cotton currently on the market and unlikely to cause allergenic or toxic effects to humans or animals.

5.2. PAT

5.2.1. 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 (Strauch *et al.*, 1988; OECD, 1999). The *pat* transgene in DAS-8191Ø-7 encodes a protein sequence that is identical to the native *S. viridochromogenes* PAT protein (UniProt Accession Number: Q57146) and is identical to that produced in DAS-68416-4 soybean and DAS-444Ø6-6 soybean (currently under USDA-APHIS review, petition numbers 09-349-01p, 11-234-01p respectively).

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001 MSPERRPVEIRPATAADMAAVCDIVNHYIETSTVNFRTEPQTPQEWIDDL
051 ERLQDRYPWLVAEVEGVVAGIAYAGPWKARNAYDWTVESTVYVSHRHQRL
101 GLGSTLYTHLLKSMEAQGFKSVVAVIGLPNDPSVRLHEALGYTARGTLRA
151 AGYKHGGWHDVGFWQRDFELPAPPRPVRPVTQI
```

Figure 39. Amino Acid Sequence of PAT

5.2.2. Mode of Action of the PAT Protein

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

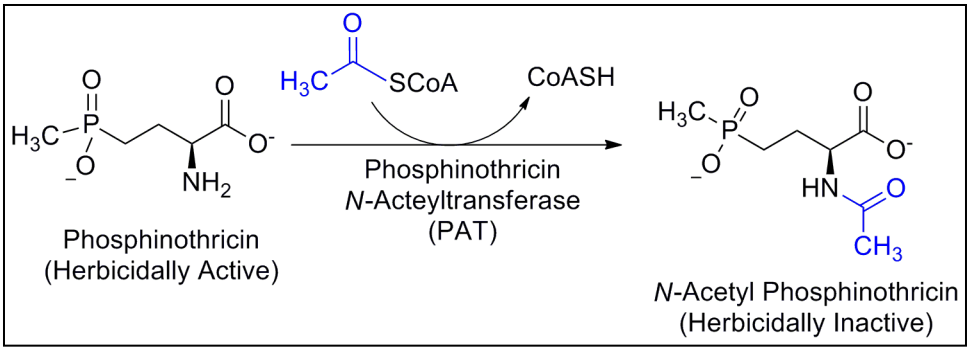


Figure 40. Mode of Action of the PAT Protein

5.2.3. 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. 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. 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 and Chang, 1995). The C-terminal peptides from DAS-8191Ø-7 cotton and *P. fluorescens* were intact and empirically determined to be identical.

5.2.4. Expression of the PAT Protein in Plant Tissues

A field expression study was conducted in the U.S. 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 (non-sprayed 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[1]	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

[1] 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. 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**.**

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

Expression Tissue	Growth Stage	PAT ng/mg Tissue Dry Weight[1,2]		
		Overall Mean	Std. Dev[3] (n = 48)	Min/Max Range[4]
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[5]-3.97

[1] Calculated from PAT expression raw data.
[2] Table represents overall results for non-sprayed and sprayed cotton tissue across all sites.
[3] Standard deviation of individual cotton samples across all sites.
[4] Represents the min and max for individual cotton samples across all sites.
[5] ND = Not Detected, expression level below LOD (Limit of Detection). A zero value was used for ND results for mean calculation.

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 non-sprayed treatments. 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. This was most likely attributed to sampling error and/or contamination.

5.2.5. Food and Feed Safety Assessment of PAT

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

The 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 (CERA - ILSI Research Foundation, 2011). Numerous transgenic crops expressing PAT have been reviewed by USDA and FDA with no concerns identified (USDA, 1996; FDA, 1998, 2001; USDA, 2001; FDA, 2004a, 2004b, 2004c; USDA, 2004, 2005)

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.

Amino Acid Sequence Comparison to Known Toxins

The PAT protein does not share any amino acid sequence similarities with known protein toxins that would present any safety concerns. Amino acid sequence similarities with known proteins were evaluated using BLASTp search algorithm against the GenBank non-redundant protein sequences (updated February 23, 2012). By their annotations, the majority proteins returned by BLASTp with statistically significant alignments are phosphinothricin acetyltransferase, other acetyltransferases, and hypothetical proteins without assigned function. Furthermore, most of the non-significant alignments are related with phosphinothricin *N*-acetyltransferase or GCN5 related *N*-acetyltransferase. None of these proteins are associated with known protein toxins that are harmful to humans or animals.

Lability in Simulated Gastric Fluid and Heat Lability

The PAT protein is rapidly degraded in simulated gastric fluid (EPA, 1997; OECD, 1999; Herouet *et al.*, 2005) 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 U.S. EPA has concluded, after reviewing data on the acute toxicity and digestibility of the PAT protein, that there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the PAT protein and the genetic material necessary for its introduction (EPA, 1997). U.S. 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, which would include DAS-8191Ø-7 cotton (40 CFR §174.522).

5.2.6. Summary of PAT Protein Characterization

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.

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.

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, and the results of the overall safety assessment of the PAT protein indicate that it is unlikely to cause allergenic or adverse effects in humans or animals.

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.

6. Composition Assessment of DAS-8191Ø-7 Cotton

6.1. DAS-8191Ø-7 Cottonseed Composition

Composition assessment of DAS-8191Ø-7 cotton was conducted to examine key nutrient and anti-nutrient levels. In one experiment, non-sprayed DAS-8191Ø-7 cottonseed were compared with the appropriate non-transgenic near isogenic isoline (control) (Figure 4) and non-transgenic reference lines. In another experiment, non-sprayed DAS-8191Ø-7 cottonseed were compared with herbicide-treated DAS-8191Ø-7 cottonseed and non-sprayed DAS-8191Ø-7 cottonseed.

The assessment was conducted using the principles and analytes in the OECD consensus document for cotton composition (OECD, 2009). 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 (Figure 4), grown concurrently, under the same field conditions; 2) natural ranges generated from an evaluation of six commercial non-transgenic cotton reference lines (ALL-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271, and Speed) grown concurrently, under the same field conditions; and 3) 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.

6.1.1. Field Study Design

Field trials were conducted at eight U.S. 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 cottonseed samples of DAS-8191Ø-7 cotton (non-sprayed and sprayed with 2,4-D plus glufosinate), near isogenic non-transgenic isoline (control) and non-transgenic cotton varieties, referred to as reference lines, for nutrient composition analysis. For the reference lines, six unique reference lines were included across all sites of the field production with three varieties per site to provide data on natural variability of each composition analyte analyzed.

Because non-transgenic cotton plants (*e.g.* isoline 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 not sprayed and contained the isoline, reference varieties and one entry of DAS-8191Ø-7 cotton. Sub-experiment two contained two entries of only DAS-8191Ø-7 cotton; one not sprayed and one sprayed with 2,4-D plus glufosinate.

The entries were arranged in a randomized complete block design within each sub-experiment. The sub-experiments were separated by 100 ft (30 m) to prevent isoline and reference entries in sub-experiment one from potential injury by 2,4-D drift from 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, isoline, 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. In sub-experiment two, 2,4-D (GF-2654) and glufosinate (Ignite 280 SL) were applied in a tank mixture as two broadcast applications to one entry of DAS-8191Ø-7 cotton. 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. Actual application rates ranged from 1108 – 1142 g ae/ha 2,4-D and 589 – 622 g ai/ha glufosinate for the 3 node application and 1061 – 1141 g ae/ha 2,4-D and 564 – 621 g ai/ha glufosinate for the 6 node application.

6.1.2. Compositional Analysis

Samples of acid delinted cottonseed were analyzed at Covance Laboratories Inc. for 73 cotton analytes (Table 11). Methods for compositional analysis are described in Appendix 5. Data from the composition analysis was statistically analyzed for each sub-experiment comparing 1) non-sprayed DAS-8191Ø-7 cotton in sub-experiment one to the appropriate non-sprayed non-transgenic near isogenic isoline and non-transgenic reference lines in sub-experiment one and 2) non-sprayed DAS-8191Ø-7 cotton in sub-experiment one to the non-sprayed DAS-8191Ø-7 cotton and to herbicide-treated DAS-8191Ø-7 cotton in sub-experiment two. The results were then placed into context with reported scientific literature values.

6.2. 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; 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 made in each sub-experiment. 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 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, the probability of falsely declaring differences based on comparison-wise P-values is very high at 95.151% ($1-0.95^{59}$) ($1-0.95^{\text{number of comparisons}}$).

Table 11. Cottonseed Composition Analytes

Proximates and Fiber (9)		
Protein	Moisture	Neutral Detergent Fiber (NDF)
Fat	Carbohydrates	Total Dietary Fiber
Ash	Acid Detergent Fiber (ADF)	Crude Fiber
Minerals (12)		
Calcium	Manganese[2]	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[1]	16:1 Palmitoleic[2]	20:0 Arachidic
10:0 Capric[1]	17:0 Heptadecanoic[1]	20:1 Eicosenoic[1]
12:0 Lauric[1]	17:1 Heptadecenoic[1]	20:2 Eicosadienoic[1]
14:0 Myristic[2]	18:0 Stearic	20:3 Eicosatrienoic[1]
14:1 Myristoleic[1]	18:1 Oleic[2]	20:4 Arachidonic[1]
15:0 Pentadecanoic[1]	18:2 Linoleic[2]	22:0 Behenic
15:1 Pentadecenoic[1]	18:3 Linolenic	
16:0 Palmitic	18:3 γ-Linolenic[1]	
Vitamins (7)		
Vitamin A (Beta Carotene) [1]	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[2]
Malvalic Acid[2]	Free Gossypol	

[1] Analytes excluded from combined site and analysis due to more than 50% of samples < LOQ

[2] Analytes with statistically significant adjusted FDR differences in the combined site analysis.

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. 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 and Hochberg, 1995), which is a commonly used approach in studies examining transgenic crops (Herman *et al.*, 2007; Coll *et al.*, 2008; Huls *et al.*, 2008; Jacobs *et al.*, 2008; Stein *et al.*, 2009; Herman *et al.*, 2010).

Therefore, the P-values from the composition 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.

6.3. Composition Analysis Results

Combined summary and statistical analysis of composition data from the non-transgenic, near isogenic isoline (control) and DAS-8191Ø-7 cotton is found in Table 12 through Table 17 and Figure 41 through Figure 46 for both sub-experiment one and two. For each analyte and entry, the least-square mean, standard error, and minimum and maximum sample values 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 (non-sprayed DAS-8191Ø-7, isoline, and reference lines), followed by a discussion of results for sub-experiment two (sprayed and non-sprayed DAS-8191Ø-7). Sub-experiment one was used to evaluate if the composition of non-sprayed DAS-8191Ø-7 cotton is equivalent to reference and isoline lines, and sub-experiment two was used to evaluate if applying 2,4-D plus glufosinate impacts the composition of DAS-8191Ø-7 cotton.

6.3.1. Sub-experiment One - Proximate and Fiber Analysis of Seed

Results from the combined site analysis of the proximate and fiber composition for the non-transgenic isoline (control) and DAS-8191Ø-7 cottonseed are provided in Table 12 and Figure 41. 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 non-sprayed for all nine analytes. In addition, mean results for all nine DAS-8191Ø-7 non-sprayed proximate and fiber analytes 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 isoline, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 12. Combined Site Analysis Results for Proximates & Fiber in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (Units)	Isoline (control)	DAS-8191Ø-7 Non-sprayed	Reference Variety Range	DAS-8191Ø-7 Non-sprayed	DAS-8191Ø-7 Sprayed[1]	Literature Range
	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
Ash (% Dry Weight)	4.29 ± 0.11 3.67 - 5.14	4.17 ± 0.11 3.62 - 4.76 (**0.047**, 0.185)	3.53 - 5.21	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)	47.5 ± 0.9 44.5 - 53.6	49.1 ± 0.9 44.7 - 57.9 (**0.007**, 0.050)	42.3 - 54.2	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)	21.7 ± 0.7 18.3 - 25.2	21.3 ± 0.7 15.6 - 26.2 (0.368, 0.642)	15.8 - 27.9	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)	26.4 ± 0.9 22.3 - 31.5	25.4 ± 0.9 22.2 - 31.1 (**0.008**, 0.055)	21.5 - 32.3	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)	8.2 ± 0.2 6.64 - 9.74	8.0 ± 0.2 6.56 - 9.39 (0.663, 0.815)	6.37 - 10.2	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[3] (% Dry Weight)	25.9 ± 0.5 23.0 - 28.6	25.3 ± 0.5 21.3 - 28.6 (0.381, 0.642)	20.4 - 29.4	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)	18.1 ± 0.3 16.0 - 21.3	17.9 ± 0.3 14.8 - 22.7 (0.677, 0.815)	15.1 - 23.5	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[4] (% Dry Weight)	34.0 ± 0.6 30.7 - 40.3	33.8 ± 0.6 28.3 - 39.7 (0.667, 0.815)	27.2 - 38.2	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)	44.8 ± 1.0 40.3 - 53.5	45.7 ± 1.0 40.7 - 56.5 (0.283, 0.591)	37.6 - 51.3	46.6 ± 1.1 40.0 - 54.0	47.2 ± 1.1 41.7 - 54.1 (0.309, 0.945)	33.69 - 47.55

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values
[3] Acid detergent fiber
[4] Neutral detergent fiber

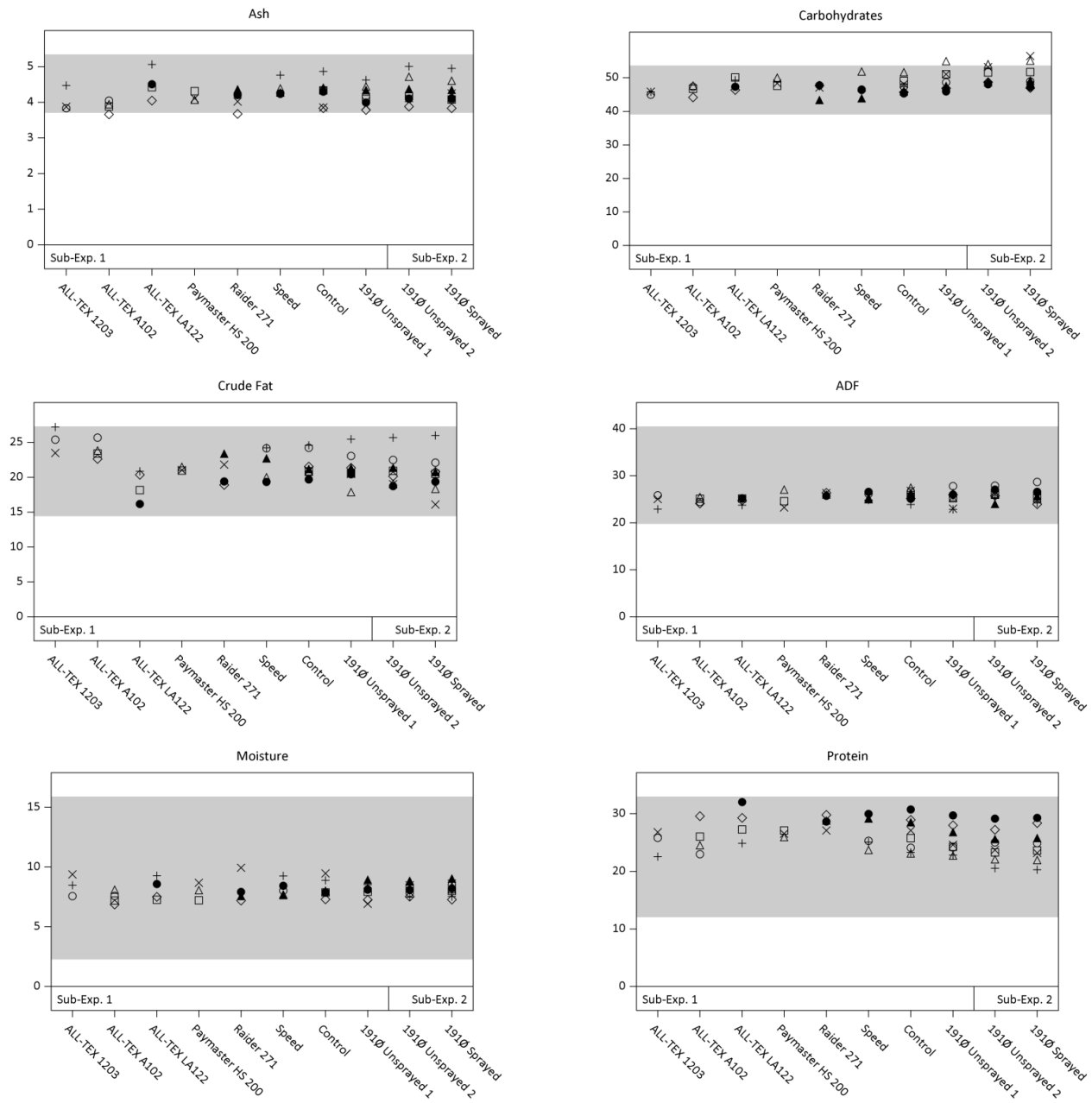


Figure 41. Proximates & Fiber in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed
Y axis: Moisture = % fresh weight, all others = % dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed 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 plus 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.

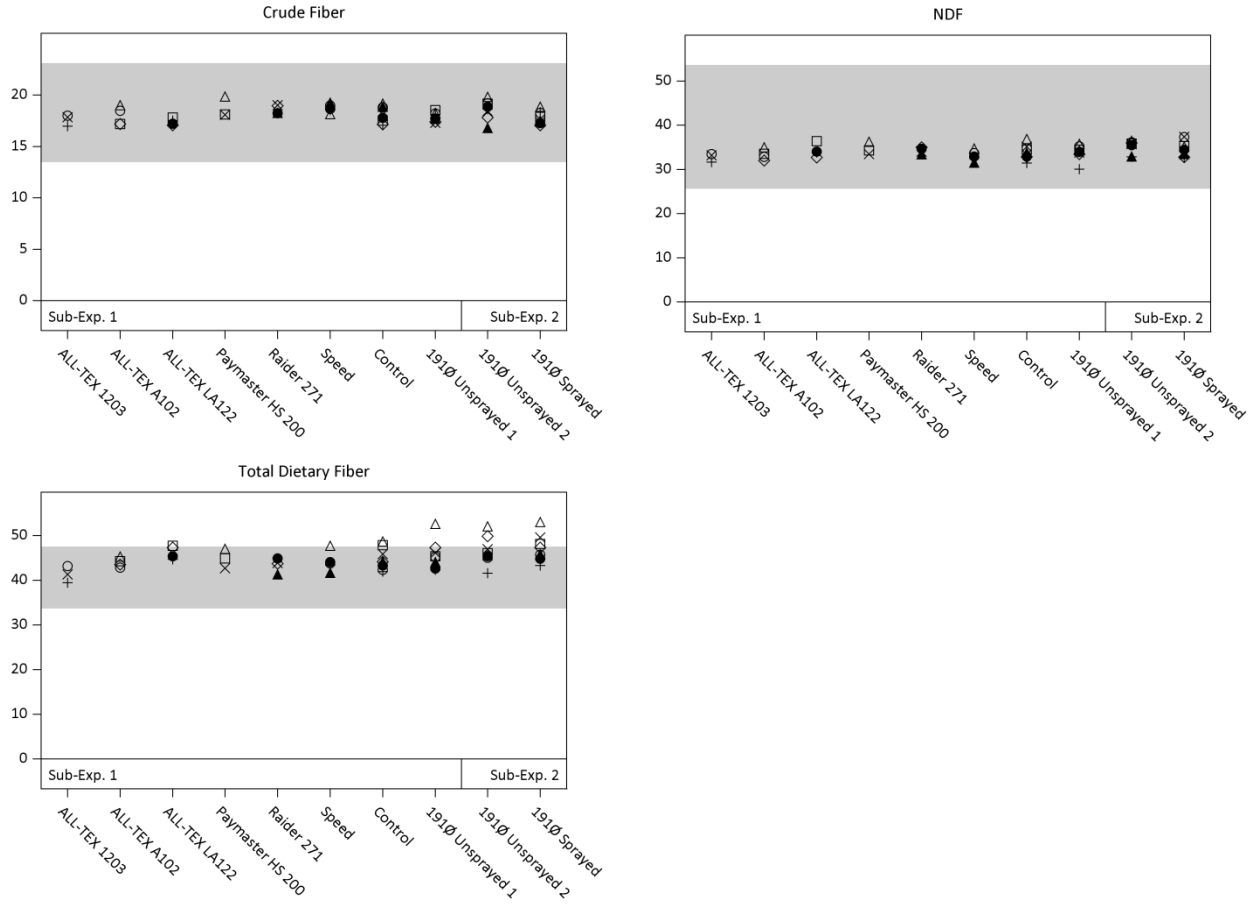


Figure 41. Proximates & Fiber in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed (continued)

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 plus 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.

6.3.2. Sub-experiment One - Mineral Analysis of Seed

Results from the combined site analysis of the mineral composition for the non-transgenic isoline (control) and DAS-8191Ø-7 cottonseed are provided in Table 13 and Figure 42. 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 non-sprayed 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 non-sprayed 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 for manganese between the mean values for DAS-8191Ø-7 non-sprayed and the non-transgenic isoline 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 isoline were not considered to be meaningful from a food, nutritional or safety perspective because they were small, less than the variability seen in the isoline, 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 non-sprayed 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 isoline, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 13. Combined Site Analysis Results for Minerals in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (mg/100 g Dry Weight)	Isoline (Control) Mean ± SE Min - Max	DAS-8191Ø-7 Non-sprayed Mean ± SE Min - Max (P-Val., Adj. P)[2]	Reference Variety Range Min - Max	DAS-8191Ø-7 Non-sprayed Mean ± SE Min - Max	DAS-8191Ø-7 Sprayed[1] Mean ± SE Min - Max (P-Val., Adj. P)[2]	Literature Range Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
Calcium	124 ± 7 88.1 - 164	133 ± 7 98.0 - 172 (**0.011**, 0.059)	78.9 - 204	136 ± 7 113 - 178	136 ± 7 114 - 178 (0.960, 0.998)	100 - 330
Copper	0.90 ± 0.07 0.525 - 1.37	0.86 ± 0.07 0.532 - 1.30 (**0.021**, 0.096)	0.466 - 1.44	0.86 ± 0.08 0.446 - 1.32	0.86 ± 0.08 0.426 - 1.41 (0.944, 0.998)	0.313 - 2.457
Iron	4.20 ± 0.24 3.24 - 6.26	4.25 ± 0.24 3.45 - 5.76 (0.546, 0.747)	3.43 - 6.45	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	387 ± 18 291 - 487	384 ± 18 282 - 488 (0.420, 0.679)	285 - 470	391 ± 19 250 - 494	388 ± 19 283 - 498 (0.278, 0.945)	340 - 493.12
Manganese	1.59 ± 0.10 1.17 - 2.36	1.35 ± 0.10 0.968 - 2.20 (**0.002**, **0.021**)	0.983 - 2.28	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	0.039 ± 0.012 0.00412 - 0.107	0.036 ± 0.012 0.00427 - 0.106 (0.460, 0.702)	0.00326 - 0.122	0.036 ± 0.011 0.00557 - 0.0980	0.034 ± 0.011 0.00539 - 0.0999 (0.331, 0.945)	NR
Phosphorus	652 ± 42 491 - 924	633 ± 42 469 - 921 (0.079, 0.259)	460 - 901	655 ± 45 412 - 936	649 ± 45 472 - 933 (0.378, 0.945)	482.54 - 991.57
Potassium	1078 ± 23 958 - 1230	1055 ± 23 937 - 1190 (0.106, 0.331)	938 - 1290	1074 ± 23 939 - 1200	1070 ± 23 934 - 1190 (0.613, 0.945)	960 - 1448.35
Selenium (ppb Dry Weight)	110 ± 30 <LOQ - 382	122 ± 30 <LOQ - 378 (0.379, 0.642)	<LOQ - 676	194 ± 95 <LOQ - 2010	180 ± 95 <LOQ - 1070 (0.670, 0.945)	NR
Sodium	123 ± 8 77.5 - 178	111 ± 8 64.7 - 188 (0.249, 0.577)	73.9 - 192	128 ± 9 78.7 - 207	136 ± 9 75.3 - 192 (0.187, 0.945)	5.4 - 740
Sulfur	495 ± 67 305 - 952	535 ± 67 311 - 1850 (0.541, 0.747)	331 - 847	520 ± 74 279 - 1430	499 ± 74 303 - 1180 (0.734, 0.945)	NR
Zinc	3.36 ± 0.13 2.63 - 3.93	3.47 ± 0.13 2.47 - 5.29 (0.290, 0.591)	2.77 - 4.26	3.51 ± 0.21 2.33 - 5.53	3.48 ± 0.21 2.23 - 5.94 (0.821, 0.998)	2.70 - 5.95

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

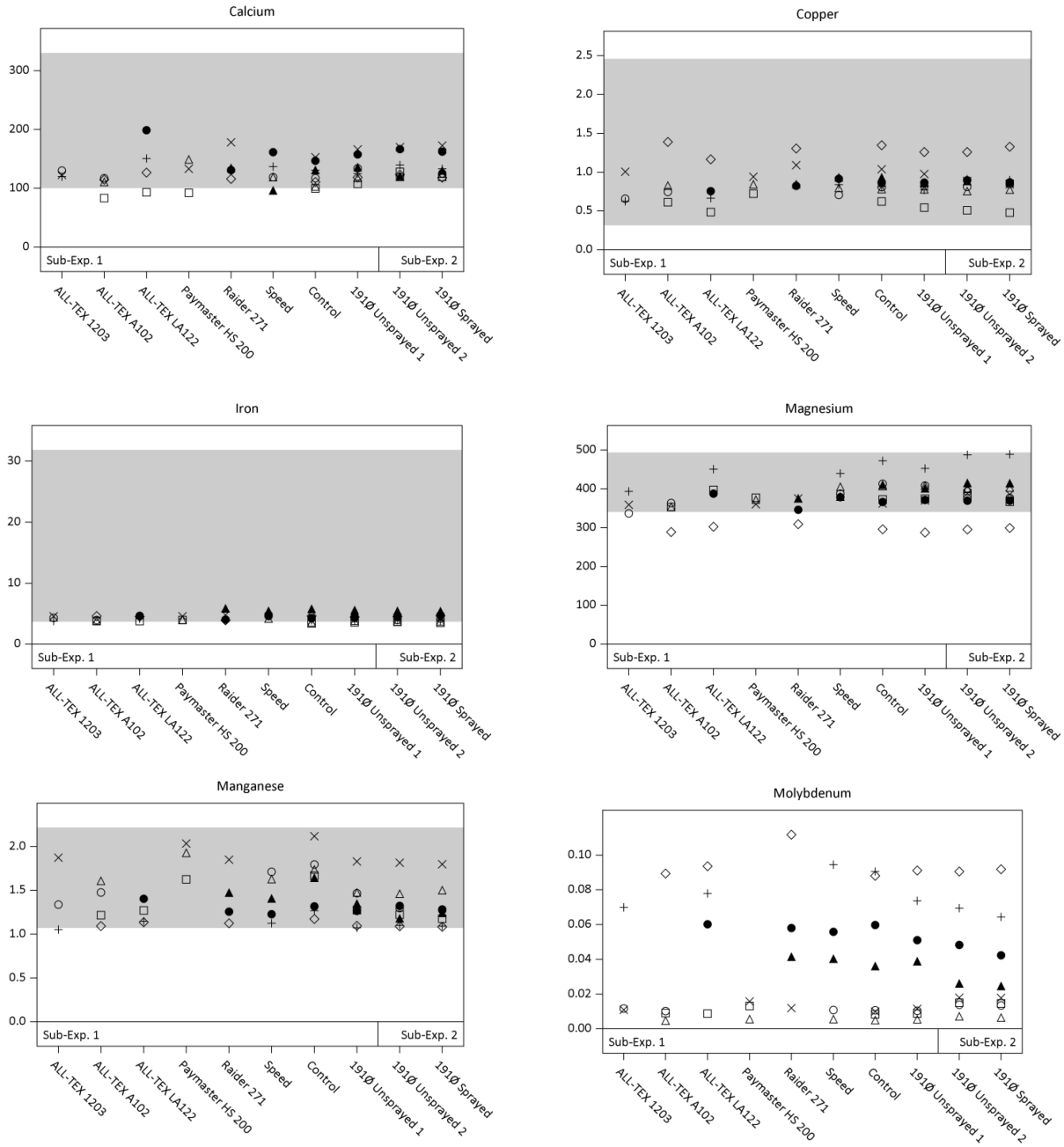


Figure 42. Minerals in Isoline, DAS-8191Ø-7 and Reference Variety CottonseedY axis: selenium = ppb dry weight, all others = mg/100 g dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D plus 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 (where available).

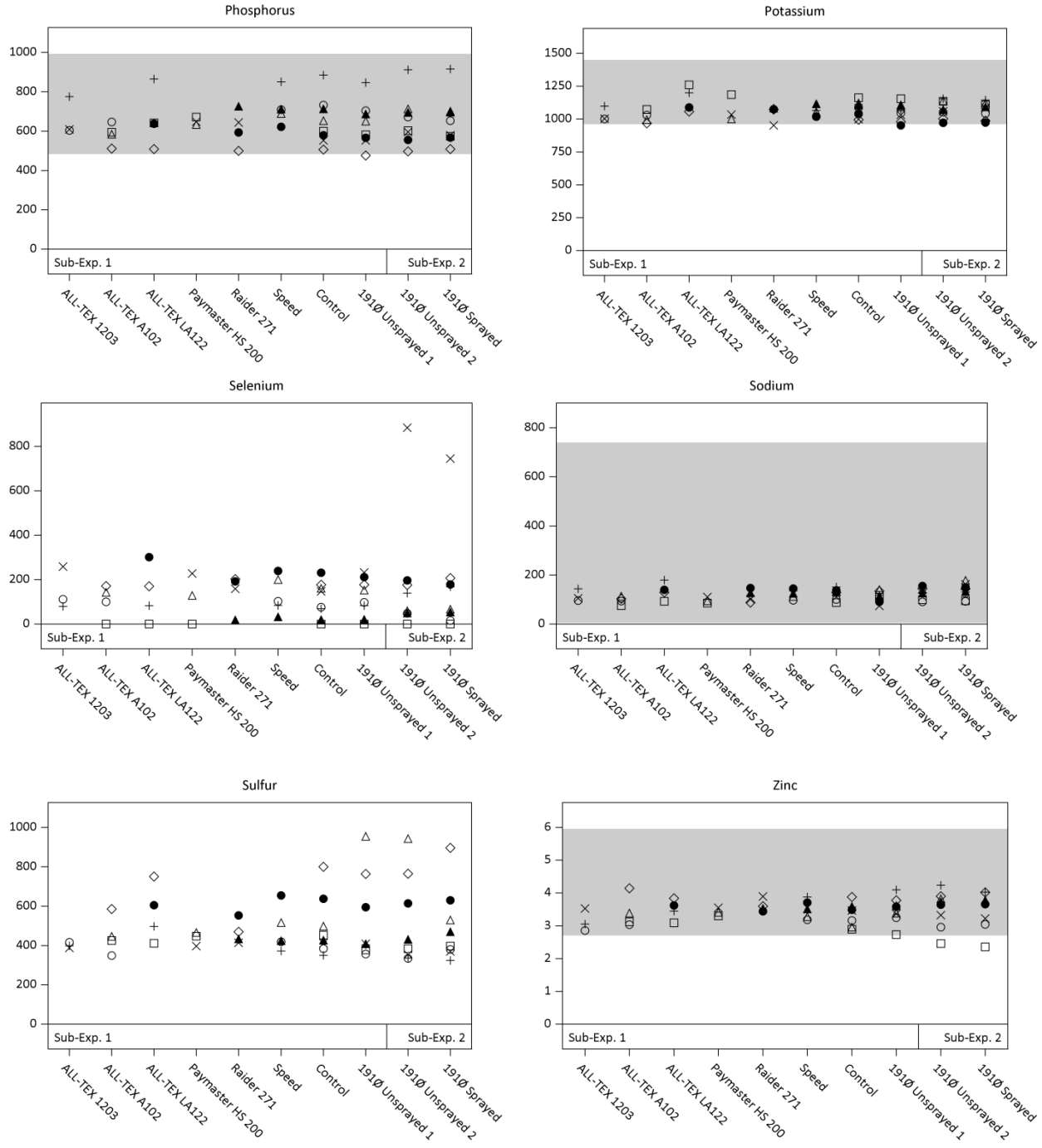


Figure 42. **Minerals in Isoline, DAS-8191Ø-7, and Reference Variety Cottonseed** (continued)
Y axis: selenium = ppb dry weight, all others = mg/100 g dry weight. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 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 plus 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 (where available).

6.3.3. Sub-experiment One - Amino Acid Analysis of Seed

Results from the combined site analysis of the amino acid composition for non-transgenic isoline (control) and DAS-8191Ø-7 cottonseed are provided in Table 14 and Figure 43. 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 non-sprayed 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 non-sprayed. In addition, mean results for all eighteen DAS-8191Ø-7 non-sprayed 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 isoline, supporting the findings of composition equivalence of DAS-8191Ø-7 cotton to non-transgenic cotton.

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

Analytical Component (% Total Amino Acids)	Isoline (Control) Mean ± SE Min - Max	DAS-8191Ø-7 Non-sprayed Mean ± SE Min - Max (P-Val., Adj. P)[2]	Reference Variety Range Min - Max	DAS-8191Ø-7 Non-sprayed Mean ± SE Min - Max	DAS-8191Ø-7 Sprayed[1] Mean ± SE Min - Max (P-Val., Adj. P)[2]	Literature Range Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
Alanine	4.44 ± 0.03 4.29 - 4.598	4.45 ± 0.03 4.22 - 4.741 (0.705, 0.815)	4.18 - 4.656	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	12.64 ± 0.16 11.87 - 13.73	12.49 ± 0.16 11.50 - 13.67 (**0.020**, 0.096)	11.57 - 13.67	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	10.09 ± 0.15 9.67 - 11.03	10.25 ± 0.15 9.75 - 12.03 (0.349, 0.642)	9.59 - 11.42	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	1.785 ± 0.040 1.563 - 2.126	1.833 ± 0.040 1.529 - 2.214 (0.145, 0.427)	1.593 - 2.339	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	20.11 ± 0.13 19.17 - 20.73	20.07 ± 0.13 18.58 - 20.72 (0.701, 0.815)	19.44 - 21.23	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	4.42 ± 0.04 4.14 - 4.61	4.41 ± 0.04 4.081 - 4.546 (0.949, 0.969)	4.10 - 4.594	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	2.841 ± 0.016 2.695 - 3.048	2.868 ± 0.016 2.604 - 3.001 (0.267, 0.583)	2.651 - 3.077	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	3.62 ± 0.02 3.284 - 3.790	3.61 ± 0.02 3.391 - 3.799 (0.746, 0.847)	3.180 - 3.817	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	6.30 ± 0.02 6.15 - 6.49	6.31 ± 0.02 6.18 - 6.45 (0.702, 0.815)	6.04 - 6.54	6.34 ± 0.04 6.15 - 6.60	6.32 ± 0.04 6.14 - 6.60 (0.153, 0.945)	6.03 - 8.11
Lysine	4.69 ± 0.06 4.36 - 5.09	4.73 ± 0.06 4.44 - 5.107 (0.254, 0.577)	4.27 - 5.03	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	1.640 ± 0.023 1.406 - 1.801	1.625 ± 0.023 1.460 - 1.774 (0.464, 0.702)	1.339 - 1.785	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	5.70 ± 0.06 5.49 - 6.12	5.68 ± 0.06 5.201 - 6.01 (0.504, 0.726)	5.44 - 6.02	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	4.04 ± 0.01 3.908 - 4.116	4.04 ± 0.01 3.895 - 4.187 (0.983, 0.983)	3.780 - 4.192	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	4.63 ± 0.04 4.352 - 4.88	4.63 ± 0.04 4.32 - 4.875 (0.878, 0.925)	4.26 - 5.05	4.63 ± 0.03 4.354 - 4.915	4.63 ± 0.03 4.291 - 4.928 (0.897, 0.998)	4.15 - 5.87

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

Table 14. Combined Site Analysis Results for Amino Acids in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges (continued)

Analytical Component	Isoline (Control)	DAS-8191Ø-7 Non-sprayed	Reference Variety Range		DAS-8191Ø-7 Non-sprayed	DAS-8191Ø-7 Sprayed[1]	Literature Range
(% Total Amino Acids)	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max		Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max
	Sub-Experiment 1 (No Herbicide)				**Sub-Experiment 2** (Herbicide Applied)		
Threonine	3.54 ± 0.03 3.312 - 3.705	3.54 ± 0.03 3.291 - 3.684 (0.849, 0.911)	3.186 - 3.756		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	1.433 ± 0.019 1.240 - 1.592	1.422 ± 0.019 1.267 - 1.651 (0.557, 0.747)	1.295 - 1.677		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	3.335 ± 0.015 3.201 - 3.457	3.313 ± 0.015 3.180 - 3.422 (0.155, 0.436)	3.190 - 3.459		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	4.75 ± 0.02 4.43 - 4.96	4.74 ± 0.02 4.50 - 4.92 (0.426, 0.679)	4.36 - 5.02		4.76 ± 0.04 4.36 - 5.023	4.74 ± 0.04 4.45 - 5.059 (0.610, 0.945)	4.49 - 6.24

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

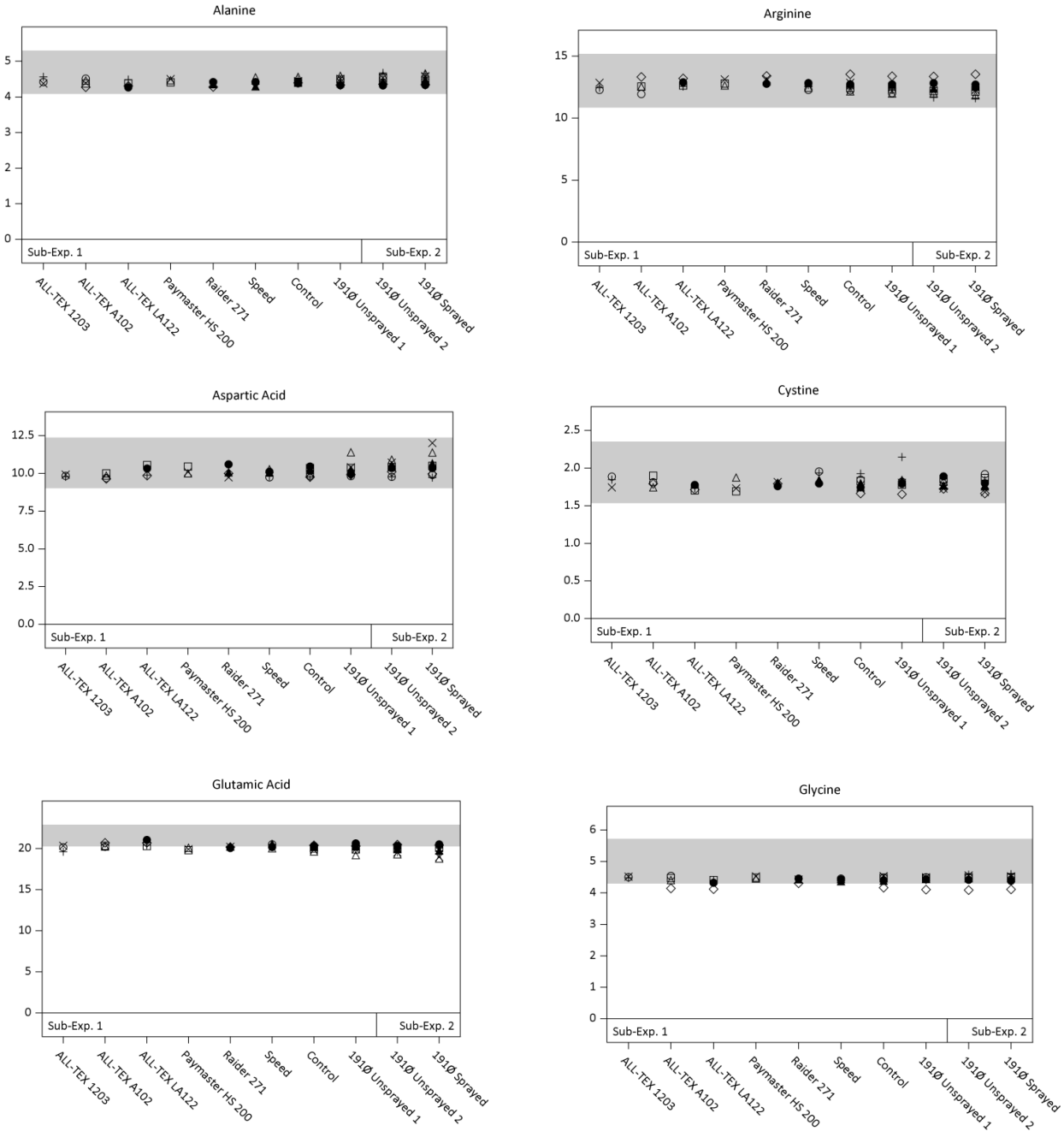


Figure 43. Percent Total Amino Acids in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed Y axis: % Total Amino Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D plus 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.

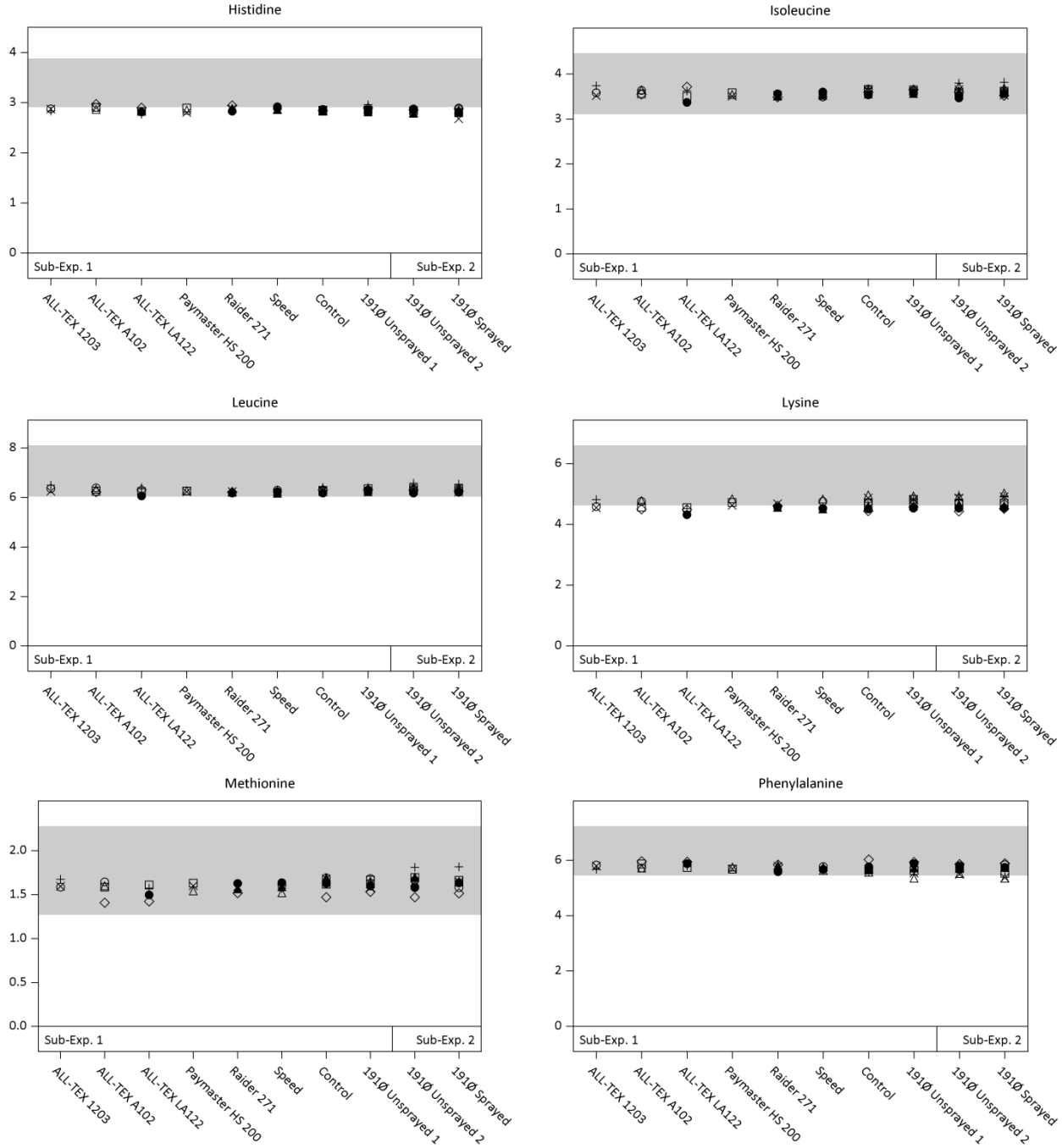


Figure 43. Percent Total Amino Acids in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed (continued)

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 plus 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

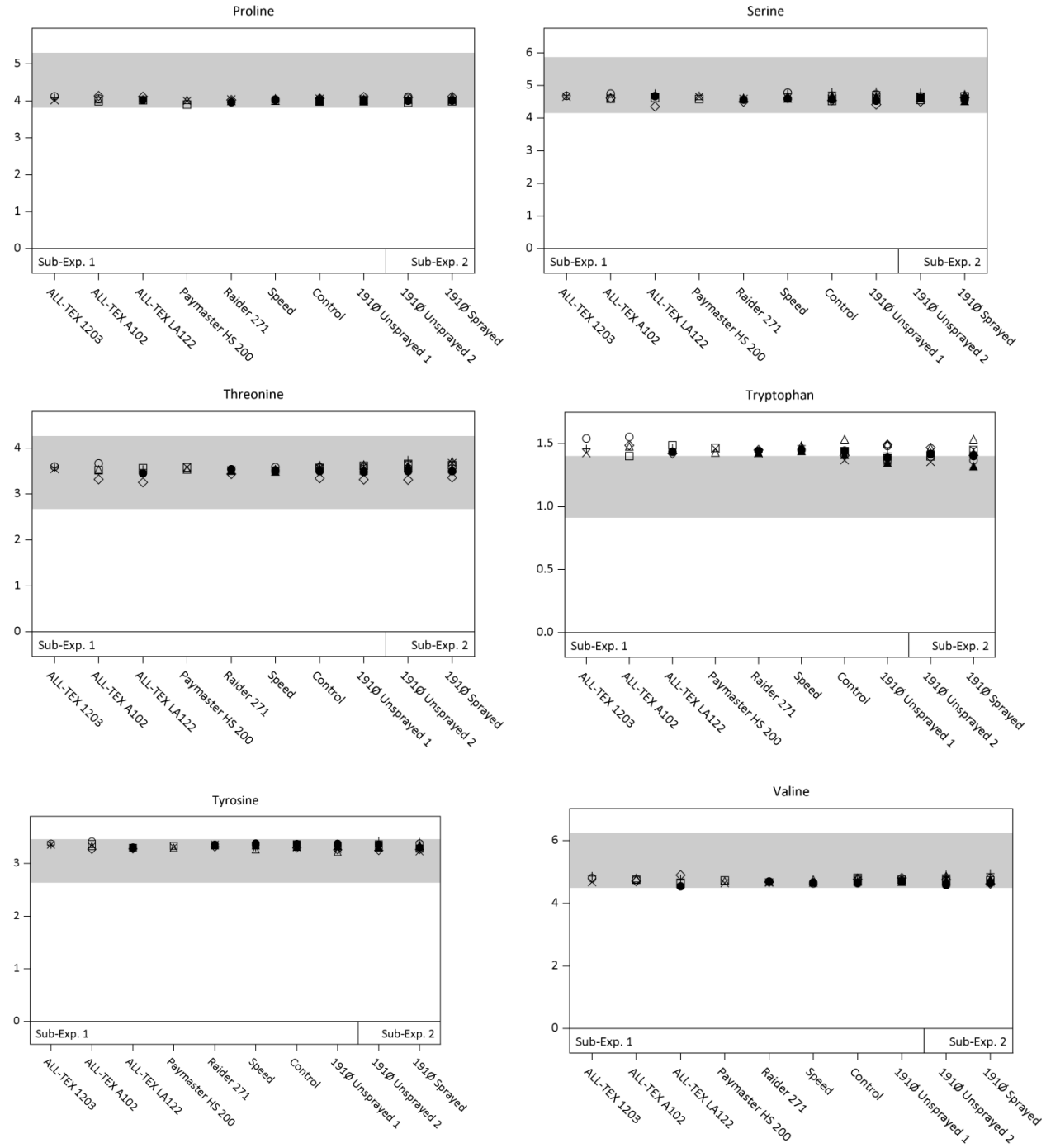


Figure 43. Percent Total Amino Acids in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed (continued)

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 plus 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

6.3.4. Sub-experiment One - Fatty Acid Analysis of Seed

Results from the combined site analysis of the fatty acid composition (22 analytes) for non-transgenic isoline (control) and DAS-8191Ø-7 cottonseed are provided in Table 15 and Figure 44. Of the 22 fatty acids analyzed (Table 15), thirteen were excluded from the analysis because the majority of the results were less than the LOQ (Table 11 and Table 15). Mean results in DAS-8191Ø-7 non-sprayed 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 difference between the mean values for non-sprayed DAS-8191Ø-7 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 0.4105 – 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 isoline were not considered to be meaningful from a food, nutritional or safety perspective because they were small, not consistently reproducible across the individual sites and the mean DAS-8191Ø-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 non-sprayed and the non-transgenic isoline 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 isoline were not considered to be meaningful from a food, nutritional or safety 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 non-sprayed fell within the reference variety ranges and literature ranges. Statistical analyses found no FDR-adjusted differences between the levels of these nutrient components in cottonseed from DAS-8191Ø-7 and the non-transgenic isoline, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

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

Analytical Component (% Total Fatty Acid)	Isoline (Control)	DAS-8191Ø-7 Non-sprayed	Reference Variety Range	DAS-8191Ø-7 Non-sprayed	DAS-8191Ø-7 Sprayed[1]	Literature Range
	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
8:0 Caprylic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
10:0 Capric	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
12:0 Lauric	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
14:0 Myristic	0.720 ± 0.041 0.545 - 0.907	0.657 ± 0.041 0.489 - 0.872 (**<0.001**, **0.002**)	0.4324 - 1.046	0.651 ± 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 <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
15:0 Pentadecanoic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	0.050 - 0.481
15:1 Pentadecenoic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
16:0 Palmitic	22.55 ± 0.58 20.37 - 25.67	22.26 ± 0.58 20.04 - 26.12 (**0.027**, 0.114)	18.76 - 26.07	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	0.494 ± 0.022 0.4105 - 0.639	0.460 ± 0.022 0.3917 - 0.588 (**<0.001**, **0.003**)	0.3787 - 0.636	0.454 ± 0.023 0.3776 - 0.587	0.451 ± 0.023 0.3728 - 0.592 (0.203, 0.945)	0.464 - 1.190
17:0 Heptadecanoic	NA <LOQ - 0.0990	NA <LOQ - 0.1046	<LOQ - 0.1085	NA <LOQ - 0.1044	NA <LOQ - 0.1030	0.092 - 0.119
17:1 Heptadecenoic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
18:0 Stearic	2.311 ± 0.068 1.951 - 2.685	2.334 ± 0.068 1.943 - 2.645 (0.373, 0.642)	1.801 - 2.962	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	14.84 ± 0.35 13.74 - 17.16	13.95 ± 0.35 12.76 - 16.50 (**<0.001**, **0.001**)	12.93 - 17.09	13.82 ± 0.34 12.54 - 16.50	13.80 ± 0.34 12.64 - 16.08 (0.698, 0.945)	12.8 - 25.3

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

Table 15. Combined Site Analysis Results for Fatty Acids in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges (continued)

Analytical Component (% Total Fatty Acid)	Isoline (Control)	DAS-8191Ø-7 Non-sprayed	Reference Variety Range	DAS-8191Ø-7 Non-sprayed	DAS-8191Ø-7 Sprayed[1]	Literature Range
	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
18:2 Linoleic	58.5 ± 0.8 54.4 - 61.1	59.7 ± 0.8 54.9 - 62.5 (<**0.001**, **0.003**)	52.36 - 63.9	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	0.2032 ± 0.0083 0.1733 - 0.2412	0.2117 ± 0.0083 0.1782 - 0.2991 (0.249, 0.577)	0.1460 - 0.2567	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 <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	0.097 - 0.232
20:0 Arachidic	0.2509 ± 0.0105 0.2088 - 0.2949	0.2492 ± 0.0105 0.2029 - 0.3063 (0.489, 0.722)	0.1855 - 0.3242	0.2530 ± 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 <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	0.095 - 0.098
20:2 Eicosadienoic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
20:3 Eicosatrienoic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
20:4 Arachidonic	NA <LOQ	NA <LOQ	<LOQ	NA <LOQ	NA <LOQ	NR
22:0 Behenic	0.1373 ± 0.0060 0.1119 - 0.1692	0.1341 ± 0.0060 0.1057 - 0.1693 (0.181, 0.464)	0.1035 - 0.1749	0.1358 ± 0.0071 0.1101 - 0.1757	0.1386 ± 0.0071 0.1092 - 0.1726 (0.373, 0.945)	0.104 - 0.295

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

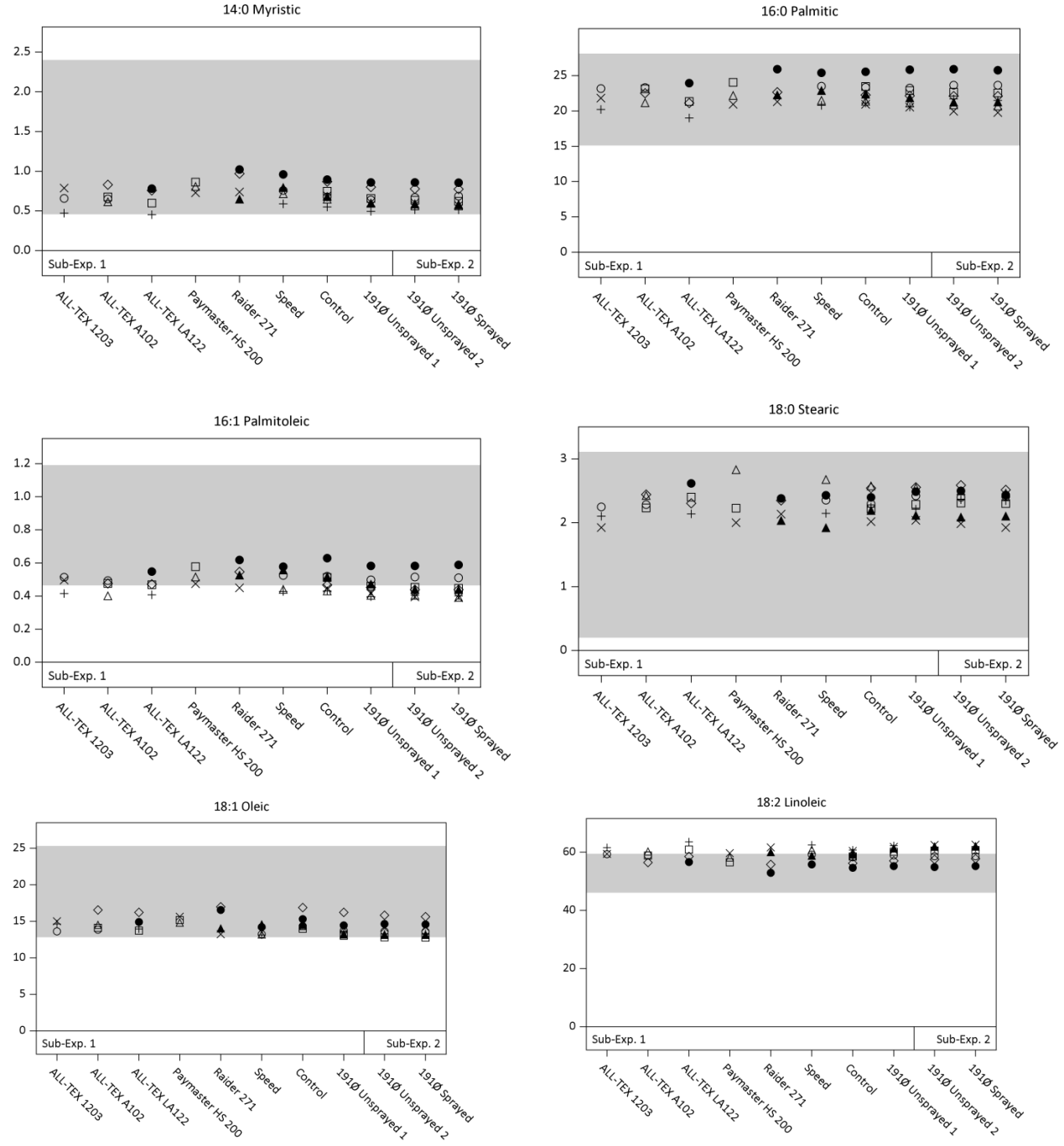


Figure 44. Percent Total Fatty Acids in Isoline, DAS-8191Ø-7 and Reference Variety CottonseedY axis: % Total Fatty Acid. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D plus 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.

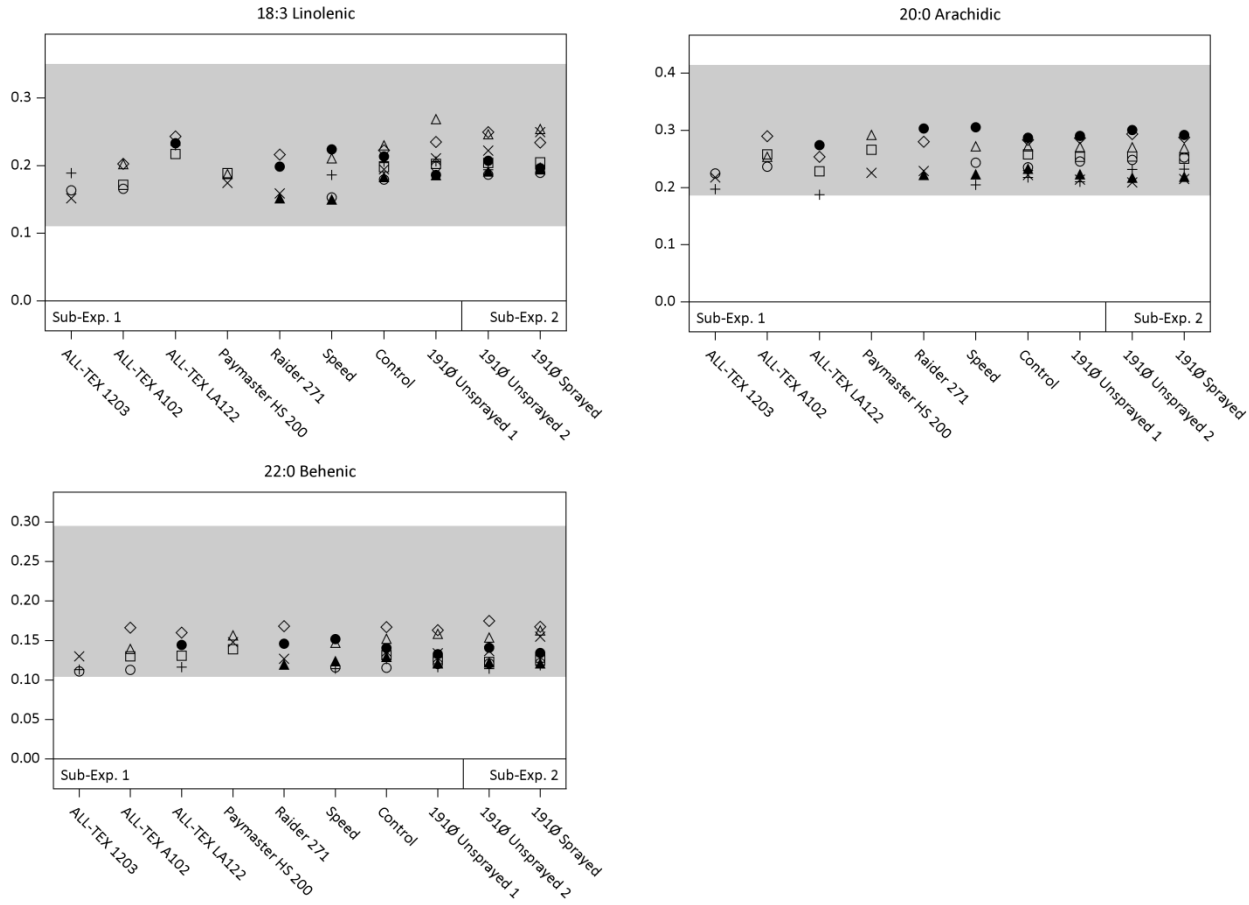


Figure 44. Percent Total Fatty Acids in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed (continued)

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 plus 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.

6.3.5. Sub-Experiment One - Vitamin Analysis of Seed

Results from the combined site analysis of the vitamin composition from non-transgenic isoline (control) and DAS-8191Ø-7 cottonseed are provided in Table 16 and Figure 45. Seven vitamin analytes were measured including vitamin E, A, B1, B2, B3, B6, and B9 (Table 16). 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 non-sprayed 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 non-sprayed 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 16. Combined Site Analysis Results for Vitamins in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (mg/kg Dry Weight)	Isoline (Control)	DAS-8191Ø-7 non-sprayed	Reference Variety Range	DAS-8191Ø-7 Non-sprayed	DAS-8191Ø-7 Sprayed[1]	Literature Range
	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
Alpha Tocopherol (Vitamin E)	90 ± 11 30.3 - 134	88 ± 11 26.7 - 136 (0.612, 0.803)	31.1 - 151	90 ± 10 20.4 - 135	89 ± 10 27.6 - 129 (0.360, 0.945)	70.825 - 197.243
Vitamin A (Beta Carotene)	NA <LOQ - 0.264	NA <LOQ - 0.323	<LOQ - 0.267	NA <LOQ - 0.249	NA <LOQ - 0.238	NR
Vitamin B1 (Thiamine HCl)	10.3 ± 0.4 7.52 - 12.6	10.3 ± 0.4 8.28 - 13.2 (0.953, 0.969)	5.54 - 14.7	10.4 ± 0.5 6.14 - 13.4	10.4 ± 0.5 7.42 - 15.4 (0.912, 0.998)	NR
Vitamin B2 (Riboflavin)	6.2 ± 0.5 3.52 - 9.95	6.3 ± 0.5 3.82 - 9.82 (0.813, 0.888)	3.44 - 9.52	6.6 ± 0.5 3.69 - 12.5	6.6 ± 0.5 3.97 - 10.0 (0.998, 0.998)	NR
Vitamin B3 (Niacin)	27.8 ± 1.2 21.7 - 35.6	28.5 ± 1.2 22.7 - 35.3 (0.315, 0.620)	20.4 - 36.8	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)	3.83 ± 0.09 3.15 - 4.49	3.72 ± 0.09 3.10 - 4.54 (0.176, 0.464)	2.84 - 5.12	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)	1.66 ± 0.07 1.14 - 2.38	1.67 ± 0.07 1.17 - 2.26 (0.806, 0.888)	1.10 - 2.40	1.56 ± 0.09 0.872 - 2.31	1.64 ± 0.09 1.15 - 2.29 (0.160, 0.945)	NR

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

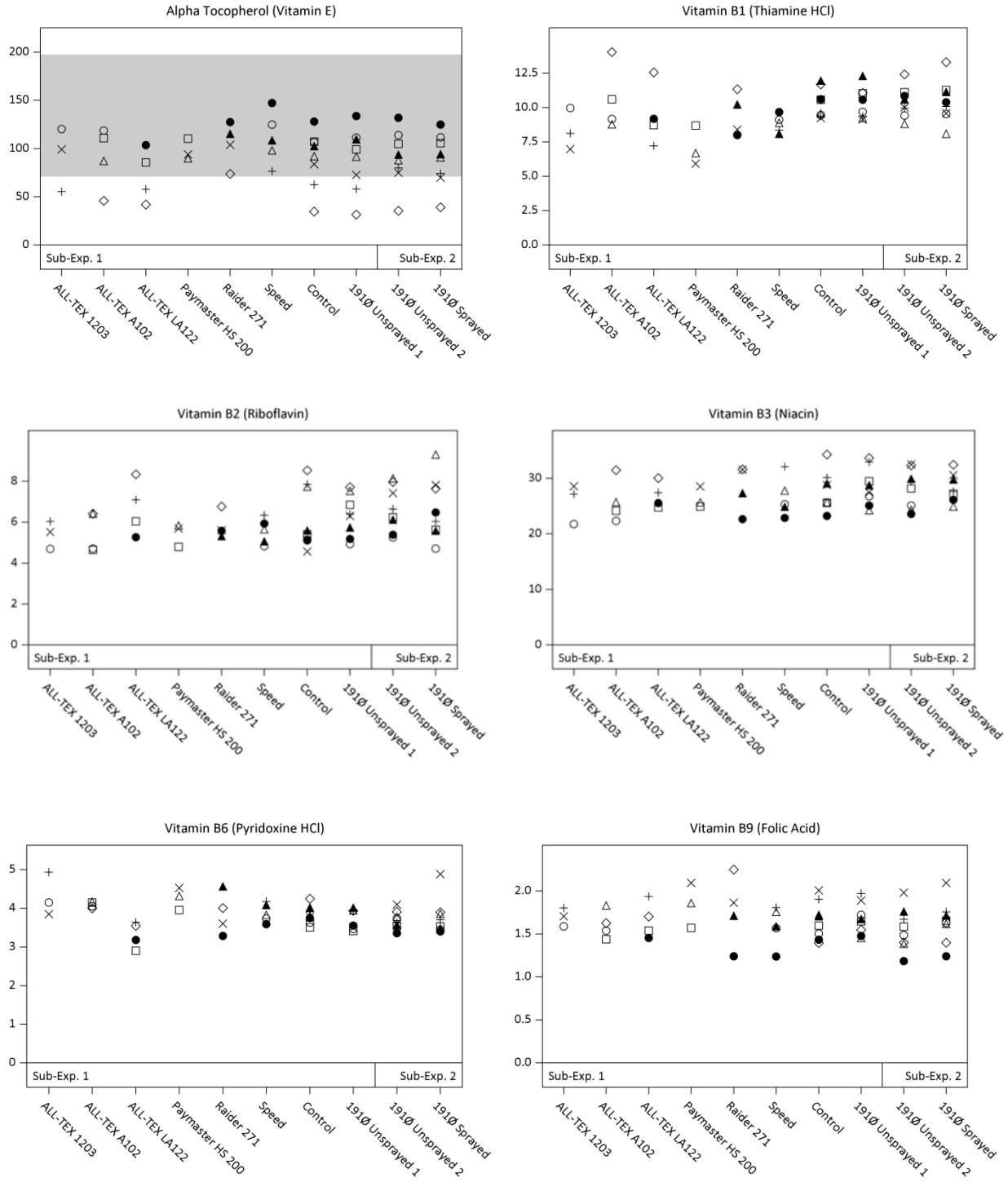


Figure 45. Vitamins in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed

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 plus 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.

6.3.6. 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 non-transgenic isoline (control) and DAS-8191Ø-7 cottonseed are provided in Table 17 and Figure 46. Mean results in DAS-8191Ø-7 non-sprayed 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 non-sprayed 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 non-sprayed and the non-transgenic isoline 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 isoline were not considered to be meaningful from a food, nutritional or safety perspective: differences were 1) small, 2) less than the variability seen in the isoline, and 3) 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 non-sprayed 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 isoline, supporting the findings of composition equivalence of DAS-8191Ø-7 to non-transgenic cotton.

Table 17. Combined Site Analysis Results for Anti-Nutrients in Isoline, DAS-8191Ø-7 and Reference Variety Cottonseed and Literature Ranges

Analytical Component (Units)	Isoline (Control)	DAS-8191Ø-7 Non-sprayed	Reference Variety Range	DAS-8191Ø-7 Non-sprayed	DAS-8191Ø-7 Sprayed[1]	Literature Range
	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max	Mean ± SE Min - Max	Mean ± SE Min - Max (P-Val., Adj. P)[2]	Min - Max
	Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)		
Dihydrosterculic Acid (% Total Fatty Acid)	0.205 ± 0.007 0.150 - 0.247	0.214 ± 0.007 0.174 - 0.261 (0.074, 0.259)	0.153 - 0.325	0.216 ± 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)	0.524 ± 0.023 0.403 - 0.645	0.577 ± 0.023 0.426 - 0.762 (**0.003**, **0.029**)	0.402 - 0.854	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)	0.275 ± 0.011 0.173 - 0.447	0.297 ± 0.011 0.209 - 0.358 (0.077, 0.259)	0.196 - 0.440	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)	0.96 ± 0.06 0.593 - 1.36	0.83 ± 0.06 0.556 - 1.17 (**0.010**, 0.059)	0.492 - 1.28	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)	1.08 ± 0.05 0.829 - 1.44	0.95 ± 0.05 0.719 - 1.19 (**0.001**, **0.010**)	0.551 - 1.41	0.92 ± 0.05 0.519 - 1.31	0.93 ± 0.05 0.624 - 1.32 (0.867, 0.998)	0.547 - 1.522

[1] Sprayed with 2,4-D and glufosinate
[2] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values

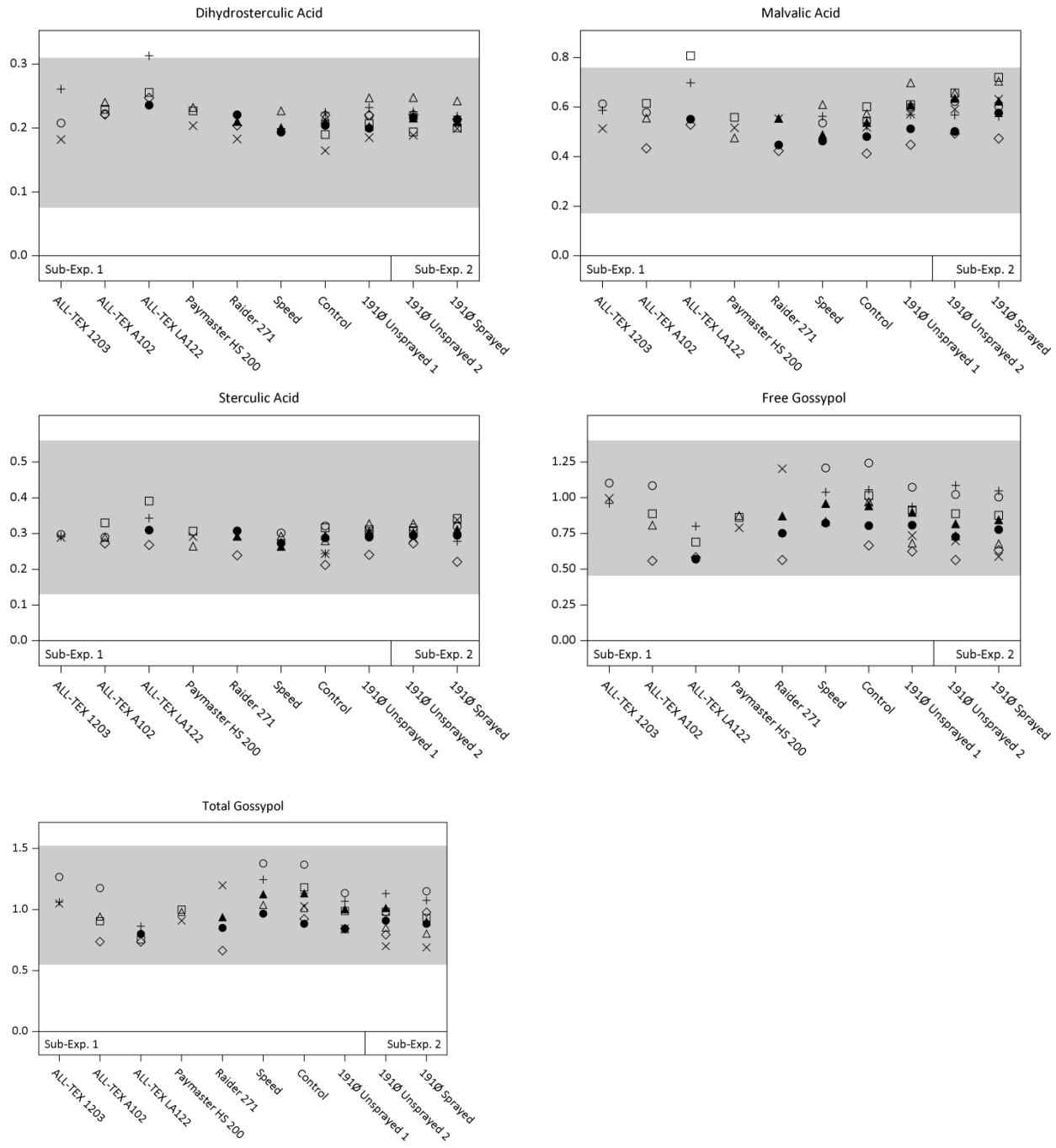


Figure 46. Anti-Nutrients in Isoline, DAS-8191Ø-7 and Reference Variety CottonseedY axis: see Table 17 for specific values. Reference Variety cottonseed: All-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271 and Speed. 191Ø Unsprayed 1 = DAS-8191Ø-7 unsprayed in sub-experiment 1, 191Ø Unsprayed 2 = DAS-8191Ø-7 unsprayed in sub-experiment 2, 191Ø Sprayed = DAS-8191Ø-7 sprayed with 2,4-D plus 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.

6.3.7. Sub-Experiment Two - Composition Results

The comparisons between sprayed and non-sprayed 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 12 - Table 17 and Figure 41 - Figure 46). In addition, comparisons of non-sprayed DAS-8191Ø-7 cotton in sub-experiment two with non-sprayed DAS-8191Ø-7 cotton in sub-experiment one indicated no statistically significant differences for all 59 analytes. Based on these comparisons, sprayed DAS-8191Ø-7 cotton in sub-experiment two is compositionally equivalent to non-sprayed DAS-8191Ø-7 cotton in sub-experiment one and indicates that spraying DAS-8191Ø-7 with 2,4-D plus glufosinate has no significant effect on cottonseed composition. Based on these results, it is concluded that DAS-8191Ø-7 (non-sprayed or sprayed with 2,4-D plus glufosinate) cottonseed is compositionally equivalent to non-transgenic cottonseed.

6.4. Composition Summary

Field trials were conducted at eight U.S. field sites in 2012 to produce cottonseed samples of DAS-8191Ø-7 cotton (non-sprayed and sprayed with 2,4-D plus glufosinate), non-transgenic isoline (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, 2009). 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 11).

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 non-sprayed cotton and the non-transgenic isoline. 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 isoline and DAS-8191Ø-7 non-sprayed and the non-transgenic control. However, the observed differences between the seven analytes in DAS-8191Ø-7 and the non-transgenic isoline were not considered to be meaningful from a food, nutritional or safety perspective because the differences between DAS-8191Ø-7 and the non-transgenic isoline were small, were less than the variability seen in the non-transgenic isoline 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 non-sprayed and DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate, which indicates that

spraying DAS-8191Ø-7 with 2,4-D plus glufosinate has no significant effect on cottonseed composition. Based on these results, it is concluded that DAS-8191Ø-7 (non-sprayed or sprayed with 2,4-D plus glufosinate) cotton is compositionally equivalent to non-transgenic cotton.

6.5. Conclusions

Overall, for DAS-8191Ø-7, mean analyte values observed to be significantly different from those of the non-transgenic isoline (control) were generally shown to be of small relative magnitudes. All combined-site mean values and individual site mean values of DAS-8191Ø-7 for all analytes measured were within the context of the natural variability of reference cotton line composition data and/or published in the scientific literature.

Additionally, no statistically significant differences were detected between DAS-8191Ø-7 non-sprayed and DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate, which indicates that spraying DAS-8191Ø-7 with 2,4-D plus glufosinate 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 cottonseed and therefore the food and feed safety and nutritional quality of this product is comparable to that of commercially cultivated cotton.

7. Phenotypic, Agronomic and Ecological Characteristics

This section provides a comparative assessment of the phenotypic, agronomic and ecological characteristics of DAS-8191Ø-7 cotton compared to non-transgenic isoline (control). As part of 7 CFR § 340.6, a detailed description of the regulated article phenotype is requested as part of the petition for determination of nonregulated status to substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived. A list of all DAS-8191Ø-7 cotton field evaluations conducted under USDA permit or notification is provided in Appendix 9.

The characterization of DAS-8191Ø-7 cotton encompasses 1) vegetative growth, 2) ecological (insect and disease) and 3) seed germination, dormancy, and emergence. The data were evaluated by individuals familiar with the production and evaluation of cotton. In each assessment, DAS-8191Ø-7 cotton was compared to an appropriate, near isogenic isoline (control) that does not contain the *add-12* or *pat* genes. The data collected here support a conclusion that DAS-8191Ø-7 cotton is substantially equivalent to non-transgenic cotton with the exception of the introduced and expression of *aad-12* and *pat* genes and therefore no more likely to pose a plant pest risk or have a significant environmental impact compared to non-transgenic cotton.

7.1. Study Design

Agronomic studies used the same field studies and plots as those for composition assessment of DAS-8191Ø-7 cotton, as described in detail in section 6.1.1. Briefly, field trials were conducted at eight U.S. sites in 2012 to evaluate phenotypic, agronomic and ecological characteristics of DAS-8191Ø-7 cotton (non-sprayed and sprayed with 2,4-D plus glufosinate), near isogenic non-transgenic isoline (control) (Figure 4) and reference variety cotton plants. For the reference lines, six unique reference lines (ALL-TEX 1203, ALL-TEX A102, ALL-TEX LA122, Paymaster HS 200, Raider 271, and Speed) were included across all sites of the field production with three varieties per site.

Because non-transgenic cotton plants (*e.g.*, isoline 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 in which 2,4-D was applied. Sub-experiment one was not sprayed and contained the isoline, reference varieties and one entry of DAS-8191Ø-7 cotton. Sub-experiment two contained two entries of only DAS-8191Ø-7 cotton; one not sprayed and one sprayed with 2,4-D plus glufosinate. The objective of sub-experiment one was to determine if non-sprayed DAS-8191Ø-7 cotton is agronomically equivalent to non-transgenic cotton, and the objective of sub-experiment two was to determine if applying 2,4-D plus glufosinate impacts the agronomic characteristics of DAS-8191Ø-7 cotton.

The entries were arranged in a randomized complete block design within each sub-experiment. The sub-experiments were separated by 100 ft (30 m) to prevent isoline and reference entries in sub-experiment one from potential injury by 2,4-D drift from applications in sub-experiment two. Both sub-experiments were present at all field testing sites. Randomization of entries within blocks was unique at each field testing site. Test, isoline, 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 cotton.

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. 2,4-D (GF-2654) and glufosinate (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. Actual application rates ranged from 1108 – 1142 g ae/ha 2,4-D and 589 – 622 g ai/ha glufosinate for the 3 node application and 1061 – 1141 g ae/ha 2,4-D and 564 – 621 g ai/ha glufosinate for the 6 node application.

7.2. Data Collection and Statistical Analysis

Evaluations of nine phenotypic, agronomic and ecological characteristics (early population, seedling vigor, flower initiation, nodes above white flower, plant height, percent open bolls, lint yield, disease incidence and insect damage) were conducted to investigate the equivalency of DAS-8191Ø-7 (non-sprayed and sprayed with 2,4-D plus glufosinate) cotton to non-transgenic cotton. In all cases, the agronomic data was collected and evaluated by individuals familiar with the production and evaluation of cotton. Details regarding the agronomic data collection of the nine agronomic characteristics measured are described in Table 18.

Analysis of variance was conducted across field testing sites for agronomic data using a mixed model (SAS Institute Inc., 2011). Entry was considered a fixed effect; 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 ($\alpha = 0.05$).

Multiplicity occurs when a number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise P-values can be high ($1\text{-}0.95^{\text{number of comparisons}}$). There were nine analytes included in the combined site statistical analysis; therefore, nine comparisons were made in each sub-experiment in this study (DAS-8191Ø-7 non-sprayed compared with the control in sub-experiment one and DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate compared with DAS-8191Ø-7 non-sprayed in sub-experiment two). As a result, the probability of declaring one or more false differences based on unadjusted P-values was 36.975% ($1\text{-}0.95^{9}$) due to multiplicity in each sub-experiment (or >60% across both sub-experiments).

One method to account for 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 in a study. An alternative 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 and Hochberg, 1995), which is a commonly used approach in studies examining transgenic crops (Herman *et al.*, 2007; Coll *et al.*, 2008; Huls *et al.*, 2008; Jacobs

et al., 2008; Stein *et al.*, 2009; Herman *et al.*, 2010). Therefore, the P-values from the contrasts were each adjusted using the FDR method to improve discrimination of true differences among treatments from random effects (false positives). Differences were considered significant if the FDR-adjusted P-value was less than 0.05.

Table 18. Measured Agronomic Characteristics and Sample Collection Details

Evaluation Category	Evaluation Timing	Description of Data	Evaluation Description (scale/measurements)
Early Population (Stand Count)	28 days after planting	Total number of emerged plants in the center two rows of each plot	Plants were considered emerged when the cotyledons had assumed an erect posture and were completely unrolled.
Seedling Vigor	28 days after planting	Visual estimate of the plant vigor in each plot	0-100% rating scale; 0 = all plants were dead, 10 = short plants with small, thin leaves, 100 = all plants were tall with robust leaves. Evaluation was not based on growth of control entries and did not consider germination/ emergence (stand count).
Flower Initiation	First white flower	1) Date when approximately 50% of plants in plot produced at least one white flower 2) Number of days from planting and the date when 50% of plants in plot have reached first white flower	Visual approximation of when 50% of the plants in each plot had produced one open white flower.
Nodes Above First White Flower	28 days after first white flower	Number of nodes above the uppermost node containing a first-position white flower on six plants per plot.	Recorded the number of the uppermost node and the total number of nodes on six cotton plants that contained a first-position white flower. The number of nodes above first white flower were calculated by subtracting the number of the uppermost node that contained a first-position white flower from the total number of nodes.
Plant Height	28 days after first white flower	Plant height from soil surface to terminal bud on six plants per plot	Recorded the height each plant in centimeters.
Percent Open Bolls	Maturity but prior to defoliation	1. Total number of bolls and the number of open bolls on six plants from each plot. 2. Calculated percent open bolls	1. Recorded the total number of bolls on six representative plants from each plot. Recorded the number of open bolls (bolls greater than 1 in.) on the same six plants. Cracked bolls were considered to be open. 2. Calculated percent open bolls: (Number of open bolls / total number of bolls)*100.
Lint Yield	Maturity	Weight of cotton lint from the center two rows of each plot.	Seedcotton from rows 2 and 3 of each plot was harvested and a sub-sample of the seedcotton yield was ginned. Gin turnout (lint/seedcotton) was multiplied by the seedcotton yield of each plot to determine lint yield per plot.
Disease Incidence	Peak bloom	Visual estimate of disease incidence	0-100%; Estimated % plant tissue/leaf area diseased over all plants in plot; did not record % of plants in plot that had detectable disease; 100% = all plant tissues in plot were diseased. Recorded type of disease.
Insect Damage	Peak bloom	Visual estimate of insect damage	0-100%; Estimated % plant tissue/leaf area damaged over all plants in plot; did not record % of plants in plot that had detectable damage; 100% = all plant tissues had feeding damage; recorded type of damage and type of insects if present.

7.2.1. Phenotypic and Agronomic Results

Seven agronomic characteristics were evaluated in this study: early population, seedling vigor, flower initiation, nodes above first white flower, plant height, percent open bolls and lint yield. A statistical analysis of agronomic data from the control and DAS-8191Ø-7 cotton entries across field testing sites was conducted. For each agronomic characteristic and entry, the least squares mean, standard error, and minimum and maximum values were reported (Table 19).

Table 19. Combined Site Analysis Results for Agronomic Characteristics

	Agronomic Characteristic (Units)	Isoline (Control)	DAS-8191Ø-7 Non-Sprayed	Reference Range	DAS-8191Ø-7 Non-Sprayed	DAS-8191Ø-7 Sprayed[1]
		Mean ± SE Min - Max[2]	Mean ± SE Min – Max[2] (P-Val., Adj. P)[3]	Min – Max[2]	Mean ± SE Min – Max[2] (P-Val., Adj. P)[3]	Mean ± SE Min – Max[2] (P-Val., Adj. P)[3]
		Sub-Experiment 1 (No Herbicide)			**Sub-Experiment 2** (Herbicide Applied)[1]	
	Early Population (Emerged Plants per Plot)	142 ± 10 91 – 194	129 ± 10 62 - 179 (0.050, 0.450)	4 - 209	137 ± 10 87 - 185	137 ± 10 84 - 179 (0.856, 0.963)
	Seedling Vigor (0 - 100% scale: 0% = short, thin plants, 100% = tall, robust plants)	85 ± 3 40 – 100	82 ± 3 62 - 100 (0.288, 0.555)	10 - 100	87 ± 3 60 - 100	84 ± 3 40 - 100 (**0.037**, 0.335)
	Flower Initiation (Days from Planting to First White Bloom)	62 ± 2 51 – 69	62 ± 2 51 - 76 (0.915, 0.995)	51 - 70	62 ± 2 51 - 70	62 ± 2 51 - 69 (0.642, 0.931)
	Nodes Above First White Flower (Number of Nodes)	8 ± 2 0 – 18	7 ± 2 0 - 17 (0.161, 0.555)	0 - 19	8 ± 2 0 - 17	8 ± 2 1 - 19 (0.625, 0.931)
	Plant Height (cm)	99 ± 9 48 – 158	99 ± 9 49 - 147 (0.774, 0.995)	45 - 164	103 ± 9 55 - 161	102 ± 9 60 - 163 (0.624, 0.931)
	Percent Open Bolls (Percent of Total Bolls)	65 ± 8 0 – 100	68 ± 8 17 - 100 (0.199, 0.555)	11 - 100	68 ± 8 15 - 100	68 ± 8 14 - 100 (0.989, 0.989)
	Lint Yield (kilograms per hectare)	762 ± 191 146 – 1991	762 ± 191 78 - 1891 (0.995, 0.995)	74 - 2225	745 ± 193 208 - 2187	687 ± 193 144 - 1585 (0.423, 0.931)
Ecological Eval.	**Disease Incidence**[4] (0 - 100%: 0% = no damage, 100% = all plants diseased)	0 ± 0 0 – 7	0 ± 0 0 - 5 (0.855, 0.995)	0 - 7	0 ± 0 0 - 3	0 ± 0 0 - 3 (0.724, 0.931)
Ecological Eval.	**Insect Damage**[4] (0 - 100%: 0% = no damage, 100% = all plants damaged)	1 ± 1 0 – 10	1 ± 1 0 - 5 (0.308, 0.555)	0 - 10	1 ± 1 0 - 5	1 ± 1 0 - 10 (0.270, 0.931)

[1] Sprayed with 2,4-D and glufosinate
[2] Min, Max value is an individual data point reported for a single test plot
[3] P-Val – unadjusted P-value, Adj. P – FDR Adjusted P-values
[4] Evaluation conducted at peak bloom

7.2.1.1. Comparison of the Isoline (Control) and Non-Sprayed DAS-8191Ø-7

For the assessment of the nine phenotypic, agronomic and environmental characteristics measured there were no statistically significant differences (Adj. P<0.05) observed between the control and non-sprayed DAS-8191Ø-7 entries (sub experiment 1); moreover, mean

results for non-sprayed DAS-8191Ø-7 entries fell within the reference variety ranges (Table 19). Therefore, non-sprayed DAS-8191Ø-7 was found to be agronomically equivalent to the isoline (control).

7.2.1.2. Comparison of Non-Sprayed and Sprayed DAS-8191Ø-7 Cotton

Comparisons between DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate and non-sprayed DAS-8191Ø-7 cotton (sub experiment 2) were not statistically significant (Adj. $P<0.05$) for the nine agronomic characteristics included in the combined site analysis based on FDR-adjusted P-values (Table 18).

The unadjusted P-value for seedling vigor in DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate was significant. Significant P-values for seedling vigor were identified at only one of the eight field locations. The relative magnitudes of the differences between the mean values for non-sprayed DAS-8191Ø-7 cotton vs. sprayed DAS-8191Ø-7 cotton for the combined site analysis were small (-3.45%). Mean results for DAS-8191Ø-7 sprayed with 2,4-D plus glufosinate fell within the reference variety ranges, and the FDR-adjusted P-value was insignificant; therefore, the statistically significant difference for seedling vigor based on the unadjusted P-value is not considered biologically meaningful. The agronomic characteristics of DAS-8191Ø-7 cotton sprayed with 2,4-D plus glufosinate are equivalent to non-sprayed DAS-8191Ø-7.

7.2.1.3. Phenotypic and Agronomics Conclusions

Phenotypic and agronomic analysis of DAS-8191Ø-7 cotton revealed no statistically significant differences between non-sprayed DAS-8191Ø-7 cotton and the isoline (control). In addition, all mean results from the isoline and DAS-8191Ø-7 entries were within the ranges observed in the non-transgenic cotton reference varieties included in the study. It was also determined that the agronomic characteristics of DAS-8191Ø-7 cotton sprayed with 2,4-D plus glufosinate are equivalent to non-sprayed DAS-8191Ø-7 cotton.

The data collected here support a conclusion that DAS-8191Ø-7 cotton is substantially equivalent to non-transgenic cotton with the exception of the introduced and expression of *aad-12* and *pat* genes and therefore no more likely to pose a plant pest risk, increase in weediness, or have an altered environmental impact compared to non-transgenic cotton.

7.2.2. Ecological Evaluations

As part of the plant characterization for DAS-8191Ø-7 cotton, evaluations of disease incidence and insect damage were conducted to determine the potential for increase in plant pest characteristics compared to non-transgenic cotton. The same field trials and plots as described for the phenotypic and agronomic analyses for DAS-8191Ø-7 cotton were monitored for both insect damage and disease incidence at peak bloom. Monitoring was performed and observed by personnel familiar with cotton cultivation practices (field station managers, field agronomists, field associates).

The personnel conducting the field tests visually monitored the incidence of plant disease and pests on DAS-8191Ø-7 cotton and non-transgenic cotton varieties included in the same trials. Disease and insect damage was rated at peak bloom on a numerical scale of 0-100%, with 0% representing no damage due to disease incidence or insect pests (Table 19). Table 20 summarizes the insects and diseases that caused the insect damage and disease incidence at each field site.

Insect damage or disease was limited, and where present, was found in both test and control plots (Table 19). The insect and disease pressure observed in these trials was typical of the growing locations. There were no statistically significant differences between non-sprayed DAS-8191Ø-7 cotton and the isoline (control) in susceptibility to and interactions with diseases and insects (Table 19). Likewise, spraying 2,4-D and glufosinate on DAS-8191Ø-7 cotton did not statistically significantly affect the occurrence of insect damage or disease incidence.

All plots were uniformly treated with pest control measures at each location based on pressure in the non-transgenic plots such that low incidence of these pests was expected. This design mimics commercial practice and allows comparison to typical cultivation conditions for non-transgenic cotton.

These data support the conclusion that DAS-8191Ø-7 cotton is substantially equivalent to non-transgenic cotton and is no more likely to be susceptible or resistant to insects or diseases typical to U.S. cotton growing regions. The data collected here support a conclusion that, with the exception of the introduced and expression of *aad-12* and *pat* genes DAS-8191Ø-7 cotton is no more likely to pose a plant pest risk, increase in weediness or have an altered environmental impact compared to non-transgenic cotton.

Table 20. Insects and Diseases Present at Field Testing Sites

Site[1]	Insects Present	Type of Insect Damage	Diseases Present
AL	Whitefly	Stippling	Leaf spot
GA	Bollworm/Tobacco Budworm	Chewing	None
LA	None	None	None
MO	None	None	None
MS	Plant bug	Square Feeding	None
NC	None	None	None
TX1	None	None	None
TX2	None	None	Alternaria

[1] Site location listed by U.S. state abbreviation with two locations shown for Texas (TX1, TX2)

7.2.3. Cottonseed Germination and Dormancy Evaluation

Seed dormancy is a characteristic that is often associated with plants that are considered weeds. Cotton, however, has had this characteristic removed through selection and breeding. Since USDA-APHIS considers weediness as a factor in the plant pest determination, an assessment of seed dormancy and germination was conducted on DAS-8191Ø-7 cottonseed. The germination and dormancy of DAS-8191Ø-7 cottonseed compared with non-transgenic isoline (control) (98M-2983XCoker 310) cottonseed under warm and cool conditions was evaluated to determine any impact of AAD-12 and PAT expression on seed germination and dormancy characteristics.

7.2.3.1. Study Design

The warm and cool germination tests consisted of four replications of each cotton entry with 100 seeds per replication in a completely randomized design.

Warm Germination Methods: One hundred acid delinted cotton seeds were evenly distributed onto two water saturated sheets of non-toxic, germination paper towels. A third water saturated towel was used to cover the seeds and all three towels were loosely rolled and placed upright in a plastic container. This process was repeated for each of four replications. The rolled towels were placed in an incubator set at 30° C. Sufficient water was added throughout the experiment to maintain a moist substrate while ensuring that a film of excess water did not accumulate around the seeds. On day four and day eight, seeds were observed for germination. This method is adapted from the AOSA Rules for Testing Seeds (AOSA, 2010a). Seedlings were evaluated in accordance with the AOSA Seedling Evaluation guidelines (AOSA, 2010b).

Cool Germination Methods: One hundred acid delinted cotton seeds were evenly distributed onto two water saturated sheets of germination non-toxic paper towels. A third water saturated towel was used to cover the seeds and all three towels were loosely rolled and placed upright in a plastic container. Prior to use all towels had been kept at 18° C for at least 16 hours. Each container was covered to retain moisture and then kept at 18° C for 7 days in the dark. On day seven, the germination was recorded. For cool germination, normal seedlings were defined as "normal seedlings having a combined hypocotyl and root length of 4 cm (1 and 10/16 in) or longer (AOSA, 2009)." All other germinated seedlings that did not meet these criteria were classified as abnormal. This method is adapted from the AOSA Seed Vigor Testing Handbook (AOSA, 2009).

Data were transformed using the arcsine of the square root of the decimal fraction of cotton seeds normally germinated per replicate and then subjected to analysis of variance based on a completely randomized design (SAS Institute Inc., 2011).

7.2.3.2. Germination and Dormancy Results

Percentages of normally germinated cotton seedlings and P-values for the significance of the effect of entry on germination are provided in Table 21. Germination of event DAS-8191Ø-7 cotton did not differ significantly ($\alpha = 0.05$) from that of the non-transgenic cotton comparator under warm ($P = 0.1189$) or cool ($P = 0.0717$) conditions, indicating that the presence of the transgenic insert in DAS-8191Ø-7 cotton did not alter cottonseed germination. These data support the conclusion that DAS-8191Ø-7 cottonseed is substantially equivalent to non-transgenic cottonseed in its germination characteristics. The data support a conclusion that with the exception of the introduced and expression of *aad-12* and *pat* genes DAS-8191Ø-7 cottonseed is no more likely to pose a plant pest risk or increase in weediness compared to non-transgenic cotton.

Table 21. Germination Percentages for DAS-8191Ø-7 and Control Cottonseed

Temperature		Entry	Cotton Germination (%)[1]					*P*-Value[2]
			Rep 1	Rep 2	Rep 3	Rep 4	Mean (±SE)	
Warm	30 °C	DAS-8191Ø-7	93	95	95	99	95.5 (±1.3)	0.1189
	30 °C	Control	89	93	91	95	92.0 (±1.3)	
Cool	18 °C	DAS-8191Ø-7	60	73	48	50	57.8 (±5.7)	0.0717
	18 °C	Control	85	81	76	59	75.3 (±5.7)	

[1] Four replications of each cotton sample at the indicated temperature with 100 seeds per replication in a completely randomized design
[2] Differences were considered significant if the P-value was less than 0.05.

7.3. Summary of Phenotypic, Agronomic and Ecologic Characteristics

A comparative assessment of the phenotypic, agronomic and ecological characteristics of DAS-8191Ø-7 cotton compared to non-transgenic isoline (control) was conducted. The characterization of DAS-8191Ø-7 cotton encompassed 1) vegetative growth, 2) ecological (insect and disease) and 3) seed germination and dormancy. The data were evaluated by individuals familiar with the production and evaluation of cotton.

In each assessment, DAS-8191Ø-7 cotton was compared to an appropriate, near isogenic isoline (control) that does not contain the *add-12* or *pat* genes (Figure 4). Phenotypic, agronomic and ecological analysis of DAS-8191Ø-7 cotton revealed no statistically significant differences between non-sprayed DAS-8191Ø-7 cotton and the isoline (control). It was also determined that the phenotypic, agronomic and ecological characteristics of DAS-8191Ø-7 cotton sprayed with 2,4-D plus glufosinate are equivalent to non-sprayed DAS-8191Ø-7 cotton. In addition, studies conducted under warm and cool conditions revealed that the germination and dormancy of DAS-8191Ø-7 cotton did not differ significantly from its non-transgenic cotton, near isogenic comparator.

The data collected here support a conclusion that DAS-8191Ø-7 cotton is substantially equivalent to non-transgenic cotton with the exception of the introduced and expression of *aad-12* and *pat* genes and therefore no more likely to pose a plant pest risk or have a significant environmental impact compared to non-transgenic cotton.

7.4. Field Efficacy

As part of the phenotypic, agronomic and ecologic field studies, herbicide injury ratings were also collected from DAS-8191Ø-7 cotton entries (non sprayed and sprayed with 2,4-D plus glufosinate). Field trial designs and herbicide application ratings are described in section 7.1. Herbicide applications were applied at the 3 node and 6 node stage and plants were evaluated 14 days after application on a scale of 0 – 100% where 0 represents no injury and 100 represents complete plant death.

Results of the herbicide injury data are shown in Table 22. DAS-8191Ø-7 cotton displayed minimal herbicide injury for applications at both the 3 node and 6 node stages. These data support the excellent tolerance of DAS-8191Ø-7 cotton at the proposed maximum herbicide use rate.

Table 22. DAS-8191Ø-7 Cotton Tolerance to Application of Herbicides

Herbicide[1]	Application Rate[2]	Application Stage	Percent Plant Injury[3] (Mean ± SE)	
			DAS-8191Ø-7 No Herbicide	DAS-8191Ø-7 Sprayed
2,4-D plus glufosinate	1120 g ae/ha (2,4-D) 596 g ai/ha (glufosinate)	3 node	0 ± 0	4 ± 2
		6 node	0 ± 0	4 ± 2

[1] 2,4-D and glufosinate were applied in combination as a tank mixture
[2] g ae/ha = grams acid equivalent per hectare, g ai/ha = grams active ingredient per hectare
[3] Means for no herbicide treatment (No Herbicide) and sprayed were not statistically compared since the data contained insufficient variability to enable ANOVA. Data from the Louisiana field testing site were excluded from this analysis since data was not collected on non sprayed plants.

8. Impact on Environmental and Agronomic Practices

As part of the plant pest assessment required by 7 CFR § 340.6(c)(4), impacts to agricultural and cultivation practices must be considered. This section provides a summary of current agronomic practices in the U.S. for producing cotton and describes potential impacts regarding the introduction of DAS-8191Ø-7 cotton into the environment, including gene flow and on non-target organisms and endangered species. In addition, this section provides an evaluation of the potential impact of DAS-8191Ø-7 cotton on current agronomic practices including cultivation, weed control, volunteer management and herbicide resistance management.

With the exception of the presence of the AAD-12 and PAT proteins, DAS-8191Ø-7 cotton is phenotypically equivalent to commercially cultivated cotton and is no more susceptible to diseases or pests than commercially cultivated cotton. Therefore, there are no likely impacts of DAS-8191Ø-7 cotton to the majority of the agronomic practices currently employed in cotton production and based on substrate specificity of AAD-12 and PAT, no effect on non-target organisms or endangered species is anticipated for DAS-8191Ø-7 cotton.

8.1. Current U.S. Agronomic Practices for Cotton

8.1.1. Cotton Production

Only the cotton boll (consisting of fibers and seeds) is useful for fiber, food and feed with the primary use of cotton in the U.S. being fibers which are mainly used in the manufacturing of a large number of textiles. Approximately 85% of cotton value is derived from the fiber. The boll is processed to remove the fiber for textile use; the remaining fuzzy seed is processed into four major food and feed products: oil, meal, hulls and linters. Cottonseed currently results in approximately 10% of the world's oilseed production.

Cotton is the world's most widely grown fiber crop, accounting for over 35% of the total world fiber use (USDA ERS, 2013a). The United States is the top exporter of raw cotton, and third in world production behind China and India (USDA ERS, 2013a). U.S. cotton production alone accounts for more than $25 billion in products and services annually and generates approximately 200,000 U.S. jobs (USDA ERS, 2013a).

Upland cotton, *Gossypium hirsutum*, accounts for approximately 97% of U.S. annual cotton crop production, with Pima (Egyptian) cotton, *G. barbadense*, accounting for the remaining 3% of cotton acreage (USDA NASS, 2013c, p.42). In the U.S., cotton is grown across the southern states in four major cotton growing regions which include the Southeast, Midsouth, Southwest and West regions (Table 23, Figure 47 & Figure 48). Over the past 10 years, harvested cotton acres have varied from around 7.5 to 13.8 million acres harvested with the lowest harvested acres recorded in 2009 and the highest in 2005 (Table 24).

Texas devotes more area to cotton production and produces more cotton than any other state, producing over five million bales in 2012 accounting for nearly 30% of all cotton production

in the U.S. (Table 23). Other states producing over one million bales in 2012 included Georgia, North Carolina, Arkansas, and California (Table 23).

In 2012, 9.4 million acres of cotton were harvested in the US; with production value estimated at just under $6 billion (USDA NASS, 2013a, 2013b). Current estimates of all cotton planted in the U.S. for 2013 are 10.2 million acres, with 10.0 million acres estimated for upland cotton and the remaining U.S. acreage dedicated to pima cotton which is mainly grown and limited to the Southwestern U.S. due to the longer required growing season compared to upland cotton (Figure 48).

Table 23. Total U.S. Cotton Growing Regions, States and 2010 – 2012 Harvest, Yield & Production

		Cotton Harvest (Acres X 1000)			Cotton Yield (lb/acre)			Cotton Production[2] (X1000)[3]		
US Cotton Regions	States	2010	2011	2012	2010	2011	2012	2010	2011	2012
Southeast	Alabama	338	443	378	682	742	946	480	685	745
	Florida	89	118	107	766	744	897	142	183	200
	Georgia	1315	1495	1280	821	791	1091	2250	2465	2910
	North Carolina	545	800	580	838	616	1014	951	1026	1225
	South Carolina	201	301	298	898	828	955	376	519	593
	Virginia	82	115	85	732	676	1118	125	162	198
	Total	**2570**	**3272**	**2728**	**4737**	**4397**	**6021**	**4324**	**5040**	**5871**
Midsouth	Arkansas	540	660	585	1045	9289	1064	1176	1277	1297
	Louisiana	249	290	225	842	846	1020	437	511	478
	Mississippi	410	605	470	993	952	1014	848	1200	993
	Missouri	308	367	330	1068	969	1063	685	741	731
	Tennessee	387	490	377	845	796	946	681	813	743
	Total	**1894**	**2412**	**1987**	**4793**	**12852**	**5107**	**3827**	**4542**	**4242**
Southwest	Kansas	50	65	54	787	510	622	82	69	70
	New Mexico	49.7	61.4	40.3	1156	1049	1060	119.7	134.2	89
	Oklahoma	270	70	140	750	597	531	422	87	155
	Texas	5366.5	2868.5	3857.5	704	592	624	7871	3540	5014.5
	Total	**5736.2**	**3064.9**	**4091.8**	**3397**	**2748**	**2837**	**8494.7**	**3830.2**	**5328.5**
West	Arizona	195.5	258	200	1509	1526	1470	614.4	820	612.3
	California	303	454	365	1337	1418	1658	844	1341	1261
	Total	**498.5**	**712**	**565**	**2846**	**2944**	**3128**	**1458.4**	**2161**	**1873.3**
Totals	17	10698.7	9460.9	9371.8	15773	22941	17093	18104.1	15573.2	17314.8

[1] USDA NASS, 2012 and USDA NASS, 2013
[2] Production ginned and to be ginned
[3] 480-pound net weight bale

Table 24. Cotton Production in the U.S., 2002 - 2013[1,2]

Year[2]	Acres Harvested (x1000)	Average Yield (lbs/acre)	Production (480-lb Bales)	Value (billions)
2013	7,703	813	13,052,800	TBD
2012	9,372	887	17,314,800	5.97
2011	9,461	790	15,573,200	6.986
2010	10,699	812	18,104,100	7.348
2009	7,529	777	12,187,500	3.788
2008	7,569	813	12,815,300	3.021
2007	10,489	879	19,206,900	5.653
2006	12,732	814	21,587,800	5.013
2005	13,803	831	23,890,200	5.695
2004	13,057	855	23,250,700	4.853
2003	12,003	730	18,255,200	5.517
2002	12,417	665	17,208,600	3.777

[1]USDA NASS, 2012

[2] 2013 estimates from USDA NASS, 2013

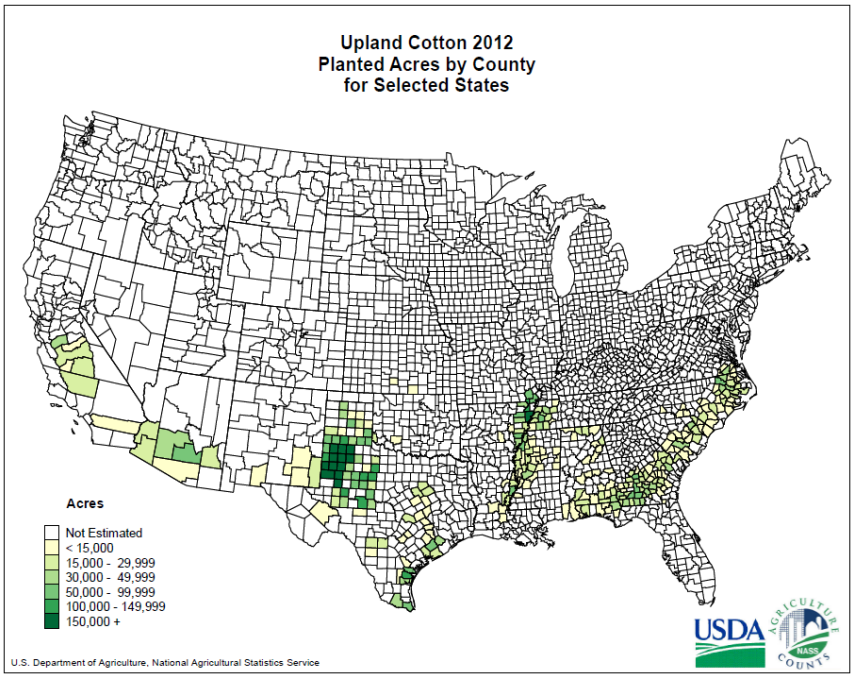


Figure 47. Planted Upland Cotton Acres by County in the U.S. in 2012 (USDA NASS, 2012b)

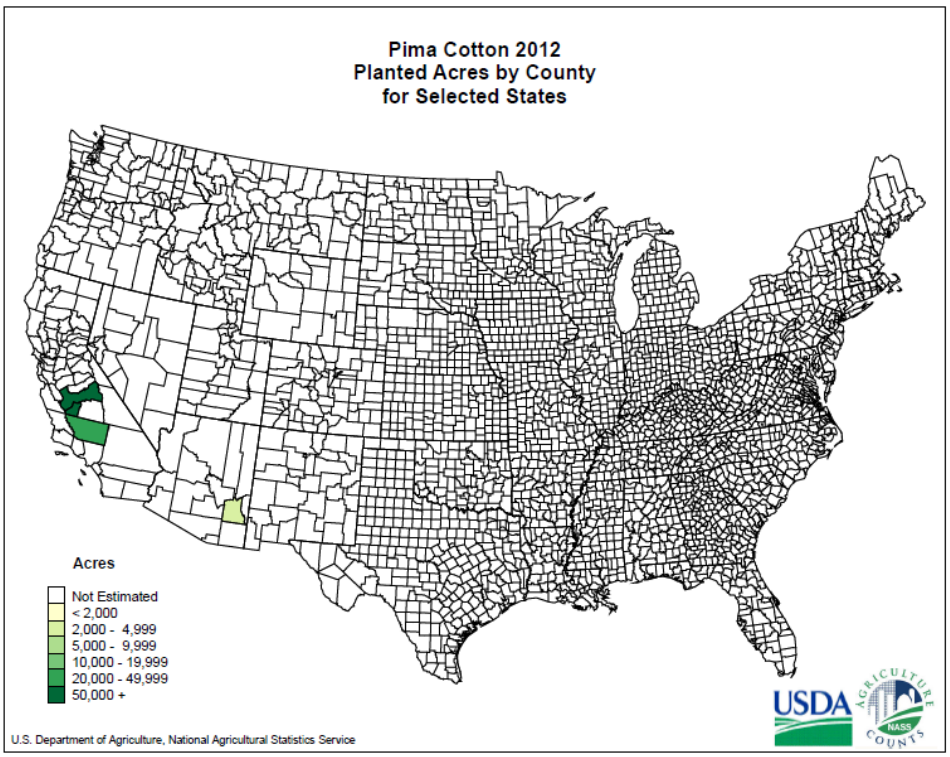


Figure 48. Planted Pima Cotton Acres by County in the U.S. in 2012 (USDA NASS, 2012a)

8.1.2. Cotton Seed Production

Seed quality, including genetic purity, vigor, and presence of weed seed, seed-borne diseases, and inert materials, is a major factor affecting crop yield. Genetic purity in commercial seed production is generally regulated through a system of seed certification which is intended to ensure that the desired traits in the seed are maintained throughout all stages in cultivation.

The U.S. Federal Seed Act, enacted in 1939, recognizes seed certification and official certifying agencies. Implementing regulations further recognize land history, field isolation, and varietal purity standards for seed. States have developed laws to regulate the quality of seed available to farmers (Bradford, 2006). Most of the laws are similar in nature and have general guidelines for providing information on the label for the following: 1) commonly accepted name of agricultural seed, 2) approximate total percentage by weight of purity, 3) approximate total percentage of weight of weed seeds, 4) name and approximate number per pound of each kind of noxious weed seeds, 5) approximate percentage of germination of the seed, and 6) month and year the seed was tested.

The Association of Official Seed Certifying Agencies (AOSCA, 2012) defines the classes of cottonseed as follows: 1) breeder seed, 2) foundation seed, 3) registered seed, and 4) certified seed. Breeder seed is seed directly controlled by the originating or sponsoring plant breeding organization. Foundation seed is the progeny of Breeder seed or Foundation seed that is handled to maintain specific genetic identity and purity. Registered seed is the progeny of Breeder or Foundation seed that is handled to maintain satisfactory genetic identity and

purity. Certified seed is the progeny of Breeder, Foundation, or Registered seed that is handled to maintain satisfactory genetic identity and purity. Commercial cotton seed sold and planted for typical cotton production is produced predominately to meet or exceed certified seed standards.

Production of all classes of certified seed requires that 1) each certifying agency shall determine that genetic purity and identity are maintained at all stages of certification including seeding, harvesting, processing, and labeling of the seed; 2) the unit of certification shall be a clearly defined field or fields; 3) one or more field inspection shall be made prior to harvest and when genetic purity and identity can best be determined; and 4) a certification sample shall be drawn in a manner approved by the certifying agency from each cleaned lot of seed eligible for certification (USDA, 2012a).

Federal regulation 7 CFR §201.76 specifies minimum land, isolation, field, and seed standards required for cotton Foundation, Registered and Certified seed. To qualify as Certified seed, only one plant of another variety in 1000 cotton plants or 0.1% of seed of other varieties or off-types are permitted (USDA, 2012b). To qualify as Registered seed, only one plant of another variety in 5000 cotton plants and 0.05% of seed of other variety or off-types are permitted. To be certified as Foundation seed, only one plant of another variety in 10,000 cotton plants and 0.03% of seed of other variety or off-types are permitted.

Agronomic practices for producing cottonseed are similar to commercial production with enhanced management practices to ensure high quality, genetically pure seeds. Commercial certified cotton seed must meet state and federal seed standards and labeling requirements. State seed certification standards vary from state to state, but can be more restrictive than standards set forth by AOSCA.

DAS-8191Ø-7 cotton does not differ from conventional cotton in agronomic characteristics (Section 7) and thus is not expected to impact U.S. cotton or seed production. Once deregulated, DAS-8191Ø-7 cottonseed will be produced in the same manner as commercially certified cottonseed, such that it will meet all state and federal seed standards and labeling requirements.

8.1.3. Planting and Cotton Development

Soil temperatures have a strong influence on the rate of germination and seedling development in cotton, which responds most favorably to soil temperatures around 64°C or greater at the appropriate seedling planting depth (Smith *et al.*, 1999). Planting cotton in soil conditions with temperatures less than 55°C will generally result in poor vigor and seedling disease problems. Under favorable conditions, germination generally occurs with five to fifteen days after planting.

Optimal cotton yield is highly dependent on achieving a satisfactory cotton stand which is dependent on location, soil conditions, environment conditions, row-spacing and the cultivar. Soil conditions can be optimized and cotton has the ability to compensate in response to row spacing and plant populations. However, cotton plants in thick stands tend to grow taller,

develop more vegetative growth and often cause delays in fruiting; this may alter the reproductive/vegetative balance of the plant (Smith *et al.*, 1999). Low plant populations can contribute to weed problems and produce inadequate fruit and impact overall yield.

The first cotton squares become visible normally five to eight weeks after planting, depending on growing conditions. The majority of cotton bolls harvested tend to come from squares which set during the first month of squaring, emphasizing the importance of proper crop management during this period including water, weed, nutrient and pest management which all have the potential to dramatically decrease crop yield if not managed properly.

Once cotton develops into the later stages of the season, management of the crop is typically orientated to harvest. Effective defoliation is an essential step in the overall process of harvesting high quality lint. Quick, efficient defoliation is accomplished by chemical means and is dependent on the plant-water status, nitrogen fertility status, insect damage, weather conditions and the chemical defoliant (Smith *et al.*, 1999). Once defoliated, the cotton is harvested mechanically.

8.1.4. Weeds in Cotton

Cotton is a slow-growing plant early in the season and competes poorly with weeds. This is especially true in the northern cotton-growing areas, when cotton is planted into cooler soils. Studies show that control of weeds during the first four to eight weeks after planting is critical to prevent weeds for competing for water, light, nutrients and other resources essential for cotton germination and growth (Smith *et al.*, 1999).

Weeds that are most common are not necessarily the most troublesome. The degree of importance depends on the expense of control, interference to cotton growth and yield, and reduction in lint quality. The type, severity, and impact of weeds in cotton vary between geographical regions. In addition, weed populations do not remain static and change over time due to reproductive ability, control methods, herbicide regimes, herbicide resistance, climate changes and other environmental factors (Smith *et al.*, 1999). It is important for proper identification of invading weed species to help develop an effective weed management program.

Major weeds in cotton include broadleaf species such as pigweeds, horseweed, and common cocklebur, as well as grass species such as Johnsongrass (*sorghum halepense*) and goosegrass (Heap, 2013b). Palmer amaranth, morning glory spp., and nutsedge species are reported as both common and troublesome (Webster and Sosnoskie, 2010). Table 25 provides a list of top weeds in cotton as reported by growers in specific cotton-growing regions, with glyphosate resistant biotypes being the most pervasive and problematic in the Southeast and parts of the Midsouth.

Table 25. Major Weeds Treated in Cotton by Region in 2011 (Descending Order)[1]

US	Southeast	Midsouth	Southwest	West
PIGWEED, REDROOT	PIGWEED, REDROOT	PIGWEED, REDROOT	PIGWEED, REDROOT	MORNINGGLORY
MORNINGGLORY	AMARANTH, PALMER	MORNINGGLORY	JOHNSONGRASS	PIGWEED, REDROOT
AMARANTH, PALMER	MORNINGGLORY	AMARANTH, PALMER	THISTLE, RUSSIAN	NIGHTSHADE, BLACK
MARESTAIL	SICKLEPOD	MARESTAIL	MORNINGGLORY	WATERGRASS
JOHNSONGRASS	COCKLEBUR	SIGNALGRASS, BROADLEAF	KOCHIA	LAMBSQUARTERS
SICKLEPOD	CRABGRASS	HENBIT	PIGWEED, TUMBLE	JOHNSONGRASS
COCKLEBUR	NUTSEDGE	CRABGRASS	MARESTAIL	AMARANTH, PALMER
CRABGRASS	PANICUM, TEXAS	JOHNSONGRASS	AMARANTH, PALMER	BINDWEED, FIELD
THISTLE, RUSSIAN	BUFFALOGRASS	GRASSES, ALL	PANICUM, TEXAS	MALLOW, COMMON
PANICUM, TEXAS	EVENINGPRIMROSE	BLUEGRASS, ANNUAL	BARNYARDGRASS	WHEAT, VOLUNTEER
KOCHIA	RYEGRASS	COCKLEBUR	SUNFLOWER	
BARNYARDGRASS	GOOSEGRASS	CHICKWEED	BINDWEED, FIELD	
SIGNALGRASS, BROADLEAF	MARESTAIL	SIDA, PRICKLY		
HENBIT	RADISH, WILD	BARNYARDGRASS		
GRASSES, ALL	GERANIUM, DOVEFOOT	RYEGRASS		
PIGWEED, TUMBLE	GRASSES, ALL			
NUTSEDGE	MUSTARD, WILD			
RYEGRASS				
BLUEGRASS, ANNUAL				

[1] Source: Third Party Proprietary Data

8.1.5. Weed Management in Cotton

Weed control in cotton uses a combination of cultural, mechanical and chemical methods (University of California Agriculture & Natural Resources, 2013c). With herbicide tolerant cotton planted on 82% of U.S. acres (USDA NASS, 2013c), herbicides are the primary basis of weed management programs. These regimens incorporate herbicides pre-emergence, during planting, post-emergence, or a combination (Burgos *et al.*, 2006). Mechanical cultivation remains common, particularly in areas of herbicide resistant weeds (University of Western Australia, 2010).

A 2010 USDA survey reports that herbicides were applied to 99% of upland cotton, with glyphosate being the most widely and heavily used (USDA NASS, 2011). Other commonly used herbicides include diuron, pendimethalin, metalochlor, pyrithriobac, and trifluralin (University of California Agriculture & Natural Resources, 2013c).

The widespread adoption of glyphosate tolerant crops and subsequent glyphosate use on a large portion of cropland has created strong selection pressure for glyphosate resistant weeds. In 1996, no weed species were known to be resistant to glyphosate; currently there are 24 confirmed glyphosate resistant weed species globally. Fourteen are confirmed in the U.S., and all occur in cotton growing states (Hurley *et al.*, 2009; Heap, 2013d). For more details see Appendix 7.

Heavy reliance on glyphosate and the resulting emergence of persistent, resistant weeds has resulted in increasing emphasis on integrated management approaches. These approaches include tillage, hand-weeding, crop rotation, and alternate herbicidal chemistries (WSSA, 2013).

Few herbicides have been developed for use on cotton as compared to corn and soybean (Kendig *et al.*, 1994). DAS-8191Ø-7 cotton and the herbicide systems it enables, provide growers needed tools for use in an integrated weed management strategy.

Weed management decisions are difficult for growers because a single tool cannot effectively control all possible weed problems. Effective weed control requires grower implementation of management practices that limit the introduction and spread of weeds, help the crop to compete with weeds, and prevent weeds from adapting. The combination of weed control practices that a grower chooses depends upon the weed spectrum, level of infestation, soil type, cropping system, weather, and time and labor available for the treatment option.

8.1.6. Crop Rotation and Tillage Practices

Cotton is commonly rotated with other crops, including corn, soybeans, sorghum, peanut and wheat (USDA APHIS, 2011). Market dynamics, geography and grower needs influence the choice of rotation crop; crop rotation is used to manage weeds, pests, diseases, erosion, and improve soil health (University of California Agriculture & Natural Resources, 2013b). While some of the cotton belt still operates under continuous cotton monoculture practice, there is evidence that rotation practice is becoming more common due to the benefits it provides (Cotton Incorporated, 2013).

Types of tillage currently used in cotton include conventional, reduced, and conservation tillage (Albers and Reinbott, 1994). Data demonstrates that new technologies, such as herbicides to control early season weeds, allowed growers to shift away from conventional tillage (University of California Agriculture & Natural Resources, 2013c). A Cotton Incorporated grower survey found a “significant increase” in conservation tillage from 1990-2004 (Smith, 2009). However, high weed pressure in recent years has resulted in a reduction in conservation tillage practice (Shaw *et al.*, 2012; Robinson, 2013).

8.2. Potential Impacts of DAS-8191Ø-7

Dow AgroSciences has developed DAS-8191Ø-7 cotton, tolerant to the herbicides 2,4-D and glufosinate. DAS-8191Ø-7 cotton will provide growers with greater flexibility in selection of herbicides for the improved control of economically important weeds; allow an increased application window for effective weed control; and provide an effective weed resistance management solution to glyphosate resistant weeds.

DAS-8191Ø-7 cotton will be combined with glyphosate tolerant cotton utilizing traditional breeding techniques. The combination of herbicide tolerant 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 modes 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 broadleaf weeds, such as pigweed, waterhemp, horseweed, and morningglory.

8.2.1. Environment – Weediness Potential, Gene Flow and Non-Target / Endangered Species

8.2.1.1. Weediness Potential

Cotton is not considered to have weedy characteristics in the U.S. and USDA-APHIS has determined that cotton (*Gossypium hirsutum*) is not a plant pest in the United States (USDA APHIS, 2011). In addition, cotton is not present on the lists of noxious weed species distributed by the federal government (7 CFR Part 360) nor does cotton possess attributes commonly associated with weeds; it is relatively slow-growing and does not compete effectively with other cultivated plants or primary colonizers (OECD, 2010). In addition, commercial varieties of cotton in the U.S. are not effective in invading established ecosystems. Cultivated cotton rarely displays any dormancy characteristics and may grow as a volunteer only under specific conditions. Volunteers are easily controlled by herbicides or mechanical means (Morgan *et al.*, 2011a; Morgan *et al.*, 2011b).

The introduction of 2,4-D, glufosinate, and glyphosate tolerance into cotton will not alter its weediness characteristics. Agronomic properties of DAS-8191Ø-7 cotton related to weediness, such as germination, emergence, seedling vigor and response to environmental stressors have been shown to be identical to conventional cotton (Section 7). If individual DAS-8191Ø-7 cotton plants were to overwinter, they can still be effectively controlled mechanically or with other herbicide modes of action such as paraquat in burndown, atrazine to control 2,4-D cotton volunteers in corn fields, and flumioxazin to control 2,4-D tolerant cotton volunteers in soybean fields (Morgan *et al.*, 2011a). Collectively, these findings support the conclusion that DAS-8191Ø-7 cotton has no increased weediness compared to commercially cultivated cotton.

8.2.1.2. Gene Flow Assessment

8.2.1.2.1. Vertical Gene Flow

Cotton is considered self-pollinating; pollen grains are large, heavy and somewhat sticky; thus dissemination by wind is absent or negligible. Pollen-mediated gene flow declines steeply, typically below 1% beyond 10 meters from the source (Van Deynze *et al.*, 2005). Thus, the potential for gene flow from DAS-8191Ø-7 cotton to cultivated, wild or feral cotton is highly unlikely.

Cotton is generally self-pollinating but some cross-pollination can occur, albeit at relatively low incidence through activity of pollinating insects (Hutmacher *et al.*, 2006). In the presence of pollinators, cross pollination may occur at low levels, with decreasing frequency as distance from the source increases (OECD, 2010). In farm scale studies using traditional Upland cotton in California, it was found that the outcrossing distance was strongly dependent on the presence of bee colonies. When only native pollinators were present in the field, 1% out-crossing was detectable over a distance of 1 meter (approximately 3 feet) and 9

meters (29.5 feet) when there was high pollinator activity (Van Deynze *et al.*, 2005). Out-crossing declined exponentially with increasing distance from the source plot (Van Deynze *et al.*, 2005). In addition, DAS-8191Ø-7 cotton would not be expected to confer a selective advantage to, or enhance the pest potential of, progeny resulting from such crosses if they were to occur. Therefore, the environmental consequences of gene flow from DAS-8191Ø-7 cotton to other cotton species is considered to be negligible.

The potential for outcrossing and gene introgression from DAS-8191Ø-7 to cultivated cotton in the U.S. (*G. hirsutum* and *G. barbadense*) is low since cotton pollen movement by wind is limited due to its large and sticky nature. In addition, studies have shown that even in the presence of pollinator activity, cross-pollination is limited by distance (Van Deynze *et al.*, 2005).

Only two 'wild' *Gossypium* species are known to persist in the U.S. including *G. thurberi* found in Arizona and feral populations of *G. hirsutum* known to occur in South Florida and Puerto Rico. In addition, *G. thurberi* is a diploid (2x) cotton species while DAS-8191Ø-7 cotton (present in *G. hirsutum*) is allotetraploid (4x) species, if pollen or gene exchange did occur, it would result in a triploid (3X) sterile plant. Such sterile plants have not been reported in the wild. Although DAS-8191Ø-7 cotton is capable of crossing with wild *G. hirsutum*, wild species of *G. hirsutum* are not commonly present in cotton growing areas of the U.S.

G. tomentosum is endemic to the Hawaiian Islands. Cultivated cotton is not grown commercially in Hawaii (USDA NASS, 2012c), but may occur in counter-season breeding nurseries. In such cases of counter-season nurseries, appropriate isolation distances and practices are required for seed productions (Federal regulation 7 CFR §201.76) and growing regulated cotton (660 feet physical isolation from non-transgenic cotton fields; or minim of 40 feet of continuous and uninterrupted non-regulated cotton border, plus 10 feet separation between the border and adjacent field; or destruction of material prior to flowering), significantly limiting any potential for gene flow between domesticated cotton and *G. tomentosum*.

Overall, the potential for DAS-8191Ø-7 cotton for crossing to cultivated, wild or feral cotton populations is low due to cotton's self-pollination nature, geographic isolation barriers and or genetic make-up. In addition, DAS-8191Ø-7 cotton would not be expected to confer a selective advantage to, or enhance the pest potential of, progeny resulting from such crosses if they were to occur. Therefore, the environmental consequences of gene flow from DAS-8191Ø-7 cotton to other cotton species is considered to be negligible.

8.2.1.2.2. Horizontal Gene Flow

DAS is unaware of any reports regarding the unintended transfer of genetic material from cotton to other sexually-incompatible species. In the unlikely event that such a transfer were to take place, the *aad-12* and/or *pat* genes from DAS-8191Ø-7 cotton would not present a human health or plant pest risk; this conclusion is based on the safety data presented in this petition. Therefore, in the highly unlikely event that horizontal gene flow were to occur, the

presence of *aad-12* and/or *pat* traits would not be expected to increase pest or weed potential in the recipient species.

8.2.1.3. Non-Target Organisms and Endangered Species

Based on substrate specificity of the AAD-12 enzyme activity, no effect on non-target organisms or endangered species is anticipated for DAS-8191Ø-7 cotton. The *aad-12* gene and expressed protein are present in nature in the soil bacterium *Delftia acidovorans*. The *pat* gene and the expressed protein are present in other crops grown in the United States with no effects on non-target organisms or endangered species. AAD-12, and PAT are not potential food allergens or toxins (Section 5.1.5.2, 5.1.5.3 and 5.2.5) and DAS-8191Ø-7 cotton has been shown to be substantially equivalent to non-transgenic cotton based on the compositional analysis of cottonseed (Section 6). Observations made during field testing of DAS-8191Ø-7 cotton revealed no effects on invertebrate populations (Section 7.2.2) and agronomic characteristics were found to be equivalent to non-transgenic cotton (Section 7).

The Endangered Species Act (ESA) (16 USC 1531) is administered by the U.S. Fish & Wildlife Service (FWS). Under ESA, Section 6 requires federal agencies who conduct activities which may affect listed species to consult with the FWS to ensure that listed species are protected should there be a potential impact. It is not anticipated that DAS-8191Ø-7 cotton will impact any currently listed species of concern since it is not anticipated that DAS-8191Ø-7 cotton will cause new cotton acres to be planted in areas that are not already in agricultural use. Habitat disruption within DAS-8191Ø-7 cotton fields would be comparable to other herbicide tolerant cropping systems.

Cultivated cotton is not invasive or weedy, and these properties are not anticipated to be altered by the insertion of the genes conferring tolerance to herbicides. Based on the data, it is reasonable to conclude that DAS-8191Ø-7 cotton will not affect threatened or endangered species or adversely affect or change designated critical habitats as compared to current commercial agricultural practices.

2,4-D is not currently registered for uses specific to cotton production. The environmental fate and ecological effects on non-target organisms for the use of 2,4-D in cotton will be addressed by the EPA as part of its review process under FIFRA.

Glufosinate is currently registered for use in cotton production as a post-emergent herbicide treatment. The environmental fate and ecological effects of these herbicides on non-target organisms and endangered species have been addressed by the EPA as part of its review process.

8.2.1.4. Potential Impacts on Boll Weevil Eradication Program

The National Boll Weevil Eradication Program (BWEP) was launched in the late 1970's by USDA's Animal and Plant Health Inspection Service (APHIS) to eradicate the boll weevil in the cotton-growing areas of the U.S. Prior to implementation of the program, boll weevils were found throughout southern cotton growing states, including California, Arizona, and New Mexico. By 2009, eradication was completed in all U.S. cotton growing regions except

for a small region in Texas (National Cotton Council of America, 2013). In 2012, boll weevils were captured in only three of the sixteen Texas eradicated zones, with a substantially reduced number of boll weevils in two of the three zones (Texas Boll Weevil Eradication Foundation Inc., 2012). Eradication continues today for a small region of Texas.

The BWEP and the Texas Boll Weevil Eradication Foundation (TBWEF) have developed comprehensive eradication programs over the past several decades to monitor, trap and treat infected boll weevil zones in the U.S. and northern Mexico (Texas Boll Weevil Eradication Foundation Inc., 2012; National Cotton Council of America, 2013). Eradication and control of boll weevil is primary based on the use of the insecticide malathion. This product has been highly efficacious in the control and eradication of boll weevil across the southern US. In addition to chemical control, the boll weevil eradication programs rely on and promotes farmers to use cultural controls such as planting during the most advantageous periods of time, harvesting early and thoroughly, and destroying crop residues and failed plantings in a timely manner (Texas Boll Weevil Eradication Foundation Inc., 2012).

Because of the warm climate and rainfall in parts of south Texas, cotton stalks left in the field after harvest can regrow. This provides hostable cotton structure (squares and bolls) that boll weevils can use as both food and reproductive harbors, potentially increasing overwintering survivability. Methods available for the management of cotton stalks include mechanical destruction or removal (shredding, tillage, and stalk pullers), flood irrigation, and herbicide application. Applied herbicides may include 2,4-D, dicamba,thifensulfuron-methyl + tribenuronmethyl and others. Based on the cotton growing zones in Texas, The Texas Department of Agriculture has provided guidelines on appropriate cotton stalk destruction methods (Texas Department of Agriculture, 2013).

In order for destruction of cotton plants to be deemed complete, both plants re-growing from stalks and those growing from seed must be eliminated. To ensure compliance of cotton crop destruction, the BWEP has extensive quality control measures in place to monitor and enforce the destruction of cotton plants post-harvest (National Cotton Council of America, 2013). Herbicides are typically the management option of choice, largely due to efficacy, convenience and cost. Among the herbicides labeled for the management of cotton stalks, sequential applications of 2,4-D are most commonly used.

Although methods other than 2,4-D are currently available for the management of cotton stalks, Dow AgroSciences recognizes the utility of 2,4-D in the BWEP and is committed to identifying other herbicides that can be used for cotton stalk control. Since 2010, Dow AgroSciences has been actively engaged in research focused on identifying alternative herbicides that meet the BWEP performance criteria of no hostable cotton structures during the prescribed period for the pest management zones of interest.

Effective treatments which result in no hostable structure have been identified including dicamba and dichlorprop and combinations of dicamba plus dichlorprop. Additional testing will be conducted over the next several years to further improve efficacy of these alternative treatments for controlling hostable structures.

The introduction of DAS-8191Ø-7 cotton in Texas is not anticipated to significantly impact the BWEP or TBWEF in the U.S or Mexico. With the exception of fields where DAS-8191Ø-7 cotton is planted, the standard management practices of the BWEP and TBWEF remain unaffected. In fields planted with DAS-8191Ø-7 cotton, identical, effective boll weevil control measures (mapping, trapping, insecticide control and framing practices) set forth by BWEP, TBWEF and Texas Department of Agriculture will remain effective measures for control and eradication of boll weevil populations. The only potential impact could be the need to use a herbicide stalk destruct method other than 2,4-D that meets the BWEP performance criteria. Collaborative research by Dow AgroSciences and public-sector scientists have identified herbicides other than 2,4-D that effectively meet the BWEP performance criteria, to mitigate this potential impact. Dow AgroSciences is committed to the long-term success of BWEP, and will continue its efforts to identify effective herbicide alternatives and management plans for cultivation of cotton in boll weevil regions of the US.

8.2.2. Agronomic Practices – cultivation/management, weed control and volunteer management

8.2.2.1. Potential Impacts on Cultivation and Management Practices

Transgenic varieties of cotton have been rapidly adopted. USDA estimates 10.3 million acres for all U.S. cotton planted in 2013, with 10 million of these acres being upland cotton (USDA NASS, 2013c). Of those acres, 90% have been planted to transgenic varieties: 8% are insect resistant varieties only, 15% are herbicide tolerant varieties only, and 67% are stacked varieties (herbicide resistance plus insect resistance) (USDA NASS, 2013c). Current cotton cultivation and management practices incorporate the management of herbicide tolerant traits. DAS-8191Ø-7 cotton is phenotypically and agronomically comparable to conventional cotton (Section 7). Ecological observations during field testing have shown no changes in insect susceptibility (Section 7.2.2); therefore no impacts are expected on insect control practices for DAS-8191Ø-7 cotton. It is anticipated that the same management practices for today for herbicide tolerant cotton will also be appropriate for DAS-8191Ø-7 cotton.

8.2.2.2. Potential Impact on Weed Control Practices

DAS-8191Ø-7 cotton will provide expanded weed management options in cotton. Post-emergence applications of 2,4-D control a broad spectrum of broadleaf weeds. 2,4-D also has some short-lived soil residual activity (approximately three weeks) which provides limited residual control of later germinating broadleaf weeds (Lajeunesse, 1997). Thus, DAS-8191Ø-7 cotton will provide greater flexibility to farmers compared to glyphosate alone in weed management systems. Inclusion of 2,4-D would contribute to the control of glyphosate resistant and hard to control broadleaf weeds, plus slow down the selection for more glyphosate resistant broadleaf weeds (Powles, 2008a).

2,4-D is not currently registered for use on cotton; however, 2,4-D is used for weed control in burndown programs in no-till fields, due to 2,4-D's efficacy against broadleaf weeds, including glyphosate resistant biotypes (University of Delaware College of Agriculture & Natural Resources, 2008). 2,4-D has approximately three weeks of residual soil activity. For

growers who choose to use 2,4-D in a burndown regimen, a 30 day waiting interval is typically required prior to planting. This time period may fluctuate slightly depending on the individual label.

In DAS-8191Ø-7 cotton, the proposed use pattern will allow application of 2,4-D at burndown or pre-emergence (1 lb ae/A or 1120 g ae/ha) without plant back restrictions, followed by one or two post-emergence (0.5 – 1.0 lb ae/A or 560-1120 g ae/ha) applications at least 12 days apart, over-the-top of the cotton, up to the mid-bloom stage of development (Figure 49). Thus the proposed maximum seasonal rate of 2,4-D on cotton is estimated to be 3.0 lbs ae/ha. DAS-8191Ø-7 cotton will allow growers to apply 2,4-D from burndown or pre-emergence up through the mid-bloom stage without risk of crop injury. This will provide new options for improved weed control during the cotton development period when weeds have the greatest potential yield impact.

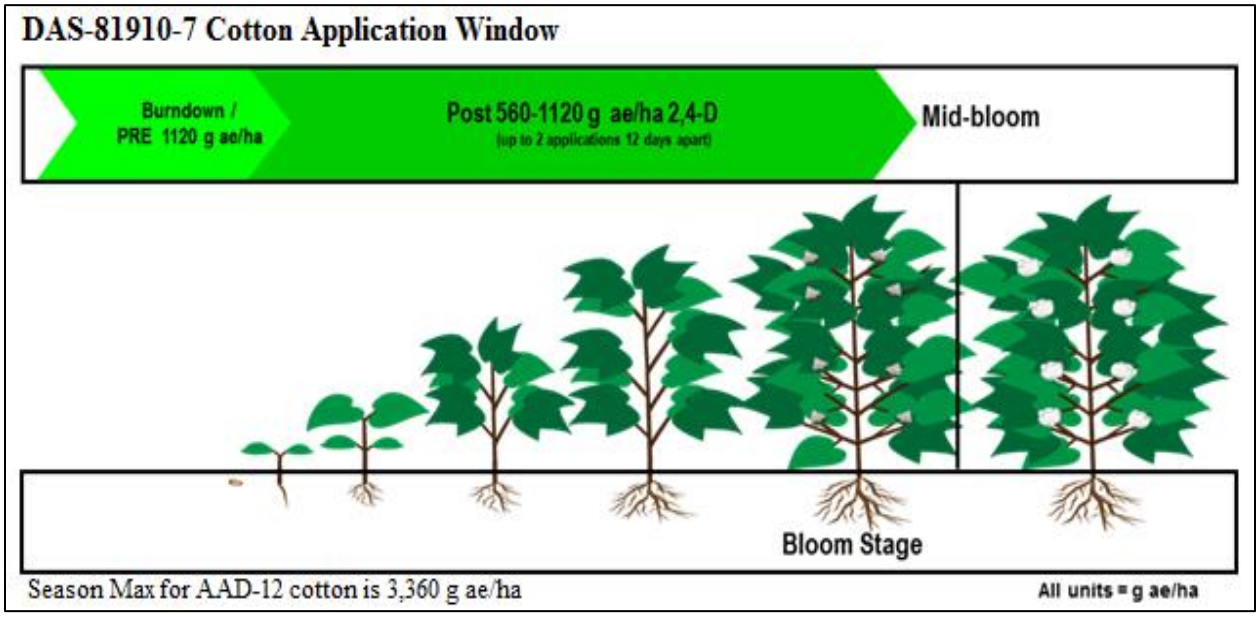


Figure 49. 2,4-D Use in DAS-8191Ø-7 Cotton

While 2,4-D is currently registered as a pesticide, supporting information on proposed label changes for its use with DAS-8191Ø-7 cotton is being provided by Dow AgroSciences to U.S. EPA for review. Application for Dow AgroSciences' 2,4-D formulation (Enlist Duo) registration on DAS-8191Ø-7 cotton and tolerance petition is scheduled for submission to U.S. EPA in early 2014 with anticipated approval in mid-2015 based on set PRIA timelines. Dow AgroSciences is also developing an extensive stewardship program that will include technological advancements in application to reduce potential for off-target movement, as well as utilizing several media venues to educate and facilitate adoption of the technology and decision management tools to ensure the proper use and stewardship of both the trait and the associated chemical technologies. DAS' stewardship program is detailed further in Appendix 8.

When combined with additional herbicide mode of actions through traditional breeding methods, DAS-8191Ø-7 cotton varieties will improve weed control by allowing use of herbicide combinations or mixtures which can provide more consistent performance in post-emergence weed control programs. They counteract glyphosate rate-creep (steady increase in the amount of glyphosate herbicide rates to effectively control weeds) needed over time to obtain effective weed control on hard to control weeds (Jaehnig, 2005). DAS-8191Ø-7 cotton will allow use of a low cost, high performance solution to reduce the escalation of glyphosate and ALS resistance in weed populations.

8.2.2.3. Volunteer Management

Commercial varieties of cotton in the U.S. are not considered weeds and are not effective in invading established ecosystems (USDA APHIS, 2011). Cultivated cotton rarely displays any dormancy characteristics and may grow as a volunteer only under specific conditions. Any volunteers are easily controlled by herbicides or mechanical means (Morgan *et al.*, 2011a; Morgan *et al.*, 2011b). Cotton is a slow grower, and does not compete effectively with other cultivated plants or primary colonizers (OECD, 2010).

Cultivated cotton is considered self-pollinating (OECD, 2010) and is not classified as a weed either in the scientific literature or by USDA (USDA APHIS, 2011). If DAS-8191Ø-7 cotton were to cross with cotton varieties expressing tolerance to herbicides with different modes of action and produce cotton volunteers with multiple herbicide tolerance, volunteers can still be effectively controlled mechanically or with other herbicide modes of action. Additionally, agronomic practices such as appropriate variety selections, crop rotation, and rotation of herbicides with different modes of action can be used to avoid or manage volunteer cotton tolerance to one or a few herbicides.

8.3. Herbicide Resistance Management

8.3.1. Herbicide Resistance

Herbicides have revolutionized weed control in most countries around the world. Herbicides are often the most reliable and least expensive method of weed control available, but reliance upon herbicides as the primary method of weed control can have unintended consequences (Appendix 7). The widespread use of herbicides can lead to weed populations that are no longer susceptible to the herbicide being used. The Weed Science Society of America defines herbicide resistance as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type" (WSSA, 2013a). Target plants with genes conferring resistance to a given herbicide can occur naturally within a population, but in extremely small numbers. Such plants remain reproductively compatible with the wild-type and can pass resistance genes on to their progeny (Mallory-Smith, 2010). Repeated use of the herbicide may allow these resistant plants to survive and reproduce. The number of resistant plants then increases in the population until the herbicide no longer effectively controls the weed. Thus, this is an evolutionary process (Moss, 2002), whereby a population changes from being susceptible to being resistant. Individual plants do not change from being susceptible to being resistant; rather, the proportion of resistant individuals within the population increases over time.

Herbicide resistant weeds have been a problem for growers for decades. During the 1970s, up to 30 different weed species were reported to be resistant to the triazine herbicides (Bandeen *et al.*, 1982). Today, there are 403 unique cases of herbicide resistant weeds globally, with 217 species: 129 dicots and 88 monocots (Heap, 2013c). A weed biotype is a sub-type or sub-population of a weed species; in this case one that has developed resistance to one or more herbicides. Report of a resistant biotype for a given weed species does not mean that weed resistance is common, widespread, or persistent in that species. There are generally many other options available to control these resistant biotypes.

Table 26 shows a tabular summary of the total number of resistant species for each herbicide mode of action as of August 2013. Two different classification systems have been developed independently by Herbicide Resistance Action Committee (HRAC) and the Weed Science Society of America (WSSA) to communicate the mode of action of herbicides; both are included in Table 26. Weeds which have developed resistance to one herbicide may also be resistant to other herbicides which have the same mode of action. Additional information on glyphosate, the evolution of herbicide resistant weeds, and 2,4-D can be found in Appendix 7.

8.3.2. Factors Impacting Herbicide Resistance

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

A matrix that can be used to evaluate the risk of selection for herbicide resistant weeds based on cropping system practices in shown in Table 27. This table assesses the risk of herbicide resistance development for each management practice as either "low", "medium", or "high". There is a high risk of resistance when limited management tools are used – for example, sole reliance on a chemical, such as glyphosate. When additional methods are used, the risk of weed resistance decreases. Cultural controls are an effective tool in battling weed resistance; these include practices such as delayed planting, cultivation or plowing prior to sowing, using certified crop seed free of weeds, and others (HRAC, 2013b).

Table 26. Number of Herbicide Resistant Weeds Reported Globally by Herbicide Group and Mode of Action[1]

Herbicide Group	Mode of Action	HRAC Group	WSSA Group	Example Herbicide	Total Count
ALS Inhibitors	Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	B	2	Chlorsulfuron	132
Photosystem II Inhibitors	Inhibition of photosynthesis at photosystem II	C1	5	Atrazine	71
ACCase Inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	A	1	Diclofop-methyl	43
Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	O	4	2,4-D	30
Bipyridiliums	Photosystem-I-electron diversion	D	24	Paraquat	28
Ureas and Amides	Inhibition of photosynthesis at photosystem II	C2	7	Chlorotoluron	23
Glycines	Inhibition of EPSP synthase	G	9	Glyphosate	24
Dinitroanilines and others	Microtubule assembly inhibition	K1	10	Trifluralin	11
Thiocarbamates and others	Inhibition of lipid synthesis – not ACCase inhibition	N	8	Triallate	8
Chloroacetamides and others	Inhibition of cell division (inhibition of very long chain fatty acids)	K3	15	Butachlor	4
Triazoles, Ureas, Isoxazolidiones	Bleaching: Inhibition of carotenoids biosynthesis (unknown target)	F3	11	Amitrole	5
4-HPPD Inhibitors	Bleaching: Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)	F2	27	Isoxaflutole	2
PPO Inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	E	14	Oxyfluorfen	6
Glutamine Synthase Inhibitors	Inhibition of glutamine synthetase	H	10	Glufosinate-ammonium	2
Nitriles and others	Inhibition of photosynthesis at photosystem II	C3	6	Bromoxynil	4
Carotenoid Biosynthesis Inhibitors	Bleaching: Inhibition of carotenoids biosynthesis at the phytoene desaturase step	F1	12	Flurtamone	3
Arylaminopropionic Acids	Unknown	Z	25	Flamprop-methyl	2
Unknown	Unknown	Z	26	(chloro) – flurenol	2
Mitosis Inhibitors	Inhibition of mitosis / microtubule polymerization inhibitor	K2	23	Propham	1
Cellulose Inhibitors	Inhibition of cell wall (cellulose) synthesis	L	27	Dichlobenil	1
Organoarsenicals	Unknown	Z	17	MSMA	1
Total herbicide resistant weeds					403

[1] (Heap, 2013a)

Table 27. Assessment of Resistance Risk by Evaluation of Cropping Systems[1]

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

[1] (Nevill *et al.*, 1998; HRAC, 2013a)

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

8.3.3. Herbicide Resistance Management

Although no cases of glyphosate resistant weeds were documented for 20 years after the launch of glyphosate (Dyer, 1994), glyphosate resistant biotypes of several weed species have now been reported in the United States (Powles, 2008b). This may be attributed to increased reliance on glyphosate for weed control after the launch of glyphosate tolerant soybeans (1996), cotton (1997), and corn (1998). The adoption of genetically engineered (GE) technology from 1996 onwards, primarily glyphosate tolerant crops, is illustrated in Figure 50: herbicide tolerant (HT) cotton is shown in dark blue; insect resistant (Bt) cotton is shown in green (USDA ERS, 2013b). The resulting evolution of glyphosate resistant weed populations threatens the ongoing sustainability of glyphosate and its contributions to world food production (Duke and Powles, 2008a).

As the number of glyphosate resistant weed species increases, it becomes increasingly important for growers to introduce greater diversity into their weed management programs (Powles, 2008a). This diversity could be achieved with herbicide rotations/sequences, mixtures of robust herbicides with different modes of action, and use of non-herbicide weed control tools. Glyphosate is increasingly being mixed with effective doses of other herbicides to manage these hard to control and resistant weed species. New herbicide tolerant traits that allow selective use of additional herbicides with a wider weed control spectrum compared to conventional herbicides can be used to control glyphosate resistant weed populations and reduce selection pressure for additional glyphosate resistant weed species. Table 28 shows that several common weeds in U.S. agriculture which are resistant to or difficult to control with glyphosate or ALS herbicides can be effectively controlled with 2,4-D.

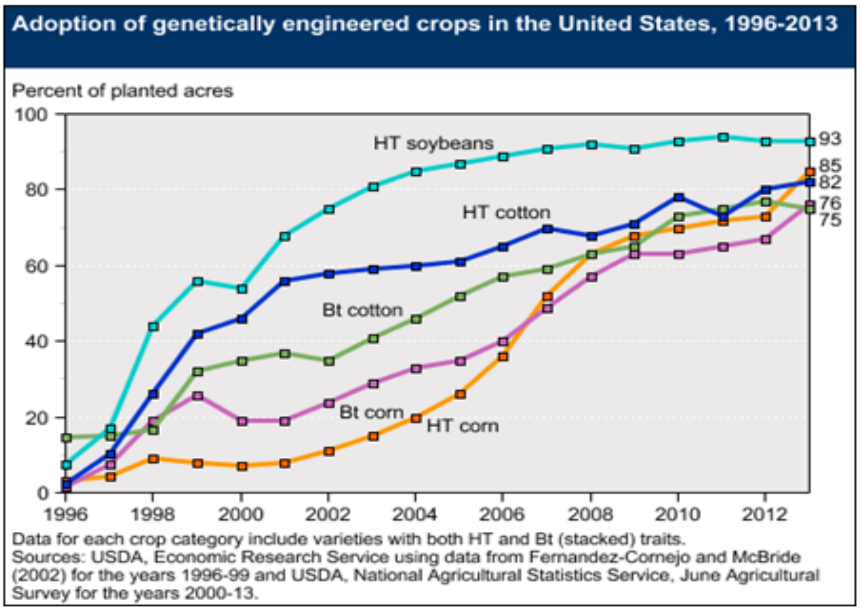


Figure 50. Adoption of GE Crops in the U.S.

Introduction of DAS-8191Ø-7 cotton will give farmers one more tool for use in their weed management programs and help ensure the long term sustainability of weed management programs, including the use of glyphosate. DAS-8191Ø-7 cotton will be combined with glyphosate tolerant cotton utilizing traditional breeding techniques. 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.

DAS-8191Ø-7 cotton will allow use of 2,4-D to control glyphosate resistant weeds and significantly delay the selection for glyphosate resistance in other weed species. The same benefits of DAS-8191Ø-7 cotton can be extended to other herbicide tolerant cropping systems, such as those with tolerance to glufosinate or ALS-inhibiting herbicides.

Table 28. Glyphosate and ALS Resistant Weeds Controlled by 2,4-D[1]

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

[1] (Heap, 2013c)
[2] Year first reported

DAS-8191Ø-7 cotton will expand the range of herbicides that can be used in herbicide tolerant cotton production systems, improving the ease and effectiveness of managing resistant and hard to control weeds and delaying the evolution of resistance to glyphosate and other herbicides.

8.4. Summary of Environmental Consequences and Impact on Agronomic Practices

Field testing results confirm that AAD-12 and PAT proteins expressed in DAS-8191Ø-7 cotton provide robust tolerance to 2,4-D and glufosinate respectively. There are no new phenotypic characteristics in DAS-8191Ø-7 cotton to indicate it is any different from conventional cotton in weediness potential, and like conventional cotton, the risk of gene flow from DAS-8191Ø-7 cotton to wild relatives in the U.S. is negligible.

No significant impact is expected on current crop management practices, non-target or endangered species, crop rotation, or volunteer management from the introduction of DAS-8191Ø-7 cotton. The availability of DAS-8191Ø-7 cotton will have a beneficial impact on weed control practices by providing growers with another tool to address their weed control needs. The availability of DAS-8191Ø-7 cotton will allow growers to proactively manage weed populations while avoiding adverse population shifts of troublesome weeds or the development of resistance.

With the exception of the presence of the AAD-12 and PAT proteins, DAS-8191Ø-7 cotton is phenotypically equivalent to commercially cultivated cotton and is no more susceptible to diseases or pests than commercially cultivated cotton. Based on the data presented throughout this petition, DAS submits that DAS-8191Ø-7 cotton is unlikely to pose a plant pest risk.

9. Adverse Consequences of Introduction

Field and laboratory testing of DAS-8191Ø-7 cotton demonstrated that the transgenic cotton is substantially equivalent to non-transgenic cotton apart from the intended change of herbicide tolerance. DAS knows of no study results or other observations indicating that there would be adverse consequences from the introduction of DAS-8191Ø-7 cotton.

10. 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. Compositional Literature Ranges for Non-Transgenic Cottonseed

Appendix 7. Evolution of Herbicide Resistant Weeds in Transgenic Crops

Appendix 8. Stewardship of Herbicide Tolerant Trait Technology for DAS-8191Ø-7 Cotton

Appendix 9. USDA Notifications for DAS-8191Ø-7 Cotton

Appendix 10. References

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 and Earle, 1991) and used as the positive control for the Southern hybridization. DIG-labeled DNA Molecular Weight Marker II and DIG-labeled 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) labeled 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^{o}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-labeled DNA molecular weight marker II and VII were used to determine the hybridizing fragment size on the Southern blots.

Detection

DIG-labeled 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 labeled probes from the membranes, allowing them to be re-hybridized with a different DNA probe.

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

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 4). 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 29.

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 30.

Table 29. 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 30. 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 analyzed 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 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 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 analyzed 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 analyzed 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 and 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 analyzed 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.

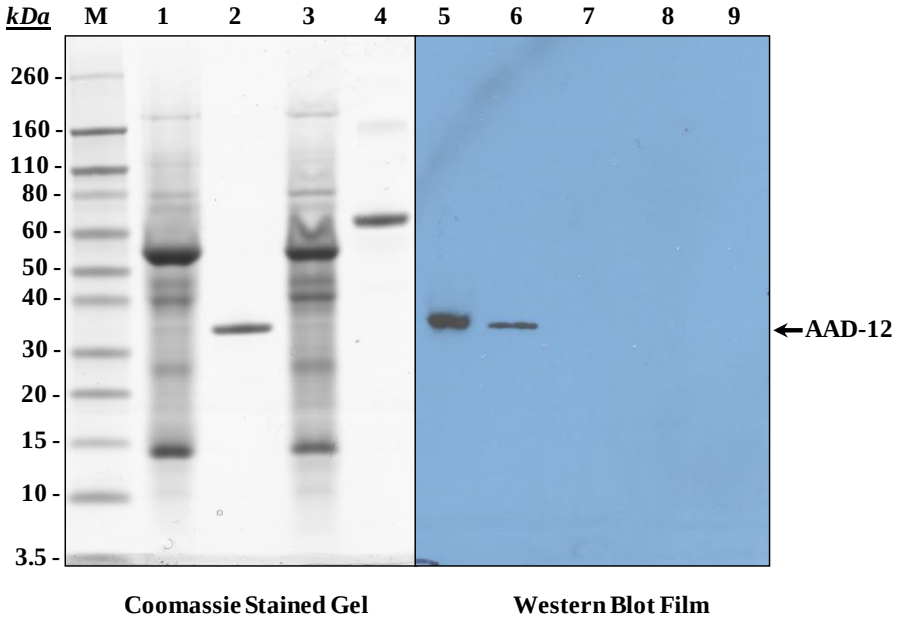
Results for Characterization of AAD-12 Protein

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 51). 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

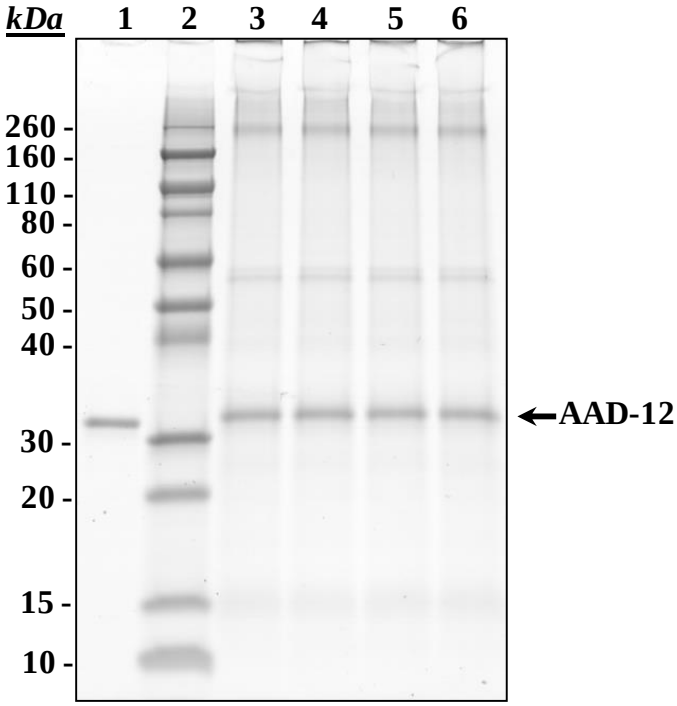
To conduct additional equivalency analysis of DAS-8191Ø-7 cotton AAD-12 to that of microbe-derived AAD-12, AAD-12 immunoaffinity precipitation was conducted on an aqueous extract of 5 – 10 grams of ground DAS-8191Ø-7 cotton lyophilized transgenic leaf tissue. Proteins bound to the anti-AAD-12 monoclonal 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 52).



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 51. 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 and 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

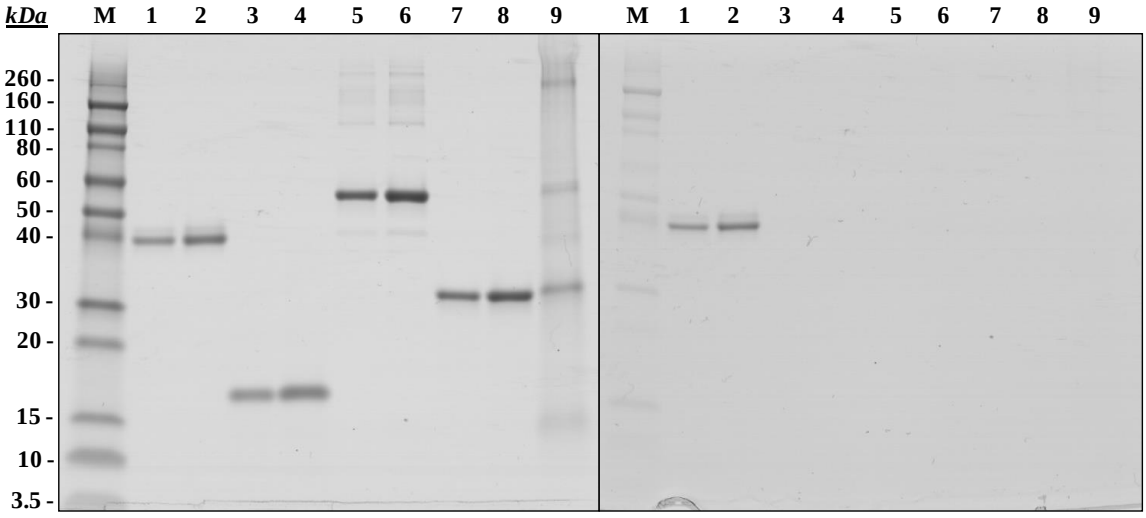
Figure 52. SDS-PAGE Analysis of Immunoaffinity Purified DAS-8191Ø-7 Cotton Derived AAD-12

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 53, 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 53. 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 52) 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 54 - Figure 56). 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 54. *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.

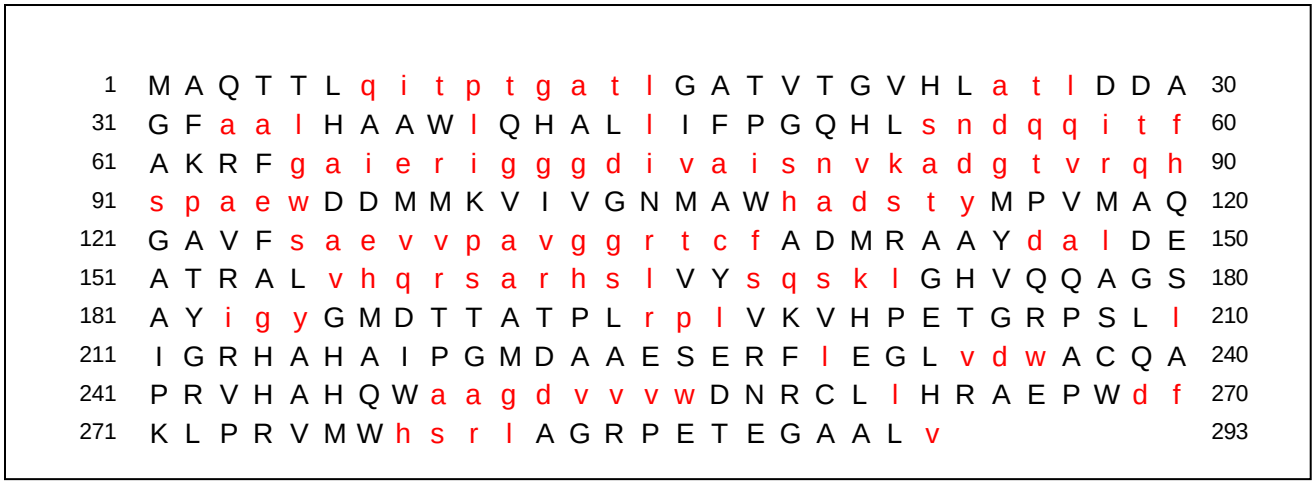


Figure 55. *In Silico* Chymotrypsin Cleavage of DAS-8191Ø-7 Cotton AAD-12
Theoretical cleavage of the AAD-12 protein with chymotrypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC. Theoretical trypsin digest peptides are indicated by the continuum of upper (black) or lower (red) case letters.

Figure 56. *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 57 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 and Hirst, 2008).

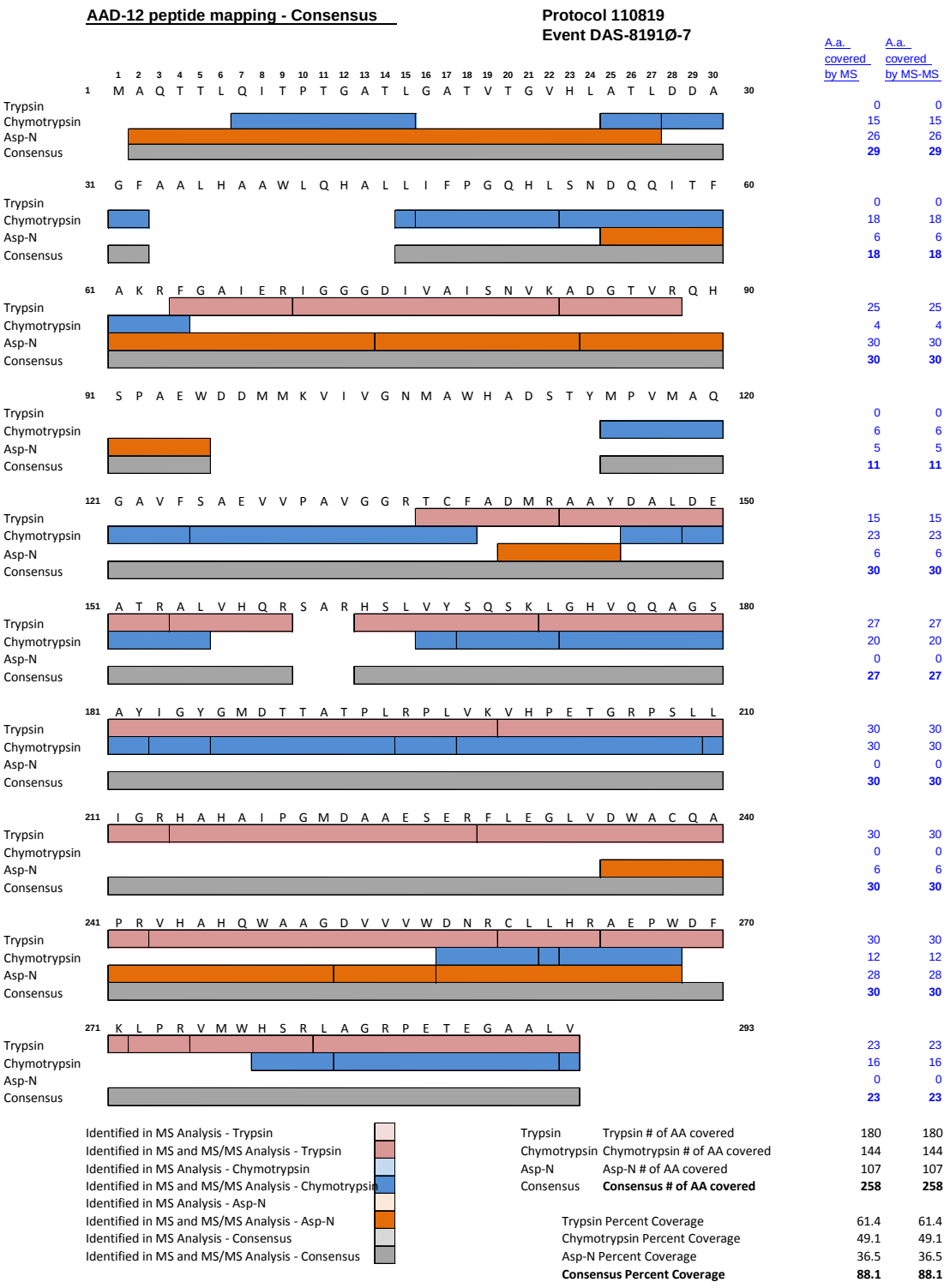


Figure 57. Comprehensive MS and MS/MS Sequence Coverage Map for Trypsin, Chymotrypsin and Asp-N Digest of DAS-8191Ø-7 Cotton-Derived AAD-12

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 31).

Table 31. 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...

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 31). 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 (Wellner *et al.*, 1990; Polevoda and Sherman, 2003). 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 and 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 and Sherman, 2003). However, examples demonstrating that N-terminal protein acetylation result in biological significance are rare (Polevoda and 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 32). 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 32. 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 58). 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.

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.

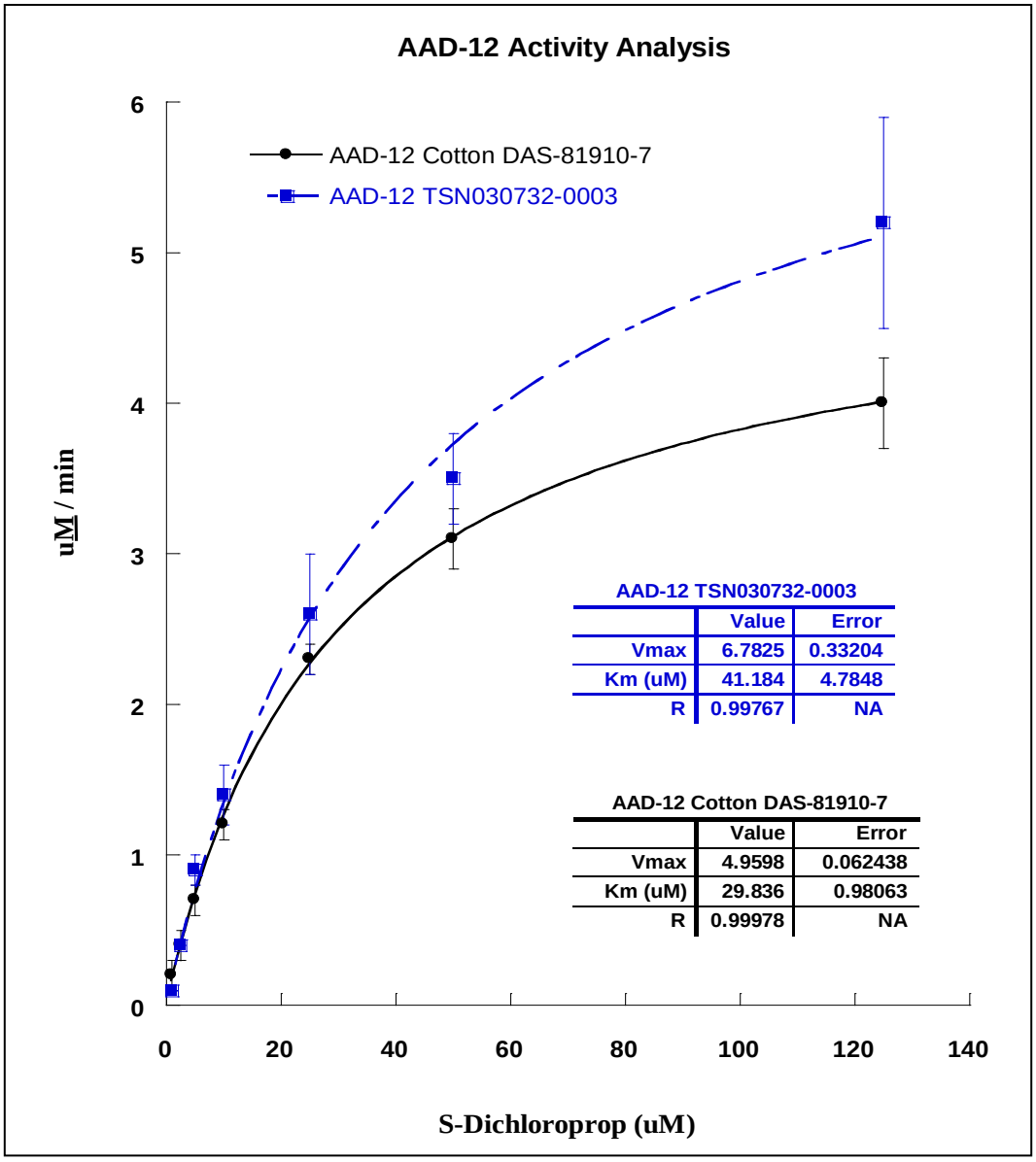


Figure 58. Kinetic Analysis of Microbe and DAS-8191Ø-7 Cotton Derived AAD-12 Proteins with S-Dichloroprop Substrate

The Michaelis-Menten plots of microbe-derived AAD-12 (black circles) and cotton-derived AAD-12 (blue squares) using S-Dichloroprop as a substrate for AAD-12. The average of three independent experiments is shown and error bars indicate the standard deviation of the analyses.

Appendix 3. Methods and Results for Characterization of PAT Protein

MaterØials & 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 4). 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 33.

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 34.

Table 33. 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 34. 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 analyzed 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 analyzed and compared with the sequence of the microbe-derived protein.

Protein purification of PAT from DAS-8191Ø-7 cotton plant tissue for functional analyses
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 (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 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. The reaction of the sulfhydryl 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) 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 analyzed using KaleidaGraph software (v.4.03).

Results for Characterization of PAT Protein

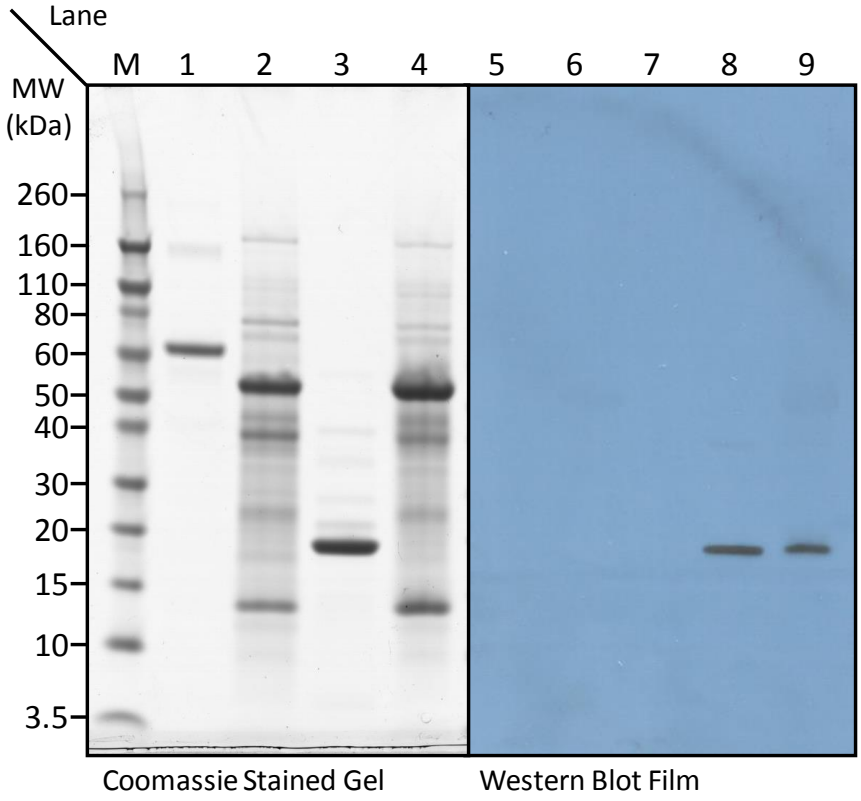
SDS-PAGE and western blot analysis of cotton leaf protein extracts

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 59) 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 59). 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

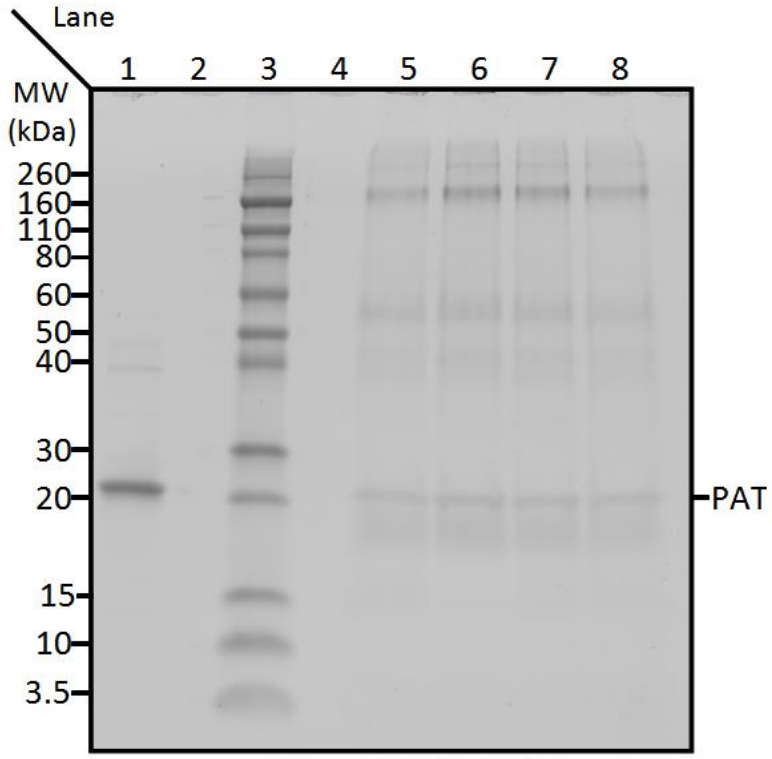
To conduct additional equivalency analysis of DAS-8191Ø-7 cotton-derived PAT to that of microbe-derived PAT, immuno-precipitation was conducted on an aqueous extract of ~6 grams of ground DAS-8191Ø-7 cotton leaf tissue to further purify the DAS-8191Ø-7 cotton-derived PAT protein. The total protein bound 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 60). 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 60) 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 59. SDS-PAGE and Western Blot Analysis of Microbe- and DAS-8191Ø-7 Cotton-Derived PAT Proteins



Coomassie Stained Gel

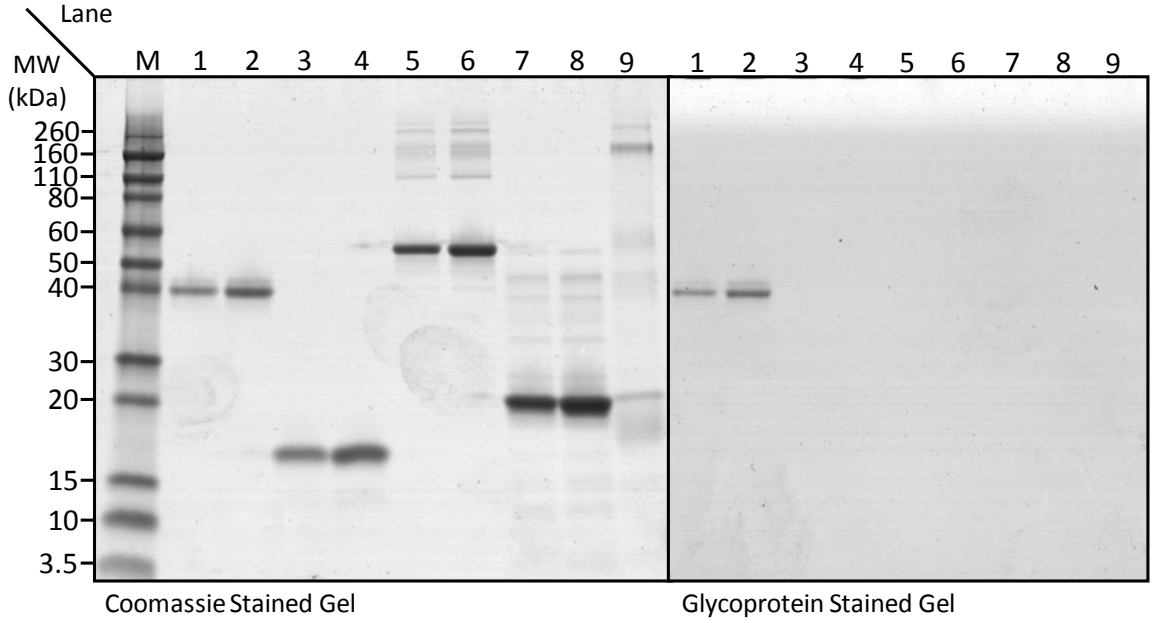
Lane	Sample	Loaded
1	Microbe-derived PAT	0.50 µg
2	No sample	---
3	Novex Sharp Pre-Stained MW marker	10.0 µL
4	No sample	---
5	DAS-8191Ø-7 immunopurified PAT	25.0 µL
6	DAS-8191Ø-7 immunopurified PAT	25.0 µL
7	DAS-8191Ø-7 immunopurified PAT	25.0 µL
8	DAS-8191Ø-7 immunopurified PAT	25.0 µL

Figure 60. 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 60). 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 and 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 61. 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 61).

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 60) and the respective bands were excised and subjected to in-gel digestion by trypsin and Asp-N endoproteases. The resulting peptide mixture was analyzed 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 62 and Figure 63 illustrate the theoretical cleavage of the PAT protein generated *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. 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 62. *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 hydrolyzes 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 63. *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 hydrolyzes 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 64). 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 35. 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 36. 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 and 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.

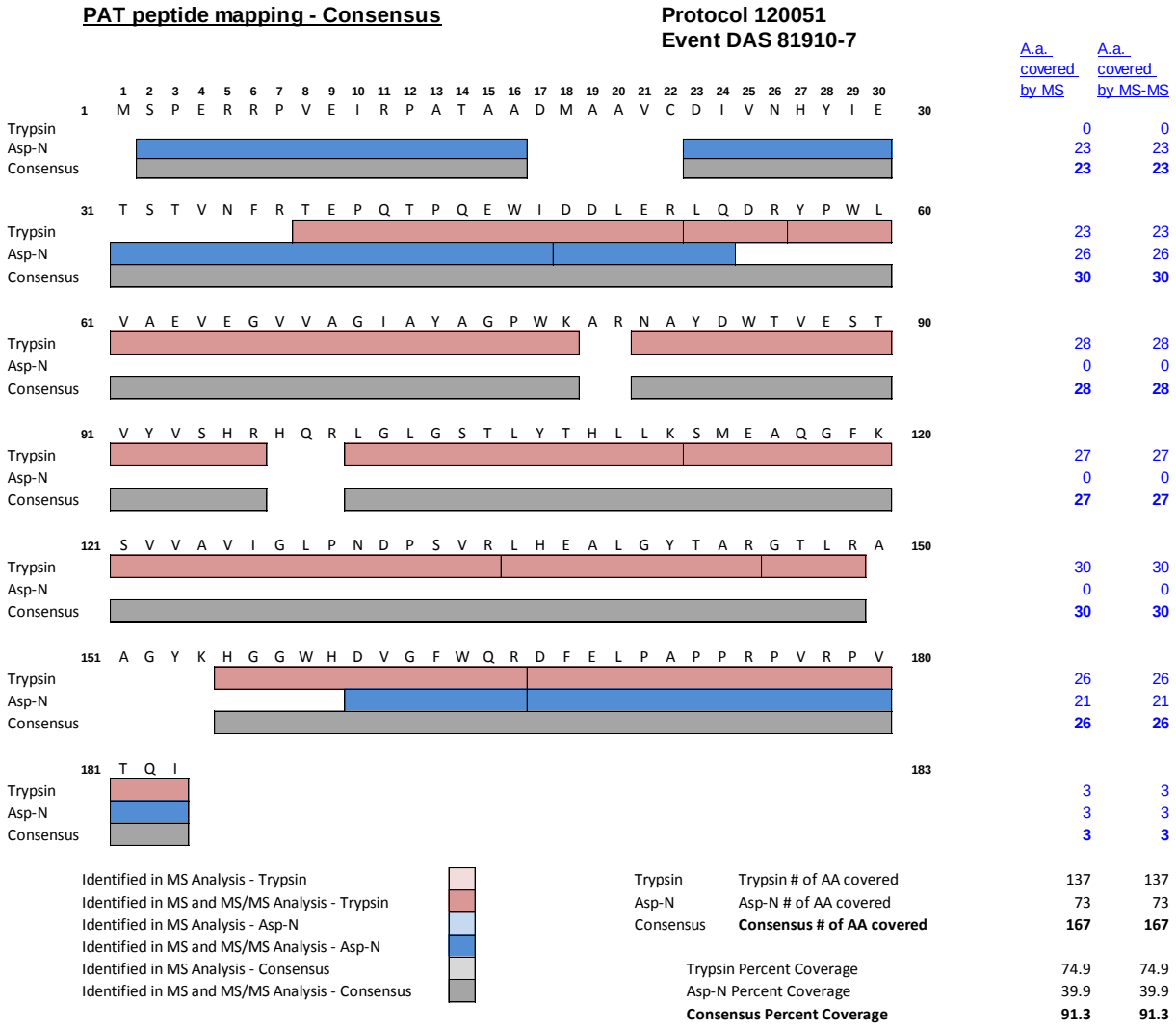


Figure 64. Comprehensive MS and MS/MS Sequence Coverage Map for Trypsin and Asp-N digests of DAS-8191Ø-7 Cotton-Derived PAT

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 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.

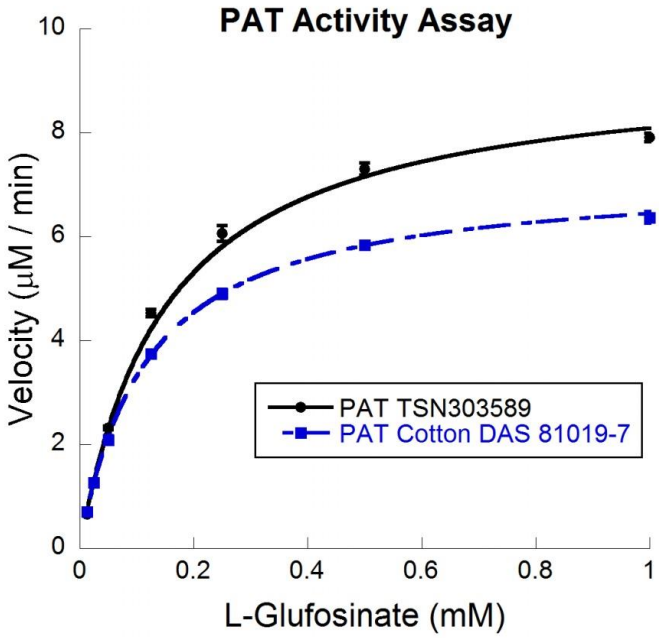


Figure 65. Kinetic Analysis of Microbe- and DAS-8191Ø-7 Cotton-Derived PAT Proteins

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 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 U.S. 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 center two rows) of each four row plot. Herbicide treatments were designed to replicate maximum label rates.

2,4-D plus glufosinate Treatment:
2,4-D (GF-2654) + Glufosinate (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.

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 analyzed using an AAD-12 ELISA kit. Briefly, an aliquot of the diluted sample and a horseradish peroxidase (HRP)/anti-AAD-12 monoclonal antibody conjugate were incubated in the wells of a microtiter plate coated with an immobilized anti-AAD-12 polyclonal antibody. These antibodies bind with AAD-12

protein in the wells and form a "sandwich" with AAD-12 protein bound between soluble and the immobilized antibodies. The unbound samples and excess conjugate were then removed from the plate by washing with PBST. Subsequent addition of an enzyme substrate generated a colored product. The reaction was stopped by adding a dilute acid solution.

Since the AAD-12 was bound in the antibody sandwich, the level of color development, determined by measuring the absorbance of the solution, was related to the concentration of AAD-12 in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm 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 analyzed 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 analyzed using a PAT ELISA kit. Briefly, an aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT monoclonal antibody and anti-PAT polyclonal antibodies coated in the wells of a 96-well plate in a sandwich ELISA format. At the end of the incubation period, the unbound reagents were removed from the plate by washing. Subsequent addition of an enzyme substrate generated a colored product. The reaction was stopped by adding a dilute acid solution.

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

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 Flower Leaf Pollen Root Seed Squares Whole Plant	0.025	0.06

Appendix 5**. Methods for Compositional Analysis**

Samples of DAS-8191Ø-7 (non-sprayed and sprayed with 2,4-D + glufosinate), near isogenic non-transgenic control (98M-2983), and reference variety cottonseed were analyzed at Covance Laboratories Inc. for 73 composition analytes. The following methods were used by Covance Laboratories to determine Proximates & Fiber (9), Minerals (12), Amino Acids (18), Fatty Acids (22), Vitamins (7) and Anti-Nutrients (5). See section 6.1 for composition analysis study design and results.

Proximates and Fiber

- **Protein**
  The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. 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 Fiber** (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 Fiber 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 fiber was determined gravimetrically.

- **Neutral Detergent Fiber** (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 Fiber 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 Fiber**
  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 fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the samples was calculated using protein and ash values and the weighed residue fractions.

- **Crude Fiber**
  Crude fiber was quantitated as the loss on ignition of dried residue remaining after digestion of the samples with 1.25% sulfuric 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	1.0
Inorganic Ventures	Sulfur	E2-S01119	100

Selenium by Inductively Coupled Plasma-Mass Spectrometry (SEICPMS)
The samples were closed-vessel microwave digested with nitric acid (HNO_3) 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 Se^{78} 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	10

Amino Acid Composition (TAALC/TRPLC)

- **Samples Analyzed (18 total)**

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

The samples were hydrolyzed 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 hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for approximately 20 hours.

The samples were analyzed 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 analyzed by gas chromatography using external standards for quantitation. Fatty acid results were converted to their triglyceride equivalents.

Fatty Acids Analyzed 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	Manufacture	Lot No.	Purity (%)
Thiamine monophosphate chloride dihydrate	Sigma-Aldrich	BCBF5554V	99.3
Thiamine hydrochloride	USP	P0K366	99.7

- **Vitamin B_2** (Riboflavin)
  The samples were hydrolyzed 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 hydrolyzed with sulfuric 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 hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the 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 hydrolyzed 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. Compositional Literature Ranges for Non-Transgenic Cottonseed

	Analyte	Units	ILSI 2010		Literature		Literature Citations	
			Min	Max	Min	Max	Min	Max
Proximates & Fiber (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 Fiber	% 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 Fiber	% 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)
	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)

Appendix 6. Compositional Literature Ranges for Non-Transgenic Cottonseed (continued)

	Analyte	Units	ILSI 2010		Literature		Literature Citations	
			Min	Max	Min	Max	Min	Max
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., 2005)	(Bertrand et al., 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)

Appendix 7. Evolution of Herbicide Resistant Weeds in Transgenic Crops

Herbicide Tolerant Crops

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

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

- the plant produces a new protein which detoxifies the herbicide (*e.g.*, glufosinate tolerance, 2,4-D tolerance), or
- the protein in the plant which is normally the target of the herbicide's action is replaced by a new protein which is unaffected by the herbicide (*e.g.*, glyphosate tolerance).

Herbicide tolerant crops available to farmers since 1996 include corn, soybean, cotton, sugar beet, alfalfa and canola among others. Initially, transgenes were only used to confer tolerance to bromoxynil, glufosinate, and glyphosate. The bromoxynil-tolerant crops are no longer sold. Until recently, this only left glyphosate and glufosinate tolerant transgenic crops, and of those, glyphosate has had the strongest impact on weed management (Duke, 2005).

Growers choose glyphosate tolerant crops because they make weed control easier and more effective, increase profit, require less tillage, and do not restrict crop rotations (Green, 2009). Thus, glyphosate tolerant corn, soybeans and cotton have experienced an unprecedented rapid adoption rate by U.S. farmers (Figure 50). The planting of glyphosate tolerant crops has increased steadily since their introduction in 1996: herbicide tolerant cotton is currently planted to 82% of U.S. acres; the vast majority is glyphosate tolerant (University of California Agriculture & Natural Resources, 2013a; USDA NASS, 2013c). Figure 66 illustrates the breakdown between herbicide tolerant (HT), insect tolerant (Bt), and stacked varieties of transgenic cotton planted in the U.S.

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

Glyphosate Resistance and Weed Shifts

Glyphosate Resistance

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

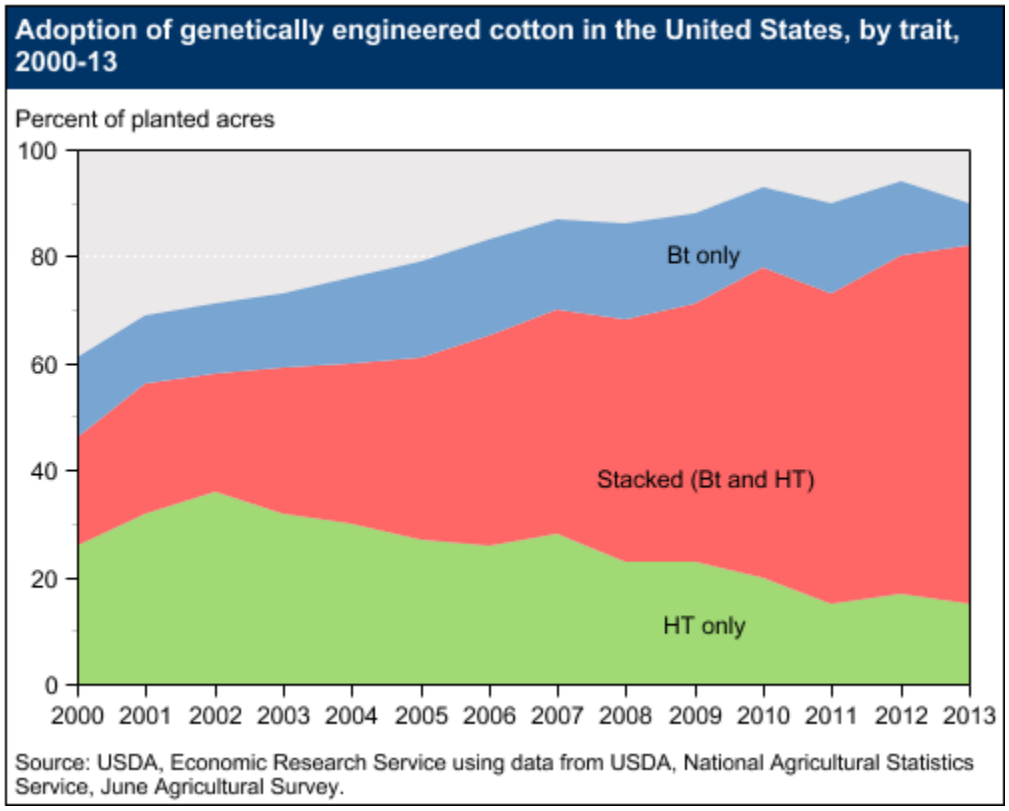


Figure 66. Adoption of GE Cotton in the U.S. (USDA ERS, 2013b)

Table 37 shows a summary of the glyphosate resistant weed species that have been reported from 1996 to August 2013. The data clearly shows that glyphosate resistance in weeds is expanding around the globe. Two of these glyphosate resistant weed species have already

become a significant problem for farmers across a large geographic area: *Conyza canadensis* (Horseweed) infests at least two million hectares of glyphosate tolerant crops in the U.S.; and *Amaranthus palmeri* (Palmer amaranth) has heavily impacted cotton growers, particularly in the Southeast.

Table 37. Weed Species with Reported Glyphosate Resistant Biotypes[1]

Common Name	Species Name	First Confirmed Report		Later Confirmed Reports
		Year	Country	
Rigid ryegrass	*Lolium rigidum*	1996	Australia	USA, S. Africa, France, Spain
Goosegrass	*Eleusine indica*	1997	Malaysia	Colombia, USA
Horseweed/Marestail	*Conyza Canadensis*	2000	USA	Brazil, China, Spain, Czech Republic
Italian ryegrass	*Lolium multiflorum*	2001	Chile	Brazil, USA, Spain, Argentina
Hairy fleabane	*Conyza bonariensis*	2003	S. Africa	Spain, Brazil, Colombia, USA
Buckhorn plantain	*Plantago lanceolata*	2003	S. Africa	
Common ragweed	*Ambrosia artemisiifolia*	2004	USA	
Giant ragweed	*Ambrosia trifida*	2004	USA	
Ragweed parthenium	*Parthenium hysterophorus*	2004	Colombia	
Palmer amaranth	*Amaranthus palmeri*	2005	USA	
Common waterhemp	*Amaranthus rudis*	2005	USA	Canada
Johnsongrass	*Sorghum halepenses*	2005	Argentina	USA
Sourgrass	*Digitaria insularis*	2006	Paraguay	Brazil
Junglerice	*Echinochloa colona*	2007	Australia	USA
Kochia	*Kochia scoparia*	2007	USA	
Liverseedgrass	*Urochloa panicoides*	2008	Australia	
Perennial ryegrass	*Lolium perenne*	2008	Argentina	
Gramilla mansa	*Cynodon hirsutus*	2008	Argentina	
Sumatran fleabane	*Coryza sumatrensis*	2009	Spain	
Australian fingergrass	*Chloris truncate*	2010	Australia	
Annual bluegrass	*Poa annua*	2010	USA	
Tropical Sprangletop	*Leptochloa virgate*	2010	Mexico	
Ripgut Brome	*Bromus diandrus*	2011	Australia	
Spiny Amaranth	*Amaranthus spinosus*	2012	USA	

[1](Heap, 2013d)

Table 38. Global Reports of Glyphosate Resistant Weed Biotypes with Resistance to Other Herbicide Modes of Action[5]

Common Name	Species Name	Year – Country (State/Providence)	Multiple Resistance to Other Herbicide Mode of Actions
Kochia	*Kochia scoparia*	2012 – CAN (Alberta)	ALS[1]
Sumatran Fleabane	*Conyza sumatrensis*	2011 – Brazil	ALS
Palmer amaranth	*Amaranthus palmeri*	2008 – U.S. (MS, GA) 2009 – U.S. (TN)	ALS
Common waterhemp	*Amaranthus rudis*	2005 – U.S. (MO) 2006 – U.S. (IL) 2011 – U.S. (IA)	ALS, PPO[2] ALS ALS, HPPD[3]
Common ragweed	*Ambrosia artemisiifolia*	2006 – U.S. (OH) 2012 – CAN (Ontario)	ALS
Hairy fleabane	*Conyza bonariensis*	2009 – U.S. (CA)	Bipyridiliums
Horseweed	*Conyza canadensis*	2003 – U.S. (OH) 2007 – U.S. (MS) 2010 – U.S. (DE) 2011 – CAN (Ontario)	ALS Bipyridiliums ALS ALS
Giant ragweed	*Ambrosia trifida*	2006 – U.S. (OH) 2008 – U.S. (MN)	ALS ALS
Goosegrass	*Eleusine indica*	1997 – Malaysia	ACCase[4]
Italian ryegrass	*Lolium multiflorum*	2002 – Chile 2006 – Chile 2007 – Chile 2010 - Argentina 2010 – Argentina 2010 – Brazil 2010 – U.S. (OR)	ALS ACCase ACCase, ALS ALS ACCase ACCase Glutamine synthase
Rigid ryegrass	*Lolium rigidum*	1999 - Australia 2003 – South Africa 2007 - Israel 2008 - Australia 2010 - Australia	ACCase, ALS, Dinitroanilines ACCase, Bipyridiliums ACCase, ALS Triazoles Bipyridiliums

[1] Acetolactase synthase inhibitors
[2] Polyphenol oxidase inhibitors
[3] Hydroxyphenyl-pyruvate-dioxygenase inhibitors
[4] Acetyl CoA carboxylase inhibitors
[5](Heap, 2013b)

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

applying glyphosate alone over wide areas on highly variable and prolific weeds made the evolution of resistant weeds inevitable (Owen, 2001; Thill and Lemerle, 2001).

Researchers have reported that eleven individual biotypes of the twenty-four glyphosate resistant species are also resistant to herbicides with other modes of action (Table 38). In recent years, chemical costs have doubled, and many growers have returned to hand weeding, in-crop cultivation, tillage, and post-harvest deep-turning to control glyphosate resistant weeds (Sosnoskie and Culpepper, 2013). Although herbicide options to control these biotypes with multiple herbicide resistance will be more limited, 2,4-D remains a viable control option for the broadleaf weeds.

Table 39. Potential Weed Shifts with Use of Glyphosate

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

Weed Shifts

When glyphosate tolerant crops are grown intensively with high reliance on glyphosate for weed control, species which possess some level of natural tolerance to glyphosate will become more prevalent. These “weed shifts” can occur more rapidly than selection for glyphosate resistance (Shaner, 2000). Some common hard to control weed species that could become “weed shifts” in U.S. cotton are listed in Table 39, with pigweed and morning glory most commonly reported by cotton growers (Duke and Powles, 2008b; Owen, 2008; Prince *et al.*, 2012).

2,4-D Resistance

2,4-D has been used continually and widely for over 70 years, and reports of resistance have been rare (Wright *et al.*, 2010; Greene and Reid, 2012). The earliest documented reports of herbicide resistant weeds were for resistance to 2,4-D in wild carrot (*Daucus carota*) (observed in 1952 but not reported until 1957) and spreading dayflower (*Commelina diffusa*) in 1957 (Heap, 2011). Today, a total of seventeen weed species have documented reports of 2,4-D resistant biotypes someplace around the globe (Table 40). Wild carrot, yellow bur-head, wild radish, musk thistle, and corn poppy are the only 2,4-D resistant weeds that have reported infestations in more than 1,000 acres. Some of these 2,4-D resistant biotypes have documented cross resistance to other auxin herbicides or multiple resistance to some ALS-inhibiting herbicides. Most of these resistant species do not appear to be spreading, as indicated by few reports of additional sites after the initial report.

Few of these auxin resistant weeds have had a significant economic impact due to the wide array of alternatives that successfully control these resistant weeds (Heap, 1997). The overall incidence of auxinic herbicide resistance after more than seventy years of use is low, particularly when compared with other herbicide families in use over shorter time periods: ALS inhibitors (imidazolinones, sulfonylureas, and sulfonamides), triazines, and ACCase herbicides.

It has been suggested by various researchers that the rarity of auxinic herbicide resistant biotypes in the field is due to: a) a commonly held belief that these herbicides have multiple sites of action in the plant (Jasieniuk *et al.*, 1996), b) redundancy in auxin receptors (AFBs) and other components of the auxin signal response (Walsh *et al.*, 2006), c) moderate selection pressure and their use in mixtures with other herbicides (Kern *et al.*, 2005), d) fitness penalties (Bourdot *et al.*, 1996), and e) quantitative inheritance of the resistance trait (Cranston *et al.*, 2001).

The mechanism of resistance to auxinic herbicides has been investigated, in varying degrees for only a few of these resistant biotypes. Resistance mechanisms in these biotypes have proven to be difficult to elucidate. A lack of differences between biotypes in auxinic herbicide absorption, translocation, and metabolism has led to the hypothesis that auxinic herbicide resistance is most often likely due to differences at the target site or differences along the signal transduction pathway (Van Eerd *et al.*, 2005).

Patterns and mechanisms of cross resistance in auxin herbicide resistant biotypes to other classes of auxin herbicides are not yet well understood. Further research is needed across a range of resistant species and biotypes to identify the potentially numerous gene mutations that cause resistance. It is also possible, but less likely, that a biotype might be resistant to all of these auxin herbicides. Due to the diversity of chemistry representing the synthetic auxin mode of action, it is unlikely plants will derive a single metabolic mechanism for tolerance to this class of herbicides.

Table 40. Weed Species with Reported 2,4-D Resistant Biotypes

Common Name	Species Name	Herbicide(s)	Year	Country / U.S. State
Wild carrot	*Daucus carota*	2,4-D	1952	Ontario
		2,4-D	1993	Michigan
		2,4-D	1994	Ohio
Dayflower	*Commelina diffusa*	2,4-D	1957	Hawaii
Field bindweed	*Convolvulus arvensis*	2,4-D	1964	Kansas
Musk thistle	*Carduus nutans*	2,4-D, MCPA	1981	New Zealand
Scentless chamomile	*Matricaria perforate*	2,4-D	1975	France
		2,4-D	1975	United Kingdom
Gooseweed	*Spenoclea zeylanica*	2,4-D	1983	Philippines
		2,4-D	1995	Malaysia
		2,4-D	2000	Thailand
Canada thistle	*Cirsium arvense*	2,4-D, MCPA	1985	Hungary
Globe fringerush	*Fimbristylis miliacea*	2,4-D	1989	Malaysia
Wild mustard	*Sinapsi arvensis*	2,4-D, most other auxins	1990	Manitoba
Corn poppy	*Papaver rhoeas*	2,4-D, tribenuron	1993	Spain
Yellow bur-head	*Limnocharis flava*	2,4-D	1995	Indonesia
		2,4-D, bensulfuron-methyl	1998	Malaysia
Italian thistle	*Carduus pycnocephalus*	2,4-D	1997	New Zealand
Wild radish	*Raphanus raphanistrum*	2,4-D	1999	Australia
Marshweed	*Limnophila erecta*	2,4-D, ALS	2002	Malaysia
Indian hedge mustard	*Sisymbrium orientale*	2,4-D, metsulfuron-methyl	2005	Australia
Prickly lettuce	*Lactuca serriola*	2,4-D, dicamba, MCPA	2007	Washington
Common Waterhemp	*Amaranthus tuberculatus*	2,4-D	2009	Nebraska

[1](Heap, 2013e)

To summarize, selection for auxin resistant weed biotypes after more than 70 years of use has been slow. Auxin resistant weeds have not shown significant spread from initial sites, nor have they demonstrated significant economic importance. Thus far, there have been no reports of auxin resistant weed biotypes in cotton fields. Use of 2,4-D in DAS-8191Ø-7 cotton should not result in 2,4-D resistant weeds becoming a significant issue in cotton. Additionally, 2,4-D will likely be used in a mixture with one or more other herbicides. Other alternative herbicides which are effective on the same weeds can be used to control any 2,4-D resistant weeds that might occur.

Glufosinate Resistance

There are currently only two reported weed biotypes that have developed resistance to glufosinate, Goosegrass (*Eleusine indica*) in Malaysia and Italian ryegrass (Oregon) (Heap, 2013a). Italian Ryegrass is also resistant to glyphosate.

Weed Resistance Management

Cotton growers in the U.S. are currently battling a number of glyphosate resistant weeds (Table 41) due to years of exclusive use of glyphosate for weed control (Foresman and Glasgow, 2008; Gustafson, 2008). As described above, overreliance on this single method of control has greatly increased selection pressure for glyphosate resistant weeds.

Table 41. U.S. Glyphosate Resistant Weeds in Cotton Growing States[1]

Number	Species	Common Name	State
1	*Amaranthus palmeri*	Palmer Amaranth	Georgia North Carolina Arkansas Tennessee New Mexico Alabama Georgia Mississippi Missouri Tennessee Louisiana Kansas Arizona California
2	*Amaranthus spinosus*	Spiny Amaranth	Mississippi
3	*Amaranthus tuberculatus (syn. rudis)*	Common Waterhemp	Missouri Kansas Mississippi Oklahoma Tennessee
4	*Ambrosia artemisiifolia*	Common Ragweed	Arkansas Missouri Kansas
5	*Ambrosia trifida*	Giant Ragweed	Arkansas Kansas Tennessee Missouri Mississippi
6	*Conyza bonariensis*	Hairy Fleabane	California
7	*Conyza canadensis*	Horseweed	Kentucky Tennessee Missouri Arkansas Mississippi North Carolina California Kansas Mississippi Oklahoma
8	*Echinochloa colona*	Junglerice	California
9	*Eleusine indica*	Goosegrass	Mississippi Tennessee
10	*Kochia scoparia*	Kochia	Kansas
11	*Lolium multiflorum*	Italian Ryegrass	Mississippi Arkansas California
12	*Lolium rigidum*	Rigid Ryegrass	California
13	*Poa annua*	Annual Bluegrass	Missouri Tennessee
14	*Sorghum halepense*	Johnsongrass	Arkansas Mississippi Louisiana

[1] (Webster and Sosnoskie, 2010)

The magnitude of current weed resistance challenges has prompted outreach efforts by industry and academics to educate growers on integrated weed management strategies. For example, the Weed Science Society of America has extensive resources on its website, including lesson modules for herbicide resistant weeds (WSSA, 2013b). These modules explain the current status of resistance, how herbicides work, what herbicide resistance is, and principles of managing herbicide resistance (WSSA, 2013c). Similarly, the Herbicide Resistance Action Committee, an international body founded by the agrochemical industry, provides herbicide resistance management strategies and principles to teach growers an integrated approach to weed resistance management (HRAC, 2013b).

One cornerstone of integrated weed resistance management is the use of herbicides with multiple modes or sites of action. DAS-8191Ø-7 cotton will be combined with glyphosate tolerant cotton utilizing traditional breeding techniques and fits into a recommended integrated weed resistance management program. Additionally, DAS-8191Ø-7 cotton is a needed tool to mitigate the current selection pressure for glyphosate resistant weed species.

Conclusions

The adoption of herbicide tolerant crops, primarily glyphosate tolerant varieties, and resulting reliance on a single mode of action for weed control, has resulted in a rapidly growing population of resistant and hard to control weed species. Growers now understand the need for an integrated weed management approach; DAS-8191Ø-7 cotton offers an alternative chemistry for control of troublesome weeds, in accordance with recommended management practices.

DAS-8191Ø-7 cotton will be combined with glyphosate tolerant cotton utilizing traditional breeding techniques. 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.

Appendix 8. Stewardship of Herbicide Tolerant Trait Technology for DAS-8191Ø-7 Cotton

Introduction

DAS-8191Ø-7 cotton is a transgenic cotton product that provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate. This herbicide tolerant cotton will provide growers with greater flexibility in selection of herbicides for the improved control of economically important weeds; allow an increased application window for effective weed control; and provide an effective resistance management prevention solution to the increased incidence of hard to control and glyphosate resistant weeds[1].

Dow AgroSciences is committed to promoting the responsible use and stewardship of this new herbicide tolerant trait technology and will implement a comprehensive stewardship program for DAS-8191Ø-7 cotton and its associated herbicide technology containing a new 2,4-D choline salt. The stewardship program will focus on educating and training retailers, growers and applicators on the appropriate use of this new technology as part of an integrated weed management program. This will be accomplished by using a multi-faceted approach, including use of a variety of tools and delivery methods, and working with customers, stakeholders and industry organizations to promote responsible use of the technology. The stewardship program is designed to:

- Provide comprehensive guidance and education on responsible use
- Promote compatibility with other crops and cropping systems
- Minimize the potential for off-target movement
- Promote Weed Resistance Management[2]
- Promote responsible use and worker safety
- Support compliance with applicable regulatory requirements

Stewardship Program Components

The comprehensive stewardship program is based on Dow AgroSciences' ongoing commitment to stewardship and input from retailers, growers, applicators, regulators, and stakeholders. Key components of the stewardship program include:

- Authorized use of DAS-8191Ø-7 cotton (and associated herbicide products) through a Technology Use Agreement (TUA)
- Herbicide technology advancements, including new 2,4-D choline based herbicide technologies
- Comprehensive product use information, including mandatory use requirements and restrictions
- Education and training for distributors, retailers, growers, and applicators, and

[1] As used in this document, a herbicide resistant weed is a member of a population within a species that has an inherited ability to survive and reproduce following exposure to a dose of a herbicide normally lethal to susceptible populations of the species. Through repeated herbicide selection, the resistant population becomes dominant in a given area. A hard-to-control weed is a weed species that is inherently able to withstand treatment with a herbicide as a result of natural factors not involving herbicide selection or genetic mutation.

[2] Stewardship information on insect resistance management will also be included, as applicable.

- Compliance monitoring.

Authorized Use of DAS-8191Ø-7 Cotton and Associated Herbicide Products Through a Technology Use Agreement

Dow AgroSciences will authorize growers of DAS-8191Ø-7 cotton through a Technology Use Agreement that requires growers to use the cotton trait technology responsibly and prohibits the use of unauthorized herbicides.

The Technology Use Agreement is a legal and contractual obligation that will require all growers of DAS-8191Ø-7 cotton to:

- Use only EPA accepted and Dow AgroSciences' authorized herbicide products for post-plant applications to DAS-8191Ø-7 cotton;
- Read and follow the Product Use Guide (PUG) requirements;
- Read and follow FIFRA pesticide product label directions, which will include requirements to use in a manner protective of the environment;
- Read and follow seed packaging information; and
- Provide access to information about use of the technology and allow on-farm assessments.

Herbicide Technology Advancements

Dow AgroSciences has discovered and developed a novel (non-ester or non-amine) 2,4-D choline[3]. Exceptionally low volatility of DAS' 2,4-D choline salt has been demonstrated in both laboratory and field studies. In a laboratory study, wheat plants treated with rates ten times higher than specified on the label of 2,4-D choline were placed in an enclosed chamber with grape and tomato plants (Ouse et al., 2010). After 24 hours of exposure in the chamber at 104°F, neither sensitive crop showed any symptoms of injury. The lack of any symptoms on the grape and tomato plants is a clear indication that there was no detectable volatility following application of 2,4-D choline salt. Field results are consistent with these laboratory findings (Hillger et al., 2010).

Comprehensive Product Use Information

Product Use Guides

Dow AgroSciences will provide DAS-8191Ø-7 cotton growers a comprehensive Product Use Guide that is a material part of the Technology Use Agreement and details the requirements for responsible use. As a standard practice, Dow AgroSciences provides a Product Use Guide to users (e.g., retailers, dealers, growers, applicators) of its technology. The Product Use Guide provides growers comprehensive information including required and recommended management practices. The Product Use Guide for DAS-8191Ø-7 cotton will include detailed requirements for responsible use of the cotton and associated herbicide products. It will include information on weed resistance management, application and management of

[3] Applications for 2,4-D choline and associated herbicides for use with DAS-8191Ø-7 cotton will be submitted to EPA.

off-target herbicide movement, as well as seed planting directions and cotton seed and by-product stewardship.

The Product Use Guide will require DAS-8191Ø-7 cotton growers to:

- Use only EPA accepted and Dow AgroSciences' authorized herbicide products with DAS-8191Ø-7 cotton
- Read and follow FIFRA pesticide product label directions
- Follow Weed Resistance Management guidelines, recommending utilization of herbicide products with additional modes of action
- Read and follow marketing guidelines
- Provide management practices to promote compatibility with other crops, which may include setbacks, consulting sensitive crop registries, and communicating with neighbors.

The Product Use Guide will promote appropriate cultural practices including diversified weed control strategies (e.g. herbicide use or crop rotation) to minimize selection for herbicide resistant weed populations. Responsible use of the cotton seed and herbicides in an integrated farming system promotes compatibility with other crops.

Herbicide Application and Management of Off-Target Movement

The new 2,4-D choline-containing herbicide products will minimize off target movement by reducing volatility and the number of driftable fines. To further minimize the potential for off-target movement with the new 2,4-D choline-containing herbicide products, the Product Use Guide will include recommendations on application equipment and methods such as following proper equipment maintenance, calibration and use.

A variety of factors including weather conditions (*e.g.*, wind direction, wind speed, temperature, and relative humidity) and method of application can influence pesticide drift. The Product Use Guide will require applicators to evaluate factors and make appropriate adjustments when applying 2,4-D choline salt for use with DAS-8191Ø-7 cotton consistent with the final product label approved by EPA. Dow AgroSciences is conducting significant research on nozzles for spray application. The Product Use Guide will include instructions on using specific nozzles that minimize driftable spray droplets and specified environmental conditions such as wind speed and field temperature that minimize the potential for off-target movement due to particle or vapor drift.

Product Labels, Seed Bags and Tags

Product Labels

Dow AgroSciences will submit a product label to EPA that includes comprehensive information and requirements for responsible use of 2,4-D choline with DAS-8191Ø-7 cotton. That label language will include detailed use directions and application requirements designed to minimize the potential for weed resistance development, minimize the potential for off-target movement, and promote worker safety. For example, the submitted label will

not allow herbicide application through any type of irrigation equipment and will prohibit aerial application.

Seed Bags and Tags

Packages of DAS-8191Ø-7 cotton seed will have product stewardship information on the seed bags and seed tags, including use requirements and a customer information telephone number. Information will be included on seed packaging to communicate the presence of herbicide tolerant traits. This information will also be included in the Product Use Guide, training, and marketing materials.

Education and Training for Retailers, Growers and Applicators

Dow AgroSciences will provide comprehensive education and training in multiple formats to distributors, retailers, growers, and applicators to reinforce the requirements for proper stewardship and responsible use. Dow AgroSciences provides comprehensive training on its portfolio of products through a variety of formats. Education and training, reinforced through product profiles, technical bulletins, sales literature, direct mailing and websites will also be presented in multiple formats to enhance learning and mastery of core concepts related to the stewardship program.

A variety of educational formats will be used to promote concept learning. Training will be provided through face-to-face meetings, retailer visits, field visits, and self-paced learning modules on:

- stewardship requirements for responsible use of DAS-8191Ø-7 cotton and associated herbicides, including
- weed resistance management.

This training will reinforce the cotton grower's obligation to adhere to product use guidelines, FIFRA Pesticide Product Labels, seed product information and all product stewardship requirements for responsible use and proper stewardship. Training is an ongoing process, with interactive learning incorporated, where applicable, to promote responsible use and stewardship.

Multiple resources and tools will be available to the growers and applicators to promote and communicate the importance of stewardship and responsible technology use. In addition to the Technology Use Agreement, Product Use Guide, and training, additional resources will be available, including:

- Customer Service
- Technical Bulletins
- AdvanceFarming.com
- On-line training library
- In-field experts
- Trait stewardship website[4]

[4] www.traitstewardship.com

- Stewardship tools

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

Compliance Monitoring

Dow AgroSciences will monitor compliance with the Technology Use Agreement and Product Use Guide through surveys and on-farm assessments. Upon request by Dow AgroSciences, DAS-8191Ø-7 cotton growers must provide information regarding the location of fields planted with DAS-8191Ø-7 cotton, and the herbicides applied to these fields.

Failure to follow the stewardship requirements will result in:

- Education and reinforcement of the stewardship requirements with growers, applicators and retailers,
- Additional education and training, and/or
- Loss of access to DAS-8191Ø-7 cotton.

Stakeholder Outreach and Industry Involvement

In addition to managing the technology in accordance with the applicable requirements of federal and state government agencies, Dow AgroSciences is a member of the American Chemistry Council's Responsible Care® initiative and Founding Member of the biotechnology industry's Excellence Through Stewardship® organization which encourages effective and comprehensive stewardship programs and quality management systems throughout a trait product's life cycle. Dow AgroSciences participates in several organizations and associations globally to promote the safe research and development, production, distribution, and responsible use of agricultural chemical and biotechnology products.

Dow AgroSciences is a participant in the Herbicide Resistance Action Committee (HRAC)[5], an industry-based group supported by CropLife International[6]. HRAC focuses on encouraging responsible herbicide usage, communicating herbicide resistance management strategies and supporting their implementation through practical guidelines. HRAC engages in active collaboration with public and private researchers, especially in the areas of problem identification and devising and implementing herbicide management strategies.

Dow AgroSciences' personnel interact with academic weed scientists in addressing weed resistance management[7] issues. Dow AgroSciences conducts joint trials at university sites as

[5] www.hracglobal.com
[6] www.croplife.org/public/resistance_management
[7] www.wssa.net/Weeds/Resistance

well as seeks input from university researchers regarding weed management. Dow AgroSciences also participates in a wide range of professional organizations including agronomy societies, seed trade groups, weed science societies, and crop commodity groups.

Conclusion

Dow AgroSciences is committed to promoting the responsible use and stewardship of DAS-8191Ø-7 cotton and associated herbicide products. The comprehensive stewardship program outlined here incorporates elements to:

- Promote responsible use of DAS-8191Ø-7 cotton and associated herbicide products,
- Promote compatibility with other crops and cropping systems,
- Promote Weed Resistance Management, and
- Support compliance with all relevant state and federal requirements.

The stewardship program, focusing on education and management practices for proper use of the cotton and herbicides, coupled with the physical attributes of DAS' 2,4-D choline formulation (e.g., ultra-low volatility, minimized potential for drift, decreased odor and improved handling characteristics) enables coexistence with organic crops, crops traditionally sensitive to 2,4-D, and non-genetically engineered cotton.

Education and training efforts are also an integral part of the stewardship effort, promoting awareness and understanding of proper use of the cotton and herbicides. The combination of EPA mandated herbicide label requirements, Dow AgroSciences' stewardship program, and associated proprietary herbicide technology will provide the grower of DAS-8191Ø-7 cotton with the tools needed to mitigate potential risk to human health and the environment without compromising farm productivity.

Appendix 9. USDA Notifications for DAS-8191Ø-7 Cotton

USDA Notification Number	Authorization Date	Expiration Date	State(s)	Number of Trials	Trial Report Status[1]
13-063-109n	4/17/2013	4/17/2014	TX	12	Pending
13-053-106n	4/1/2013	4/1/2014	TX	3	Pending
13-063-110n	3/25/2013	3/25/2014	AR, CA, GA, LA, MS, NC, OK, SC	27	Pending
12-089-109n	4/26/2012	4/26/2013	LA	1	Pending
12-089-106n	4/26/2012	4/26/2013	TX	2	Pending
12-089-109n	4/26/2012	4/26/2013	AL, AR, CA, FL, GA, MO, MS, NC	9	Pending
12-089-101n	4/15/2012	4/15/2013	TX	13	Pending
13-066-104n	4/12/2012	4/12/2013	AL, AR, CA, GA, LA, MO, MS, NC	10	Pending
12-081-113n	4/6/2012	4/6/2013	AR, CA, GA, MO, MS, NC	13	Pending
12-062-102n	4/5/2012	4/5/2013	AR, CA, GA, LA, MO, MS, NC, OK, SC, TN	50	Pending
12-081-114n	4/4/2012	4/4/2013	TX	8	Pending
11-249-101n	11/1/2011	11/1/2012	PR	1	Submitted (Apr 2013)
11-147-101n	6/10/2011	6/10/2012	IN	1	Submitted (Nov 2012)
11-087-112n	4/29/2011	4/29/2012	AR, CA, GA, LA, MS, NC, SC, TN, TX	24	Submitted (Oct 2012)
10-076-102n	4/19/2010	4/19/2011	AL, AR, CA, GA, LA, MS, NC, SC, TN	29	Submitted (Oct 2011)

[1] Pending reports to be submitted within six months of the notification expiration date

Appendix 10. References

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