Dow AgroSciences

RECEIVED
By APHIS BRS Document Control Officer at 3:22 pm, Dec 20, 2012

Petition for Determination of Nonregulated Status for Insect-Resistant DAS-81419-2 Soybean

OECD Unique Identifier: DAS-81419-2

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

Submitting Company:

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268

Submitted by:

Lei Han, Ph.D.
Regulatory Manager

Regulatory Sciences & Government Affairs
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
Phone: (317) 337-3398
Fax: (317) 337-4523
Email: lhan@dow.com

September 26, 2012
Revised November 2, 2012
Revised December 19, 2012

Contains No Confidential Business Information

Release of Information

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

Lei Han, Ph.D.
Regulatory Manager

Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268
Phone: (317) 337-3398
Fax: (317) 337-4523
Email: lhan@dow.com

Summary

Dow AgroSciences LLC (herein referred to as "DAS") is submitting a Petition for Determination of Nonregulated Status for DAS-81419-2 soybean. DAS requests a determination from USDA Animal and Plant Health Inspection Service (APHIS) that soybean transformation event DAS-81419-2 and any soybean lines derived from crosses between DAS-81419-2 soybean and conventional soybean or biotechnology-derived soybean granted non-regulated status by APHIS no longer be considered regulated articles under 7 CFR Part 340.

DAS-81419-2 Soybean
DAS-81419-2 soybean is a transgenic soybean that expresses the insecticidal proteins Cry1Ac and Cry1F originally from the naturally-occurring soil bacterium, *Bacillus thuringiensis*. Cry1Ac and Cry1F provide protection against several lepidopteran pests of soybean, including soybean looper (*Chrysodeixis includens*, formerly *Pseudoplusia includens*), velvetbean caterpillar (*Anticarsia gemmatalis*), fall armyworm (*Spodoptera frugiperda*) and tobacco budworm (*Heliothis virescens*). In addition, DAS-81419-2 soybean expresses the phosphinothricin acetyltransferase (PAT) protein from the soil bacterium *Streptomyces viridochromogenes.* The PAT protein provides tolerance to the herbicide glufosinate and was used as a selectable marker during the development of DAS-81419-2 soybean. The transgenes for Cry1Ac, Cry1F and PAT expression were introduced into soybean via *Agrobacterium*-mediated transformation to create DAS-81419-2 soybean.

DAS-81419-2 soybean provides an effective pest management tool for lepidopteran pests that could reduce or replace current insecticide applications in regions where these insect pests cause significant yield loss. In the U.S., the majority of soybeans are grown in the north central states and the remaining in the southeastern and Mississippi delta region. Insect pressure is generally greatest in the southeast, where climate allows the major soybean insect pests to overwinter and facilitates multiple generations per year.

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), are subject to regulation by the U.S. Environmental Protection Agency (EPA). Pesticides produced in planta, known as plant-incorporated protectants (PIPs), are also subject to regulation by the EPA under FIFRA. DAS has submitted an application for a FIFRA Section 3 seed increase registration with EPA to support future breeding and seed increase activities in the U.S. Under a Section 3 seed increase registration, commercial sale of DAS-81419-2 soybean in the U.S. is prohibited. At present, DAS does not plan to commercialize DAS-81419-2 soybean in the U.S. due to the limited number of acres that consistently experience sufficient lepidopteran insect pressure. DAS will apply for a FIFRA Section 3 commercial use registration if DAS decides to commercialize DAS-81419-2 soybean in the U.S.

Evaluation of Plant Pest Potential of DAS-81419-2 Soybean
The data and information presented in this petition demonstrate that DAS-81419-2 soybean is no more likely to become a plant pest than conventional soybean or to have an adverse environmental impact. This conclusion is based on the outcome of extensive evaluations including i) detailed molecular characterization of the inserted DNA, ii) detailed biochemical characterization of the introduced proteins, iii) compositional assessment of forage and grain, iv)

agronomic and phenotypic characteristics and ecological interactions assessments, and v) assessment on the potential impact on non-target organisms and threatened and endangered species.

DAS-81419-2 soybean was genetically modified to express two insecticidal proteins, Cry1Ac and Cry1F, and phosphinothricin acetyltransferase (PAT) protein. Cry1Ac consists of 1156 amino acids from three components: the N-terminal toxin core from Cry1Ac1 originating from *B. thuringiensis* subsp. *kurstaki,* followed by a very small portion of Cry1Ca3 originating from *B. thuringiensis* subsp. *aizawai*, and the C-terminal sequence from Cry1Ab1 originating from *B. thuringiensis* subsp. *Berliner.* Cry1F consists of 1148 amino acids from three components: the N-terminal toxin core from Cry1Fa2 following by a very small portion of Cry1Ca3 originating from *B. thuringiensis* subsp. *aizawai,* and the C-terminal sequence from Cry1Ab1 originating from *B. thuringiensis* subsp. *Berliner.* Cry1Ac and Cry1F expressed in DAS-81419-2 soybean are 100% identical in amino acid sequence to Cry1Ac and Cry1F expressed in the deregulated events comprising WideStrike® cotton; DAS-21023-5 (also described as 3006-210-23 expressing Cry1Ac) and DAS-24236-5 (also described as 281-24-236 expressing Cry1F). The PAT enzyme, originating from *Streptomyces viridochromogenes*, acetylates the primary amino group of phosphinothricin rendering it inactive. The PAT enzyme expressed in DAS-81419-2 soybean is 100% identical in amino acid sequence to PAT expressed in a number of deregulated events, including LibertyLink® soybean ACS-GMØØ5-3 (also described as A2704-12) (USDA, 1996), Herculex® I corn DAS-01507-1 (also described as TC1507) (USDA, 2001), DAS-21023-5 (also described as 3006-210-23) and DAS-24236-5 (also described as 281-24-236) comprising WideStrike® cotton (USDA, 2004), and Herculex® RW DAS-59122-7 (USDA, 2005).

The transgenes *cry1Ac(synpro), cry1Fv3* and *pat* expressing Cry1Ac, Cry1F and PAT proteins were introduced into DAS-81419-2 soybean using *Agrobacterium*-mediated transformation. Molecular characterization by Southern blot analyses of DAS-81419-2 soybean confirmed that a single, intact DNA insert containing the *cry1Ac(synpro), cry1Fv3,* and *pat* gene expression cassettes was integrated into the soybean genome and the intact DNA insert was stably inherited in the five breeding generations tested. Southern blot analyses confirmed the absence of the plasmid backbone DNA in DAS-81419-2 soybean. Analyses of the segregating generations confirmed that segregation of the DNA insert followed the predicted Mendelian inheritance pattern. These data confirmed the stability of DAS-81419-2 soybean during traditional breeding procedures.

The expression levels of Cry1Ac, Cry1F and PAT proteins in DAS-81419-2 soybean were determined by protein-specific enzyme linked immunosorbent assays (ELISA). Protein expression was analyzed in leaf, root, whole-plant and grain tissues collected throughout the growing season from DAS-81419-2 soybean plants grown in the major soybean production regions in the U.S. Mean protein expression levels across sites ranged from 0.39 ng/mg dry weight (dwt) in root to 25.44 ng/mg dwt in V5 leaf tissue for Cry1Ac, 5.23 ng/mg dwt in root to 56.75 ng/mg dwt in V5 leaf tissue for Cry1F, and 0.63 ng/mg dwt in root to 5.60 ng/mg dwt in V10-12 leaf tissue for PAT. Expression of the Cry1Ac and Cry1F proteins peaked during the

® WideStrike and Herculex are registered trademarks of Dow AgroSciences LLC.
® LibertyLink is a registered trademark of Bayer.

vegetative growth stage, providing protection against target lepidopteran insect pests and potential yield loss as a result of plant damage. The overall low levels of Cry1Ac, Cry1F and PAT proteins in DAS-81419-2 soybean indicate a low exposure to humans and animals.

Cry1Ac and Cry1F have a long history of safe use. The proteins originate from the naturally occurring soil bacterium *B. thuringiensis.* The safety of the proteins has been demonstrated in sprayable Bt formulations for pest control in agriculture for over half a century (Mendelsohn *et al.*, 2003; EPA, 2011; Sanahuja *et al.*, 2011). Both proteins are expressed in WideStrike® cotton which is authorized for cultivation in the U.S. and Brazil and for food and feed use in Australia, Brazil, Canada, European Union, Japan, Korea, Mexico, New Zealand and U.S. (www.biotradestatus.com). Bt corn and Bt cotton expressing variations of Cry1Ac or Cry1F have been cultivated for commercial use in the U.S. and other countries for more than a decade. In 1997, EPA established an exemption from the requirement of a tolerance for the plant-incorporated protectant Cry1Ac in all plants (40 CFR §174.510). Later, EPA established an exemption from the requirement of a tolerance for the plant-incorporated protectant Cry1F in cotton (40 CFR §174.504) and in corn (40 CFR §174.520). The exemptions were based on safety assessments of the proteins including digestibility in simulated gastric fluid, lack of homology to known allergens and protein toxins, and lack of mammalian toxicity as demonstrated by acute oral mouse gavage studies. DAS has filed a petition with EPA for an exemption from the requirement of a tolerance for Cry1F as expressed in soybean in 2012.

An extensive set of biochemical evaluations confirmed the identity of the Cry1Ac and Cry1F proteins produced in DAS-81419-2 soybean. Moreover DAS-81419-2 soybean-derived Cry1Ac and Cry1F were determined to be biochemically equivalent to the corresponding proteins purified from a microbial expression host organism *Pseudomonas fluorescens.* The Cry1Ac and Cry1F purified from *P. fluorescens* had been extensively assessed to establish the safety of the proteins. The assessments included acute oral toxicity in mice and protein digestability in simulated gastric fluid. The proteins have a very low acute toxicity potential and are rapidly degraded in simulated gastric fluid. Updated bioinformatic analyses showed that neither Cry1Ac nor Cry1F share meaningful amino acid sequence similarities with known allergens. No significant homology was identified when either protein sequence was compared with known allergen using the search criteria of either a match of eight or more contiguous identical amino acids, or greater than 35% identity over 80 amino acid residues. Likewise, neither protein share meaningful amino acid sequence similarities with known protein toxins.

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

Nutrient compositional analyses of forage and grain were conducted to compare the composition of DAS-81419-2 soybean with that of a non-transgenic control. Compositional analyses were used to evaluate any changes in the levels of key nutrients and anti-nutrients in DAS-81419-2 soybean. A total of 88 analytes were evaluated, nine in forage and the remaining 79 in seed including protein, fat, ash, moisture, carbohydrate, mineral, amino acids, fatty acids, vitamins, and bioactives. Seventeen analytes were excluded from statistical analysis because more than 50% of the samples were below the limit of quantitation. Of the remaining 71 analytes, the results indicate that there were no statistical differences between DAS-81419-2 and the non-transgenic control for 61 analytes based on overall treatment effects and pair-wise comparisons. The statistical differences observed for the remaining ten analytes based on unadjusted P-values were non-existent after adjustment for multiplicity using the false discovery rate method. The numerical differences in mean values observed for the ten analytes between DAS-81419-2 and non-transgenic control were small relative to natural variation. The mean values of DAS-81419-2 soybean were within the literature ranges and/or within the range of the reference varieties included in the study. Nutrient compositional analyses indicate that DAS-81419-2 soybean is substantially equivalent to conventional soybean and will not exhibit unexpected or unintended effects with respect to plant pest risk.

DAS-81419-2 soybean has been field tested in the major soybean growing regions in the U.S. All field tests were conducted under USDA APHIS notifications. Agronomic performance and phenotypic assessments were conducted on DAS-81419-2 soybean in multi-site field studies to measure characteristics such as emergence, seedling vigor, days to flowering, days to maturity, lodging, plant height, plant morphology, final population, shattering, and yield. All field trials were also observed for opportunistic disease or insect stressors as well as normal phenotypic characteristics. Laboratory studies were carried out to evaluate DAS-81419-2 soybean for seed dormancy and germination characteristics and to compare to that of the non-transgenic control. Agronomic, phenotypic and ecological evaluations indicate that DAS-81419-2 soybean is substantially equivalent to conventional soybean and does not possess a selective advantage that would result in increased plant pest risk compared to conventional soybean.

A review of potential environmental impact indicates that DAS-81419-2 soybean 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 including Cry protein specificity and long history of safe use, Cry protein content in DAS-81419-2 plant tissues and estimated likelihood for exposure, environmental fate, hazard characterization for representative non-target organisms including vertebrate and invertebrate species, significant margins of exposure, and non-target arthropod field trials comparing DAS-81419-2 soybean with non-transgenic soybean.

Because DAS-81419-2 soybean is agronomically and compositionally similar to conventional soybean, and Cry1Ac, Cry1F and PAT proteins have a history of safe use, no significant impact is expected on current crop production practices, non-target or endangered species, crop rotation, volunteer management, or commodity food and feed soybean products. The availability of DAS-81419-2 soybean is expected to have a beneficial impact on insect pest management by providing another tool to address insect control needs.

Information collected during field trials and laboratory analyses presented herein demonstrate that DAS-81419-2 soybean exhibits no plant pathogenic properties or weediness characteristics. DAS-81419-2 soybean is no more likely to become a plant pest than conventional soybean, and the Cry1Ac, Cry1F and PAT proteins are unlikely to increase the weediness potential of any other cultivated plant or wild species.

DAS hereby requests a determination from APHIS that insect-resistant DAS-81419-2 soybean and all progeny derived from crosses between DAS-81419-2 soybean and conventional soybean or biotechnology-derived soybean granted non-regulated status by APHIS no longer be considered regulated articles under 7 CFR Part 340.

Table of Contents

List of Figures

List of Tables

Acronyms and Scientific Terms

ADF	Acid detergent fiber
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service, USDA
AtUbi10	Ubiquitin promoter from *Arabidopsis thaliana*
AtuORF1	Open reading frame 1 of *Agrobacterium tumefaciens* pTi15955
AtuORF23	Open reading frame 23 of *Agrobacterium tumefaciens* pTi15955
bp	Base pair
Bt	*Bacillus thuringiensis*
CFR	US Code of Federal Regulations
Cry1	Insecticidal proteins from *Bacillus thuringiensis*
Cry1Ac	Cry1Ac insecticidal protein derived from *Bacillus thuringiensis* subsp. *kurstaki*
cry1Ac(synpro)	Coding sequence for Cry1Ac protein
Cry1F	Cry1F insecticidal protein derived from *Bacillus thuringiensis* subsp. *aizawai*
cry1Fv3	Coding sequence for Cry1F protein
CsVMV	Promoter from cassava vein mosaic virus
DAS	Dow AgroSciences LLC
DAS-21023-5	OECD identifier for the cotton event expressing the Cry1Ac and PAT proteins. DAS-21023-5 is also described as 3006-210-23
DAS-24236-5	OECD identifier for the cotton event expressing the Cry1F and PAT proteins. DAS-24236-5 is also described as 281-24-236
DAS-81419-2	OECD identifier for the soybean event expressing the Cry1Ac, Cry1F, and PAT proteins
DNA	Deoxyribonucleic acid
dwt	Dry weight of tissue
EEC	Estimated environmental concentration
ELISA	Enzyme-linked immunosorbent assay
EPA	US Environmental Protection Agency
ESI-LC/MS	Electrospray ionization-liquid chromatography mass spectrometry
FDA	US Food and Drug Administration
FDR	False Discovery Rate
FESTF	FIFRA Endangered Species Task Force
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
HAFT	Highest average field trial
HEEE	High-end exposure estimate
ILSI	International Life Sciences Institute
IPM	Integrated Pest Management
IRM	Insect Resistance Management
Kb	Kilobase pair
kDa	Kilodalton
L	Liter
LOD	Limit of Detection
LOQ	Limit of Quantitation

MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Maverick	Publicly available soybean line used in transformation to produce DAS-81419-2 soybean
MOE	Margin of exposure
NDF	Neutral detergent fiber
NOEC	No observed effect concentration
NTO	Non-target organism
OECD	Organisation for Economic Co-operation and Development
ORF	Open reading frame
Ori	Origin of replication
pat	Gene from *Streptomyces viridochromogenes* which encodes the PAT protein
PAT	Phosphinothricin *N*-acetyltransferase
PBN	US FDA Pre-market Biotechnology Notice
PCR	Polymerase chain reaction
pDAB9582	DNA vector carrying the *cry1Ac(synpro)*, *cry1Fv3* and *pat* expression cassettes
PIP	Plant-incorporated protectant
PPT	Phosphinothricin
PTU	Plant transcription unit consisting of promoter, gene, and terminator sequences
RCB	Randomized complete block
SCN	Soybean cyst nematode
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SGF	Simulated gastric fluid
Std. Dev.	Standard deviation
STMR	Supervised trials mean residue
subsp	subspecies
T-DNA	Transfer DNA
USDA	United States Department of Agriculture
USDA ARS	United States Department of Agriculture Agricultural Research Service
USDA ERS	United States Department of Agriculture Economic Research Service
USDA NASS	United States Department of Agriculture National Agricultural Statistics Service
UTR	Untranslated region
w/v	Weight per volume

1. Rationale for the Development of DAS-81419-2

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 genetically engineered insect-resistant DAS-81419-2 soybean and requests a determination from APHIS that DAS-81419-2 soybean and crosses of DAS-81419-2 with nonregulated soybean lines no longer be considered regulated articles under 7 CFR 340.

1.2. Rationale for the Development of DAS-81419-2 Soybean and Benefits

Large scale soybean production began in the U.S. in the 1920s. Since then, the acres planted to soybeans have expanded rapidly due to ideal planting conditions, increased crop rotation, rising yield, and lower production cost (USDA ERS, 2012). With the exception of 2007, over the last 15 years soybeans have become the second most planted field crop in the U.S. after corn with more than 70 million acres (USDA NASS, 2012). Soybeans are the dominant oilseed in the U.S. accounting for about 90 percent of U.S. oilseed production. In 2011 soybeans were planted on 75 million acres producing 3.056 billion bushels of soybeans with a farm value of more than $35.7 billion. About 1.615 billion bushels were crushed to yield soybean meal and soybean oil for domestic use and 1.275 billion bushels were processed for export worldwide. These sales accounted for 37% of the world's soybean trade valued at $21.5 billion (USDA ERS, 2012; USDA NASS, 2012).

In the U.S. the majority of soybeans are grown in the north central states with the remaining primarily grown in the southeastern and Mississippi delta region. Insect injury has not traditionally resulted in substantial economic losses in most North American growing regions. However, in recent years, losses have been severe in some southern regions of the U.S. (Catchot, 2011). Higher market prices for soybeans have lowered economic thresholds for mitigating damage caused by soybean pests, resulting in expansion of the area and number of insecticide treatments applied to soybeans (USDA ERS, 2011). Recent increases in pest insect populations in soybean have been observed; increases are attributed by some to global warming or insect adaptation to the soybean plant (Way, 1994). Lower economic thresholds coupled with higher insect populations favor more aggressive pest management strategies (Pedigo, 1996).

Insect pressure on soybeans in the southern U.S. states is derived primarily from four lepidopteran insect pests. Of these four, velvetbean caterpillar and soybean looper are the most damaging defoliating insects in the south (Way, 1994; Gianessi, 2009). On average about one-third of the soybean acres in Georgia, Louisiana, and North Carolina have been sprayed regularly with insecticides since 1991. Insecticides are used on approximately 50% of the soybean acreage in Georgia for lepidopteran pests with velvetbean caterpillar being the most targeted pest (Gianessi *et al.*, 2002). Approximately 40% of the soybean acreage in Louisiana is treated with

insecticides for lepidopteran pests, with soybean looper being the main target (Gianessi *et al.*, 2002).

Chemical insecticides can have limited efficacy in controlling lepidopteran infestations in soybean. Narrow application windows, the emergence of insecticide resistance, and public pressure for reduced pesticide use limit the desirability of this approach to pest management (Thomas and Boethel, 1994). Soybean looper has developed extensive insecticide resistance (Thomas and Boethel, 1994); resistance to pyrethroids is widespread across the southern U.S. (Felland *et al.*, 1990; Leonard *et al.*, 1990). Insecticides remain effective against velvetbean caterpillar. However, infestations can quickly reach damaging levels and cause economic loss if insecticides are not applied promptly.

Bt crops expressing one or more insecticidal proteins of *Bacillus thuringiensis* are effective in targeting specific insect pests. Increasing adoption rates of Bt corn and cotton since 1996 have coincided with increased yields (USDA ERS, 2006). Pesticide use has also decreased since the introduction of these insect-resistant Bt crops with one study showing an 8% reduction in insecticide use for adopters of Bt corn as compared to non-adopters (USDA ERS, 2006). Importantly, Bt crops have also been credited with increasing yields, even when economic thresholds have not been reached in either Bt- or non-Bt-fields due to regional insect population reductions (Hutchison *et al.*, 2010).

DAS developed a transgenic soybean that is resistant to target lepidopteran pests. The unique identifier for the transgenic soybean, in accordance with the Organization for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2004), is DAS-81419-2.

DAS-81419-2 soybean expresses two insecticidal proteins, Cry1Ac and Cry1F, originally from the naturally-occurring soil bacterium, *Bacillus thuringiensis.* Cry1Ac and Cry1F provide plant protection against several target lepidopteran pests. In addition, DAS-81419-2 soybean expresses the phosphinothricin acetyltransferase (PAT) protein from the soil bacterium *Streptomyces viridochromogenes.* The PAT protein provides tolerance to the herbicide glufosinate and was used as a selectable marker during the development of DAS-81419-2 soybean. The transgenes for Cry1Ac, Cry1F and PAT expression were introduced into soybean via *Agrobacterium*-mediated transformation to create DAS-81419-2 soybean. Cry1Ac and Cry1F are expressed in the leaf tissues of DAS-81419-2 soybean plants throughout the growing season, providing excellent control of target lepidopteran pests of soybeans. DAS-81419-2 soybean offers several potential benefits, in particular:

- Efficient and environmentally sound alternative to chemical insecticides. DAS-81419-2 soybean offers effective control of multiple lepidopteran pests of soybean. The availability of DAS-81419-2 soybean could reduce the need for insect scouting, preserve beneficial insect populations, and provide increased convenience and greater performance consistency.
- Greater durability and improved insect resistance management. DAS-81419-2 soybean expresses two insecticidal proteins, Cry1Ac and Cry1F, whose modes of action differ with respect to receptor binding. For example, Cry1Ac and Cry1F have been shown to bind to different receptors in the midgut of the target soybean insect pest tobacco

budworm (*H. virescens*) (Jurat-Fuentes and Adang, 2001). Cry1Ac binds to at least three sets of receptors while Cry1F binds to at least two, only one of which also binds Cry1Ac. The major receptor for Cry1Ac is not recognized by Cry1F (Jurat-Fuentes and Adang, 2006). Such incomplete shared binding is expected to lead to incomplete cross-resistance when resistance is mediated by receptor changes. Bt gene pyramiding offered by DAS-81419-2 soybean offers greater durability than Bt crops carrying a single Bt trait and provides protection against the development of insect resistance.

Due to narrow commercial applicability in the U.S., DAS submitted an application for a FIFRA Section 3 seed increase registration with EPA to support future breeding and seed increase activities in the U.S. Under a Section 3 seed increase registration, commercial sale of DAS-81419-2 soybean in the U.S. is prohibited. If DAS chooses to market DAS-81419-2 soybean for commercial purposes in the U.S. in the future, DAS would seek a FIFRA Section 3 commercial use registration from EPA. DAS-81419-2 soybean is targeted for commercialization in Brazil and Argentina where insect pressure is heavy, resulting in significant yield loss. DAS expects to make submissions beginning 2012 to the regulatory authorities of trade partners for import clearance and cultivation approval. Deregulation of DAS-81419-2 soybean by APHIS is a prerequisite in achieving regulatory approvals in many of these countries.

1.3. Submission to Other Regulatory Agencies

DAS-81419-2 soybean 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 intends to submit a pre-market biotechnology notification (PBN) to FDA in 2012.

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) [7 U.S.C. §136(u)], are subject to regulation by the Environmental Protection Agency (EPA). Pesticides produced *in planta*, referred to as plant-incorporated protectants (PIPs), are also subject to regulation by the EPA under FIFRA. DAS submitted an application for a FIFRA Section 3 seed increase registration of DAS-81419-2 soybean on July 27th, 2012. Concurrent with the Section 3 application, DAS also petitioned the EPA for a permanent exemption from the requirement of a tolerance for Cry1F and the genetic material required for its production in soybean, under Federal Food, Drug and Cosmetic Act (FFDCA) Section 408. An exemption from the requirement of tolerance for Cry1Ac in all crops has already been established by the EPA and is published in the Code of Federal Regulations (40 CFR §174.510). Under seed increase registration, commercial sale of DAS-81419-2 soybean in the U.S. is prohibited by law. If DAS chooses to cultivate DAS-81419-2 soybean for commercial purposes in the U.S. in the future, DAS will apply for a FIFRA Section 3 commercial use registration with EPA and will develop a specific IRM program in connection with the commercial use registration.

DAS intends to submit dossiers beginning in 2012 to the regulatory authorities of trade partners for import clearance and production approval which may include Canada, Japan, Korea, Taiwan, European Union, Australia/New Zealand, South Africa, Brazil, Argentina and Mexico.

2. The Biology of Soybean

2.1. Overview of Soybean Biology

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

- general description, including taxonomy, morphology, and the uses of soybean as a crop plant
- agronomic practices
- centers of origin
- reproductive biology
- cultivated *Glycine max* as a volunteer weed
- ability to cross inter-species/genus, introgressions into relatives, and interactions with other organisms
- summary of the ecology of *Glycine max*

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

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

2.2. Characterization of the Recipient Soybean Cultivar

The publicly available cultivar ‘Maverick’ was used as the recipient line for the generation of DAS-81419-2 soybean.

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

3. Development of DAS-81419-2 Soybean

3.1. Description of the Transformation System

Transgenic soybean (*Glycine max*) was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. The disarmed *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986), carrying the binary vector, pDAB9582, with the selectable marker (*pat*) and the genes of interest (*cry1Ac(synpro)* and *cry1Fv3*) within the T-DNA region, was used to initiate transformation.

Agrobacterium-mediated transformation was carried out using a modified procedure of Zeng *et al.* (2004). Briefly, soybean seeds (cv Maverick) were germinated on basal media and cotyledonary nodes were isolated and infected with *Agrobacterium* EHA101 carrying pDAB9582. After infection with *Agrobacterium*, cotyledonary nodes were cultured on the cocultivation medium for 5 days before transferring to shoot initiation medium. All media, including shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin to inhibit the growth of *Agrobacterium*. Glufosinate selection (3-8 mg/L) was also employed in those media to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

Terminal leaflets of regenerated plantlets were painted with glufosinate (0.05% - 2% w/v) to screen for putative transformants. Those plantlets exhibiting tolerance were transferred to the greenhouse, allowed to acclimate and then leaf painted again with glufosinate (0.05% - 2% w/v) to reconfirm herbicide tolerance. The glufosinate tolerant plants were sampled and analyzed at the molecular level to confirm the presence of the selectable marker gene and/or the genes of interest. Specifically, for T0 plants, PCR analysis was performed to verify the absence of the spectinomycin sequence as well as the presence of the *cry1Ac(synpro)* and *cry1Fv3* genes. Invader® assay (Kwiatkowski *et al.*, 1999) was carried out for copy number detection for *pat*, *cry1Ac(synpro)*, and *cry1Fv3* genes. Selected T0 plants were allowed to self-fertilize in the greenhouse to give rise to T1 seed. For T1 plants, Invader assay and Southern blot analyses were performed to determine copy number, integration number, and PTU integrity.

Figure 1 shows a plasmid map of pDAB9582.

Figure 2 shows a diagram of the intended T-DNA insert in plasmid pDAB9582.

Figure 3 outlines the development of DAS-81419-2 soybean.

Figure 4 shows a breeding diagram for DAS-81419-2 soybean including identification of the generations used in various safety assessment studies.

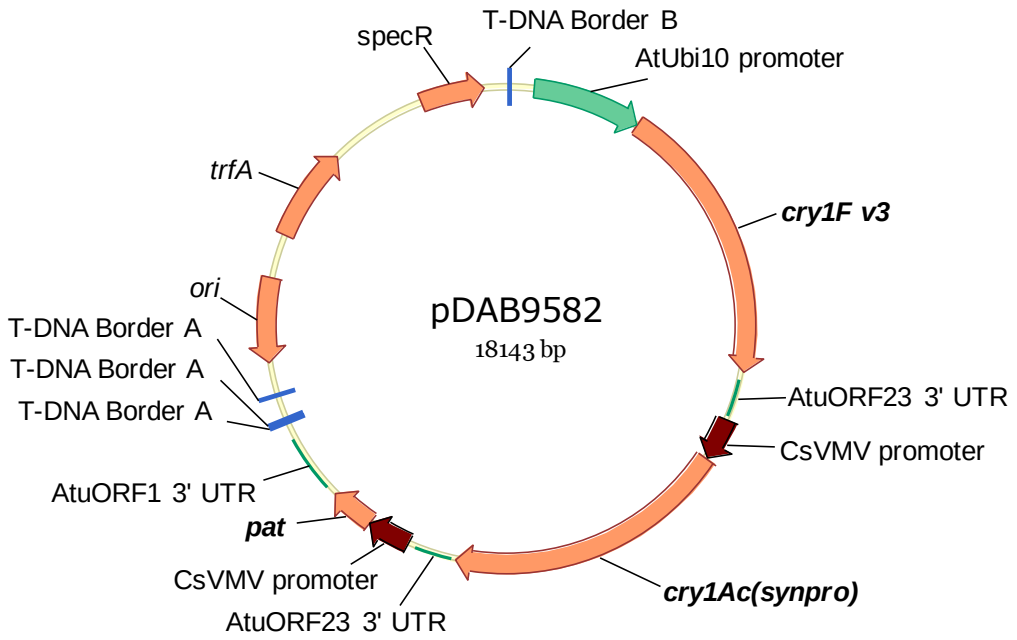


Figure 1. Plasmid map of pDAB9582.

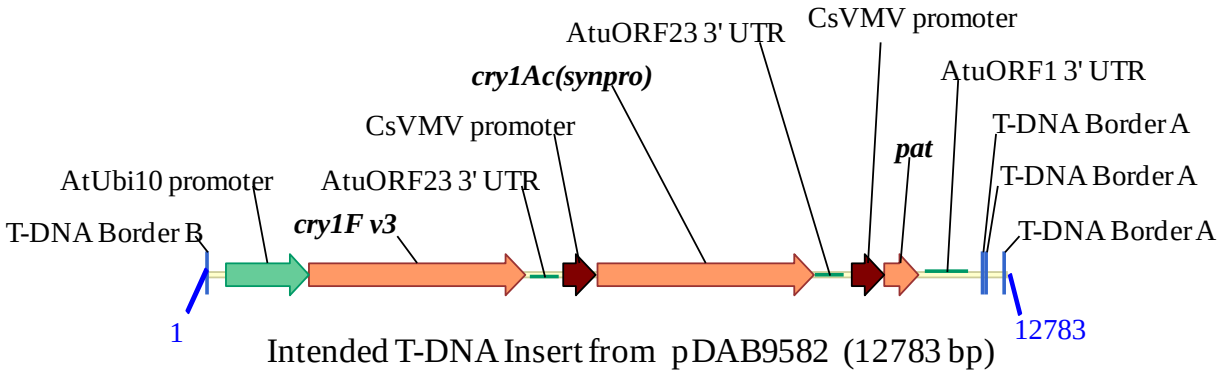


Figure 2. Diagram of intended T-DNA insert from plasmid pDAB9582.

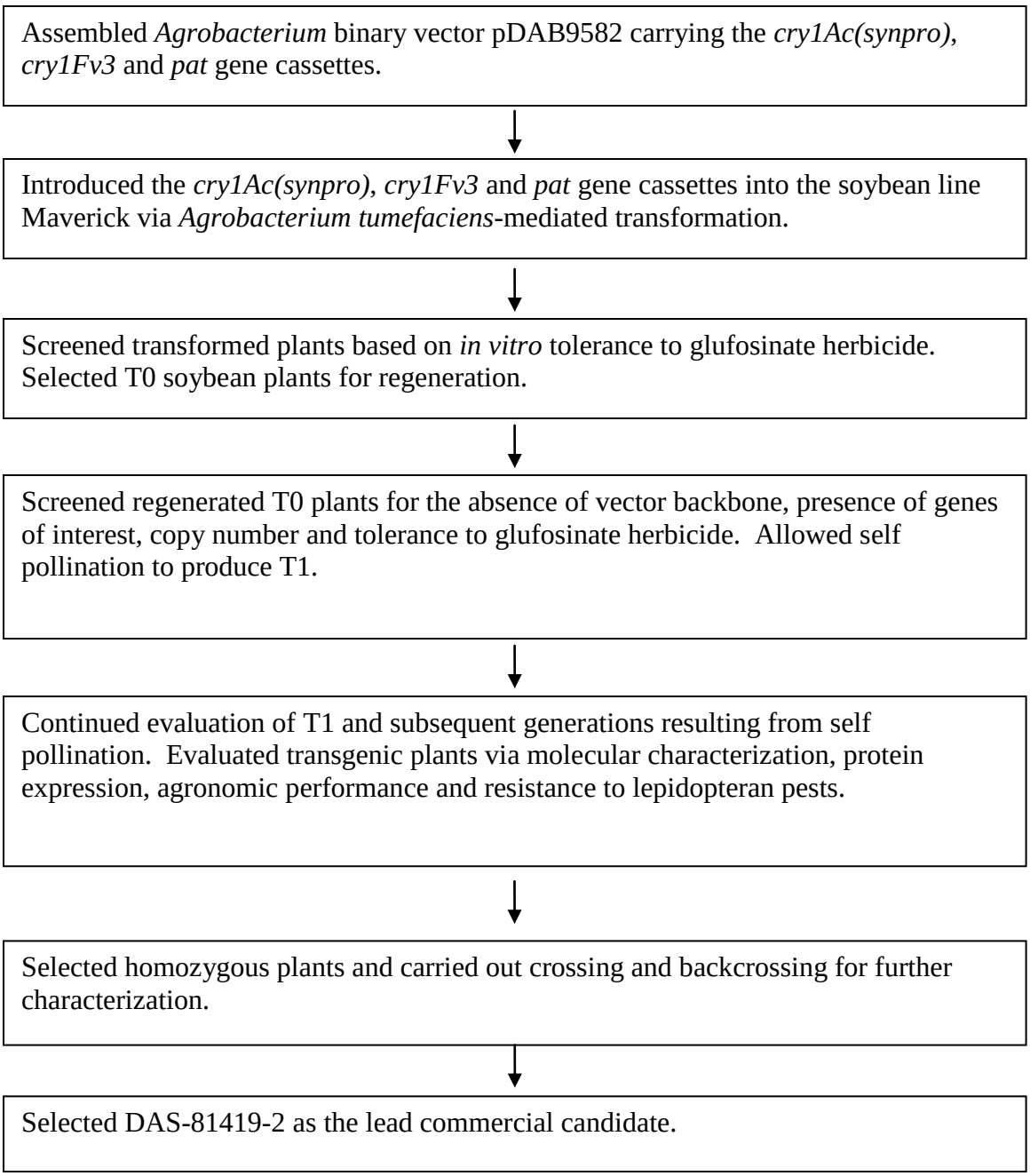
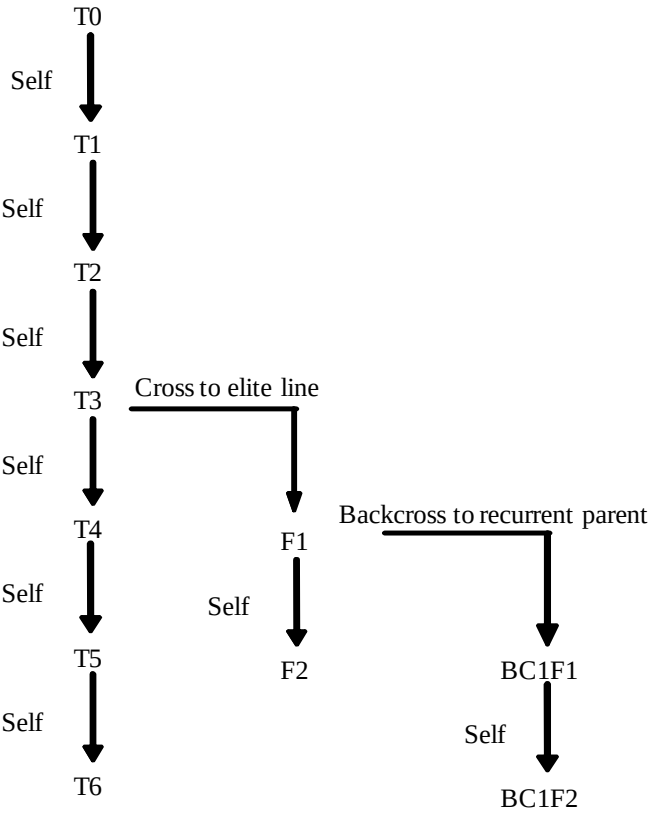


Figure 3. Event sorting and selection process for DAS-81419-2 soybean.



Analysis	Petition Section(s)	DAS-81419-2 Soybean Generations	Control
Genetic Characterization	5.2	T1, T2, T3, T4, F2	Non-transgenic soybean Maverick
Segregation Analysis	5.3	F2, BC1F2	None
Protein Characterization	6.1.3, 6.1.6, 6.2.3	T4, T5	Non-transgenic soybean Maverick
Protein Expression	6.1.4, 6.1.7, 6.2.4	T4	Non-transgenic soybean Maverick
Agronomics	8	T4	Non-transgenic soybean Maverick
Germination/Dormancy	8.3	T4	Non-transgenic soybean Maverick
Composition	7	T4	Non-transgenic soybean Maverick
NTO	9.1.2	T4	Non-transgenic soybean Maverick
Efficacy	9.1.1	T4	Non-transgenic soybean Maverick

Figure 4. Breeding diagram of DAS-81419-2 soybean.

3.2. Selection of Comparators for DAS-81419-2 Soybean

To ensure the accurate assessment of the impact of transgene insertion on various characteristics of DAS-81419-2 soybean, a proper selection of comparator plants is important.

The control plants should have a genetic background similar to that of DAS-81419-2 soybean but lack the transgenic insert. In all cases, the non-transgenic variety Maverick was used as the control. Maverick is the recipient variety that was transformed to generate DAS-81419-2 soybean. The T0 plants and all subsequent self-pollinated generations derived post-transformation were essentially genetically identical to Maverick soybean with the exception of the transgenic insert DNA.

4. Donor Genes and Regulatory Sequences

4.1. Identity and Source of Genetic Material in pDAB9582

DAS-81419-2 soybean was generated by *Agrobacterium*-mediated transformation using plasmid pDAB9582 (Figure 1). The T-DNA insert in the plasmid contains two synthetic genes from *Bacillus thuringiensis*, *cry1Ac(synpro)* and *cry1Fv3*, as well as a *pat* gene from *Streptomyces viridochromogenes* (Figure 2). A summary of the genetic elements in pDAB9582 is provided in Table 1.

Three gene expression cassettes are present in the T-DNA region of plasmid pDAB9582 for insertion into soybean. The *cry1Fv3* expression cassette is designed to express a synthetic version of the Cry1F protein. The *cry1Fv3* gene is comprised of three parts; at the 5′ end, a toxin core that was optimized from the native *cry1Fa2* gene originally isolated from *Bacillus thuringiensis* subsp. *aizawai* strain PS811; in the middle, a very small portion of *cry1Ca3* which was originally isolated from *B. thuringiensis* subsp. *aizawai* strain PS811; and at the 3′ end, a tail that was optimized from the native *cry1Ab1* tail originally isolated from *B. thuringiensis* subsp. *Berliner* 1715. The *cry1Fv3* gene encodes the Cry1F protein that is comprised of 1148 amino acids and has a molecular weight of ~130.2 kDa. The amino acid sequence of the Cry1F protein is identical to that expressed in cotton event DAS-24236-5 (also described as 281-24-236, expressing Cry1F) which was deregulated by USDA APHIS in 2004 (USDA, 2004).

Expression of *cry1Fv3* is controlled by the AtUbi10 promoter from *Arabidopsis thaliana* and the AtuORF23 3′UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The AtUbi10 promoter is known to drive constitutive expression of the genes it controls (Norris *et al.*, 1993). The function of AtuORF23 (GenBank Accession: CAA25184) in pTi15955 (GenBank Accession: X00493) was not identified (Barker *et al.*, 1983).

The *cry1Ac(synpro)* expression cassette is designed to express a synthetic version of the Cry1Ac protein. The *cry1Ac(synpro)* gene is comprised of three parts; at the 5′ end, a toxin core that was optimized from the native *cry1Ac1* gene originally isolated from *B. thuringiensis* subsp. *kurstaki* strain HD73; in the middle, a very small portion of *cry1Ca3* which was originally isolated from *B. thuringiensis* subsp. *aizawai* strain PS811; and at the 3′ end, a tail that was optimized from the native *cry1Ab1* tail originally isolated from *B. thuringiensis* subsp. *Berliner* 1715. The *cry1Ac(synpro)* gene encodes the Cry1Ac protein that is comprised of 1156 amino acids and has a molecular weight of ~130.7 kDa. The *cry1Ac(synpro)* gene sequence and the corresponding Cry1Ac amino acid sequence are identical to that expressed in cotton event DAS-21023-5 (also described as 3006-210-23, expressing Cry1Ac) which was deregulated by USDA APHIS in 2004 (USDA, 2004).

Expression of *cry1Ac(synpro)* is controlled by the CsVMV promoter from Cassava Vein Mosaic virus and the AtuORF23 3′UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The cassava vein mosaic virus is a double stranded DNA virus which infects cassava plants (*Manihot esculenta* Crantz) and has been characterized as a plant pararetrovirus belonging to the caulimovirus subgroup. The CsVMV promoter is known to drive constitutive expression of the genes it controls (Verdaguer *et al.*, 1996). The function of AtuORF23 (GenBank Accession: CAA25184) in pTi15955 (GenBank Accession: X00493) was not identified (Barker *et al.*, 1983).

The *pat* expression cassette is designed to express the phosphinothricin *N*-acetyltransferase (PAT) protein. The *pat* gene originates from the common soil bacterium *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988). Expression of PAT protein in soybean plants confers tolerance to glufosinate and was used as a selectable marker during DAS-81419-2 soybean development. The *pat* gene encodes a protein of 183 amino acids that has a molecular weight of approximately 20.6 kDa. The *pat* gene has been widely used both as a selectable marker and herbicide tolerance trait in previously deregulated products (USDA, 1996, 2001, 2004, 2005).

Expression of the *pat* gene is controlled by the CsVMV promoter from cassava vein mosaic virus and AtuORF1 3′ UTR sequence from *Agrobacterium tumefaciens* plasmid pTi15955. The CsVMV promoter driving *pat* expression is the same as that driving expression of *cry1Ac(synpro).* The function of AtuORF1 (GenBank Accession: CAA25163) in pTi15955 (GenBank Accession: X00493) was not identified (Barker *et al.*, 1983), but its translated amino acid sequence has a significant similarity with an indole-3-lactate synthase (GenBank Accession: AAK90967) from *Agrobacterium tumefaciens* str. C58.

Table 1. Genetic elements from plasmid pDAB9582.

Feature Name	Feature Start	Feature Stop	Feature Length	Description
T-DNA Border B	1	24	24	T-DNA Border B sequence required for transfer of DNA from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)
Intervening sequence	25	295	271	Non-specific DNA sequences necessary for cloning
AtUbi10 promoter	296	1617	1322	AtUbi10 promoter along with the 5' untranslated region and intron from *Arabidopsis thaliana* polyubiquitin 10 (UBQ10) gene (Norris *et al.*, 1993)
Intervening sequence	1618	1625	8	Non-specific DNA sequences necessary for cloning
cry1F v3	1626	5072	3447	*cry1F v3* (synthetic version of the *cry1F* gene from *Bacillus thuringiensis* subsp. *aizawai* strain PS811) (Cardineau *et al.*, 2001; Gao *et al.*, 2006)
Intervening sequence	5073	5174	102	Non-specific DNA sequences necessary for cloning
AtuORF23 3' UTR	5175	5631	457	AtuORF23 3' UTR (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	5632	5694	63	Non-specific DNA sequences necessary for cloning
CsVMV promoter	5695	6211	517	CsVMV promoter along with the 5' untranslated region derived from Cassava Vein Mosaic virus (Verdaguer *et al.*, 1996)
Intervening sequence	6212	6220	9	Non-specific DNA sequences necessary for cloning
cry1Ac(synpro)	6221	9691	3471	*cry1Ac (synpro)* (synthetic version of the *cry1Ac* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD73) (Adang *et al.*, 1985; Gilroy and Wilcox, 1992; Cardineau *et al.*, 2001)
Intervening sequence	9692	9724	33	Non-specific DNA sequences necessary for cloning
AtuORF23 3' UTR	9725	10181	457	AtuORF23 3' UTR (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	10182	10295	114	Non-specific DNA sequences necessary for cloning
CsVMV promoter	10296	10812	517	CsVMV promoter along with the 5' untranslated region derived from Cassava Vein Mosaic virus (Verdaguer *et al.*, 1996)
Intervening sequence	10813	10819	7	Non-specific DNA sequences necessary for cloning

Feature Name	Feature Start	Feature Stop	Feature Length	Description
pat	10820	11371	552	*pat* (synthetic version of the phosphinothricin acetyl transferase gene from *Streptomyces viridochromogenes*) (Wohlleben *et al.*, 1988)
Intervening sequence	11372	11473	102	Non-specific DNA sequences necessary for cloning
AtuORF1 3' UTR	11474	12177	704	AtuORF1 3' UTR (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	12178	12405	228	Non-specific DNA sequences necessary for cloning
T-DNA Border A	12406	12429	24	T-DNA Border A sequence required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)
Intervening sequence	12430	12448	19	Non-specific DNA sequences necessary for cloning
T-DNA Border A	12449	12472	24	T-DNA Border A sequence required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)
Intervening sequence	12473	12759	287	Non-specific DNA sequences necessary for cloning
T-DNA Border A	12760	12783	24	T-DNA Border A sequence required for transfer of T-DNA insert from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)
Intervening sequence	12784	13162	379	Plasmid backbone sequences from gram-negative bacteria broad host-range RK2 plasmid (Stalker *et al.*, 1981)
ori	13163	14182	1020	*ori* (Replication origin sequences from gram-negative bacteria broad host-range RK2 plasmid) (Stalker *et al.*, 1981)
Intervening sequence	14183	14727	545	Plasmid backbone sequences from gram-negative bacteria broad host-range RK2 plasmid (Stalker *et al.*, 1981)
trfA	14728	15876	1149	*trfA* (Plasmid replication sequences for Trf A protein from gram-negative bacteria broad host-range RK2 plasmid) (Stalker *et al.*, 1981)
Intervening sequence	15877	17080	1204	Plasmid backbone sequences from gram-negative bacteria broad host-range RK2 plasmid (Stalker *et al.*, 1981)
*Spec*R	17081	17869	789	*Spec*R (spectinomycin resistance gene from *Escherichia coli* Tn7 transposon) (Fling *et al.*, 1985)
Intervening sequence	17870	18143	274	Plasmid backbone sequences for cloning

5. Genetic Characterization

5.1. Overview of Genetic Characterization

Molecular characterization of DAS-81419-2 soybean was conducted by Southern blot analyses and DNA sequencing. The results demonstrated that the transgene insert in DAS-81419-2 soybean occurred as a single integration of the T-DNA insert from plasmid pDAB9582, including a single, intact copy of each of the plant transcription units (PTUs) for the *cry1Fv3, cry1Ac(synpro),* and *pat* genes. In addition, a minor (<100 bp) fragment of the *cry1Ac(synpro)* gene was identified at the 5′ end of the T-DNA insert in a complementary orientation. The sequence of the insert in DAS-81419-2 soybean was confirmed and the genetic elements identified in DAS-81419-2 soybean are provided in Table 2. The full insert was stably integrated and inherited across breeding generations, and no plasmid backbone sequences are present in DAS-81419-2 soybean.

Detailed Southern blot analyses were conducted using probes specific to the genes, promoters, terminators, and other regulatory elements contained in the transformation plasmid pDAB9582. Locations of each probe on plasmid pDAB9582 are described in Table 3 and Figure 5. The expected and observed fragment sizes with specific digest and probe combinations, based on the known restriction enzyme sites of plasmid pDAB9582 and the actual T-DNA insert in DAS-81419-2 soybean are shown in Figure 6 and Figure 7, respectively. The Southern blot analyses described here made use of two types of restriction fragments: a) internal fragments generated by known restriction enzyme recognition sites contained within the T-DNA insert of pDAB9582, and b) border fragments generated by one known restriction enzyme recognition site located within the T-DNA insert and another site located in the soybean genome flanking the insert. Border fragment sizes vary by events because they rely on the location of the restriction enzyme recognition sites in the DNA sequence of flanking genomic region. Since integration sites are unique for each event, border fragments provide a means to determine the number of transgene insertions and to specifically identify the event.

Genomic DNA for Southern blot analyses was prepared from leaf material of individual DAS-81419-2 soybean plants from five distinct breeding generations. Genomic DNA from leaves of non-transgenic variety Maverick was used as the control material. Plasmid DNA of pDAB9582 added to genomic DNA from the non-transgenic variety Maverick served as the positive control for Southern blot analyses. 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 7. Southern blot analysis results are shown in Figure 8 to Figure 28. Southern blot analyses showed that DAS-81419-2 soybean contains a single intact copy of each of the PTUs for the *cry1Fv3, cry1Ac(synpro),* and *pat* genes from pDAB9582 (Section 5.2.1 and Section 5.2.2). In addition, a minor (<100 bp) fragment of the *cry1Ac(synpro)* gene was identified at the 5′ end of the T-DNA insert. No plasmid backbone sequences were detected in DAS-81419-2 soybean (Section 5.2.3). The hybridization patterns across five generations of DAS-81419-2 soybean (T1, T2, T3, T4, and F2) were identical, indicating that the insert is stably integrated in the soybean genome (Section 5.2.4). The inheritance of DAS-81419-2 soybean insert in segregating generations was investigated using event-specific PCR and detection of the PAT protein expression. All results confirmed the predicted inheritance of the transgene in a single locus (Section 5.3).

Table 2. Genetic elements in DAS-81419-2 soybean.

Feature Name	Feature Start	Feature Stop	Feature Length	Description
5' Flanking border	1	1297	1297	Soybean genomic DNA flanking the 5' end of the insert in DAS-81419-2 soybean
Re-arranged sequence	1298	1321	24	Re-arranged DNA fragment at the 5' end of the insert
cry1Ac(synpro) partial fragment	1322	1419	98	Complementary *cry1Ac(synpro*) partial fragment at the 5' end of the insert that is 99% identical to 1990 - 2087 bp of the full-length *cry1Ac(synpro)* gene
Re-arranged sequence	1420	1432	13	Re-arranged DNA fragment at the 5' end of the insert
Partial T-DNA Border B	1433	1433	1	Last nucleotide from T-DNA Border B which is required for transfer of DNA from *Agrobacterium tumefaciens* into plant cells (Barker *et al.*, 1983)
Intervening sequence	1434	1704	271	Non-specific DNA sequences necessary for cloning
AtUbi10 promoter	1705	3026	1322	AtUbi10 promoter along with the 5' untranslated region and intron from *Arabidopsis thaliana* polyubiquitin 10 (UBQ10) gene (Norris *et al.*, 1993)
Intervening sequence	3027	3034	8	Non-specific DNA sequences necessary for cloning
cry1F v3	3035	6481	3447	*cry1F v3* (synthetic version of the *cry1F* gene from *Bacillus thuringiensis* subsp. *aizawai* strain PS811) (Cardineau *et al.*, 2001; Gao *et al.*, 2006)
Intervening sequence	6482	6583	102	Non-specific DNA sequences necessary for cloning
AtuORF23 3' UTR	6584	7040	457	AtuORF23 3' UTR (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	7041	7103	63	Non-specific DNA sequences necessary for cloning
CsVMV promoter	7104	7620	517	CsVMV promoter along with the 5' untranslated region derived from Cassava Vein Mosaic virus (Verdaguer *et al.*, 1996)
Intervening sequence	7621	7629	9	Non-specific DNA sequences necessary for cloning
cry1Ac(synpro)	7630	11100	3471	*cry1Ac (synpro)* (synthetic version of the *cry1Ac* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD73) (Adang *et al.*, 1985; Gilroy and Wilcox, 1992; Cardineau *et al.*, 2001)
Intervening sequence	11101	11133	33	Non-specific DNA sequences necessary for cloning

Feature Name	Feature Start	Feature Stop	Feature Length	Description
AtuORF23 3' UTR	11134	11590	457	AtuORF23 3' UTR (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	11591	11704	114	Non-specific DNA sequences necessary for cloning
CsVMV promoter	11705	12221	517	CsVMV promoter along with the 5' untranslated region derived from Cassava Vein Mosaic virus (Verdaguer *et al.*, 1996)
Intervening sequence	12222	12228	7	Non-specific DNA sequences necessary for cloning
pat	12229	12780	552	*pat* (synthetic version of phosphinothricin acetyl transferase gene from *Streptomyces viridochromogenes*) (Wohlleben *et al.*, 1988)
Intervening sequence	12781	12882	102	Non-specific DNA sequences necessary for cloning
AtuORF1 3' UTR	12883	13586	704	AtuORF1 3' UTR (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	13587	13784	198	Non-specific DNA sequences necessary for cloning
Re-arranged sequence	13785	13793	9	Re-arranged DNA fragment at the 3' end of the insert
3' Flanking border	13794	15172	1379	Soybean genomic DNA flanking the 3' end of the insert in DAS-81419-2 soybean

Table 3. List of probes and their positions in plasmid pDAB9582.

Probe Name	Location in pDAB9582	Size (bp)
AtUbi10 Promoter	29-1620	1592
cry1F-5′	1631-3376	1746
cry1F-3′A	3354-3874	521
cry1F-3′B	3851-5081	1231
AtuORF23-A 3′ UTR	5080-5630	551
CsVMV Promoter	5664-6250	587
cry1Ac-5′	6221-7940	1720
cry1Ac-3′A	7918-8352	435
cry1Ac-3′B	8329-9778	1450
AtuORF23-B 3′ UTR	9714-10180	467
CsVMV Promoter	10292-10817	526
pat	10820-11371	552
AtuORF1 3′ UTR	11342-12412	1071
Backbone 3	12389-13178	790
Ori	12804-14182	1379
Backbone 2	14149-15876	1728
Backbone 1	15846-17079	1234
SpecR	17081-18116	1036
Backbone 4	17426-18143	718

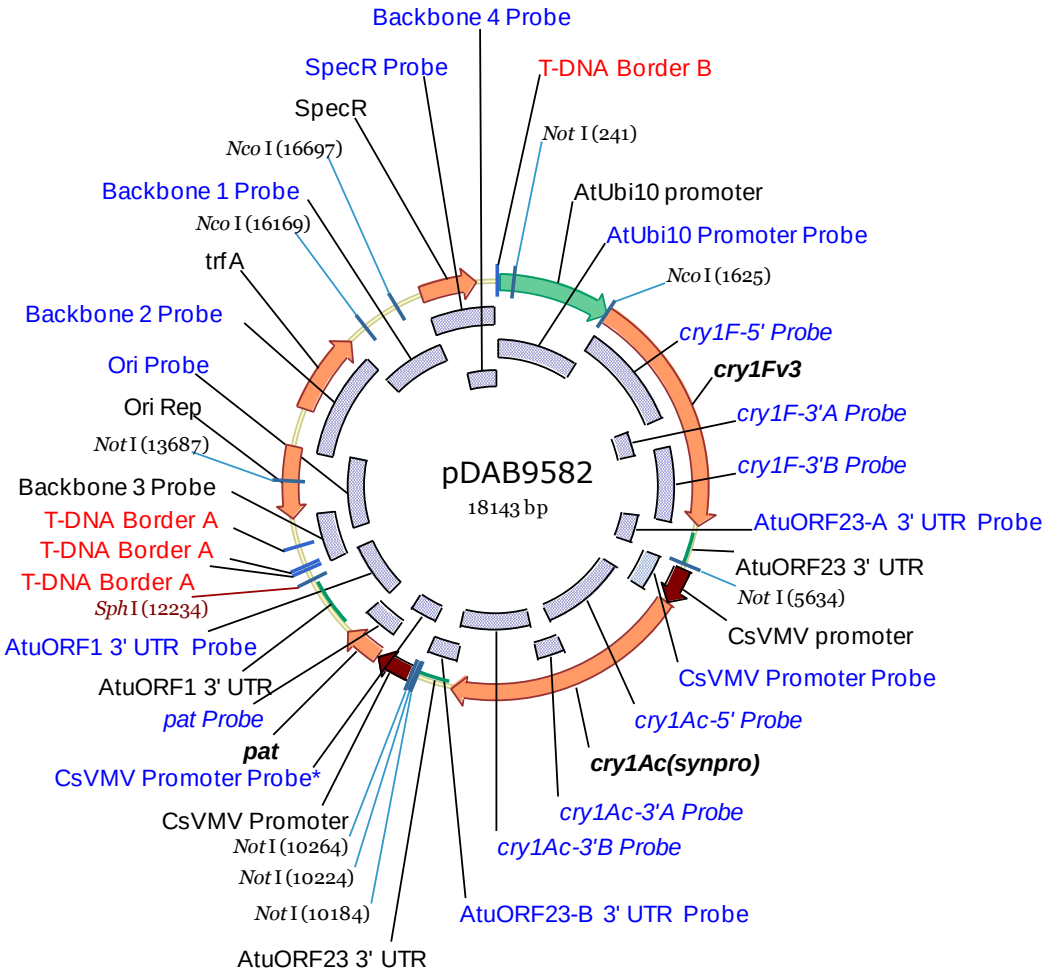


Figure 5. Location of the probes on pDAB9582 used in Southern blot analysis of DAS-81419-2 soybean.

*Note: * Since the CsVMV promoter element in the cry1Ac(synpro) and pat PTUs are 100% identical based on the nucleotide sequences, CsVMV Promoter probe generated from cry1Ac(synpro) PTU was used to hybridize CsVMV promoter in pat PTU.*

Table 4. Predicted and observed sizes of hybridizing fragments in Southern blot analyses of DAS-81419-2 soybean.

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
AtUbi10 promoter	*Nco*I	pDAB9582	2	3071	~3100	8A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>1737	~7500	
	*Sph*I	pDAB9582	2	18143	~18100	8B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1F-5′	*Nco*I	pDAB9582	2	14544	~14500	9A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	9B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1F-3′A	*Nco*I	pDAB9582	2	14544	~14500	10A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	10B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1F-3′B	*Nco*I	pDAB9582	2	14544	~14500	11A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	11B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
AtuORF23 -A 3′ UTR	*Nco*I	pDAB9582	2	14544	~14500	12A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	12B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
CsVMV Promoter	*Nco*I	pDAB9582	2	14544	~14500	13A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	13B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1Ac-5′	*Nco*I	pDAB9582	2	14544	~14500	14A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	14B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1Ac-3′A	*Nco*I	pDAB9582	2	14544	~14500	15A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759, >1737	~14500, ~7500	
	*Sph*I	pDAB9582	2	18143	~18100	15B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1Ac-3′B	*Nco*I	pDAB9582	2	14544	~14500	16A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	16B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
AtuORF23 -B 3′ UTR	*Nco*I	pDAB9582	2	14544	~14500	17A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	17B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
pat	*Nco*I	pDAB9582	2	14544	~14500	18A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	18B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
AtuORF1 3′ UTR	*Nco*I	pDAB9582	2	14544	~14500	19A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	19B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346, >150	~18100, ~4100	
AtUbi10 promoter	*Not*I/*Sph*I (Release *cry1Fv3* PTU)	pDAB9582	2	5393, 4697	~5400, ~4700	20A
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, >353	~5400, ~6500	
cry1F-5′		pDAB9582	2	5393	~5400	20B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393	~5400	
cry1F-3′A		pDAB9582	2	5393, 4550	~5400, ~4600	21A
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
cry1F-3′B		pDAB9582	2	5393, 4550	~5400, ~4600	21B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
AtuORF23 -A 3′ UTR		pDAB9582	2	5393, 4550	~5400, ~4600	22A
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
CsVMV Promoter	*Not*I/*Sph*I (Release *cry1Ac(syn pro)* PTU)	pDAB9582	2	4550, 1970	~4600, ~2000	22B
		Maverick	3	none	none	
		DAS-81419-2	4-18	4550, 1970	~4600, ~2000	
cry1Ac-5′		pDAB9582	2	4550	~4600	23A
		Maverick	3	none	none	
		DAS-81419-2	4-18	4550	~4600	
cry1Ac-3′A		pDAB9582	2	5393, 4550	~5400, ~4600	24A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>353, 4550, 5393	~6500, ~4600, ~5400	
cry1Ac-3′B		pDAB9582	2	5393, 4550	~5400, ~4600	24B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
AtuORF23 -B 3′ UTR		pDAB9582	2	5393, 4550	~5400, ~4600	23B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
pat	*Not*I/*Sph*I (Release *pat* PTU)	pDAB9582	2	1970	~2000	25A
		Maverick	3	none	none	
		DAS-81419-2	4-18	1970	~2000	
AtuORF1 3′ UTR		pDAB9582	2	1453, 1970,	~1500, ~2000	25B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>150, 1970	~4000, ~2000	
Ori	*Sph*I	pDAB9582	2	18143	~18100	26A
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
SpecR	*Nco*I	pDAB9582	2	3071	~3100	26B
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
Backbone 1	*Nco*I	pDAB9582	2	14544, 3071, 528	~14500, ~3100, ~500	27A
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
Backbone 2	*Sph*I	pDAB9582	2	18143	~18100	27B
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
Backbone 3 + 4	*Nco*I	pDAB9582	2	14544, 3071	~14500, ~3100	28
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	

[1]Expected fragment sizes are based on the plasmid map of pDAB9582 (Figure 6) and the T-DNA insert in DAS-81419-2 soybean (Figure 7).
[2]Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker fragments. Due to the incorporation of DIG molecules for visualization, the Marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.

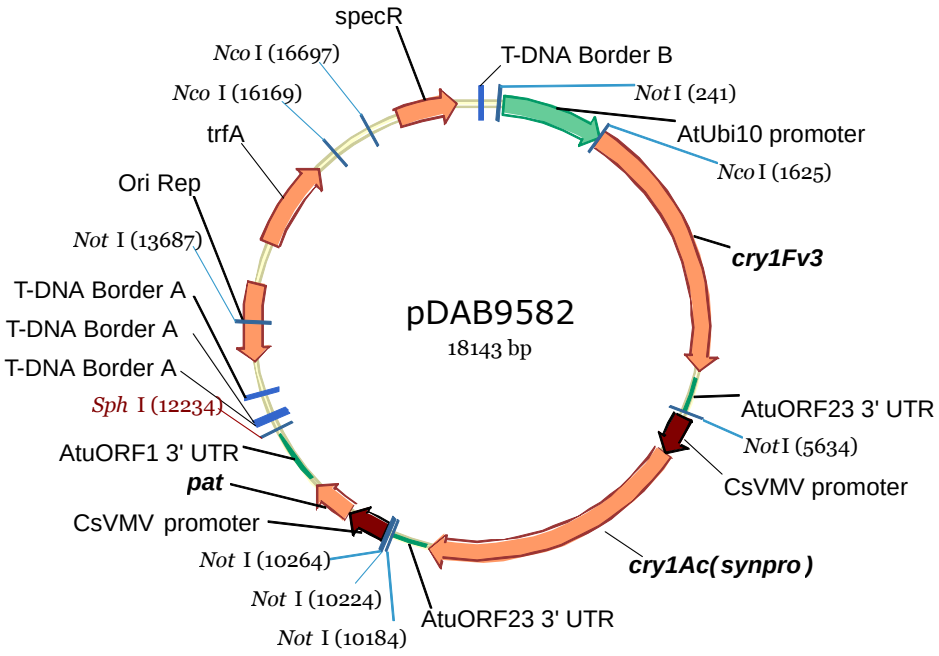


Figure 6. Plasmid map of pDAB9582 with selected restriction enzyme sites used for Southern analysis.

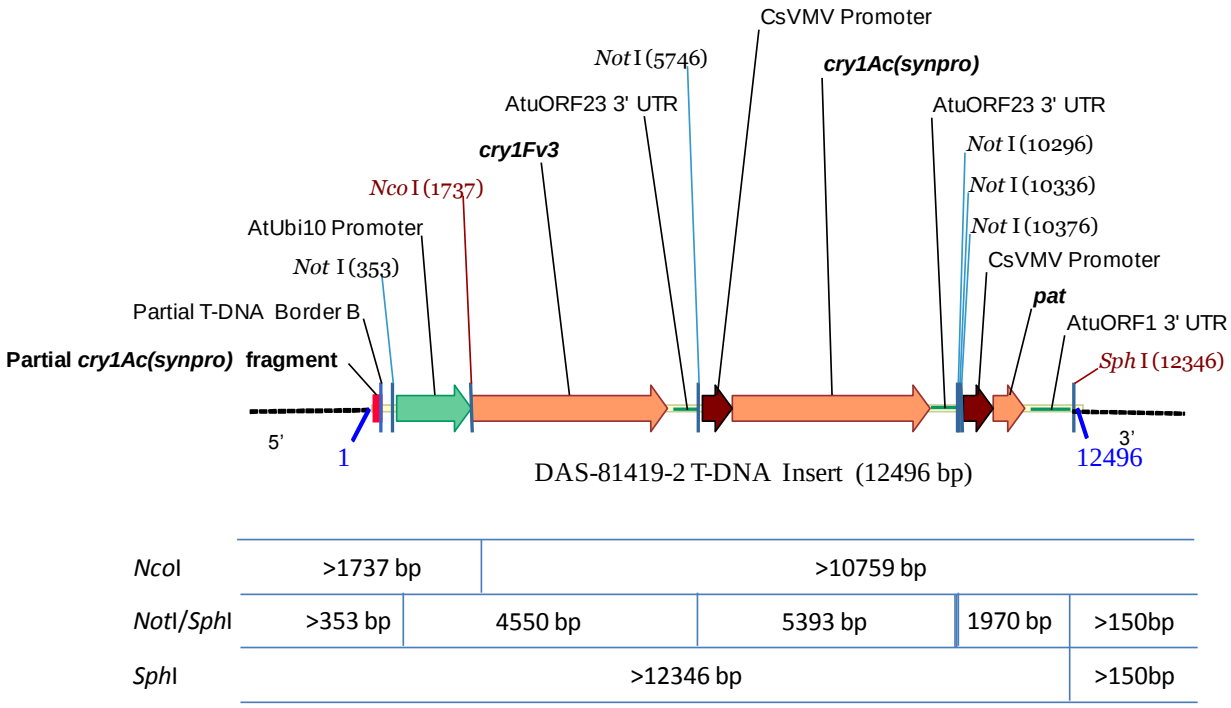


Figure 7. Actual T-DNA insert in DAS-81419-2 soybean with restriction enzymes used for DNA digestion and sizes of the expected hybridization bands.

5.2. Analysis of the Insert and Its Genetic Elements

5.2.1. Number of Insertion Sites

The restriction enzymes *Nco*I *and Sph*I were chosen to determine the number of insertions in DAS-81419-2 soybean (Figure 7). Probes derived from the DNA sequences of AtUbi10 Promoter, *cry1Fv3* (split into three overlapping regions *cry1F*-5′, *cry1F*-3′A, and *cry1F*-3′B), AtuORF23 3′UTR (probes A and B contain small pieces of different intervening sequences at the 5′ and 3′ ends of the AtuORF23 3′UTR sequence), CsVMV promoter, *cry1Ac(synpro)* (split into three overlapping regions *cry1Ac*-5′, *cry1Ac*-3′A, and *cry1Ac*-3′B), *pat*, and AtuORF1 3′UTR were then hybridized to the digested genomic DNA to determine the number of insertion sites in DAS-81419-2 soybean.

When digested with restriction enzyme *Nco*I and independently hybridized with the *cry1F*-5′, *cry1F*-3′A, *cry1F*-3′B, AtuORF23-A 3′ UTR, CsVMV Promoter, *cry1Ac*-5′, *cry1Ac*-3′A, *cry1Ac*-3′B, AtuORF23-B 3′ UTR, *pat*, and AtuORF1 3′ UTR probes, a single hybridization band of ~14500 bp was detected in DAS-81419-2 soybean samples, consistent with the predicted size of >10759 bp for the *Nco*I fragment as shown in Table 4 and Figure 9A - Figure 19A. When *Nco*I digested samples were hybridized with AtUbi10 Promoter, a single band of ~7500 bp was detected in DAS-81419-2 soybean samples, consistent with the expected result of >1737 bp (Figure 8A).

When digested with restriction enzyme *Sph*I and probed with the AtUbi10 Promoter, *cry1F*-5′, *cry1F*-3′A, *cry1F*-3′B, AtuORF23-A 3′ UTR, CsVMV Promoter, *cry1Ac*-5′, *cry1Ac*-3′A,

cry1Ac-3′B, AtuORF23-B 3′ UTR, and *pat* probes individually, a single band of ~18100 bp was detected in DAS-81419-2 soybean samples, consistent with the expected result of >12346 bp (Table 4, Figure 8B - Figure 18B). When *Sph*I digested samples were hybridized with AtuORF1 3′ UTR probe, two fragments of ~18100 bp and ~4100 bp were detected in DAS-81419-2 soybean samples, consistent with the expected result of >12346 bp and >150 bp (Figure 19B).

Specific hybridization bands were detected in all of the positive samples at the expected sizes, while no specific hybridization bands were detected in any of the negative control samples. All these data indicate that there is a single insertion of the T-DNA containing all the expected elements from pDAB9582 in DAS-81419-2 soybean genome.

5.2.2. Structure of the Insert and Genetic Elements

According to the plasmid and T-DNA restriction maps shown in Figure 6 and Figure 7, the plant transcription units (PTUs) for *cry1Fv3*, *cry1Ac(synpro)*, and *pat* could be released by restriction digestion with *Not*I/*Sph*I. These digestions were performed to characterize the structure of the insert in DAS-81419-2 soybean.

When digested with *Not*I/*Sph*I and hybridized with AtUbi10 Promoter probe, two bands of ~5400 bp and ~6500 bp were detected in all DAS-81419-2 soybean and the positive control samples, consistent with the expected sizes of 5393 bp and > 353 bp for the *cry1Fv3* PTU and the border fragment that the AtUbi10 promoter probe extended into, respectively (Figure 20A). When digested with *Not*I/*Sph*I and hybridized with *cry1F*-5′ probe, a single band of ~5400 bp was detected in DAS-81419-2 soybean and the positive control samples, consistent with the expected size of 5393 bp for the *cry1Fv3* PTU (Figure 20B).

Since both *cry1Fv3 and cry1Ac(synpro)* genes contain synthetic *cry1Ab* sequence with 89% identity at nucleotide level, it is expected that the probes derived from the 3′ region of the *cry1F* gene (*cry1F*-3′A, *cry1F*-3′ B) would cross-hybridize with the respective DNA fragments containing the 3′ region of the *cry1Ac* gene. When digested with *Not*I/*Sph*I and hybridized with *cry1F*-3′A and *cry1F*-3′B probes individually, two bands of ~5400 bp and ~4600 bp which are consistent with the expected bands with sizes of 5393 bp for *cry1Fv3* PTU and 4550 bp for *cry1Ac(synpro)* PTU (Figure 21), were detected in DAS-81419-2 soybean and the positive control samples. Since the AtuORF23 3′ UTR element in the *cry1Fv3* and *cry1Ac(synpro)* PTUs are 100% identical based on the nucleotide sequences (Figure 5), it is expected that probes derived from AtuORF23 3′ UTR element (AtuORF23-A 3′ UTR and AtuORF23-B 3′ UTR) would cross-hybridize with the respective DNA fragments containing the AtuORF23 3′ UTR. When digested with *Not*I/*Sph*I and hybridized with AtuORF23-A 3′ UTR probe, two hybridization bands of 5393 bp and 4550 bp are expected for DAS-81419-2 soybean and positive control samples. As shown in Figure 22A, two bands of ~5400 bp and ~4600 bp were detected in DAS-81419-2 soybean and the positive control samples, consistent with the predicted sizes for *cry1Fv3* and *cry1Ac(synpro)* PTUs. These data suggest that an intact *cry1Fv3* PTU is present in all generations tested.

Since the CsVMV promoter element in the *cry1Ac(synpro*) and *pat* PTUs are 100% identical based on the nucleotide sequences (Figure 5), it is expected that probe derived from CsVMV promoter (CsVMV Promoter probe) would cross-hybridize with the respective DNA fragments containing the CsVMV promoter element. When digested with *Not*I/*Sph*I and hybridized with

CsVMV Promoter probe, two hybridization bands of 4550 bp and 1970 bp are expected for DAS-81419-2 soybean and the positive control samples. As shown in Figure 22B, two bands of ~4600 bp and ~2000 bp were detected in DAS-81419-2 soybean and the positive control samples, consistent with the predicted sizes for *cry1Ac(synpro)* and *pat* PTUs. When digested with *Not*I/*Sph*I and hybridized with *cry1Ac-5′* probe, a single band of ~4600 bp was detected in DAS-81419-2 soybean and the positive control samples, consistent with the expected size of 4550 bp for the *cry1Ac(synpro)* PTU (Figure 23A).

According to the sequence information, a 98-bp partial *cry1Ac(synpro)* fragment was also identified at the 5′end of the T-DNA insert in DAS-81419-2 soybean (Figure 7). This 98-bp partial *cry1Ac(synpro)* fragment is 99% identical to 1990 - 2087 bp region of the *cry1Ac(synpro)* gene in a complementary orientation. As a consequence, when digested with *Not*I/*Sph*I and hybridized with the *cry1Ac*-3′A probe, two hybridization bands, one of ~4600 bp which is consistent with the expected size of 4550 bp for full length *cry1Ac(synpro)* PTU and the other one of ~ 6500 bp which is consistent with the expected result of >353 bp for the DNA fragment containing the partial *cry1Ac(synpro)*, were detected in DAS-81419-2 soybean samples In addition, a very faint band of ~5400 bp was also detected in DAS-81419-2 soybean and the positive control samples, consistent with the expected size of 5393 bp for *cry1Fv3* PTU which is expected to be cross-hybridized by the *cry1Ac*-3′A probe (Figure 24A).

When digested with *Not*I/*Sph*I and hybridized with *cry1Ac*-3′ B probe, two hybridization bands of 5393 bp and 4550 bp are expected for DAS-81419-2 soybean samples along with positive control sample. As shown in Figure 24B, two bands of ~5400 bp and ~4600 bp were detected in DAS-81419-2 soybean and positive control samples, consistent with the predicted sizes for *cry1Ac(synpro)* and *cry1Fv3* PTUs. When digested with *Not*I/*Sph*I and hybridized with AtuORF23-B 3′ UTR probe, two bands of 5393 bp and 4550 bp are expected for DAS-81419-2 soybean and positive control samples. As shown in Figure 23B, two bands of ~5400 bp and ~4600 bp were detected in DAS-81419-2 soybean and positive control samples, consistent with the predicted sizes for *cry1Fv3* and *cry1Ac(synpro)* PTUs. These data suggest that an intact *cry1Ac(synpro)* PTU and a small partial *cry1Ac(synpro)* fragment is present in all generations tested.

When digested with *Not*I/*Sph*I and hybridized with *pat* probe, a single band of ~2000 bp was detected in all DAS-81419-2 soybean and the positive control samples, consistent with the predicted size of 1970 bp for the *pat* PTU (Figure 25A). When digested with *Not*I/*Sph*I and hybridized with AtuORF1 3′ UTR probe, two bands of ~4000 bp and ~2000 bp were detected in all DAS-81419-2 soybean and the positive control samples, consistent with expected bands of >150 bp and 1970 bp (Figure 25B). These data suggest that an intact *pat* PTU is present in all generations tested.

Hybridization bands of the expected sizes were detected in all of the positive samples, while no specific hybridization band was detected in the non-transgenic soybean negative control samples as expected. 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 the single insert in DAS-81419-2 soybean contains an intact PTU for each of the *cry1Fv3, cry1Ac(synpro),* and *pat* genes along with a partial *cry1Ac(synpro)* fragment at the 5′ end of the insert.

5.2.3. Absence of Plasmid Backbone Sequences

To verify that no plasmid vector backbone sequences exist in DAS-81419-2 soybean, six probes covering the entire region outside of the T-DNA in pDAB9582 were hybridized with *Nco*I or *Sph*I digested samples (Table 3, Figure 5). The hybridization results demonstrated that no specific hybridization signals were detected in any DAS-81419-2 samples or the negative control samples, while hybridization fragments of the expected size were detected in the respective positive controls (Figure 26 - Figure 28). These data indicate that no backbone sequences from pDAB9582 have been integrated into DAS-81419-2 soybean.

5.2.4. Stability of the Insert Across Generations

Southern blot analyses were conducted with samples across five distinct generations (T1, T2, T3, T4, and F2) of DAS-81419-2 soybean. Prior to initiation of Southern blot analyses, all plants were tested for PAT protein expression using a lateral flow strip assay to identify PAT expression-positive plants. All of the genetic element probes: AtUbi10 Promoter, *cry1F*-5′, *cry1F*-3′A, *cry1F*-3′B, AtuORF23-A 3′ UTR, CsVMV Promoter, *cry1Ac*-5′, *cry1Ac*-3′A, *cry1Ac*-3′B, AtuORF23-B 3′ UTR, *pat*, and AtuORF1 3′ UTR, as well as the probes covering the entire backbone regions of plasmid pDAB9582, were hybridized with the DAS-81419-2 soybean samples. Results across all DAS-81419-2 soybean samples in the five generations were consistent with what were expected (Table 4), indicating stable integration and inheritance of the intact, single copy transgene insert across multiple generations of DAS-81419-2 soybean.

5.2.5. Conclusions

The Southern blot analyses confirm that DAS-81419-2 soybean contains a single insertion of the T-DNA from plasmid pDAB9582 with each of the intact PTUs for *cry1Fv3*, *cry1Ac(synpro),* and *pat* genes. In addition, a minor (<100 bp) fragment of the *cry1Ac(synpro)* gene was identified at the 5′ end of the T-DNA insert. Identical hybridization patterns were observed across five distinct generations of DAS-81419-2 soybean, indicating stable inheritance of the transgene insert across multiple generations. Moreover, the absence of the backbone sequences from plasmid pDAB9582 in DAS-81419-2 soybean was confirmed using probes covering the entire region flanking the T-DNA insert.

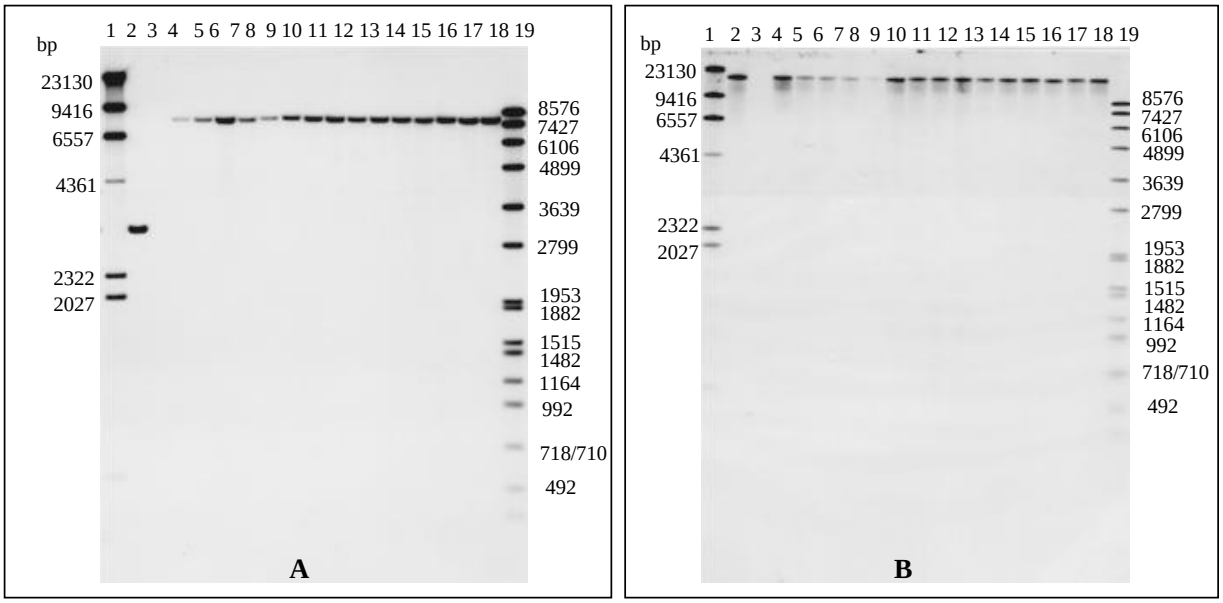


Figure 8. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtUbi10 promoter probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Bands in lanes 7-9 of panel B may not be clearly visible on printed report.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

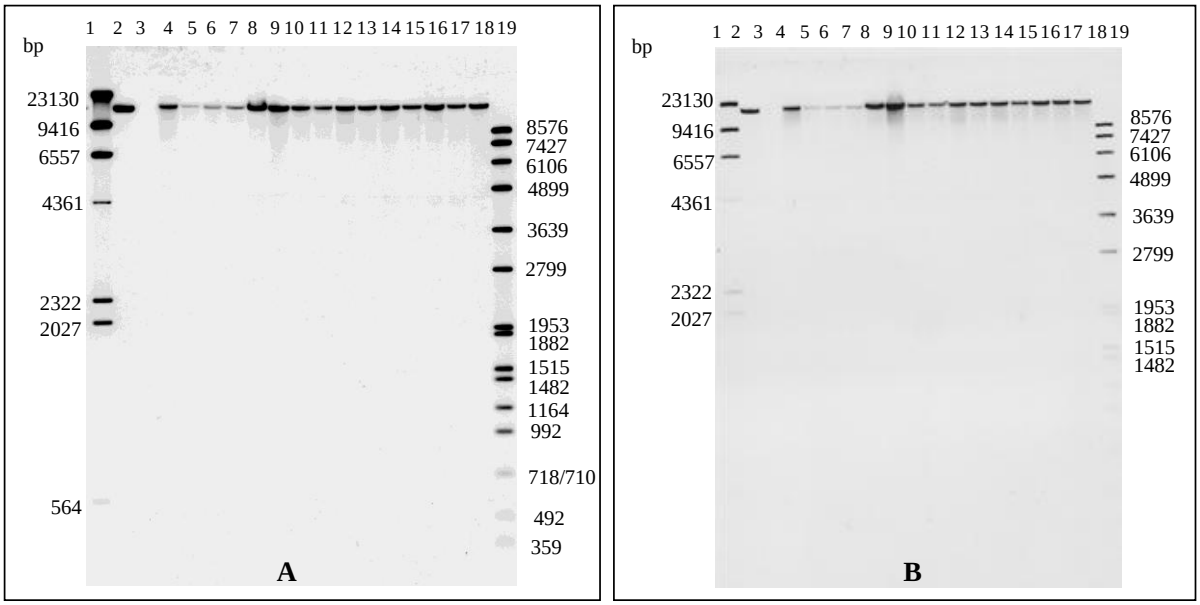


Figure 9. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1F-5′* probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

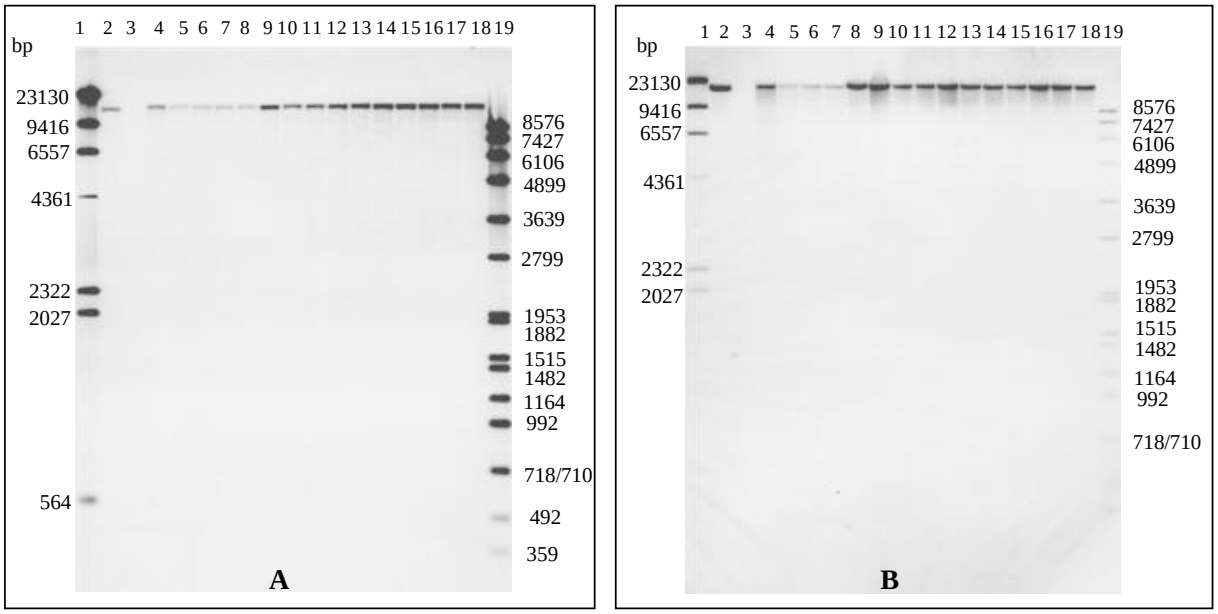


Figure 10. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1F-3′*A probe.

Approximately 10 μg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 μg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

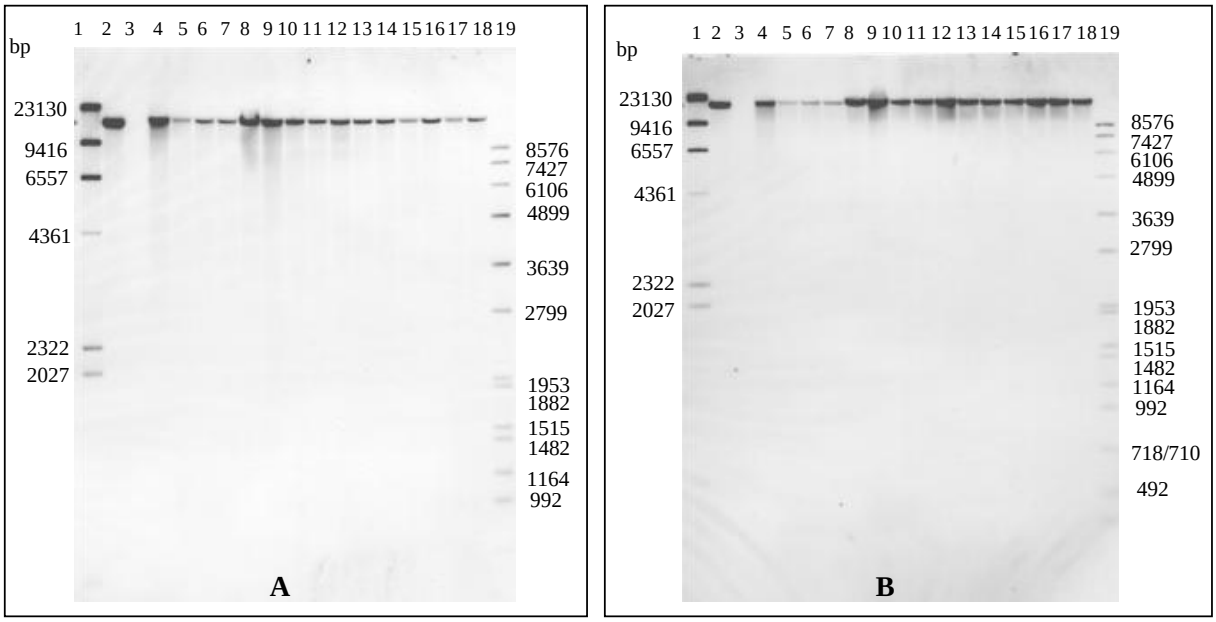


Figure 11. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1F-3′* B probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

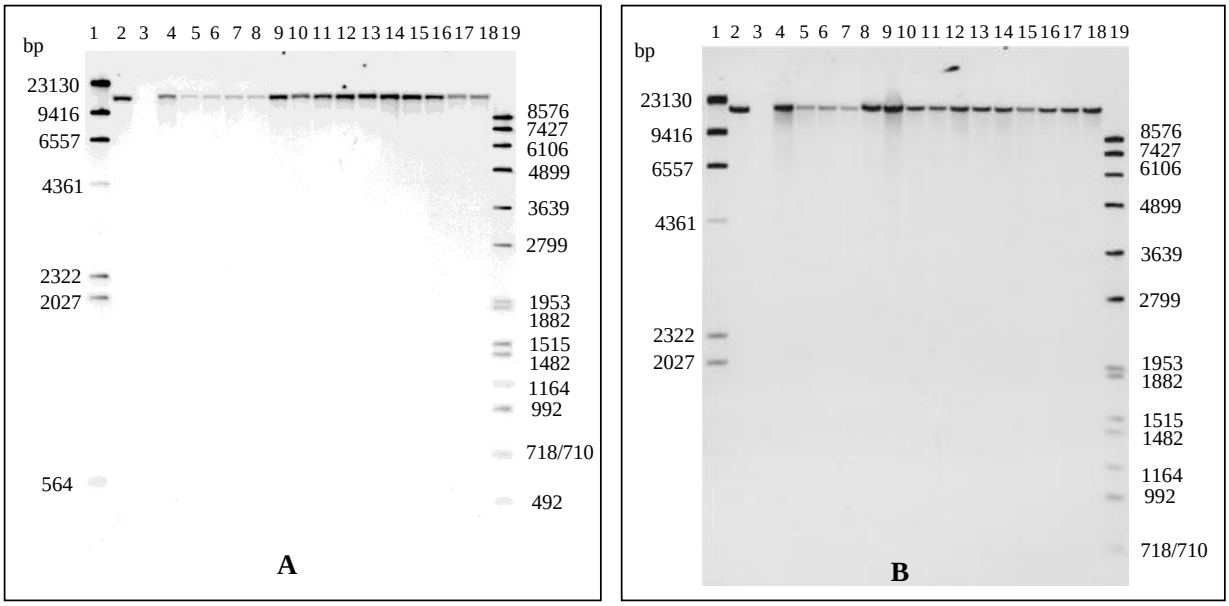


Figure 12. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtuORF23-A 3′UTR probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

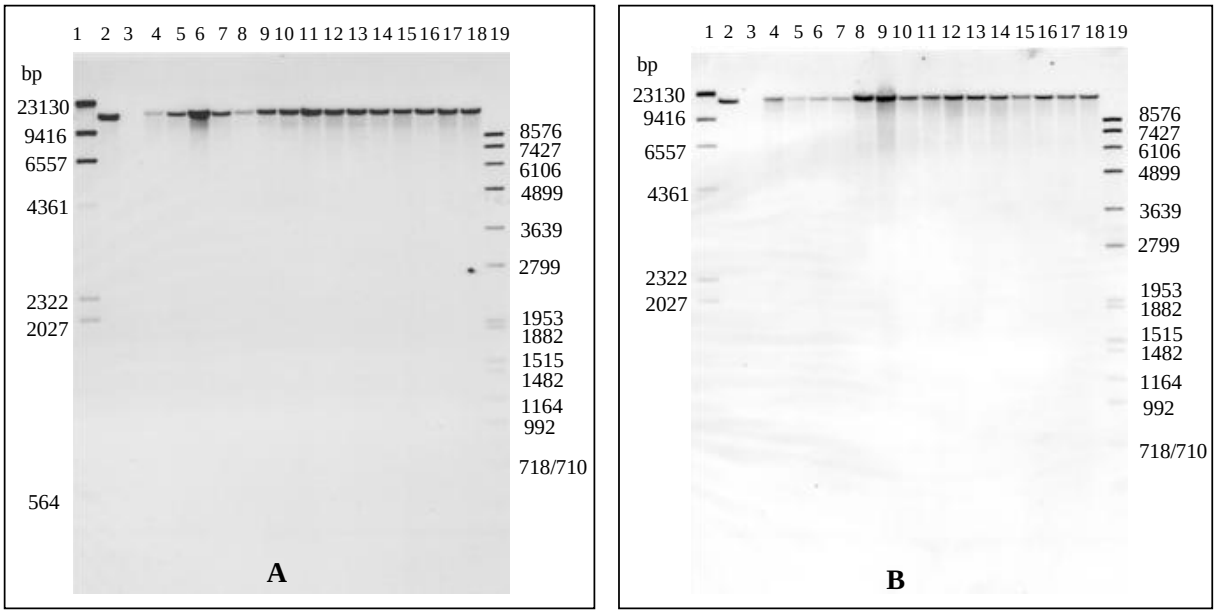


Figure 13. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); CsVMV promoter probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

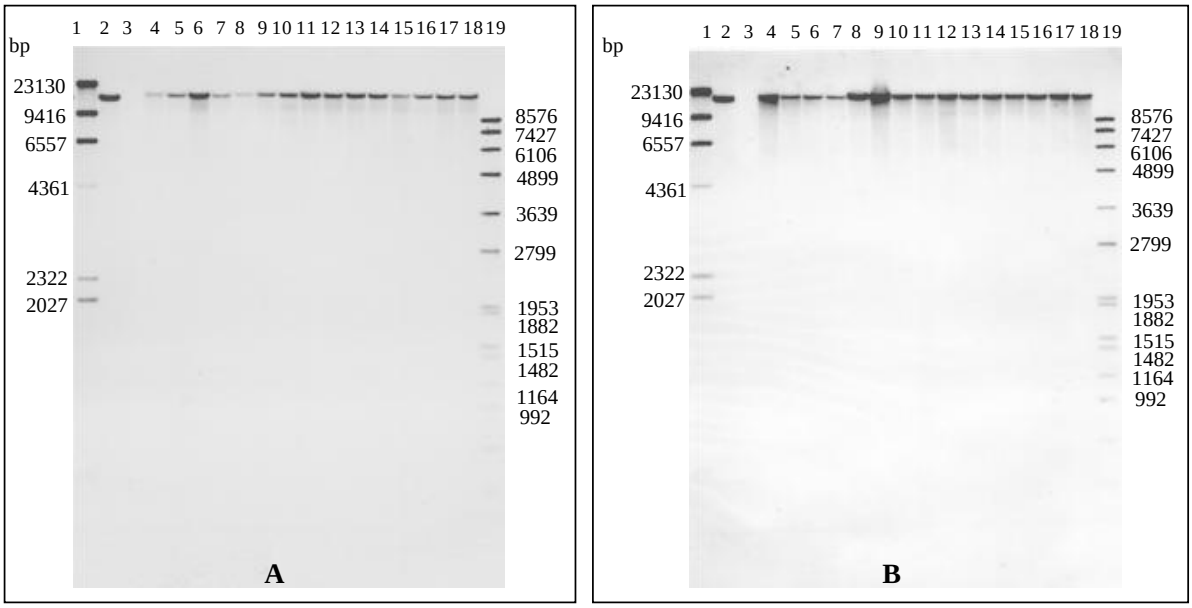


Figure 14. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1Ac*-5′ probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

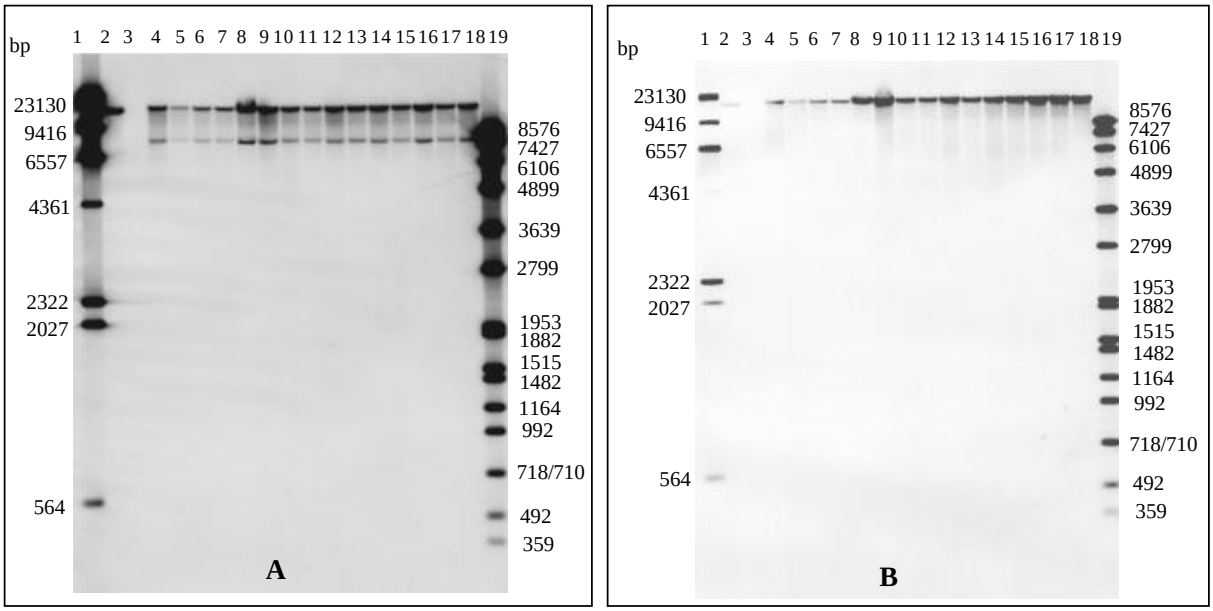


Figure 15. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1Ac*-3′A probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Fuzzy smear bands at ~18000 bp in lanes 4-18 of panel A may be a result of DNA degradation and/or incomplete digestion.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

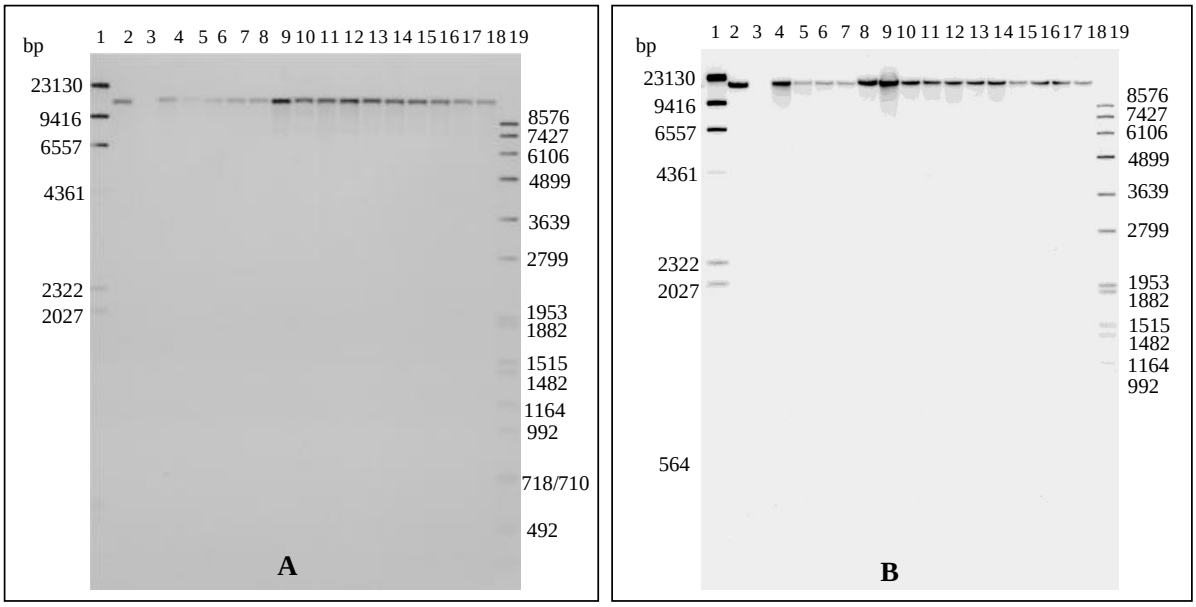


Figure 16. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1Ac*-3′B probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Bands in lane 8 of panel A may not be clearly visible on printed report.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

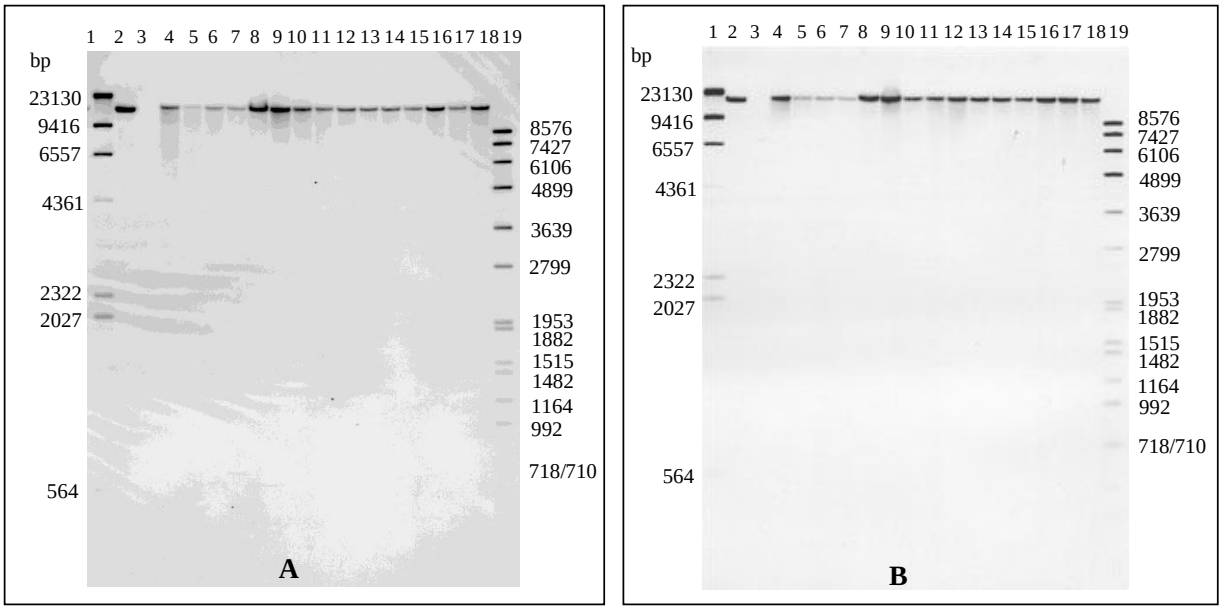


Figure 17. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtuORF23-B 3′UTR probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

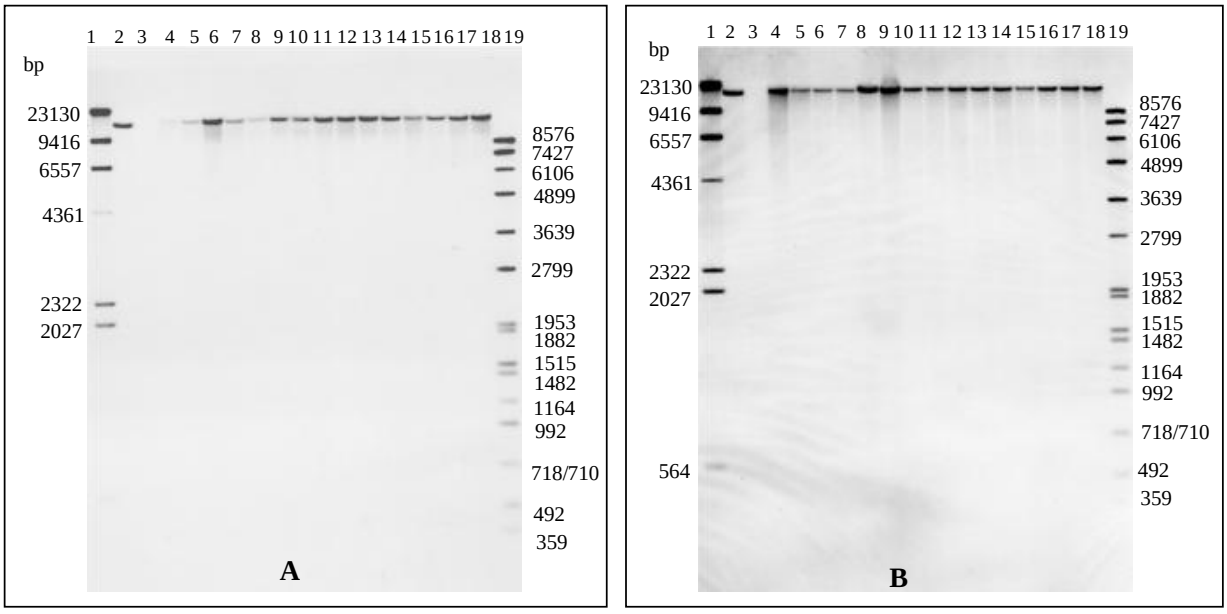


Figure 18. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *pat* probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Bands in lane 4 of panel A may not be clearly visible on printed report.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

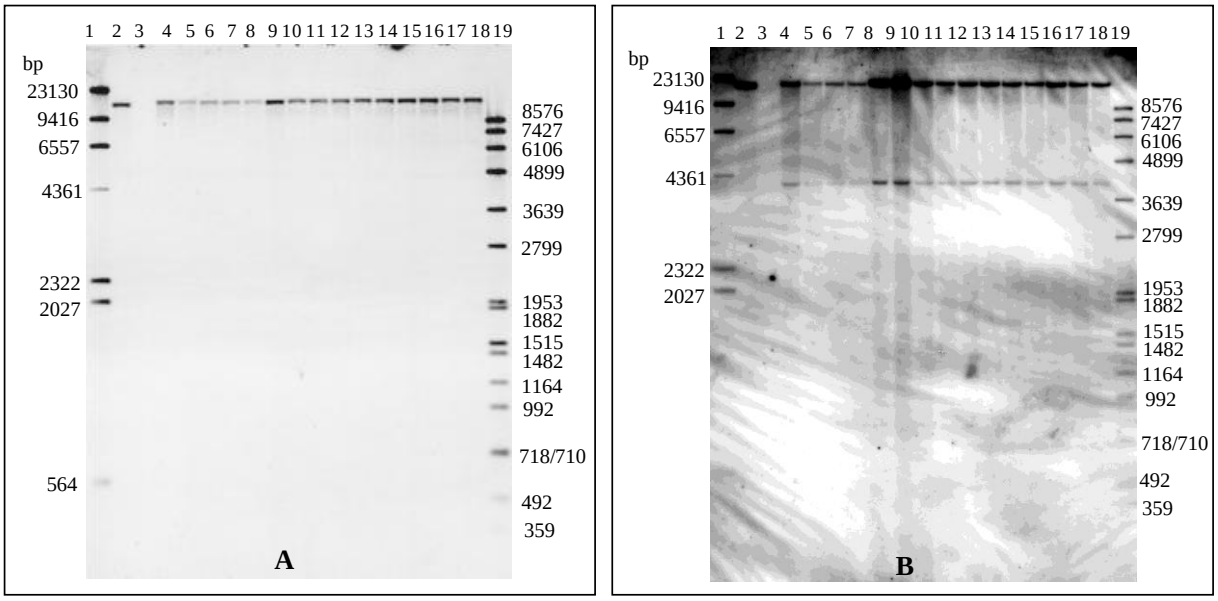


Figure 19. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtuORF1 3′ UTR probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Bands in lanes 7-8 of panel B may not be clearly visible on printed report.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

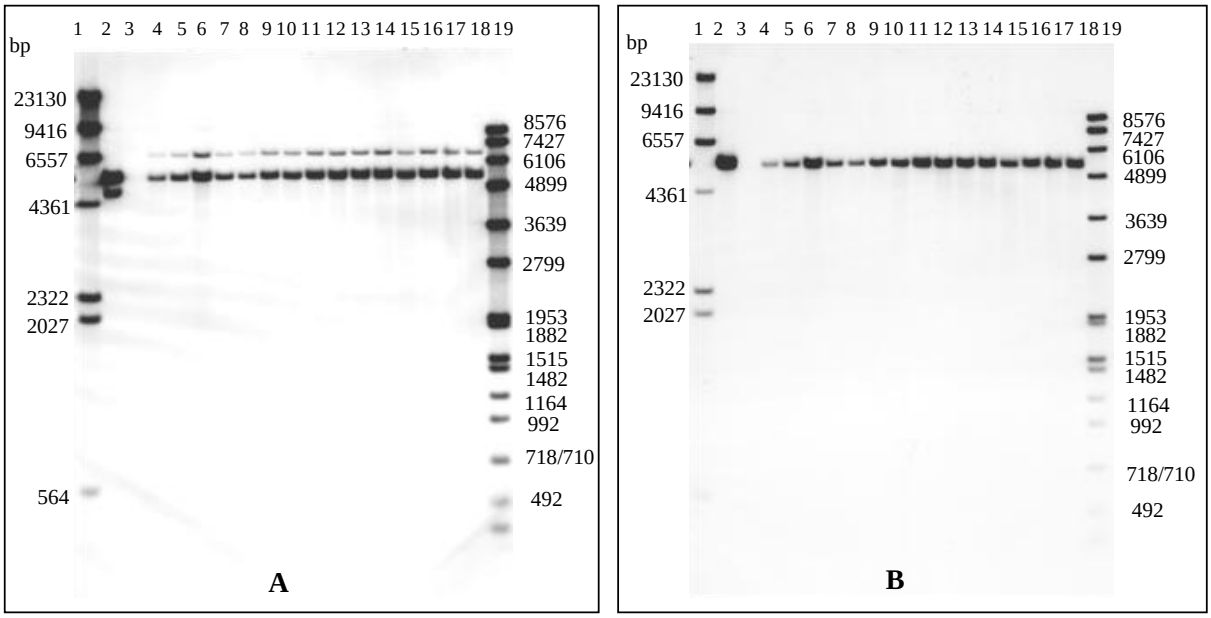


Figure 20. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) AtUbi10 promoter probe, (B) *cry1F-5′* probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

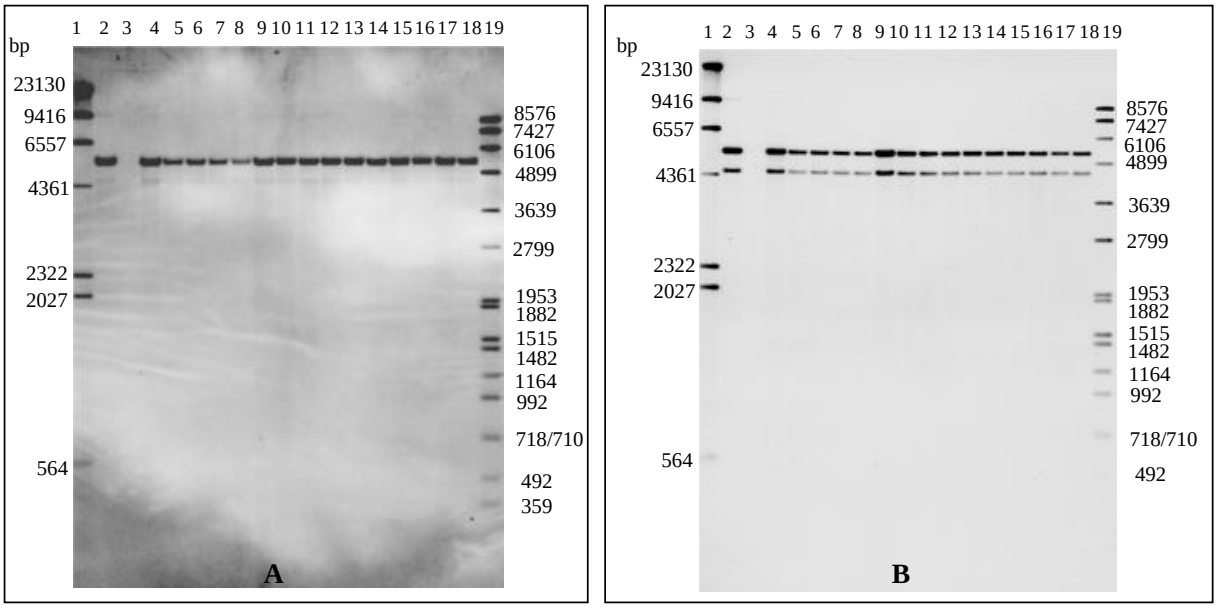


Figure 21. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *cry1F*-3′A probe, (B) *cry1F*-3′B probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Faint bands of ~ 4600 bp in positive control and DAS-81419-2 soybean samples observed in panel A are the expected cross-hybridization results of the cry1F-3'A probe binding to the cry1Ac(synpro) PTU.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

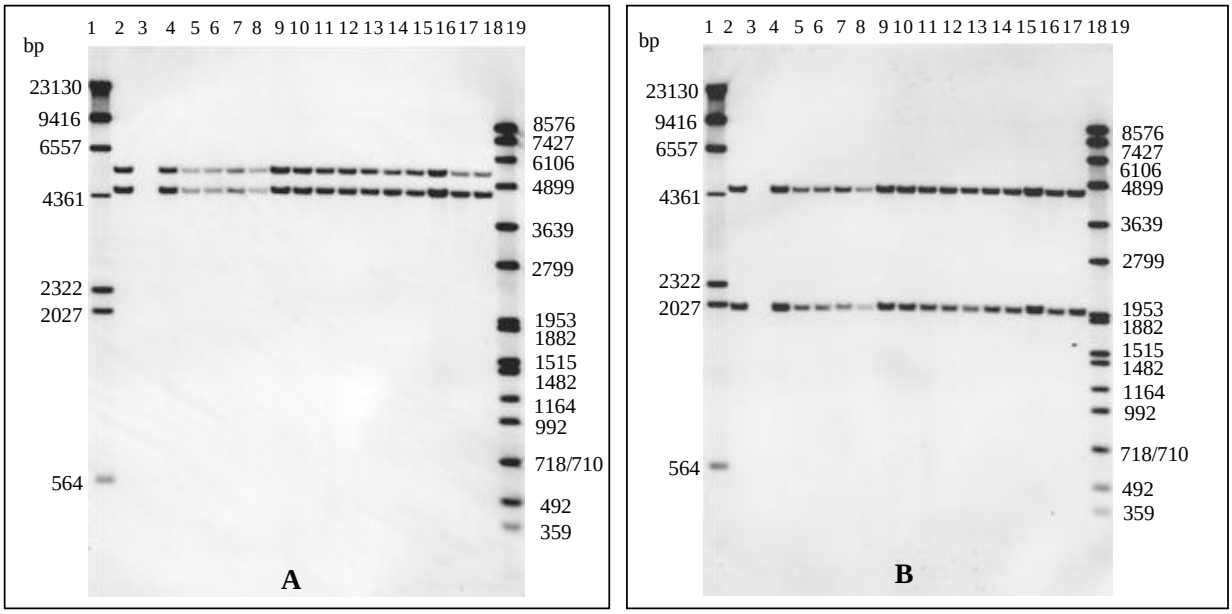


Figure 22. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) AtuORF23-A 3′UTR probe, (B) CsVMV promoter probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

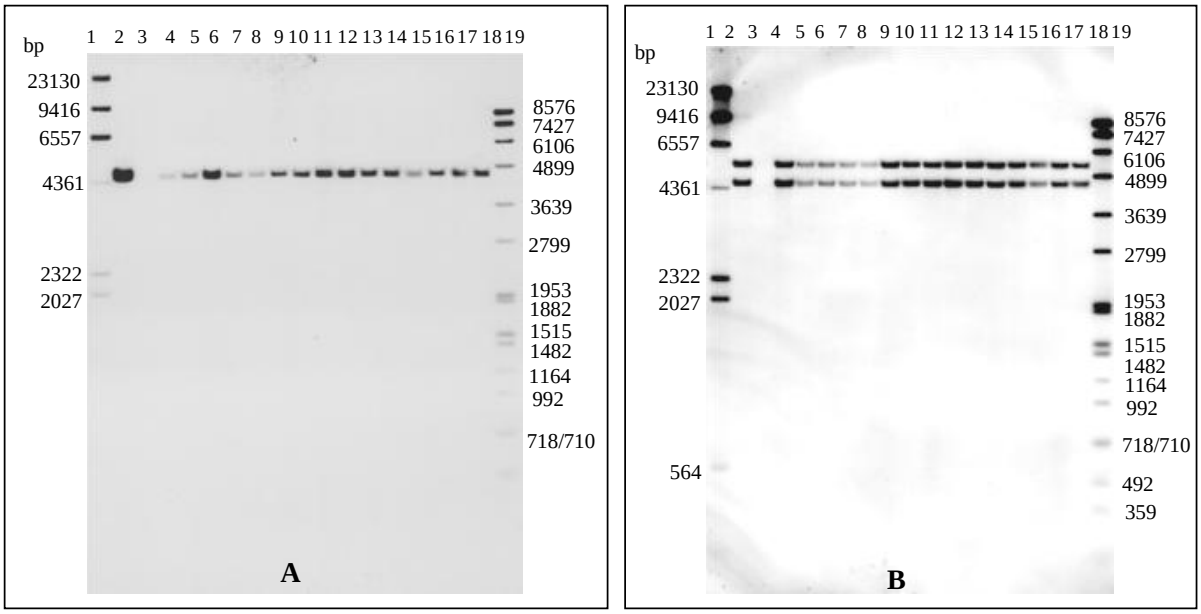


Figure 23. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *cry1Ac*-5′ probe, (B) AtuORF23-B 3′UTR probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

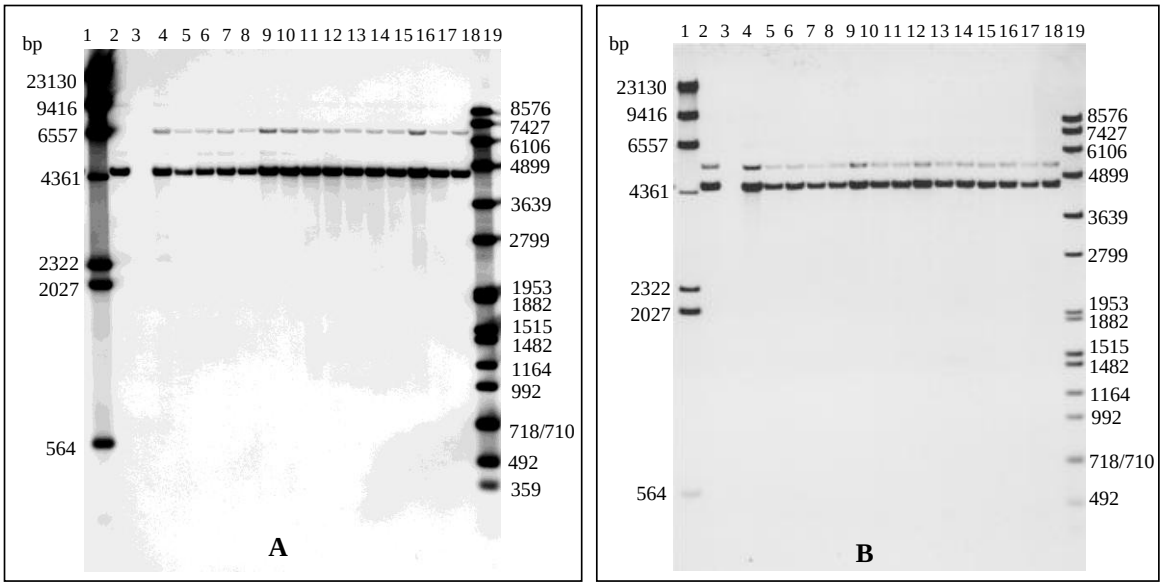


Figure 24. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *cry1Ac*-3′A probe, (B) *cry1Ac*-3′B probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image. *Note: Faint bands of ~ 5400 bp in positive control and DAS-81419-2 soybean samples observed in panel A are the expected cross-hybridization bands of the cry1Ac-3'A probe binding to the cry1Fv3 PTU. Faint bands at ~9500 bp observed in lanes 2 and 4 in panel A are most likely a result of incomplete digestion.*

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

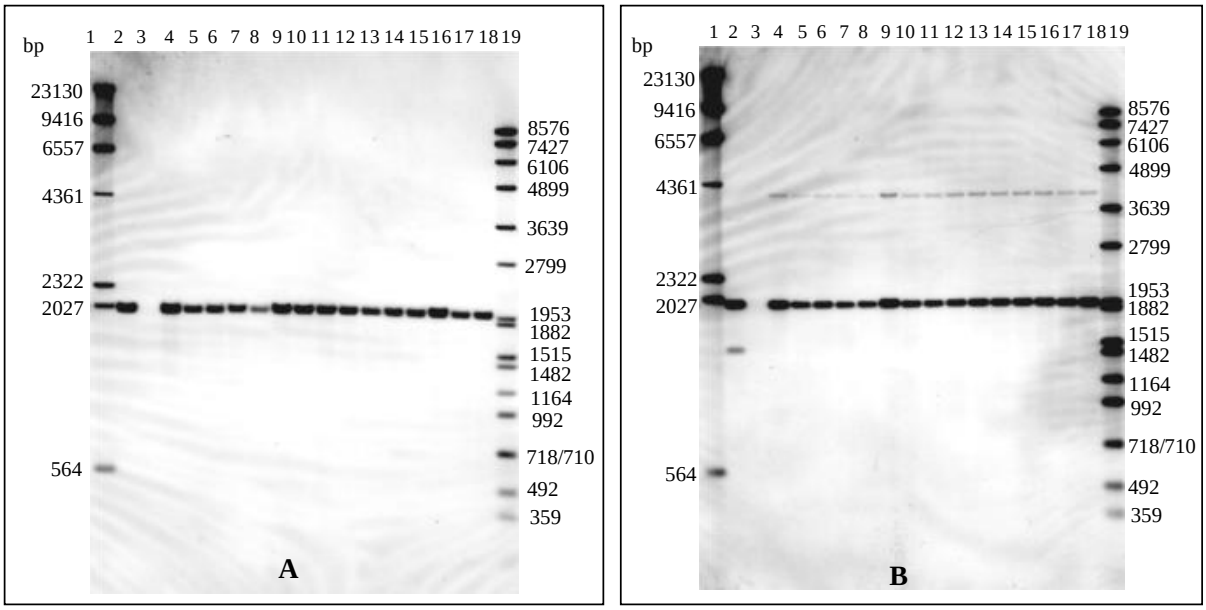


Figure 25. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *pat* probe, (B) AtuORF1 3′UTR probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

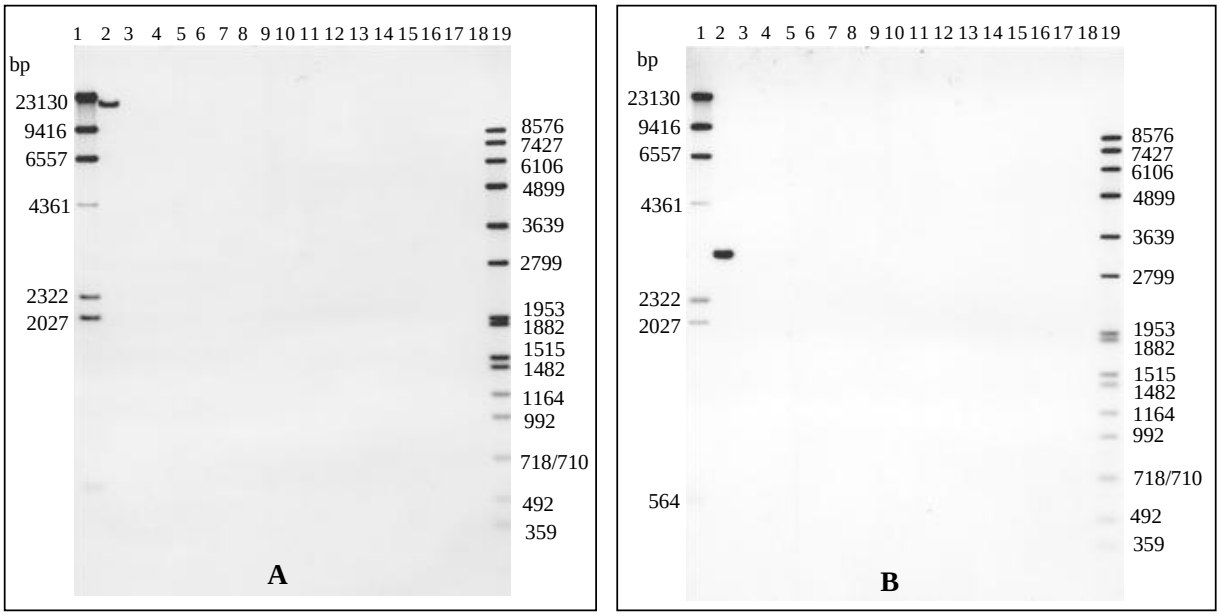


Figure 26. Southern blot analysis of DAS-81419-2 soybean digested with (A) *Sph*I; Ori probe, (B) *Nco*I; SpecR probe.

Approximately 10 μg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 μg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

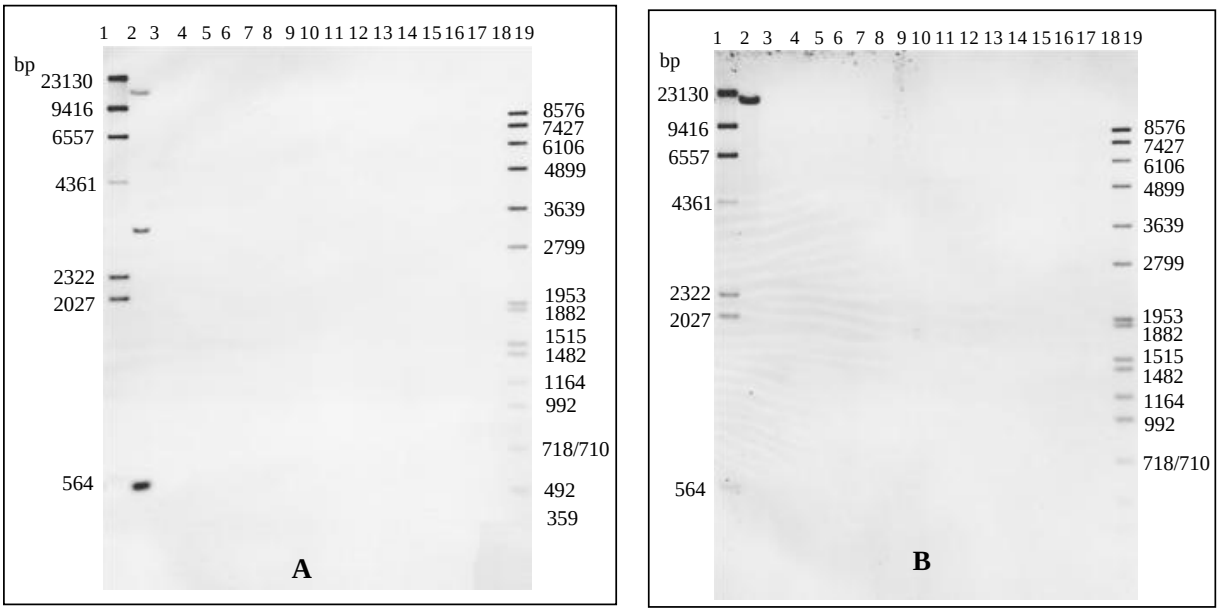


Figure 27. Southern blot analysis of DAS-81419-2 soybean digested with (A) *Nco*I; Backbone 1 probe, (B) *Sph*I; Backbone 2 probe.

Approximately 10 µg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 µg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

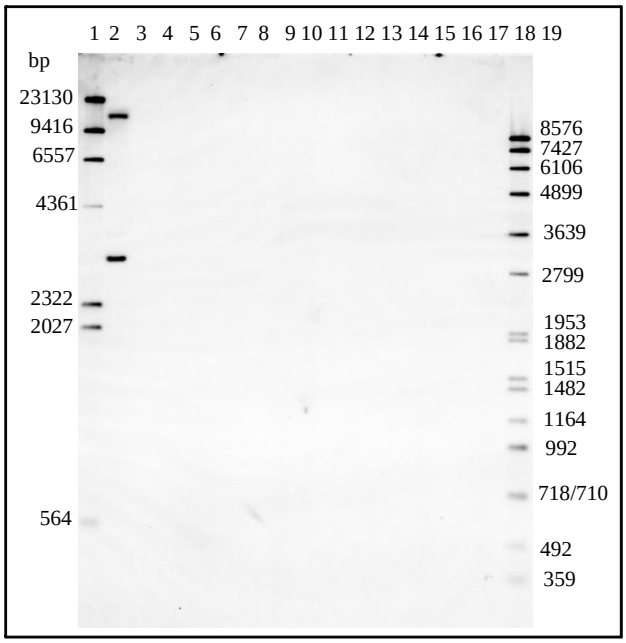


Figure 28. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I; Backbone 3 and 4 probes.

Approximately 10 μg of genomic DNA was used in the digestion. The positive control included plasmid pDAB9582 at approximately one transgene copy per soybean genome and 10 μg genomic DNA from non-transgenic soybean Maverick. Fragment sizes in bp of the DIG-labeled DNA Molecular Weight Marker II and DIG-labeled DNA Molecular Weight Marker VII are indicated adjacent to image.

Lane	Sample	Lane	Sample
1	DIG Molecular Weight Marker II	11	DAS-81419-2-T2-2
2	Maverick + pDAB9582	12	DAS-81419-2-T2-3
3	Maverick	13	DAS-81419-2-T3-1
4	DAS-81419-2-F2-1	14	DAS-81419-2-T3-2
5	DAS-81419-2-F2-2	15	DAS-81419-2-T3-3
6	DAS-81419-2-F2-3	16	DAS-81419-2-T4-1
7	DAS-81419-2-T1-1	17	DAS-81419-2-T4-2
8	DAS-81419-2-T1-2	18	DAS-81419-2-T4-3
9	DAS-81419-2-T1-3	19	DIG Molecular Weight Marker VII
10	DAS-81419-2-T2-1		

5.3. Segregation Analysis

5.3.1. Analysis of a Segregating Generation

The inheritance pattern of the transgene insert within a segregating generation was demonstrated with a lateral flow strip assay and event-specific PCR analyses of individual plants within a F2 generation of DAS-81419-2 soybean. The F1 generation was generated by crossing homozygous T3 plants of DAS-81419-2 soybean with a non-transgenic soybean line. The F1 plants were self pollinated to produce the F2 seeds.

A total of 123 plants from the F2 generation of DAS-81419-2 soybean were tested for PAT protein expression by a lateral flow strip assay. Of the 123 F2 plants tested, 91 plants were positive and 32 were negative (segregated nulls) for PAT protein expression. One plant died before sufficient tissue sample was harvested for genomic DNA extraction and thus was not subjected to event-specific PCR analysis. As a consequence, a total of 122 F2 plants were tested by event-specific PCR to determine the presence or absence of the DAS-81419-2 transgene insert. Of the 122 plants tested, 90 plants were positive for the presence of DAS-81419-2 transgene insert, and the remaining 32 plants were negative (segregated null). All plants that tested positive for PAT protein expression by a lateral flow strip assay were also positive for the DAS-81419-2 transgene insert by event-specific PCR analysis. Similarly, all plants that tested negative for PAT protein expression were also negative for the presence of the DAS-81419-2 transgene insert by event-specific PCR (Table 5). This result confirmed that the phenotypic segregation matched the genotypic makeup of the tested F2 generation. Statistical analysis using a chi-square goodness of fit test indicated the ratio of 91 positive to 32 null segregants for the LFS test, and the ratio of 90 positive to 32 null segregants for the event-specific PCR analysis, did not significantly differ from the expected Mendelian 3:1 segregation pattern for a single independent locus.

Table 5. Results of F2 individual plants tested with LFS and event-specific PCR.

Tested Method	Total plants tested	Positive	Negative	Expected ratio	P-value[a]
LFS	123	91	32	3:1	0.7946
Event-Specific PCR	122	90	32	3:1	0.7538

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

5.3.2. Segregation Analysis of Breeding Generations

Chi-square goodness of fit analyses of trait inheritance data from three populations of the BC1F2 breeding generation was conducted to determine the Mendelian inheritance of the transgene insert in DAS-81419-2 soybean. The presence or absence of the transgene insert was determined using an event-specific PCR assay for DAS-81419-2 soybean. The expected segregation ratio of 3:1 for plants containing the transgene insert versus plants that do not contain the transgene insert (segregated nulls) was observed (Table 6).

Table 6. Results of the individual plants from DAS-81419-2 soybean tested with event-specific PCR within segregating BC1F2 generations.

Generation	Total plants tested	Transgene insert positive (with event-specific PCR)	Transgene insert negative (with event-specific PCR)	Expected ratio	P-value[a]
BC1F2	48	34	14	3:1	0.5050
BC1F2	30	22	8	3:1	0.8330
BC1F2	294	220	74	3:1	0.9463

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

5.4. Summary of the Genetic Characterization

Molecular characterization of DAS-81419-2 soybean by Southern blot analyses confirmed that a single T-DNA insert containing each of the intact PTUs for the *cry1Fv3*, *cry1Ac(synpro)*, and *pat* genes from plasmid pDAB9582, were integrated into DAS-81419-2 soybean. In addition to the full-length insert, a minor (<100 bp) fragment of the *cry1Ac(synpro)* gene was identified on the 5′ end of the T-DNA insert. The inserted DNA was stably inherited across the five generations (T1, T2, T3, T4, and F2) evaluated. No transformation plasmid backbone sequences were identified in DAS-81419-2 soybean as demonstrated by Southern blot analyses using probes covering the entire region of the plasmid flanking the T-DNA insert. Moreover, the T-DNA insert displayed the expected Mendelian inheritance pattern for a single independent insert/locus in segregating generations (F2 and BC1F2).

6. Characterization of the Introduced Proteins

6.1. Cry1Ac and Cry1F

6.1.1. Cry1 Protein Mode of Action

Cry1 insecticidal crystal proteins from *Bacillus thuringiensis* are a class of structurally related delta endotoxins having three distinct structural domains. These proteins are generally toxic to a subset of lepidopteran larvae, and this class of Cry proteins has been investigated most thoroughly with regard to their mode of action. The most widely accepted hypothesis for Cry1 mode of action is the two-receptor model proposed by (Bravo *et al.*, 2004). In this model, upon ingestion, the protein is solubilized in the midgut of the insect and is processed to an active core structure by proteases located within the midgut. The processed core then binds to a cadherin protein through the interaction of specific residues on domains II and III of the protein. Cadherins are a superfamily of proteins made up of a series of 5 to 34 calcium binding repeat elements. The first report of a cadherin interacting with a Cry protein was in *Manduca sexta* L. (Lepidoptera: Sphingidae) (Vadlamudi *et al.*, 1993). The cadherin was shown to bind Cry1Ac, Cry1Aa and Cry1Ab. Cry1Aa and Cry1Ac competed with Cry1Ab binding, suggesting a common epitope within the cadherin protein. Subsequently, cadherins have been implicated in Cry1 susceptibility in a number of different insect species (Gahan *et al.*, 2001; Morin *et al.*, 2003; Coates *et al.*, 2005; Yang *et al.*, 2011). Upon binding to the cadherin receptor, structural changes in the Cry1 active core allow for further processing of the N-terminus enabling it to oligomerize to a tetramer. In addition to cadherins, receptors in the aminophosphatase-N family have been identified in several lepidopteran species while alkaline phosphatases are also believed to be involved. Thus the mode of action of Cry1 protein corresponds to proteolytic processing of the protein and sequential multiple receptor binding interactions leading to pore formation in the insect gut.

6.1.2. Identity of the Cry1Ac Protein

The Cry1Ac protein expressed in DAS-81419-2 soybean is a synthetic version of Cry1Ac1, from *B. thuringiensis* subsp. *kurstaki* strain HD73. In this synthetic version, the first 612 amino acids are comprised of the insect-active portion of Cry1Ac1; the remaining portion consists of C-terminal sequences from Cry1Ca3 (*B. thuringiensis* subsp. *aizawai* PS81I), and Cry1Ab1 (*B. thuringiensis* subsp. *Berliner* 1715). Together, the portions of Cry1Ca3 and Cry1Ab1 that comprise the chimeric C-terminal domain are approximately those removed by alkaline proteases in the lepidopteran mid-gut during formation of the active Cry1Ac core toxin. The synthetic Cry1Ac protein is comprised of 1156 amino acids and has a molecular weight of ~130.7 kDa (Figure 29). The amino acid sequence of the Cry1Ac protein expressed in DAS-81419-2 soybean is identical to that expressed in WideStrike® cotton event DAS-21023-5 (also described as 3006-210-23 expressing Cry1Ac) (Dow AgroSciences, 2003a).

```
   1 MDNNPNINECIPYNCLSNPEVEVLGGERIE 30
  31 TGYTPIDISLSLTQFLLSEFVPGAGFVLGL 60
  61 VDIIWGIFGPSQWDAFLVQIEQLINQRIEE 90
  91 FARNQAISRLEGLSNLYQIYAESFREWEAD 120
 121 PTNPALREEMRIQFNDMNSALTTAIPLFAV 150
 151 QNYQVPLLSVYVQAANLHLSVLRDVSVFGQ 180
 181 RWGFDAATINSRYNDLTRLIGNYTDYAVRW 210
 211 YNTGLERVWGPDSRDWVRYNQFRRELTLTV 240
 241 LDIVALFPNYDSRRYPIRTVSQLTREIYTN 270
 271 PVLENFDGSFRGSAQGIERSIRSPHLMDIL 300
 301 NSITIYTDAHRGYYYWSGHQIMASPVGFSG 330
 331 PEFTFPLYGTMGNAAPQQRIVAQLGQGVYR 360
 361 TLSSTLYRRPFNIGINNQQLSVLDGTEFAY 390
 391 GTSSNLPSAVYRKSGTVDSLDEIPPQNNNV 420
 421 PPRQGFSHRLSHVSMFRSGFSNSSVSIIRA 450
 451 PMFSWIHRSAEFNNIIASDSITQIPAVKGN 480
 481 FLFNGSVISGPGFTGGDLVRLNSSGNNIQN 510
 511 RGYIEVPIHFPSTSTRYRVRVRYASVTPIH 540
 541 LNVNWGNSSIFSNTVPATATSLDNLQSSDF 570
 571 GYFESANAFTSSLGNIVGVRNFSGTAGVII 600
 601 DRFEFIPVTATLEAESDLERAQKAVNALFT 630
 631 SSNQIGLKTDVTDYHIDRVSNLVECLSDEF 660
 661 CLDEKKELSEKVKHAKRLSDERNLLQDPNF 690
 691 RGINRQLDRGWRGSTDITIQGGDDVFKENY 720
 721 VTLLGTFDECYPTYLYQKIDESKLKAYTRY 750
 751 QLRGYIEDSQDLEIYLIRYNAKHETVNVPG 780
 781 TGSLWPLSAPSPIGKCAHHSHHFSLDIDVG 810
 811 CTDLNEDLGVWVIFKIKTQDGHARLGNLEF 840
 841 LEEKPLVGEALARVKRAEKKWRDKREKLEW 870
 871 ETNIVYKEAKESVDALFVNSQYDRLQADTN 900
 901 IAMIHAADKRVHSIREAYLPELSVIPGVNA 930
 931 AIFEELEGRIFTAFSLYDARNVIKNGDFNN 960
 961 GLSCWNVKGHVDVEEQNNHRSVLVVPEWEA 990
 991 EVSQEVRVCPGRGYILRVTAYKEGYGEGCV 1020
1021 TIHEIENNTDELKFSNCVEEEVYPNNTVTC 1050
1051 NDYTATQEEYEGTYTSRNRGYDGAYESNSS 1080
1081 VPADYASAYEEKAYTDGRRDNPCESNRGYG 1110
1111 DYTPLPAGYVTKELEYFPETDKVWIEIGET 1140
1141 EGTFIVDSVELLLMEE              1156
```

Figure 29. Amino acid sequence of Cry1Ac protein.

6.1.3. Biochemical Characterization of the Cry1Ac Protein

Large quantities of purified Cry1Ac protein are required to perform safety assessment studies. As it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans, 2004), the Cry1Ac protein was produced in *Pseudomonas fluorescens*. Characterization studies were performed to confirm the equivalency of the Cry1Ac protein expressed in DAS-81419-2 soybean with the *P. fluorescens*-derived Cry1Ac protein.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, glycoprotein detection, and protein sequence analysis by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF MS/MS were used to characterize the biochemical properties of the proteins. Using these methods, the Cry1Ac protein from *P. fluorescens* and DAS-81419-2 soybean were shown to be biochemically equivalent, thereby supporting the use of the microbe-derived protein in safety assessment studies.

The methods and results of the biochemical characterization of DAS-81419-2 soybean- and microbe-derived Cry1Ac proteins are described in detail in Appendix 2. Briefly, both the plant and *P. fluorescens*-derived Cry1Ac proteins showed the expected molecular weight of ~130 kDa by SDS-PAGE and were immunoreactive to Cry1Ac protein-specific polyclonal antibodies by Western blot analysis. There was no evidence of any post-translational modifications (i.e. glycosylation) of the DAS-81419-2 soybean-derived Cry1Ac protein. The amino acid sequence was confirmed by enzymatic peptide mass fingerprinting using MALDI-TOF MS and MALDI-TOF MS/MS and was shown to be as expected and was identical to the protein expressed in *P. fluorescens*. The result is consistent with those for the Cry1Ac protein expressed in WideStrike® cotton event DAS-21023-5 (also described as 3006-210-23) (Dow AgroSciences, 2003a).

6.1.4. Expression of the Cry1Ac Protein in Plant Tissues

A field expression study was conducted at ten locations in U.S. during 2011 (Appendix 5). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at V5 and V10-12 stages, and root and forage were collected at the R3 stage of development. The grain was collected at the R8 stage of development (Gaska, 2006). The soluble, extractable Cry1Ac protein was measured using a validated enzyme-linked immunosorbent assay (ELISA). Cry1Ac protein levels for all tissue types were calculated on ng/mg dry weight basis. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed in Appendix 5.

A summary of the Cry1Ac protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 7. Average expression values ranged from 0.39 ng/mg dry weight in R3 stage root to 25.44 ng/mg dry weight in V5 stage leaf tissue. No Cry1Ac protein was detected in the control (Maverick) tissues across the ten locations.

Table 7. Expression of Cry1Ac in DAS-81419-2 soybean.

Matrix	Description	Cry1Ac ng/mg Tissue Dry Weight				
		Overall Mean	Std. Dev. (n=10)	Min/Max Range	STMR[a]	HAFT[b]
V5 Leaf	DAS-81419-2	25.44	6.61	12.10 - 40.20	26.90	35.25
V10-12 Leaf	DAS-81419-2	23.16	6.17	10.70 - 37.45	22.15	34.61
Forage	DAS-81419-2	5.54	2.54	1.38 - 11.83	5.44	10.28
Root	DAS-81419-2	0.39	0.24	[0.12]* - 1.12	0.32	0.97
Grain	DAS-81419-2	1.04	0.10	0.79 - 1.40	1.04	1.20

[a] Supervised Trials Mean Residue is calculated as the median of all the individual results across different sites.
[b] Highest Average Field Trial is the maximum value of all the means across different sites.
* Expression level below LOQ

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the Cry1Ac ELISA in the tissue matrices were as follows:

	Cry1Ac (ng/mg sample dry weight)	
Matrix	LOD	LOQ
Leaf V5	0.1	0.2
Leaf V10-12	0.1	0.2
Root	0.1	0.2
Forage	0.1	0.2
Grain	0.1	0.2

6.1.5. Identity of the Cry1F Protein

The Cry1F protein expressed in DAS-81419-2 soybean is a synthetic version of Cry1F from *Bacillus thuringiensis* subsp. *aizawai* strain PS81I. In this synthetic version, the first 604 amino acids are comprised of the insect-active portion of Cry1F; the remaining portion consists of C-terminal sequences from Cry1Ca3 (*B. thuringiensis* subsp. *aizawai* PS81I), and Cry1Ab1 (*B. thuringiensis* subsp. *Berliner* 1715). Together, the portions of Cry1Ca3 and Cry1Ab1 that comprise the chimeric C-terminal domain are approximately those removed by alkaline proteases in the lepidopteran mid-gut during the formation of the active Cry1F core toxin. The synthetic Cry1F protein is comprised of 1148 amino acids and has a molecular weight of ~130.2 kDa (Figure 30). The amino acid sequence of the Cry1F protein expressed in DAS-81419-2 soybean is identical to that expressed in WideStrike® cotton event DAS-24236-5 (also described as 281-24-236) (Dow AgroSciences, 2003b).

```
   1 MENNIQNQCVPYNCLNNPEVEILNEERSTG 30
  31 RLPLDISLSLTRFLLSEFVPGVGVAFGLFD 60
  61 LIWGFITPSDWSLFLLQIEQLIEQRIETLE 90
  91 RNRAITTLRGLADSYEIYIEALREWEANPN 120
 121 NAQLREDVRIRFANTDDALITAINNFTLTS 150
 151 FEIPLLSVYVQAANLHLSLLRDAVSFGQGW 180
 181 GLDIATVNNHYNRLINLIHRYTKHCLDTYN 210
 211 QGLENLRGTNTRQWARFNQFRRDLTLTVLD 240
 241 IVALFPNYDVRTYPIQTSSQLTREIYTSSV 270
 271 IEDSPVSANIPNGFNRAEFGVRPPHLMDFM 300
 301 NSLFVTAETVRSQTVWGGHLVSSRNTAGNR 330
 331 INFPSYGVFNPGGAIWIADEDPRPFYRTLS 360
 361 DPVFVRGGFGNPHYVLGLRGVAFQQTGTNH 390
 391 TRTFRNSGTIDSLDEIPPQDNSGAPWNDYS 420
 421 HVLNHVTFVRWPGEISGSDSWRAPMFSWTH 450
 451 RSATPTNTIDPERITQIPLVKAHTLQSGTT 480
 481 VVRGPGFTGGDILRRTSGGPFAYTIVNING 510
 511 QLPQRYRARIRYASTTNLRIYVTVAGERIF 540
 541 AGQFNKTMDTGDPLTFQSFSYATINTAFTF 570
 571 PMSQSSFTVGADTFSSGNEVYIDRFELIPV 600
 601 TATLEAESDLERAQKAVNALFTSSNQIGLK 630
 631 TDVTDYHIDRVSNLVECLSDEFCLDEKKEL 660
 661 SEKVKHAKRLSDERNLLQDPNFRGINRQLD 690
 691 RGWRGSTDITIQGGDDVFKENYVTLLGTFD 720
 721 ECYPTYLYQKIDESKLKAYTRYQLRGYIED 750
 751 SQDLEIYLIRYNAKHETVNVPGTGSLWPLS 780
 781 APSPIGKCAHHSHHFSLDIDVGCTDLNEDL 810
 811 GVWVIFKIKTQDGHARLGNLEFLEEKPLVG 840
 841 EALARVKRAEKKWRDKREKLEWETNIVYKE 870
 871 AKESVDALFVNSQYDRLQADTNIAMIHAAD 900
 901 KRVHSIREAYLPELSVIPGVNAAIFEELEG 930
 931 RIFTAFSLYDARNVIKNGDFNNGLSCWNVK 960
 961 GHVDVEEQNNHRSVLVVPEWEAEVSQEVRV 990
 991 CPGRGYILRVTAYKEGYGEGCVTIHEIENN 1020
1021 TDELKFSNCVEEEVYPNNTVTCNDYTATQE 1050
1051 EYEGTYTSRNRGYDGAYESNSSVPADYASA 1080
1081 YEEKAYTDGRRDNPCESNRGYGDYTPLPAG 1110
1111 YVTKELEYFPETDKVWIEIGETEGTFIVDS 1140
1141 VELLLMEE
```

Figure 30. Amino acid sequence of Cry1F protein.

6.1.6. Biochemical Characterization of the Cry1F Protein

Large quantities of purified Cry1F protein are required to perform safety assessment studies. As it is technically infeasible to extract and purify sufficient amounts of recombinant protein from transgenic plants (Evans, 2004), the Cry1F protein was produced in *Pseudomonas fluorescens*. Characterization studies were performed to confirm the equivalency of the Cry1F protein expressed in DAS-81419-2 soybean with the *P. fluorescens*-derived Cry1F protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, glycoprotein detection, and protein sequence analysis by matrix assisted laser desorption/ionization time-of-

flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF MS/MS were used to characterize the biochemical properties of the proteins (Gao *et al.*, 2006). Using these methods, the Cry1F protein from *P. fluorescens* and DAS-81419-2 soybean were shown to be biochemically equivalent, thereby supporting the use of the microbe-derived protein in safety assessment studies.

The methods and results of the biochemical characterization of DAS-81419-2 soybean- and microbe-derived Cry1F proteins are described in detail in Appendix 3. Briefly, both the plant and *P. fluorescens*-derived Cry1F proteins showed the expected molecular weight of ~130 kDa by SDS-PAGE and were immunoreactive to Cry1F protein-specific polyclonal antibodies by Western blot analysis. There was no evidence of any post-translational modifications (i.e. glycosylation) of the DAS-81419-2 soybean-derived Cry1F protein. The amino acid sequence was confirmed by enzymatic peptide mass fingerprinting using MALDI-TOF MS and MALDI-TOF MS/MS and was shown to be as expected and was identical to the protein expressed in *P. fluorescens*. The result is consistent with those for the Cry1F protein expressed in WideStrike® cotton event DAS-24236-5 (also described as 281-24-236) (Dow AgroSciences, 2000; Gao *et al.*, 2006).

6.1.7. Expression of the Cry1F Protein in Plant Tissues

A field expression study was conducted at ten locations in U.S. during 2011 (Appendix 5). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at V5 and V10-12 stages, and root and forage were collected at the R3 stage of development. The grain was collected at the R8 stage of development (Gaska, 2006). The soluble, extractable Cry1F protein was measured using a validated enzyme-linked immunosorbent assay (ELISA). Cry1F protein levels for all tissue types were calculated on ng/mg dry weight basis. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed in Appendix 5.

A summary of the Cry1F protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 8. Average expression values ranged from 5.23 ng/mg dry weight in R3 stage root to 56.75 ng/mg dry weight in V5 stage leaf tissue. No Cry1F protein was detected in the control (Maverick) tissues across the ten locations.

Table 8. Expression of Cry1F in DAS-81419-2 soybean.

Matrix	Description	Cry1F ng/mg Tissue Dry Weight				
		Overall Mean	Std. Dev. (n=10)	Min/Max Range	STMR[a]	HAFT[b]
V5 Leaf	DAS-81419-2	56.75	15.03	24.60 - 99.50	56.30	76.05
V10-12 Leaf	DAS-81419-2	39.07	16.60	12.75 - 76.71	38.70	59.98
Forage	DAS-81419-2	20.28	11.29	5.34 - 44.62	20.64	40.23
Root	DAS-81419-2	5.23	3.74	1.09 - 16.08	4.12	14.21
Grain	DAS-81419-2	13.80	1.24	10.41 - 16.95	13.71	16.21

[a] Supervised Trials Mean Residue is calculated as the median of all the individual results across different sites.
[b] Highest Average Field Trial is the maximum value of all the means across different sites.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the Cry1F ELISA in the tissue matrices were as follows:

	Cry1F (ng/mg sample dry weight)	
Matrix	LOD	LOQ
Leaf V5	0.25	0.5
Leaf V10-12	0.25	0.5
Root	0.15	0.3
Forage	0.05	0.1
Grain	0.2	0.4

6.1.8. Food and Feed Safety Assessment of Cry1Ac and Cry1F

The food and feed safety assessment of the Cry1Ac and Cry1F proteins expressed in DAS-81419-2 soybean 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. Results of the overall safety assessments of Cry1Ac and Cry1F indicate that each protein is unlikely to cause an allergenic reaction in humans or be a toxin to humans or animals.

The donor organism, *Bacillus thuringiensis*, is a naturally occurring microorganism commonly found in soil. *B. thuringiensis* was first registered as a pesticide in the U.S. in 1961. Today there are more than 100 registered pesticide products containing *B. thuringiensis* as an active ingredient (EPA, 2011). An exemption from the requirement of tolerance for residues of *B. thuringiensis* was first established by FDA in 1960 and was republished and then amended after EPA was established.

Cry1Ac and Cry1F have a long history of safe use. The safety of the proteins has been demonstrated in sprayable Bt formulations for pest control in agriculture for over half a century (Mendelsohn *et al.*, 2003; EPA, 2011; Sanahuja *et al.*, 2011). Both proteins are expressed in events comprising Dow AgroSciences' WideStrike® cotton authorized for cultivation in the U.S. and Brazil and for food and feed use in Australia, Brazil, Canada, European Union, Japan, Korea, Mexico, New Zealand and U.S. (www.biotradestatus.com). Bt corn and Bt cotton expressing variations of either Cry1Ac or Cry1F have been cultivated for commercial use in the U.S. and other countries for more than a decade. In 1997, EPA established an exemption from the requirement of a tolerance for the plant-incorporated protectant Cry1Ac in all plants (40 CFR §174.510). Later EPA established an exemption from the requirement of a tolerance for the plant-incorporated protectant Cry1F in cotton (40 CFR §174.504) and in corn (40 CFR §174.520). The exemptions were based on safety assessments of the proteins including digestibility in simulated gastric fluid, lack of homology to known allergens and protein toxins, and lack of mammalian toxicity as demonstrated by acute oral mouse gavage studies. DAS has filed a petition with EPA for an exemption of a tolerance for Cry1F as expressed in soybean in 2012.

Allergenicity assessments of Cry1Ac and Cry1F found no evidence of allergenic potential for either protein. Both Cry1Ac and Cry1F were rapidly digested in simulated gastric fluids in less than one minute indicating that the proteins are unlikely to elicit allergenic reactions when consumed. Glycosylation analysis revealed no detectable covalently linked carbohydrates in the Cry1Ac and Cry1F proteins expressed in DAS-81419-2 soybean. Bioinformatics analysis of Cry1Ac and Cry1F amino acid sequences using an updated allergen database (Food Allergy Research and Resource Program (FARRP), www.allergenonline.org, Allergen Database Version 12, Released February, 2012) demonstrated that the proteins do not share any amino acid sequence similarities with known allergens. No significant homology was identified when either protein sequence was compared with known allergen using the search criteria of either a match of eight or more contiguous identical amino acids, or greater than 35% identity over 80 amino acid residues.

Toxicity assessments identified no potential for toxic effects in humans or animals for Cry1Ac or Cry1F. Mouse acute oral toxicity studies demonstrated that neither protein was acutely toxic or caused any adverse effects with doses up to 700 mg/kg body weight and 600 mg/kg body weight for Cry1Ac and Cry1F, respectively. BLASTp search of Cry1Ac and Cry1F amino acid sequences against an up-to-date GenBank non-redundant protein sequences (nr) database (www.ncbi.nlm.nih.gov/genbank; updated to May 18, 2012) demonstrated that neither Cry1Ac nor Cry1F shared any amino acid sequence similarities with known protein toxins.

In 2005, EPA concluded, based upon the human health assessment of Cry1Ac and Cry1F expressed in WideStrike® cotton events DAS-21023-5 (also described as 3006-210-23 expressing Cry1Ac) and DAS-24236-5 (also described as 281-24-236 expressing Cry1F), that "there is a reasonable certainty that no harm will result from exposure to Cry1F and Cry1Ac" and "data provided by Dow AgroSciences support issuance of a tolerance exemption for the *Bacillus thuringiensis* Cry1F protein and the genetic material necessary for its productin in or on cotton" (EPA, 2005). Cry1Ac and Cry1F expressed in DAS-81419-2 soybean are identical in amino acid sequence to Cry1Ac and Cry1F expressed in WideStrike® cotton events DAS-21023-5 and DAS-24236-5 (Sections 6.1.2 and 6.1.5). The Cry proteins expressed in DAS-81419-2

soybean are biochemically equivalent to Cry1Ac and Cry1F (Sections 6.1.3 and 6.1.6) that had been previously assessed in terms of allergenic and toxicity potential.

Potential dietary risk to humans and livestock from the consumption of the Cry1Ac and Cry1F proteins in food and feed derived from DAS-81419-2 soybean were evaluated by determining margins of exposure (MOE). MOE is the ratio of the No Observable Effect Level (NOEL) of Cry1Ac or Cry1F determined by the mouse acute oral toxicity study (700 mg/kg body weight for Cry1Ac, 600 mg/kg body weight for Cry1F) to estimated dietary intake of the respective Cry protein. To evaluate dietary risk for human food consumption, MOEs were determined for the overall U.S. population and for several subpopulations based on mean protein expression levels in the seed. The dietary risk assessments revealed that the MOEs for the overall U.S. population were greater than 1067073 and 68918 for Cry1Ac and Cry1F, respectively. The MOEs for infants (an age group with highest soybean intake) were greater than 268817 and 17366 for Cry1Ac and Cry1F, respectively. It is a common regulatory practice that MOE values greater than 100 are considered protective when using animal data to protect human health. Large MOE values for the overall U.S. population and for each subpopulation indicate there is no concern for Cry1Ac and Cry1F from acute dietary exposure through DAS-81419-2 soybean. The U.S. livestock dietary assessments considered several soybean commodity forms as potential animal feeds including seed, meal, hulls, aspirated grain fractions and optional forage. For the typical diet without soybean forage, the highest exposed animal in the U.S. is beef cow. For the worst-case diet with soybean forage, the highest exposed animal in the U.S. is dairy cow. Using the worst-case diet for assessments the MOEs were greater than 2941 and 596 for Cry1Ac and Cry1F, respectively, indicating an adequate margin of safety for livestock.

6.1.9. Summary of Cry1Ac and Cry1F Characterization

The Cry1Ac protein expressed in DAS-81419-2 soybean is a synthetic version of Cry1Ac1, from *B. thuringiensis* subsp. *kurstaki* strain HD73. The Cry1Ac protein is comprised of 1156 amino acids and has a molecular weight of ~130.7 kDa. Detailed biochemical characterization of the Cry1Ac protein derived from DAS-81419-2 soybean confirmed the identity of the protein. Moreover, biochemical characterization demonstrated that the DAS-81419-2 soybean-derived and *Pseudomonas*-derived Cry1Ac proteins are equivalent thereby supporting the use of *Pseudomonas*-derived Cry1Ac protein for safety assessment. Additionally, characterization of Cry1Ac protein expression in DAS-81419-2 soybean over the growing season was determined by analyzing leaf, root, whole plant, and grain tissues.

The Cry1F protein expressed in DAS-81419-2 soybean is a synthetic version of Cry1F from *B. thuringiensis* subsp. *aizawai* strain PS81I. The Cry1F protein is comprised of 1148 amino acids and has a molecular weight of ~130.2 kDa. Detailed biochemical characterization of the Cry1F protein derived from DAS-81419-2 soybean confirmed the identity of the protein. Moreover, biochemical characterization demonstrated that the DAS-81419-2 soybean-derived and *Pseudomonas*-derived Cry1F proteins are equivalent, thereby supporting the use of *Pseudomonas*-derived Cry1F protein for safety assessment. Additionally, characterization of Cry1F protein expression in DAS-81419-2 soybean over the growing season was determined by analyzing leaf, root, whole plant, and grain tissues.

A step-wise, weight-of-evidence approach was used to assess the potential for toxic or allergenic effects from the Cry1Ac and Cry1F proteins. Bioinformatic analyses revealed no meaningful

homologies to known or putative allergens or toxins for the Cry1Ac and Cry1F amino acid sequence. Both Cry1Ac and Cry1F hydrolyzed rapidly in simulated gastric fluid and glycosylation analysis revealed no detectable covalently linked carbohydrates in Cry1Ac and Cry1F. Additionaly, neither protein caused adverse effects in mouse acute oral toxicity studies. Therefore, the low level Cry1Ac and Cry1F content in DAS-81419-2 soybean relative to total plant proteins presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the Cry1Ac and Cry1F proteins indicate that the proteins are unlikely to cause allergenic or toxic effects in humans or animals.

6.2. PAT

6.2.1. Mode of Action of the PAT Protein

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

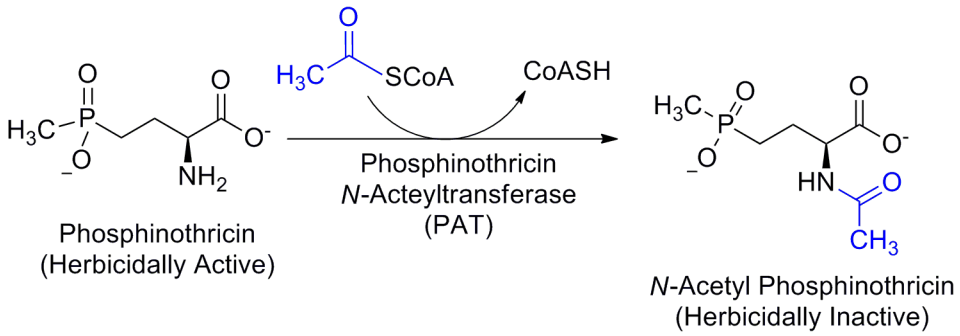


Figure 31. Mode of action of the PAT protein.

6.2.2. Identity of the PAT Protein

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium (Strauch *et al.*, 1988; OECD, 1999). The *pat* transgene in DAS-81419-2 encodes a protein sequence that is identical to the native PAT protein (UniProt Accession Number: Q57146). PAT is comprised of 183 amino acids and has a molecular weight of ~20.6 kDa (Figure 32).

```
  1 MSPERRPVEIRPATAADMAAVCDIVNHYIE 30
 31 TSTVNFRTEPQTPQEWIDDLERLQDRYPWL 60
 61 VAEVEGVVAGIAYAGPWKARNAYDWTVEST 90
 91 VYVSHRHQRLGLGSTLYTHLLKSMEAQGFK 120
121 SVVAVIGLPNDPSVRLHEALGYTARGTLRA 150
151 AGYKHGGWHDVGFWQRDFELPAPPRPVRPV 180
181 TQI
```

Figure 32. Amino acid sequence of the PAT protein.

6.2.3. Biochemical Characterization of the PAT Protein

Characterization of the biochemical properties of the DAS-81419-2 soybean-derived PAT protein was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE), Western blot analysis, protein sequence alignment, and a lateral flow strip assay. The methods and results are described in Appendix 4. Using these methods, the PAT protein produced in DAS-81419-2 soybean was shown to be substantially equivalent to that produced in *Escherichia coli.* Furthermore, the sequence of the PAT protein is identical to the PAT protein expressed in other deregulated transgenic crops (USDA, 1996, 2001, 2004, 2005).

6.2.4. Expression of the PAT Protein in Plant Tissues

A field expression study was conducted at ten locations in U.S. during 2011 (Appendix 5). The test sites represented regions of diverse agronomic practices and environmental conditions for soybean in North America. Plant tissues sampled included leaf, grain, root, and forage. Leaf tissues were collected at V5 and V10-12 stages, and root and forage were collected at the R3 stage of development. The grain was collected at the R8 stage of development (Gaska, 2006). The soluble, extractable PAT protein was measured using a validated enzyme-linked immunosorbent assay (ELISA) method. PAT protein levels for all tissue types were calculated on ng/mg dry weight basis. Methods used for tissue sampling and quantification of protein expression by ELISA are detailed in Appendix 5.

A summary of the PAT protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 9. Average expression values ranged from 0.63 ng/mg dry weight in R3 stage root to 5.60 ng/mg dry weight in V10-12 stage leaf tissue. No PAT protein was detected in the control (Maverick) tissues across the ten locations.

Table 9. Expression of PAT in DAS-81419-2 soybean.

		PAT ng/mg Tissue Dry Weight				
Matrix	Description	Overall Mean	Std. Dev.	Min/Max Range	STMR[a]	HAFT[b]
V5 Leaf	DAS-81419-2	5.23	0.88	3.25 - 7.35	5.30	6.93
V10-12 Leaf	DAS-81419-2	5.60	1.14	2.55 - 7.56	5.76	7.32
Forage	DAS-81419-2	4.06	1.30	1.24 - 6.12	4.02	5.69
Root	DAS-81419-2	0.63	0.12	0.44 - 1.05	0.63	0.85
Grain	DAS-81419-2	0.86	0.13	0.63 - 1.12	0.83	1.06

[a] Supervised Trials Mean Residue is calculated as the median of all the individual results across different sites.
[b] Highest Average Field Trial is the maximum value of all the means across different sites.

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

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

	PAT (ng/mg sample dry weight)	
Matrix	LOD	LOQ
Root	0.06	0.12
Forage	0.06	0.12
Grain	0.06	0.12

6.2.5. Food and Feed Safety Assessment for PAT Protein

The PAT protein expressed in DAS-81419-2 soybean originates from a common soil bacterium *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988). The protein is 100% identical in amino acid sequence to PAT expressed in other transgenic crops that have been previously deregulated by USDA (USDA, 1996, 2001, 2004, 2005). The food and feed safety of PAT was assessed in these products and in published findings (OECD, 1999; Herouet *et al.*, 2005) and shown to present no significant food or feed safety risk. The PAT protein is hydrolyzed rapidly in simulated gastric fluid and there was no evidence of acute toxicity in mice at a dose of 5000 mg/kg body weight of PAT protein (OECD, 1999). Additionally, updated bioinformatic analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence.

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

The dietary risk assessments showed large margins of exposure for the overall U.S. populations as well as for infants (age group with the highest soybean consumption). The analyses also revealed large margins of exposure for poultry and livestock. Large margins of exposure indicate lack of meaningful risks to humans and animals from dietary exposure.

6.2.6. Summary of PAT Protein Characterization

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium. PAT is comprised of 183 amino acids and has a molecular weight of ~20.6 kDa. The PAT protein, as expressed in DAS-81419-2, has the same amino acid sequence as the PAT expressed in several other transgenic events. Western blot analysis and lateral flow strip assays demonstrated that the PAT protein expressed in DAS-81419-2 soybean had the expected molecular weight and immunoreactivity. Characterization of PAT protein expression in DAS-81419-2 soybean was determined by analyzing leaf, root, whole plant, and grain tissues over the growing season. The PAT protein has a long history of safe use and the food and feed safety of PAT has previously been assessed in other products and in published findings. The low level expression of the PAT protein in DAS-81419-2 soybean presents a low exposure risk to humans and animals, and the results of the overall safety assessment of the PAT protein indicate that it is unlikely to cause allergenic or toxic effects in humans or animals.

7. Grain and Forage Composition

Field trials with DAS-81419-2 soybean, the non-transgenic control (Maverick), and reference lines were conducted in 2011 at ten sites located in the U.S. This study used the same plots that were used for protein expression (Section 6) and agronomic characterization (Section 8) studies. No biologically meaningful unintended compositional differences were observed between the non-transgenic near-isogenic control (Maverick) and DAS-81419-2 soybean. Results from this study demonstrate compositional equivalence between event DAS-81419-2 soybean and non-transgenic soybean.

7.1. Field Study Design and Data Analysis

A crop composition study with DAS-81419-2 soybean, a near-isogenic non-transgenic control (Maverick) and six non-transgenic reference lines (IL 3503, Porter 75148, DSR 75213-72, Pioneer 93M62, HiSOY 38C60, Williams 82) was conducted in 2011 at ten sites located in Richland, Iowa; Atlantic, Iowa; Carlyle, Illinois; Wyoming, Illinois; Frankfort, Indiana; Fisk, Missouri; La Plata, Missouri; York, Nebraska; Brunswick, Nebraska; and Germansville, Pennsylvania.

Each trial site included event DAS-81419-2, the non-transgenic near-isogenic control (Maverick), and three non-transgenic reference lines. At each of the ten sites, all entries were arranged in a randomized complete block design with four blocks. Across all sites, each control (Maverick) and DAS-81419-2 entry was represented by a total of 40 plots (10 sites, 4 replicate plots per entry at each site). Three of the six reference lines were included at each site by randomizing across sites in a balanced incomplete-block design. Each of the six reference lines was assigned to five sites; therefore, each reference line was represented by a total of 20 plots across sites (5 sites per reference line, 4 replicate plots per entry at each site).

At each site, four replicate plots of each entry were established, with each plot consisting of four 25 ft (7.62 m) rows. Soybean seeds were planted at a seeding rate of approximately 125 seeds per row with seed spacing within each row of approximately 2.4 inches (6 cm). Each soybean plot was bordered by two rows of a non-transgenic soybean cultivar of similar maturity. The entire trial site was surrounded by a minimum of four rows (10 ft or 3.0 m) of a non-transgenic soybean cultivar of similar maturity. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop. Soybean forage samples used for composition analysis were composed of the above-ground portion of three whole plants that collected from the center two rows of each four row plot at the R3 growth stage. Soybean seed samples used for composition analysis were composed of a representative 500 g sample of the soybean seed harvested from the center two rows of each four row plot.

Composition Analysis

Samples of soybean forage and seed were analyzed for nutrient and antinutrient content (Appendix 6). The analytes examined are presented in Table 10.

Table 10. Composition analytes.

Matrix	Category	Analyte
Forage	Proximate	Protein
Forage	Proximate	Fat
Forage	Proximate	Ash
Forage	Proximate	Moisture
Forage	Proximate	Carbohydrates
Forage	Fiber	ADF
Forage	Fiber	NDF
Forage	Mineral	Calcium
Forage	Mineral	Phosphorus
Grain	Proximate	Protein
Grain	Proximate	Fat
Grain	Proximate	Ash
Grain	Proximate	Moisture
Grain	Proximate	Carbohydrates
Grain	Fiber	ADF
Grain	Fiber	NDF
Grain	Fiber	Total Dietary Fiber
Grain	Mineral	Calcium
Grain	Mineral	Copper
Grain	Mineral	Iron
Grain	Mineral	Magnesium
Grain	Mineral	Manganese
Grain	Mineral	Phosphorus
Grain	Mineral	Potassium
Grain	Mineral	Selenium
Grain	Mineral	Sodium
Grain	Mineral	Zinc
Grain	Amino Acid	Alanine
Grain	Amino Acid	Arginine
Grain	Amino Acid	Aspartic Acid
Grain	Amino Acid	Cystine
Grain	Amino Acid	Glutamic Acid
Grain	Amino Acid	Glycine
Grain	Amino Acid	Histidine
Grain	Amino Acid	Isoleucine
Grain	Amino Acid	Leucine
Grain	Amino Acid	Lysine
Grain	Amino Acid	Methionine
Grain	Amino Acid	Phenylalanine
Grain	Amino Acid	Proline
Grain	Amino Acid	Serine
Grain	Amino Acid	Threonine
Grain	Amino Acid	Tryptophan
Grain	Amino Acid	Tyrosine
Grain	Amino Acid	Valine
Grain	Fatty Acid	8:0 Caprylic
Grain	Fatty Acid	10:0 Capric
Grain	Fatty Acid	12:0 Lauric
Grain	Fatty Acid	14:0 Myristic
Grain	Fatty Acid	14:1 Myristoleic
Grain	Fatty Acid	15:0 Pentadecanoic
Grain	Fatty Acid	15:1 Pentadecenoic
Grain	Fatty Acid	16:0 Palmitic
Grain	Fatty Acid	16:1 Palmitoleic
Grain	Fatty Acid	17:0 Heptadecanoic
Grain	Fatty Acid	17:1 Heptadecenoic
Grain	Fatty Acid	18:0 Stearic
Grain	Fatty Acid	18:1 Oleic
Grain	Fatty Acid	18:2 Linoleic
Grain	Fatty Acid	18:3 Linolenic
Grain	Fatty Acid	18:3 γ-Linolenic
Grain	Fatty Acid	20:0 Arachidic
Grain	Fatty Acid	20:1 Eicosenoic
Grain	Fatty Acid	20:2 Eicosadienoic
Grain	Fatty Acid	20:3 Eicosatrienoic
Grain	Fatty Acid	20:4 Arachidonic
Grain	Fatty Acid	22:0 Behenic
Grain	Vitamin	Vitamin A (Beta Carotene)
Grain	Vitamin	Vitamin B1 (Thiamine HCl)
Grain	Vitamin	Vitamin B2 (Riboflavin)
Grain	Vitamin	Vitamin B3 (Niacin)
Grain	Vitamin	Vitamin B5 (Pantothenic Acid)
Grain	Vitamin	Vitamin B6 (Pyridoxine HCl)
Grain	Vitamin	Vitamin B9 (Folic Acid)
Grain	Vitamin	Vitamin C (Ascorbic Acid)
Grain	Vitamin	α-tocopherol (Vitamin E)
Grain	Vitamin	β-tocopherol
Grain	Vitamin	γ-tocopherol
Grain	Vitamin	δ-tocopherol
Grain	Vitamin	Total tocopherol
Grain	Bioactive	Lectin
Grain	Bioactive	Phytic Acid
Grain	Bioactive	Raffinose
Grain	Bioactive	Stachyose
Grain	Bioactive	Trypsin Inhibitor
Grain	Bioactive	Total Daidzein Equivalent
Grain	Bioactive	Total Genistein Equivalent
Grain	Bioactive	Total Glycitein Equivalent

The results of the compositional analysis for soybean forage and seed were compared with values reported in the literature (Kakade *et al.*, 1972; Iskander, 1987; Hartwig and Kilen, 1991; Padgette *et al.*, 1996; Taylor *et al.*, 1999; OECD, 2001; McCann *et al.*, 2005; Harrigan *et al.*, 2007; Bilyeu *et al.*, 2008; Lundry *et al.*, 2008; Berman *et al.*, 2009, 2010; ILSI, 2010; Berman *et al.*, 2011; Zhou *et al.*, 2011). A summary of the compositional data from the literature can be found in Appendix 7.

Statistical Analysis

Analysis of variance was conducted across field sites (combined-site analysis) for composition data using a mixed model (SAS Institute Inc., 2009). Entry was considered a fixed effect, and location, block within location, and location-by-entry, were designated as random effects. Significant differences were declared at the 95% confidence level. Data were not rounded off for statistical analysis. The significance of an overall treatment effect was estimated using an F-test. Comparisons were made between DAS-81419-2 and the control entry using t-tests.

Due to the large number of comparisons made in this study, multiplicity was an issue. Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise P-values is very high ($1-0.95^{\text{number of comparisons}}$). In this study there were 71 analytes analyzed for composition; therefore, 71 comparisons were made in the combined-site composition analysis. As a result, the probability of declaring one or more false differences based on unadjusted P-values was 97.38% ($1-0.95^{71}$).

One method to account for multiplicity is to adjust P-values to control the experiment-wise error rate, but when many comparisons are made in a study, the power for detecting specific effects can be reduced significantly. An alternative with much greater power is to adjust P-values to control the probability that each declared difference is significant (Curran-Everett, 2000). This can be accomplished using a False Discovery Rate (FDR) control procedure (Benjamini and Hochberg, 1995); FDR methods are commonly applied in studies examining transgenic crops (Herman *et al.*, 2007; Coll *et al.*, 2008; Huls *et al.*, 2008; Jacobs *et al.*, 2008; Stein *et al.*, 2009; Herman *et al.*, 2010). Therefore, the P-values from the composition contrasts were each adjusted using the FDR method to improve discrimination of true differences among treatments from random effects (false positives). Differences were considered significant if the FDR-adjusted P-value was less than 0.05.

7.2. Composition Analysis Results

An across-site summary and statistical analysis of composition data from the non-transgenic near-isogenic control (Maverick) and DAS-81419-2 soybean is found in Table 11 to Table 17 and Figure 33 to Figure 39. For each analyte and entry, the least-square mean, standard error, and minimum and maximum sample value are reported. Also for comparison, the minimum and maximum values for the six reference lines and literature range are reported (values are for individual plot results except where noted). Arithmetic means from each field site are plotted in figures and literature ranges are shaded (literature ranges reported as not detected or <LOD are plotted as zeros).

7.2.1. Proximate, Fiber, and Mineral Analysis of Forage

Soybean forage samples from the non-transgenic control (Maverick), DAS-81419-2, and reference variety entries were analyzed for proximates, fiber, and minerals (nine analytes). A summary of the results across all locations is presented in Table 11 and Figure 33. No statistical differences were observed in the combined-site analysis between the control and DAS-81419-2 entries for all proximate, fiber, and mineral analytes tested. Additionally, all mean values were within literature ranges (when available) and within ranges for reference varieties included in the study.

Table 11. Summary of the proximate, fiber, and mineral analysis of soybean forage from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Proximate					
Protein	0.786	20.4 ± 0.8	20.6 ± 0.8	14.0 - 35.5	11.2 - 24.71
		13.9 - 29.8	15.8 - 25.8		
(% DW)			(0.786, 0.828)		
Fat	0.274	2.49 ± 0.19	2.70 ± 0.20	0.685 - 5.32	1.01 - 9.87
		0.898 - 3.89	0.857 - 4.32		
(% DW)			(0.274, 0.589)		
Ash	0.926	8.96 ± 0.31	8.99 ± 0.32	6.80 - 15.2	4.68 - 10.782
		7.01 - 16.9	7.51 - 10.9		
(% DW)			(0.926, 0.931)		
Moisture	0.578	79.7 ± 0.8	79.4 ± 0.8	75.3 - 86.6	32.05 - 84.60
		75.8 - 84.3	75.5 - 83.6		
(% FW)			(0.578, 0.719)		
Carbohydrates[e]	0.510	68.2 ± 1.1	67.7 ± 1.1	50.1 - 75.6	59.8 - 80.18
		57.2 - 76.4	60.3 - 75.7		
(% DW)			(0.510, 0.696)		
Fiber					
Acid Detergent Fiber (ADF)	0.310	34.7 ± 2.1	33.3 ± 2.1	19.4 - 64.1	22.72 - 59.03
		22.4 - 56.7	22.2 - 45.4		
(% DW)			(0.310, 0.595)		
Neutral Detergent Fiber (NDF)	0.300	41.6 ± 2.6	39.9 ± 2.6	25.2 - 82.0	19.61 - 73.05
		27.8 - 70.9	27.2 - 59.3		
(% DW)			(0.300, 0.592)		
Mineral					
Calcium	0.333	1378 ± 64	1401 ± 64	874 - 2000	NR
		940 - 1840	908 - 1740		
(mg/100g dry wt.)			(0.333, 0.615)		
Phosphorus	0.793	266 ± 7	268 ± 7	187 - 381	NR
		206 - 327	201 - 342		
(mg/100g dry wt.)			(0.793, 0.828)		

Abbreviations: NR = Not Reported.

[a] Unit of measure was not converted prior to analysis, except for calcium and phosphorus. Unit of measure for calcium and phosphorus was converted from % DW to mg/100g dry wt. prior to analysis.

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

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

[d] Combined range from Appendix 7.

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

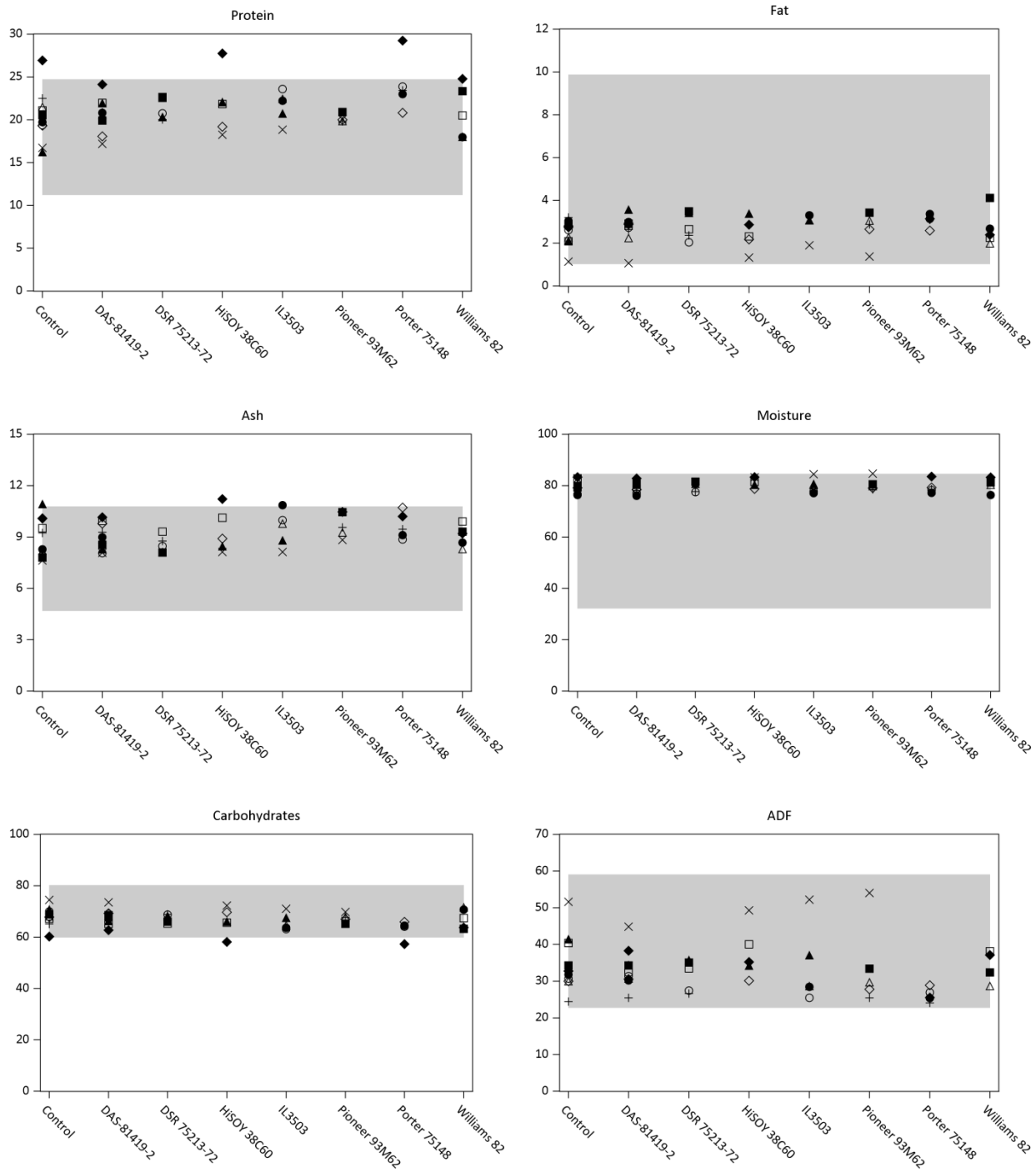


Figure 33. Proximates, fiber and minerals in control, DAS-81419-2 and reference variety soybean forage.

moisture = % fresh weight, calcium and phosphorous = mg/100g dry wt., all others = % dry weight

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

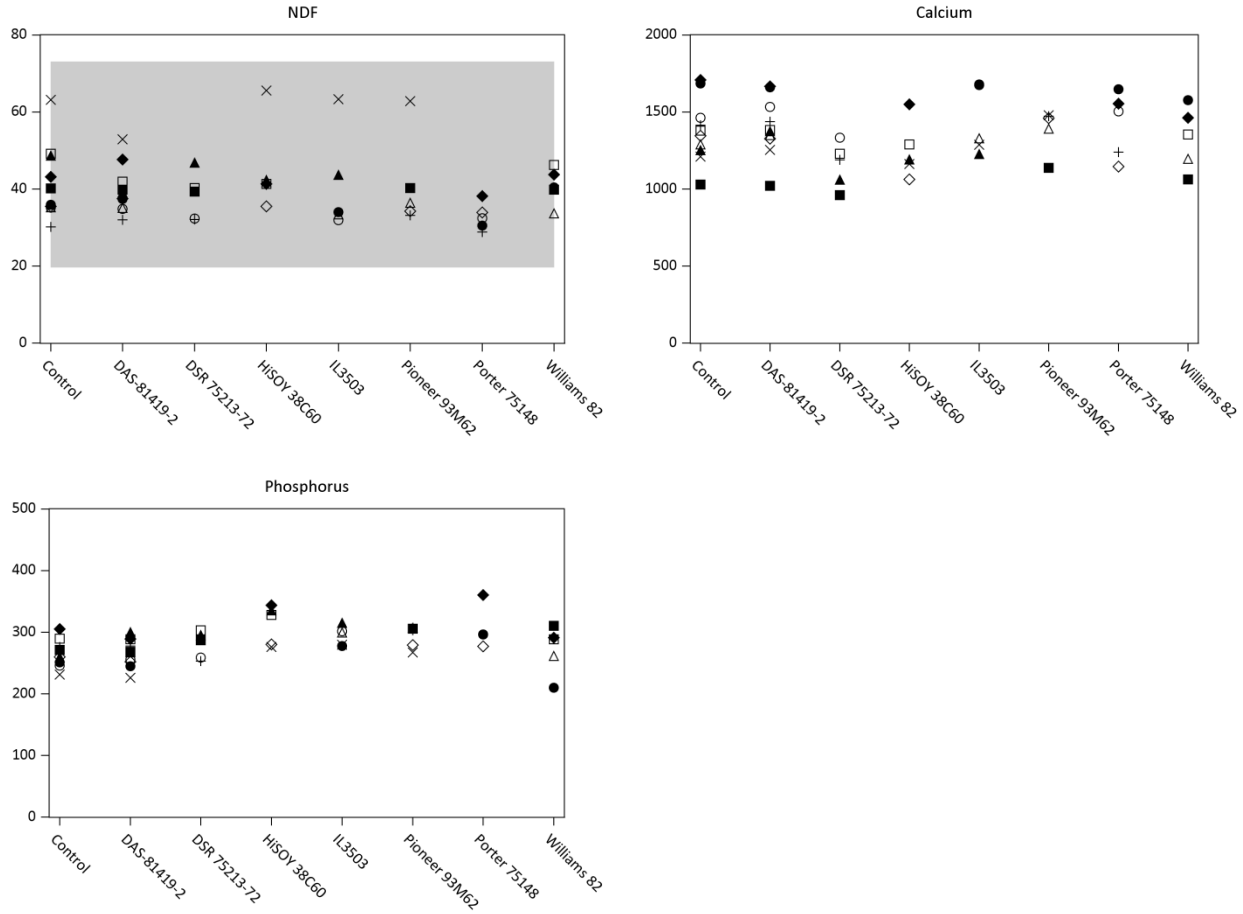


Figure 33 (Cont). Proximates, fiber and minerals in control, DAS-81419-2 and reference variety soybean forage.

moisture = % fresh weight, calcium and phosphorous = mg/100g dry wt., all others = % dry weight

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range (when available) is shaded for each analyte.

7.2.2. Proximate and Fiber Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for proximates and fiber (eight analytes). A summary of the results across all locations is presented in Table 12 and Figure 34. All mean results were within literature ranges (when available) and within ranges for reference varieties included in the study. Statistically significant overall treatment effects were found for fat, ash, and moisture, and pair-wise comparisons between the control and DAS-81419-2 were also significant for those analytes based upon unadjusted P-values (but not after adjustment for multiplicity using FDR methods). However, these differences were small relative to natural variation and not biologically meaningful, as all results were within literature ranges and within the ranges of the reference varieties included in this study.

Table 12. Summary of the proximate and fiber analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Proximate					
Protein	0.442	37.9 ± 0.6 34.3 - 41.9	38.1 ± 0.6 32.9 - 42.8 (0.442, 0.662)	34.4 - 46.0	32 - 48.4
(% DW)					
Fat	**0.008**	18.2 ± 0.5 15.4 - 21.5	17.7 ± 0.5 14.2 - 21.0 (**0.008**, 0.102)	14.1 - 22.7	8.104 - 24.7
(% DW)					
Ash	**0.026**	5.06 ± 0.07 4.62 - 5.68	5.18 ± 0.07 4.57 - 6.05 (**0.026**, 0.233)	3.79 - 6.79	3.885 - 6.994
(% DW)					
Moisture	**0.019**	12.3 ± 0.9 8.29 - 19.2	11.7 ± 0.9 7.56 - 17.9 (**0.019**, 0.196)	7.91 - 22.7	4.7 - 34.4
(% FW)					
Carbohydrates[e]	0.372	38.8 ± 0.7 33.6 - 41.2	39.0 ± 0.7 33.3 - 43.3 (0.372, 0.643)	29.9 - 40.8	29.3 - 50.2
(% DW)					
Fiber					
Acid Detergent Fiber (ADF)	0.813	15.3 ± 0.7 10.6 - 22.8	15.2 ± 0.7 10.5 - 22.5 (0.813, 0.837)	10.2 - 21.0	7.81 - 26.26
(% DW)					
Neutral Detergent Fiber (NDF)	0.576	17.5 ± 0.8 11.7 - 24.2	17.7 ± 0.8 11.6 - 25.5 (0.576, 0.719)	10.6 - 22.6	8.53 - 23.90
(% DW)					
Total Dietary Fiber	0.689	23.8 ± 0.9 17.6 - 29.1	24 .0 ± 0.9 17.4 - 31.3 (0.689, 0.765)	16.1 - 29.5	NR
(% DW)					

Abbreviations: NR = Not Reported.

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

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

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

[d] Combined range from Appendix 7.

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

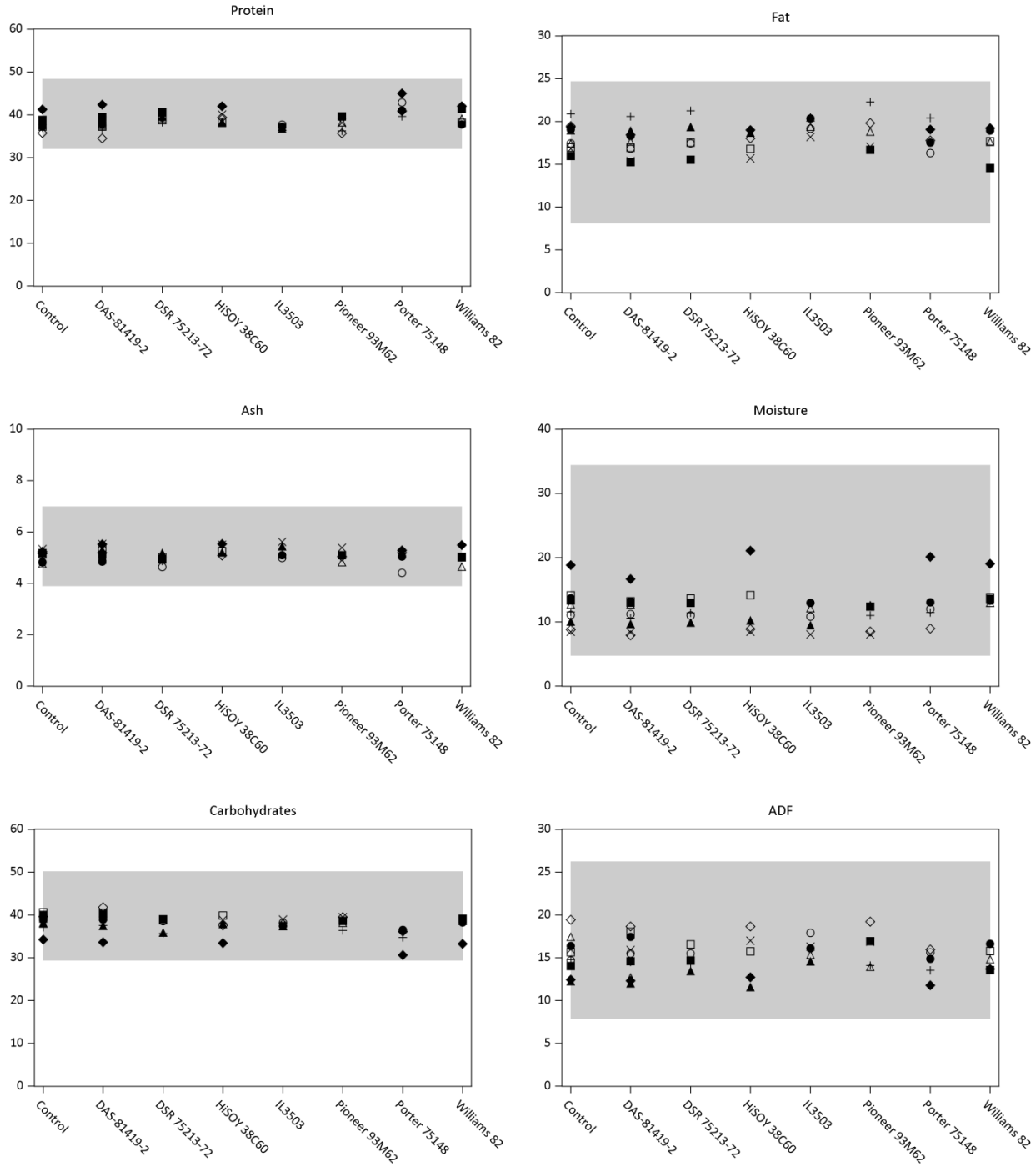


Figure 34. Proximates and fiber in control, DAS-81419-2, and reference variety soybean seed.

moisture = % fresh weight, all others = % dry weight

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

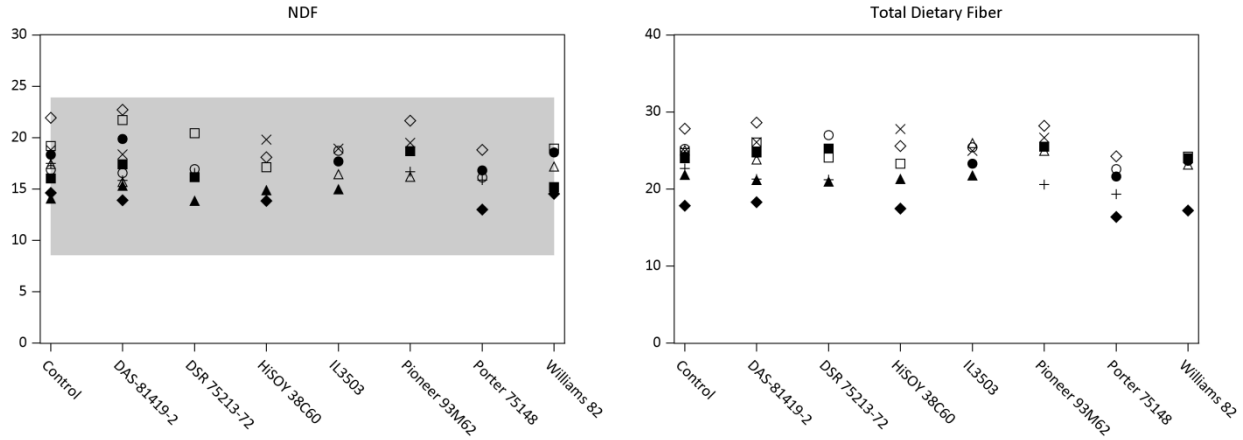


Figure 34 (Cont). Proximates and fiber in control, DAS-81419-2, and reference variety soybean seed.

moisture = % fresh weight, all others = % dry weight

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range (when available) is shaded for each analyte.

7.2.3. Mineral Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for mineral content (10 analytes). A summary of the results across all locations is presented in Table 13 and Figure 35. For sodium, statistical analysis was not performed because more than 50% of the samples were found to be below the LOQ. All mean results were within literature ranges (when available) and/or within ranges for reference varieties included in the study. Additionally, no statistical differences were observed in the combined-site analysis between the control and DAS-81419-2 entries for all mineral analytes.

Table 13. Summary of the mineral analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Calcium	0.137	270 ± 8 233 - 335	267 ± 8 205 - 328 (0.137, 0.456)	181 - 339	116.55 - 510
(mg/100 g DW)					
Copper	0.655	1.32 ± 0.06 0.922 - 1.64	1.33 ± 0.06 0.894 - 1.72 (0.655, 0.747)	0.693 - 1.86	0.632 - 1.092
(mg/100 g DW)					
Iron	0.500	9.56 ± 1.40 6.61 - 27.3	10.26 ± 1.40 6.21 - 42.9 (0.500, 0.696)	6.33 - 151	3.734 - 10.954
(mg/100 g DW)					
Magnesium	0.663	233 ± 4 204 - 256	232 ± 4 197 - 257 (0.663, 0.747)	205 - 278	219.40 - 312.84
(mg/100 g DW)					
Manganese	0.548	2.64 ± 0.09 2.13 - 3.09	2.67 ± 0.09 2.01 - 3.96 (0.548, 0.719)	2.22 - 7.18	2.52 - 3.876
(mg/100 g DW)					
Phosphorus	0.118	607 ± 14 536 - 704	619 ± 14 494 - 708 (0.118, 0.456)	471 - 759	506.74 - 935.24
(mg/100 g DW)					
Potassium	0.168	1799 ± 21 1660 - 1940	1819 ± 21 1490 - 1980 (0.168, 0.459)	1650 - 2050	1868.01 - 2510
(mg/100 g DW)					
Selenium	0.401	468 ± 188 <LOQ - 2370	507 ± 187 <LOQ - 2560 (0.401, 0.647)	<LOQ - 3060	NR
(ppb DW)					
Sodium	NA	NA <LOQ	NA <LOQ	<LOQ - 22.2	4.05 - 30
(mg/100 g DW)					
Zinc	0.066	4.53 ± 0.15 3.66 - 5.73	4.63 ± 0.15 3.66 - 5.83 (0.066, 0.381)	3.12 - 6.33	4.98 - 7.578
(mg/100 g DW)					

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

NR = Not Reported

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

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

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

[d] Combined range from Appendix 7.

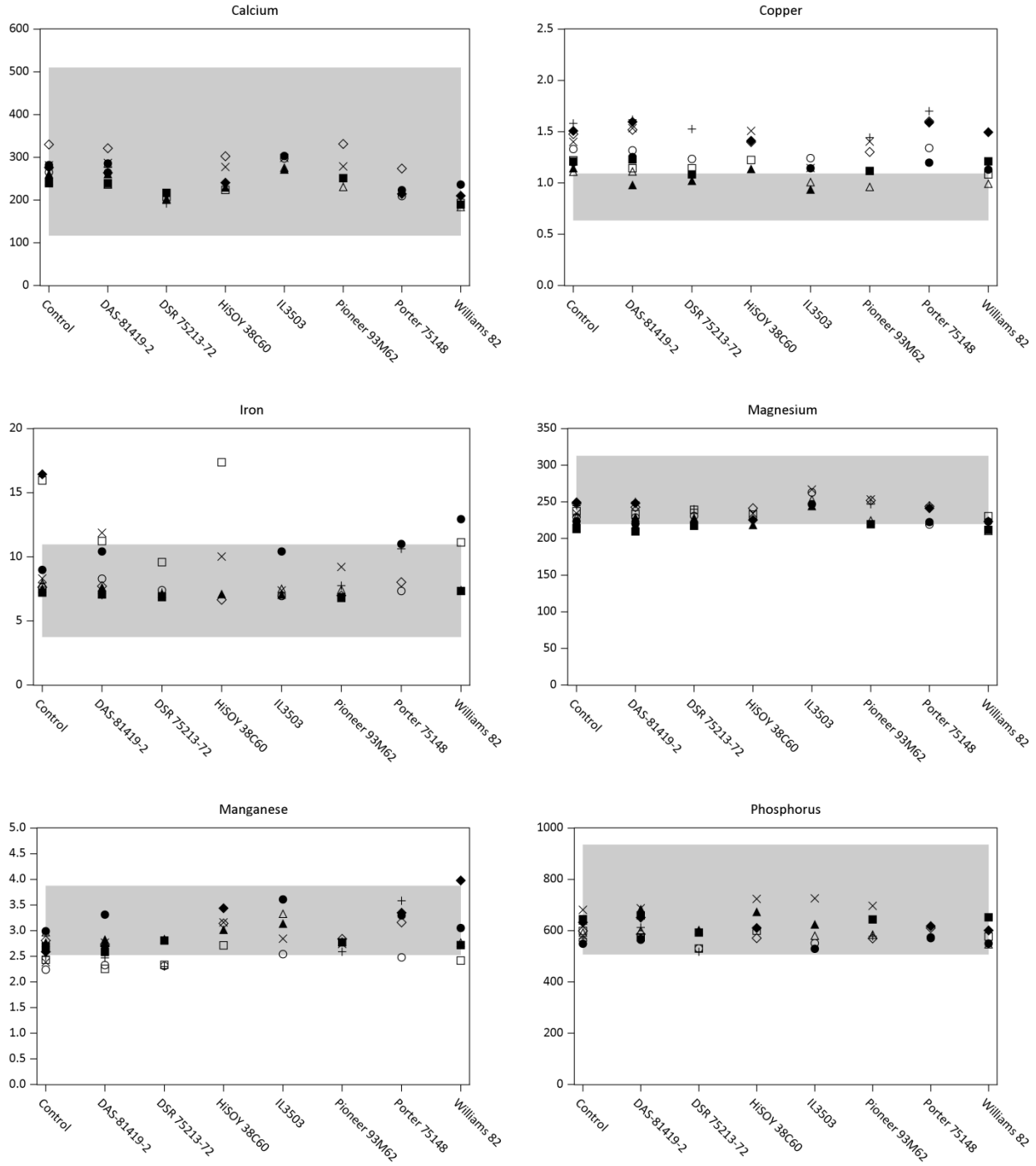


Figure 35. Minerals in control, DAS-81419-2, and reference variety soybean seed.
selenium = ppb dry weight, all others = mg/100 g dry weight
Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

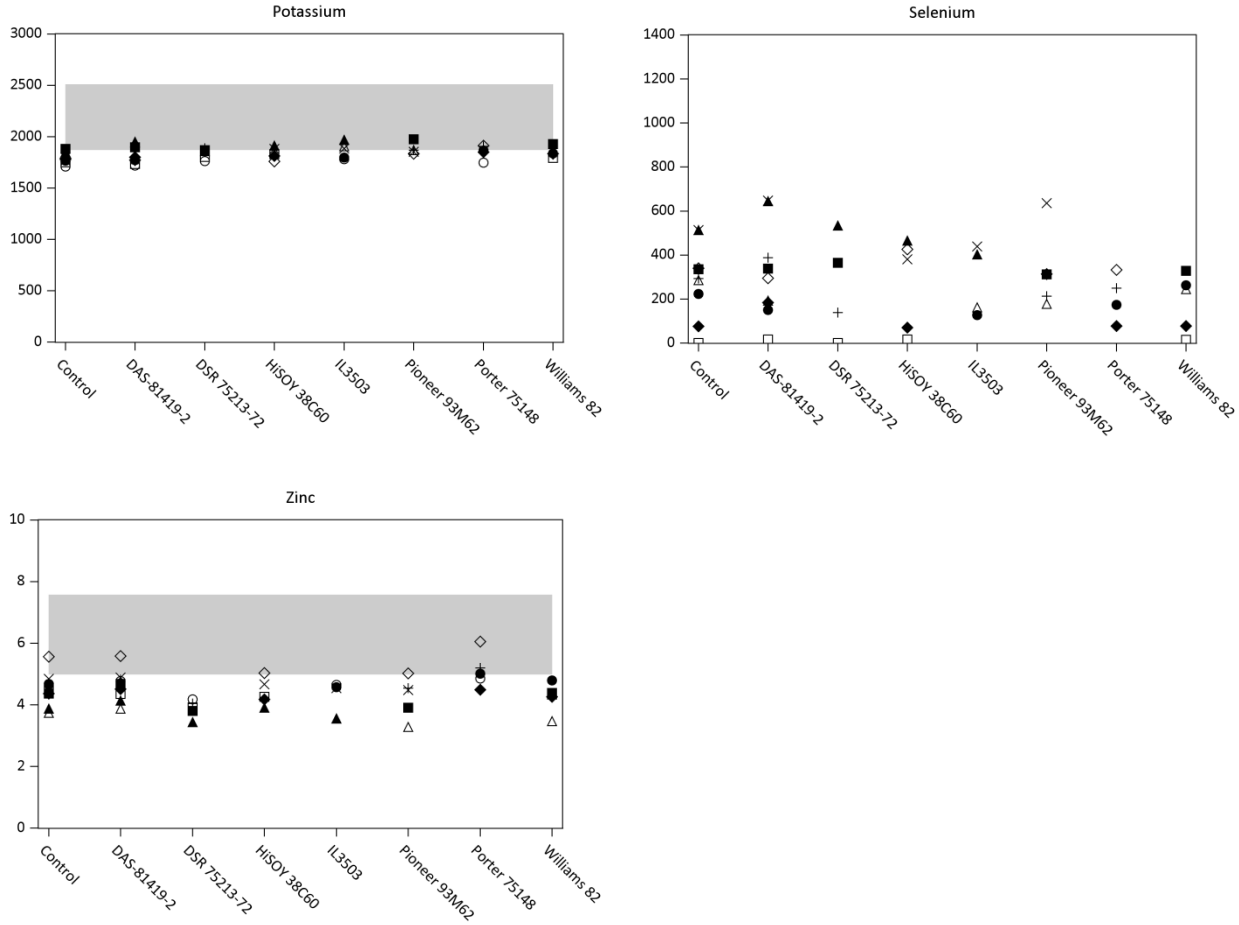


Figure 35 (Cont). Minerals in control, DAS-81419-2, and reference variety soybean seed.
selenium = ppb dry weight, all others = mg/100 g dry weight
Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range (when available) is shaded for each analyte.

7.2.4. Amino Acid Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for the content of 18 amino acids. A summary of the results across all locations is presented in Table 14 and Figure 36. All mean results were within literature ranges (when available) and/or within ranges for reference varieties included in the study. With the exception of phenylalanine, no statistical differences were observed in the combined-site analysis between the control and DAS-81419-2 entries for amino acid content. The overall treatment effect and pair-wise comparison between the control and DAS-81419-2 were significant for phenylalanine based on unadjusted P-values (but not after adjustment for multiplicity using FDR methods). However, these differences were small relative to natural variation and not biologically meaningful, as all results were within literature ranges and within the range of the reference varieties included in this study.

Table 14. Summary of the amino acid analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Alanine	0.212	4.57 ± 0.03 4.31 - 4.76	4.59 ± 0.03 4.30 - 4.82	4.28 - 4.75	4.16 - 4.74
(% total amino acid)			(0.212, 0.520)		
Arginine	0.111	7.49 ± 0.05 7.04 - 7.79	7.44 ± 0.05 7.05 - 7.74	7.22 - 8.20	6.41 - 8.41
(% total amino acid)			(0.111, 0.456)		
Aspartic Acid	0.099	11.51 ± 0.01 11.29 - 11.64	11.48 ± 0.01 11.30 - 11.76	9.99 - 11.74	11.37 - 12.68
(% total amino acid)			(0.099, 0.440)		
Cystine	0.283	1.610 ± 0.028 1.432 - 1.946	1.629 ± 0.028 1.448 - 1.861	1.240 - 1.792	1.02 - 1.87
(% total amino acid)			(0.283, 0.591)		
Glutamic Acid	0.161	17.32 ± 0.11 16.29 - 18.01	17.23 ± 0.11 16.28 - 17.98	17.04 - 18.56	17.71 - 20.48
(% total amino acid)			(0.161, 0.456)		
Glycine	0.745	4.50 ± 0.02 4.34 - 4.67	4.50 ± 0.02 4.30 - 4.66	4.14 - 4.54	4.19 - 4.62
(% total amino acid)			(0.745, 0.802)		
Histidine	0.567	2.714 ± 0.016 2.583 - 2.818	2.703 ± 0.016 2.165 - 2.836	2.434 - 2.776	2.49 - 2.89
(% total amino acid)			(0.567, 0.719)		
Isoleucine	0.931	4.80 ± 0.02 4.65 - 4.96	4.80 ± 0.02 4.52 - 4.99	4.61 - 4.99	4.13 - 5.11
(% total amino acid)			(0.931, 0.931)		
Leucine	0.605	7.65 ± 0.01 7.51 - 7.83	7.64 ± 0.01 7.49 - 7.75	7.45 - 7.98	7.46 - 8.29
(% total amino acid)			(0.605, 0.728)		

[a] Unit of measure was converted from % DW to % total amino acid prior to analysis.

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

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

[d] Combined range from Appendix 7.

Table 14 (Cont). Summary of the amino acid analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Lysine		6.32 ± 0.08	6.44 ± 0.08		
	0.151	5.90 - 7.51	5.92 - 7.41	5.61 - 7.29	6.23 - 7.38
(% total amino acid)			(0.151, 0.456)		
Methionine		1.419 ± 0.011	1.435 ± 0.011		
	0.211	1.305 - 1.608	1.325 - 1.607	1.223 - 1.618	1.18 - 1.71
(% total amino acid)			(0.211, 0.520)		
Phenylalanine		5.15 ± 0.01	5.11 ± 0.01		
	0.009	4.99 - 5.25	4.92 - 5.22	4.88 - 5.37	4.91 - 5.44
(% total amino acid)			(**0.009**, 0.102)		
Proline		5.14 ± 0.04	5.20 ± 0.04		
	0.240	4.86 - 5.97	4.91 - 5.73	4.80 - 6.02	4.75 - 5.62
(% total amino acid)			(0.240, 0.550)		
Serine		5.13 ± 0.02	5.16 ± 0.02		
	0.466	4.74 - 5.37	4.85 - 5.38	4.81 - 5.53	3.25 - 6.04
(% total amino acid)			(0.466, 0.674)		
Threonine		4.19 ± 0.03	4.20 ± 0.03		
	0.381	3.99 - 4.38	3.96 - 4.44	3.86 - 4.25	3.15 - 4.24
(% total amino acid)			(0.381, 0.643)		
Tryptophan		1.519 ± 0.016	1.524 ± 0.016		
	0.650	1.423 - 1.640	1.401 - 1.653	1.271 - 1.686	0.95 - 1.49
(% total amino acid)			(0.650, 0.747)		
Tyrosine		3.97 ± 0.01	3.96 ± 0.01		
	0.418	3.88 - 4.10	3.83 - 4.06	3.82 - 4.16	2.62 - 3.72
(% total amino acid)			(0.418, 0.659)		
Valine		4.98 ± 0.02	4.96 ± 0.02		
	0.532	4.74 - 5.22	4.70 - 5.32	4.59 - 5.18	4.28 - 5.57
(% total amino acid)			(0.532, 0.712)		

[a] Unit of measure was converted from % DW to % total amino acid prior to analysis.

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

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

[d] Combined range from Appendix 7.

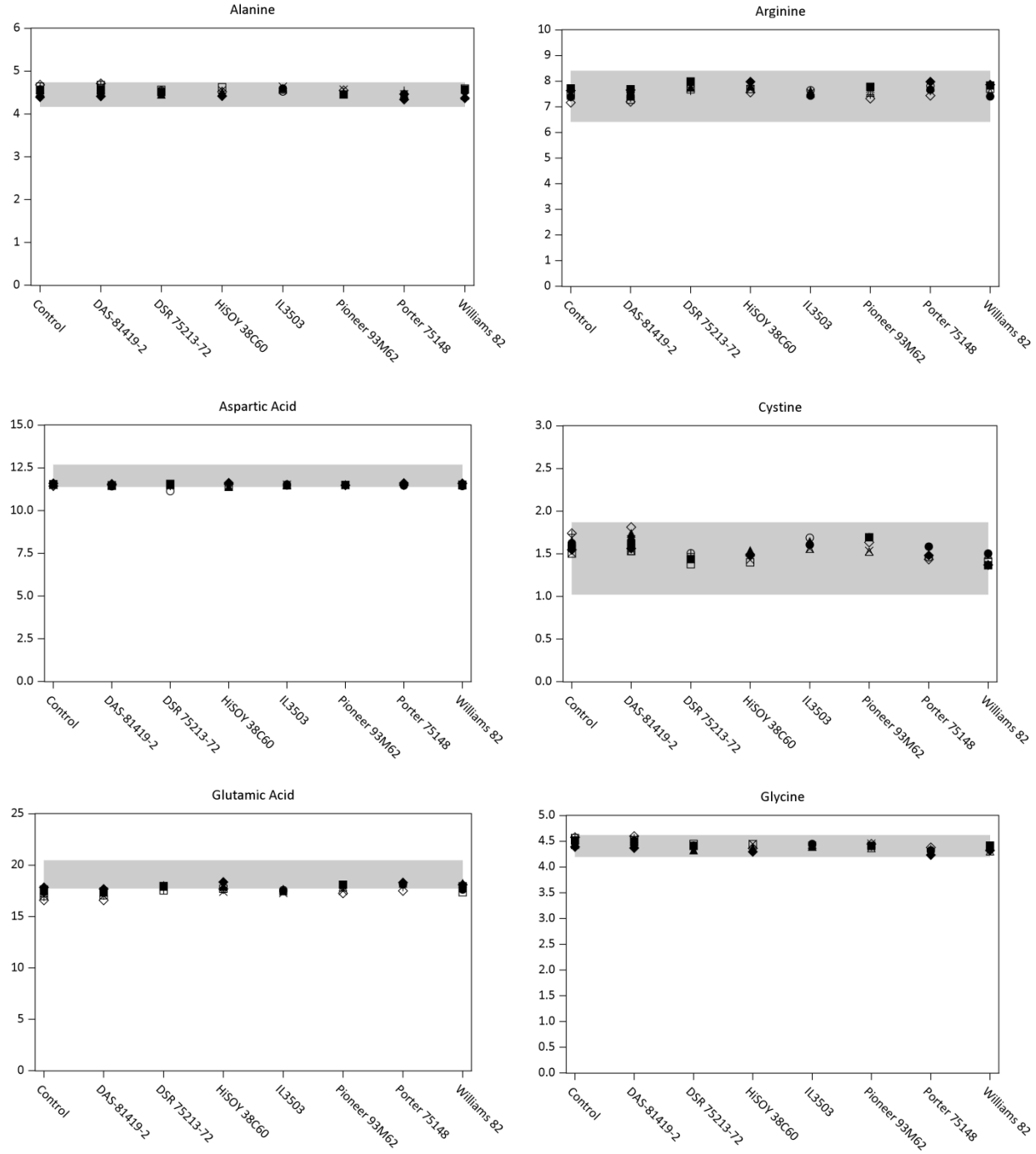


Figure 36. Amino acids in control, DAS-81419-2, and reference variety soybean seed (% total amino acid).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

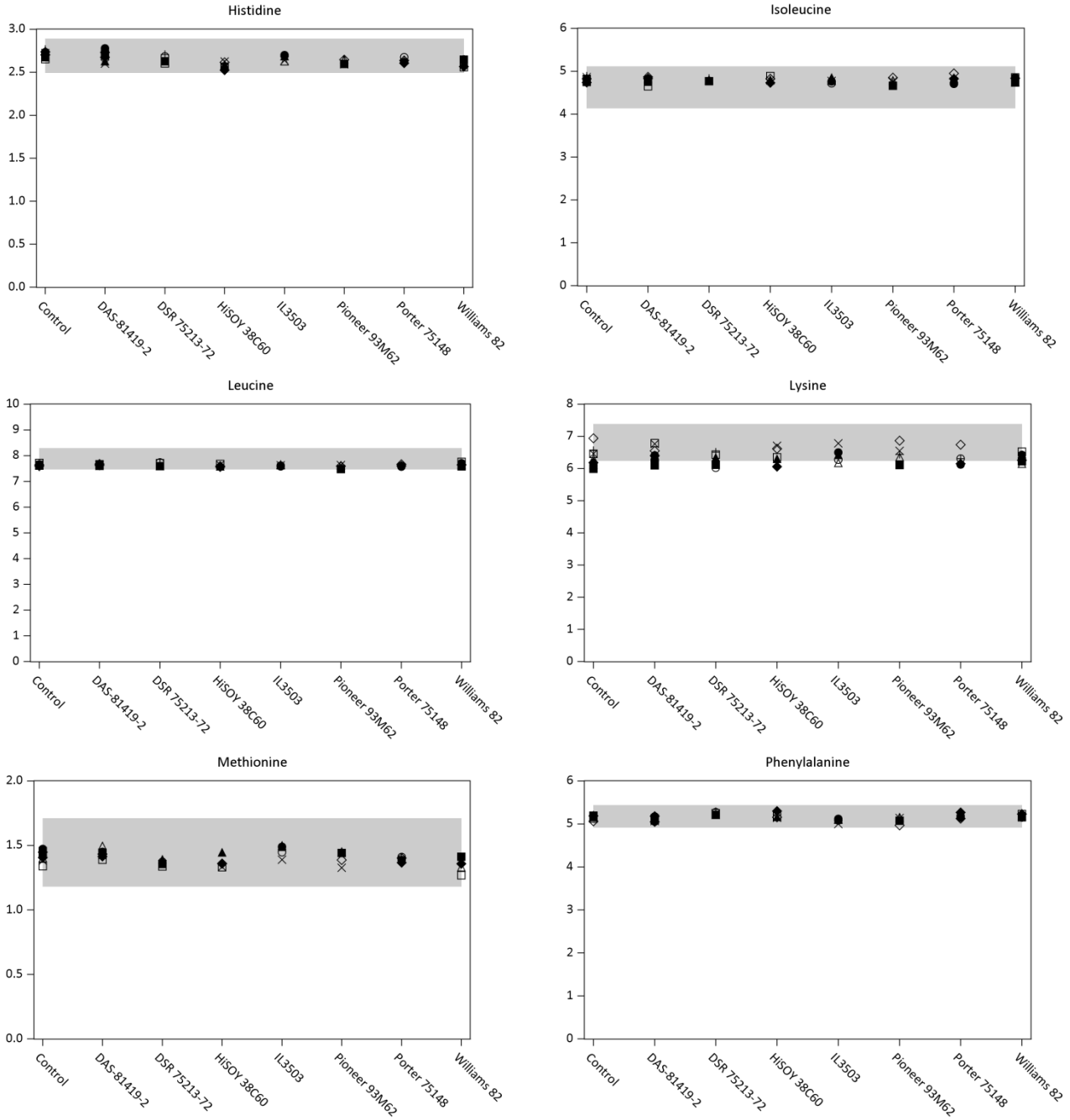


Figure 36 (Cont). Amino acids in control, DAS-81419-2, and reference variety soybean seed (% total amino acid).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

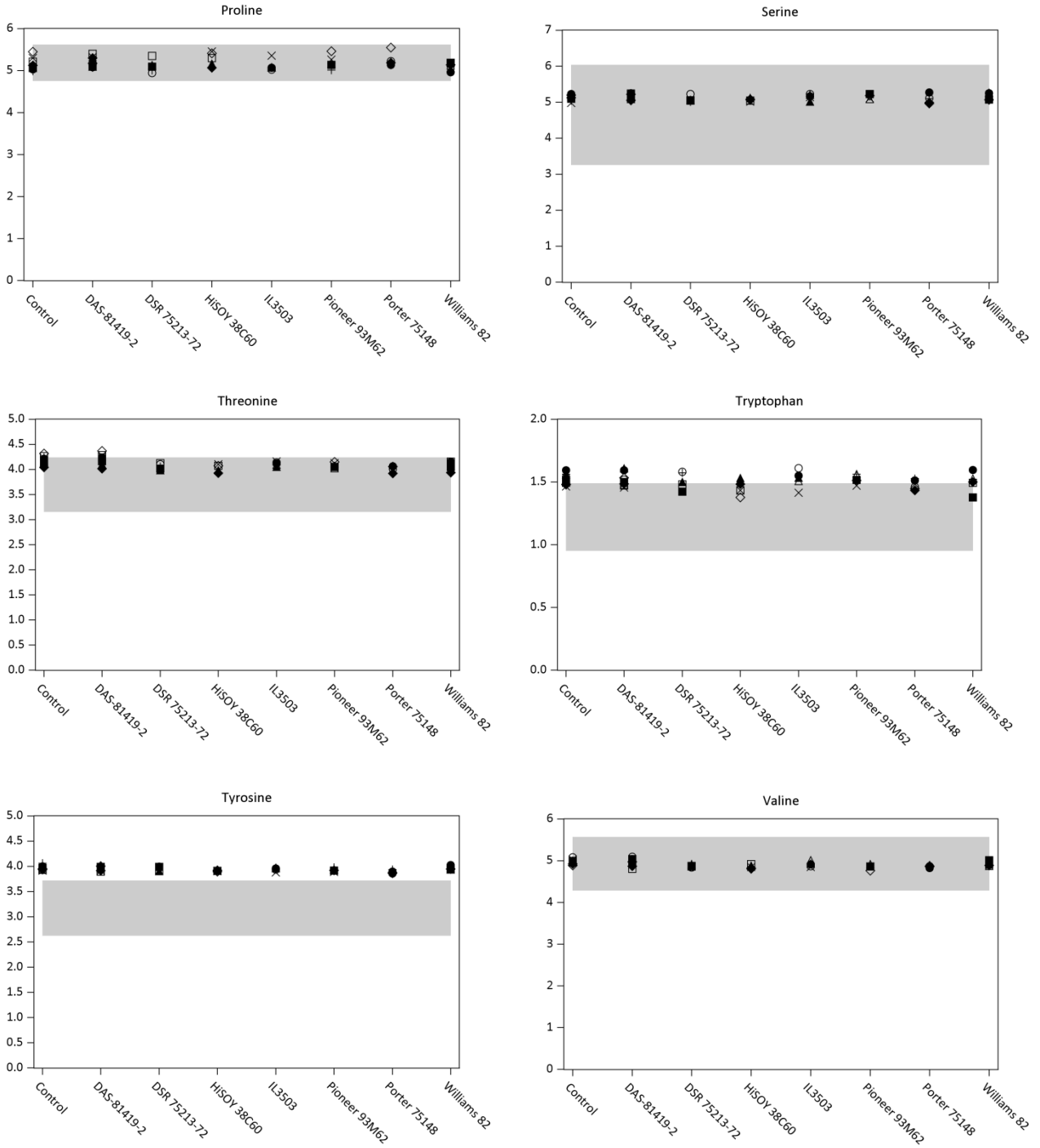


Figure 36 (Cont). Amino acids in control, DAS-81419-2, and reference variety soybean seed (% total amino acid).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

7.2.5. Fatty Acid Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for the content of 22 fatty acids. A summary of the results across all locations is presented in Table 15 and Figure 37. Statistical analysis was not performed on the following fatty acids because greater than 50% of the samples were found to be below the LOQ of 0.02% fresh weight: 8:0 caprylic, 10:0 capric, 12:0 lauric, 14:0 myristic, 14:1 myristoleic, 15:0 pentadecanoic, 15:1 pentadecenoic, 16:1 palmitoleic, 17:0 heptadecanoic, 17:1 heptadecenoic, 18:3 γ-linolenic, 20:2 eicosadienoic, 20:3 eicosatrienoic, 20:4 arachidonic. The mean values of the remaining fatty acids were within literature ranges (when available) and/or within ranges for reference varieties included in the study. Overall treatment effects and pair-wise comparisons between the control and DAS-81419-2 were significant for 16:0 palmitic, 18:3 linolenic, and 20:1 eicosenoic. However, these differences were small relative to natural variation and not biologically meaningful, as all results were within literature ranges and/or within the range of the reference varieties included in this study.

Table 15. Summary of the fatty acid analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
8:0 Caprylic	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.148
(% total fatty acid)					
10:0 Capric	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.27
(% total fatty acid)					
12:0 Lauric	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.132
(% total fatty acid)					
14:0 Myristic	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.238
(% total fatty acid)					
14:1 Myristoleic	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.125
(% total fatty acid)					
15:0 Pentadecanoic	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
(% total fatty acid)					
15:1 Pentadecenoic	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
(% total fatty acid)					
16:0 Palmitic	**<0.001**	11.12 ± 0.08 10.04 - 11.59	11.56 ± 0.08 10.88 - 12.27 (**<0.001, <0.001**)	9.12 - 11.53	1.40 - 15.77
(% total fatty acid)					

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

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

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

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

[d] Combined range from Appendix 7.

Table 15 (Cont). Summary of the fatty acid analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
16:1 Palmitoleic	NA	NA <LOQ	NA <LOQ - 0.236	<LOQ	<LOQ - 0.194
(% total fatty acid)					
17:0 Heptadecanoic	NA	NA <LOQ - 0.128	NA <LOQ - 0.132	<LOQ - 0.133	<LOQ - 0.146
(% total fatty acid)					
17:1 Heptadecenoic	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.087
(% total fatty acid)					
18:0 Stearic	0.18	4.40 ± 0.12 3.67 - 5.27	4.46 ± 0.12 3.62 - 5.22 (0.180, 0.474)	3.19 - 5.07	0.50 - 5.88
(% total fatty acid)					
18:1 Oleic	0.08	21.6 ± 0.4 19.6 - 25.2	21.2 ± 0.4 19.8 - 25.5 (0.080, 0.404)	18.8 - 24.6	2.60 - 45.68
(% total fatty acid)					
18:2 Linoleic	0.124	54.1 ± 0.4 51.2 - 55.8	53.8 ± 0.4 50.4 - 55.7 (0.124, 0.456)	53.6 - 57.5	7.58 - 58.8
(% total fatty acid)					
18:3 Linolenic	**0.04**	7.97 ± 0.15 6.91 - 8.76	8.17 ± 0.15 7.32 - 8.94 (**0.040**, 0.314)	6.58 - 9.88	1.27 - 12.52
(% total fatty acid)					

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

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

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

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

[d] Combined range from Appendix 7.

Table 15 (Cont). Summary of the fatty acid analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
18:3 γ-Linolenic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
20:0 Arachidic (% total fatty acid)	0.068	0.319 ± 0.007 0.276 - 0.370	0.325 ± 0.007 0.282 - 0.372 (0.068, 0.381)	0.254 - 0.383	0.038 - 0.57
20:1 Eicosenoic (% total fatty acid)	**0.048**	0.157 ± 0.004 <LOQ - 0.189	0.153 ± 0.004 <LOQ - 0.171 (**0.048**, 0.339)	<LOQ - 0.196	0.024 - 0.35
20:2 Eicosadienoic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.245
20:3 Eicosatrienoic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
20:4 Arachidonic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
22:0 Behenic (% total fatty acid)	0.159	0.317 ± 0.003 0.283 - 0.347	0.321 ± 0.003 0.263 - 0.345 (0.159, 0.456)	0.277 - 0.390	0.043 - 0.65

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

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

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

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

[d] Combined range from Appendix 7.

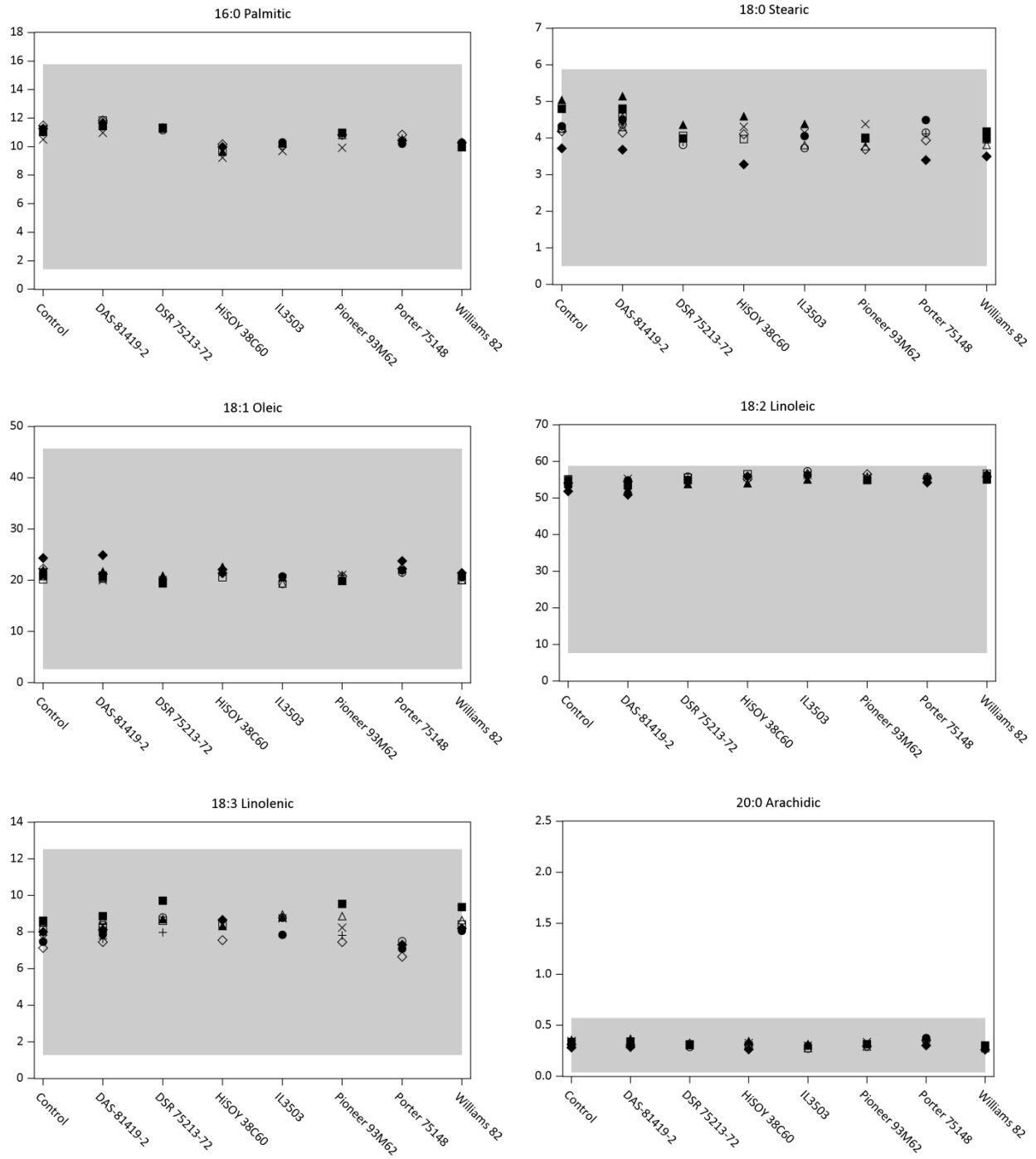


Figure 37. Fatty acids in control, DAS-81419-2, and reference variety soybean seed (% total fatty acid).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

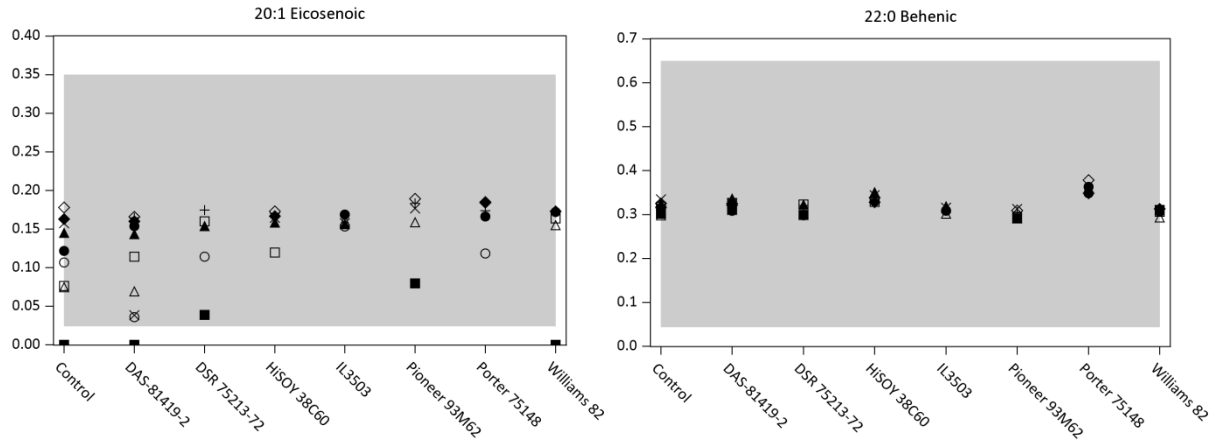


Figure 37 (Cont). Fatty acids in control, DAS-81419-2, and reference variety soybean seed (% total fatty acid).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

7.2.6. Vitamin Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for the content of 13 vitamins. A summary of the results across all locations is presented in Table 16 and Figure 38. Statistical analysis was not performed on Vitamin A because greater than 50% of the samples were found to be below the LOQ. All mean results were within literature ranges (when available) and/or within ranges for reference varieties included in the study. With the exception of γ-tocopherol and Vitamin B5, no statistical differences were observed in the combined-site analysis between the control and DAS-81419-2 entries for vitamin content. The overall treatment effect and pair-wise comparison between the control and DAS-81419-2 were significant for γ-tocopherol and Vitamin B5 based on unadjusted P-values (but not after adjustment for multiplicity using FDR methods). However, these differences were small relative to natural variation and not biologically meaningful, as all results were within the range of the reference varieties included in this study.

Table 16. Summary of the vitamin analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Vitamin A (β-Carotene) (mg/kg DW)	NA	NA <LOQ	NA <LOQ	<LOQ - 0.244	NR
Vitamin B1 (Thiamine) (mg/kg DW)	0.447	3.51 ± 0.24 2.35 - 5.44	3.43 ± 0.24 2.20 - 5.16 (0.447, 0.662)	1.82 - 4.92	1.01 - 2.54
Vitamin B2 (Riboflavin) (mg/kg DW)	0.146	3.40 ± 0.08 2.63 - 4.65	3.51 ± 0.08 2.58 - 4.64 (0.146, 0.456)	2.42 - 5.00	1.90 - 3.21
Vitamin B3 (Niacin) (mg/kg DW)	0.07	25.0 ± 0.7 20.2 - 30.5	25.6 ± 0.7 20.3 - 32.1 (0.070, 0.381)	20.5 - 29.0	NR
Vitamin B5 (Pantothenic Acid) (mg/kg DW)	**0.004**	14.8 ± 0.5 12.3 - 19.5	14 ± 0.5 11.8 - 16.8 (**0.004**, 0.074)	8.97 - 18.0	NR
Vitamin B6 (Pyridoxine) (mg/kg DW)	0.721	5.23 ± 0.11 4.42 - 6.43	5.18 ± 0.11 4.53 - 6 (0.721, 0.787)	3.01 - 6.36	NR
Vitamin B9 (Folic Acid) (mg/kg DW)	0.599	4.21 ± 0.20 3.05 - 5.62	4.15 ± 0.20 2.75 - 5.51 (0.599, 0.728)	2.94 - 5.59	2.386 - 4.709
Vitamin C (Ascorbic Acid) (mg/kg DW)	0.135	141 ± 13 75.2 - 231	133 ± 13 74 - 230 (0.135, 0.456)	49.2 - 210	NR

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

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

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

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

[d] Combined range from Appendix 7.

Table 16 (Cont). Summary of the vitamin analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
α-Tocopherol (mg/kg DW)	0.267	14.3 ± 1.3 8.56 - 29.3	13.6 ± 1.3 9.62 - 26.6 (0.267, 0.589)	6.51 - 25.0	1.934 - 84.9
β-Tocopherol (mg/kg DW)	NA	NA <LOQ	NA <LOQ	<LOQ	NR
γ-Tocopherol (mg/kg DW)	**0.004**	69.4 ± 2.8 41.3 - 83.1	74.7 ± 2.8 45.4 - 90.8 (**0.004**, 0.074)	49.7 - 104.0	NR
δ-Tocopherol (mg/kg DW)	0.338	168 ± 8 97 - 220	172 ± 8 118 - 219 (0.338, 0.615)	77.5 - 240	NR
Total Tocopherol[e] (mg/kg DW)	0.096	237.5 ± 6.9 159.5 - 280.5	247.1 ± 6.9 194.7 - 282.3 (0.096, 0.440)	150.5 - 299.9	NR

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

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

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

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

[d] Combined range from Appendix 7.

[e] Total Tocopherol = α-Tocopherol + β-Tocopherol + γ-Tocopherol + δ-Tocopherol

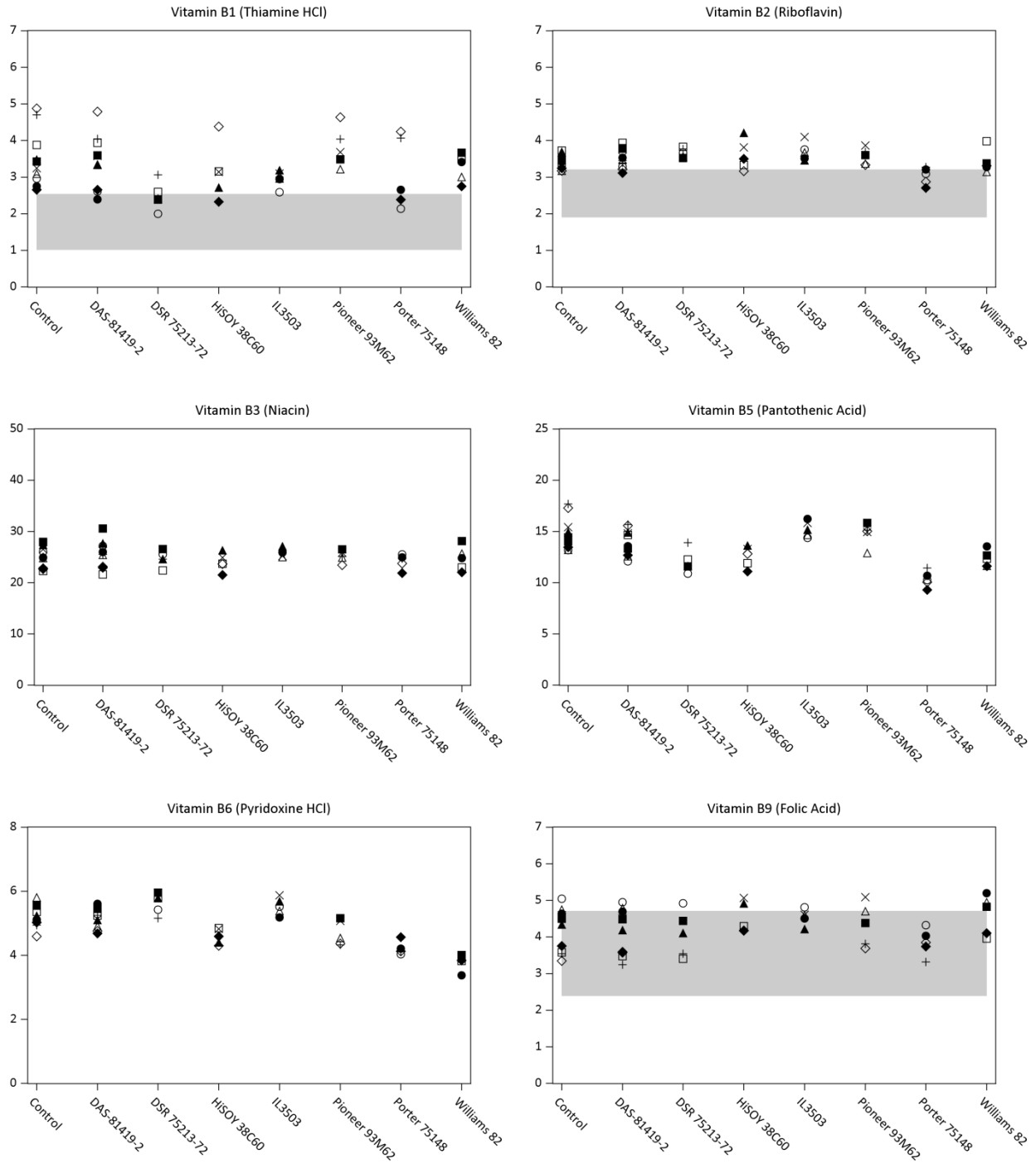


Figure 38. Vitamins in control, DAS-81419-2, and reference variety soybean seed (mg/kg dry weight).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range (when available) is shaded for each analyte.

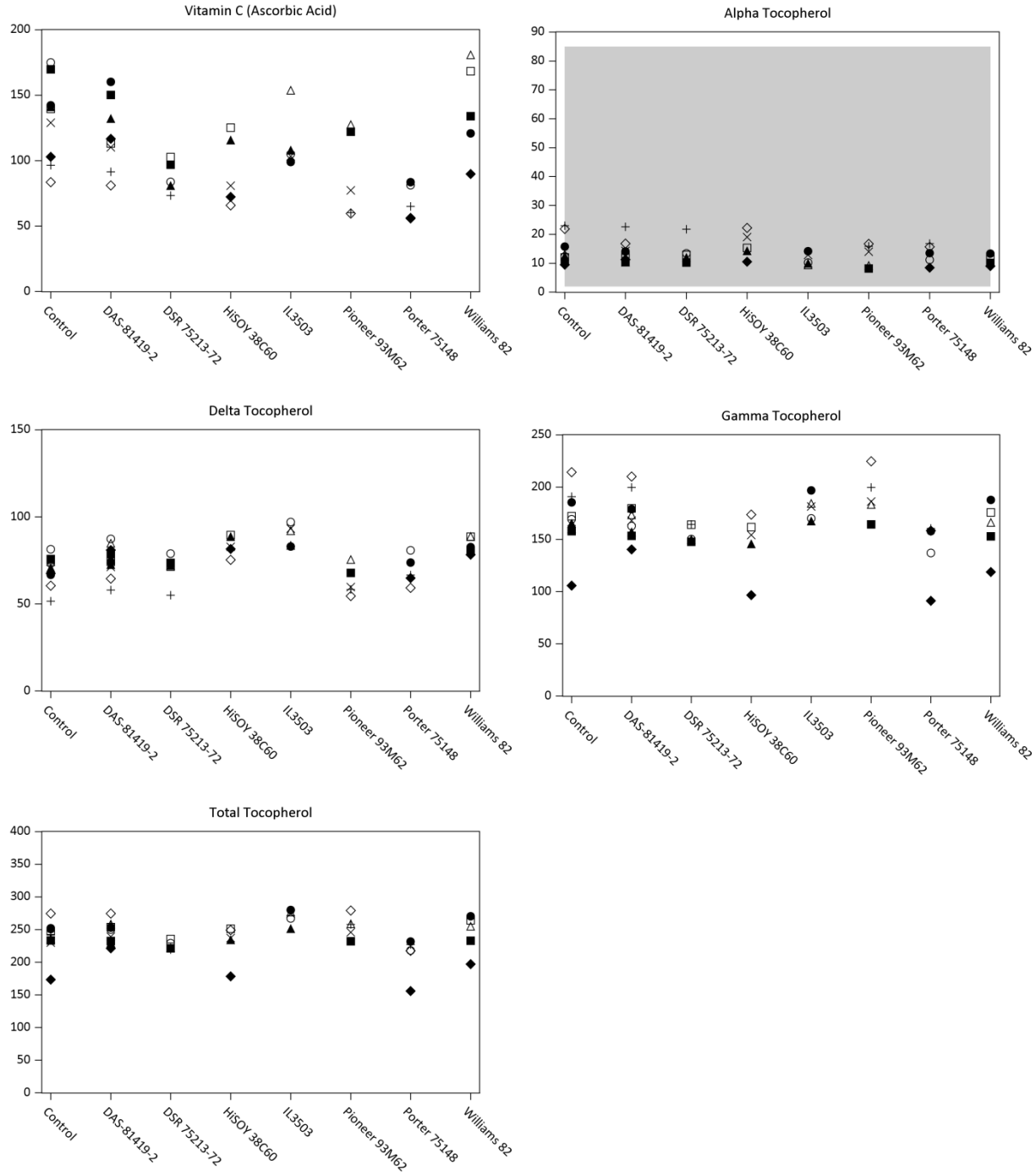


Figure 38 (Cont). Vitamins in control, DAS-81419-2, and reference variety soybean seed (mg/kg dry weight).

Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range (when available) is shaded for each analyte.

7.2.7. Bioactive Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for the content of eight bioactives. A summary of the results across all locations is presented in Table 17 and Figure 39. All mean results were within literature ranges and/or within ranges for reference varieties included in this study. With the exception of total glycitein equivalent, no statistical differences were observed in the combined-site analysis between the control and DAS-81419-2 entries for bioactive content. The overall treatment effect and pair-wise comparison between the control and DAS-81419-2 were significant for total glycitein equivalent based on unadjusted P-values (but not after adjustment for multiplicity using FDR methods). However, these differences were small relative to natural variation and not biologically meaningful, as all results were within the literature range and the range of the reference varieties included in this study.

Table 17. Summary of the bioactive analysis of soybean seed from all sites and literature range.

Analytical Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max	Combined Literature Range[d] Min - Max
Lectin (H.U./mg protein DW)	0.401	30.8 ± 2.1 13.9 - 50.1	32.2 ± 2.1 12.4 - 52.6 (0.401, 0.647)	7.89 - 45.2	37 - 323
Phytic Acid (% DW)	0.447	1.22 ± 0.06 0.857 - 2.02	1.24 ± 0.06 0.911 - 1.52 (0.447, 0.662)	0.678 - 1.71	0.41 - 2.68
Raffinose (% DW)	0.475	0.750 ± 0.038 0.505 - 1.02	0.766 ± 0.038 0.475 - 0.977 (0.475, 0.674)	0.570 - 1.16	0.212 - 1.85
Stachyose (% DW)	0.648	3.68 ± 0.08 3.19 - 4.14	3.69 ± 0.08 3.15 - 4.29 (0.648, 0.747)	3.01 - 5.28	1.21 - 6.65
Trypsin Inhibitor (TIU/mg)	0.226	29.1 ± 1.2 22.2 - 46.5	30.2 ± 1.2 21.3 - 49.9 (0.226, 0.536)	19.5 - 53.8	18.14 - 118.68
Total Daidzein Equivalent (mcg/g DW)	0.299	950 ± 48 462 - 1200	932 ± 48 504 - 1190 (0.299, 0.592)	585 - 1460	25 - 2453.5
Total Genistein Equivalent (mcg/g DW)	0.362	1296 ± 63 808 - 1680	1276 ± 63 922 - 1620 (0.362, 0.643)	753 - 1950	28 - 2837.2
Total Glycitein Equivalent (mcg/g DW)	**0.002**	197 ± 6 156 - 266	180 ± 6 140 - 237 (**0.002**, 0.074)	40.3 - 259	15.3 - 349.19

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

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

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

[d] Combined range from Appendix 7.

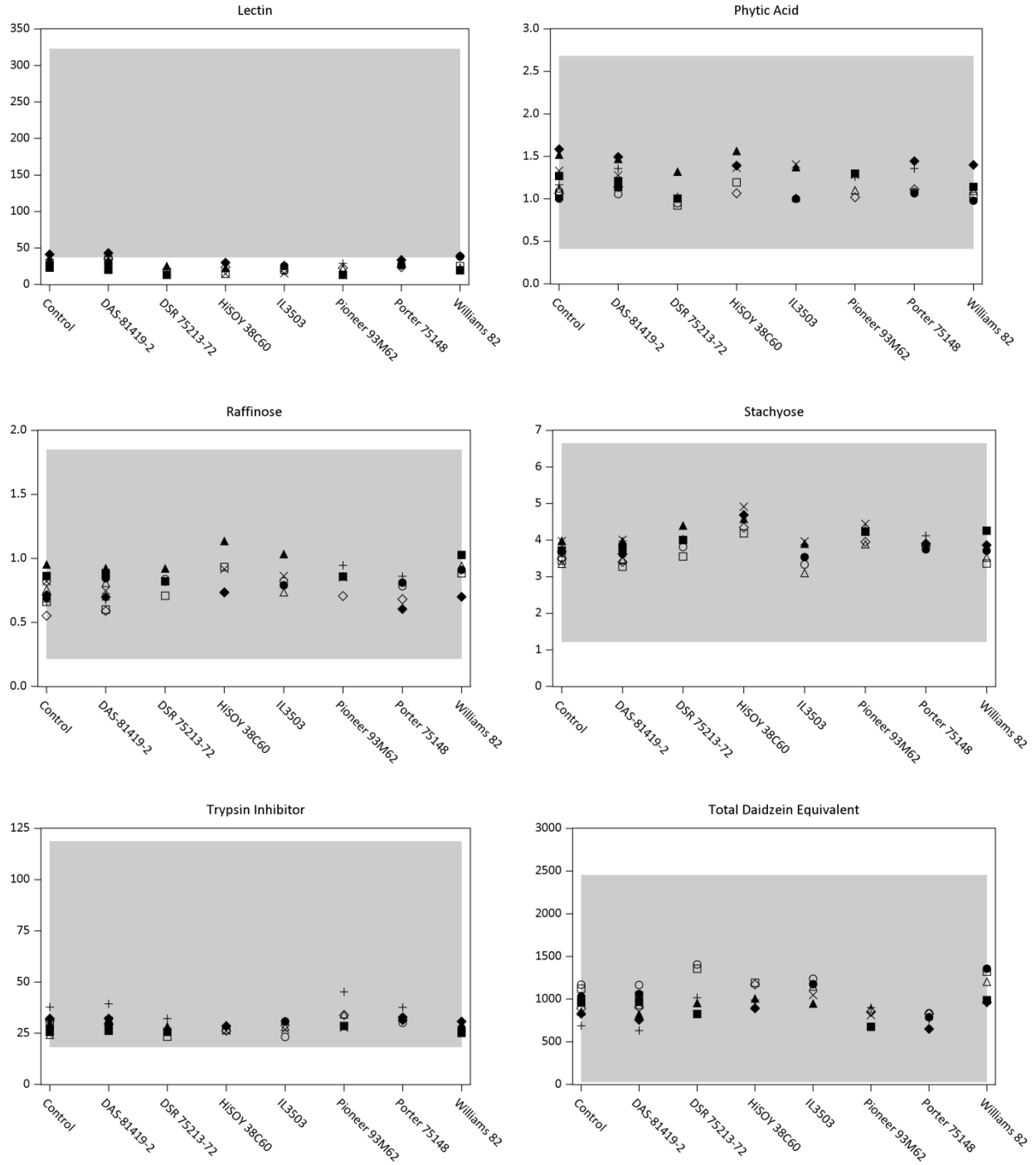


Figure 39. Bioactives in control, DAS-81419-2, and reference variety soybean seed.
Lectin = H.U./mg protein dry weight, Trypsin inhibitor = TIU/mg, total daidzein equivalent, total genistein equivalent, and total glycitein equivalent = mcg/gram dry weight, all others = % dry weight
Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

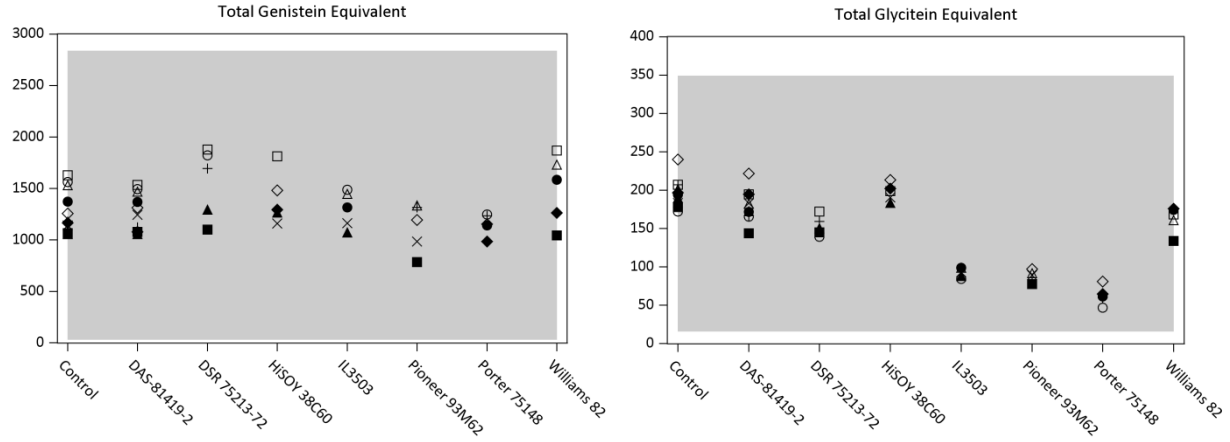


Figure 39 (Cont). Bioactives in control, DAS-81419-2, and reference variety soybean seed.
Lectin = H.U./mg protein dry weight, Trypsin inhibitor = TIU/mg, total daidzein equivalent, total genistein equivalent, and total glycitein equivalent = mcg/gram dry weight, all others = % dry weight
Symbols for each location shown: open circle = IA1, × = IA2, + = IL1, open triangle = IL2, open square = IN, open diamond = MO1, filled circle = MO2, filled triangle = NE1, filled square = NE2, filled diamond = PA. Literature range is shaded for each analyte.

7.3. Summary of Composition Analysis

All overall mean values for the control (Maverick) and DAS-81419-2 were within literature ranges (when available) for soybean and/or within ranges for non-transgenic soybean reference varieties included in the study. A limited number of statistically significant differences were observed between DAS-81419-2 and the control; however, these differences were not biologically meaningful because the differences were small relative to natural variation and the results were within ranges found for non-transgenic soybean. In conclusion, forage and grain derived from DAS-81419-2 soybean are compositionally equivalent to those of non-transgenic soybean.

8. Agronomic Characteristics

Field trials with DAS-81419-2 soybean, a non-transgenic control (Maverick), and reference varieties were conducted in 2011 at ten sites located in Iowa (2 sites), Illinois (2 sites), Indiana, Missouri (2 sites), Nebraska (2 sites), and Pennsylvania. This study used the same plots that were used for protein expression (Section 6) and nutrient composition studies (Section 7). No biologically relevant differences, other than resistance to target insect pests, were identified between DAS-81419-2 soybean and non-transgenic soybean. Agronomic characteristics of DAS-81419-2 were statistically indistinguishable from the non-transgenic control Maverick or were within the reference variety ranges for non-transgenic soybean. Ecological evaluations indicated that there were no statistically significant differences between DAS-81419-2 soybean and the non-transgenic control Maverick in susceptibility to and interactions with diseases and non-target insects. The germination and dormancy evaluation indicated that seed dormancy characteristics have not been altered in DAS-81419-2 soybean. Taken together, results from the field and laboratory studies demonstrate agronomic equivalence between DAS-81419-2 soybean and non-transgenic soybean.

8.1. Agronomic Study

An agronomic study with DAS-81419-2 soybean, a non-transgenic near-isogenic control (Maverick) and six non-transgenic reference lines (IL 3503, Porter 75148, DSR 75213-72, Pioneer 93M62, HiSOY 38C60, Williams 82) was conducted in 2011 at ten sites located in Richland, Iowa; Atlantic, Iowa; Carlyle, Illinois; Wyoming, Illinois; Frankfort, Indiana; Fisk, Missouri; La Plata, Missouri; York, Nebraska; Brunswick, Nebraska; and Germansville, Pennsylvania.

Each trial site included event DAS-81419-2, the non-transgenic near-isogenic control (Maverick), and three non-transgenic reference lines. At each of the ten sites, all entries were arranged in a randomized complete block design with four blocks. Across all sites, each control (Maverick) and DAS-81419-2 was represented by a total of 40 plots (10 sites, 4 replicate plots per entry at each site). Three of the six reference lines were included at each site by randomizing across sites in a balanced incomplete-block design. Each of the six reference lines was assigned to five sites; therefore, each reference line was represented by a total of 20 plots across sites (5 sites per reference line, 4 replicate plots per entry at each site).

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

Agronomic Data Collection

The following agronomic characteristics were measured and recorded for all test entries at each location on a per plot basis (Table 18).

Table 18. Agronomic characteristics.

Trait	Evaluation Timing	Description	Scale
Early Population (Stand Count)	≈V2	Number of plants in a representative 1 meter section of one row per plot	Number of emerged plants in 1 meter
Seedling Vigor	≈V2	Visual estimate of average vigor of plants in each plot	0-100% rating scale (e.g., 0 = all plants are short with small, thin leaves; 100 = all plants are tall with robust leaves). Not based on growth of the control entries; Did not consider germination/ emergence (stand count)
Days to 50% Flowering	≈R1/R2	Date at which ≈50% of plants are flowering	Recorded the date when ≈50% of the plants in each plot were flowering. Days since planting calculated.
Disease Incidence	≈R6	Visual estimate of disease incidence	0-100%; Estimated % plant tissue/leaf area diseased over all plants in plot; did not record % of plants in plot that had detectable disease; 100% = all plant tissues in plot were diseased; recorded type of disease
Insect Damage	≈R6	Visual estimate of insect damage	0-100%; Estimated % plant tissue/leaf area damaged over all plants in plot; Did not record % of plants in plot that had detectable damage; 100% = all plant tissues had feeding damage; Recorded type of damage, e.g. chewing, stippling, distortion; Recorded type of insect(s) if present
Days to Maturity	≈R8	Date at which ≈95% of plants had reached physiological maturity/dry down color	Recorded the date when ≈95% of the plants in each plot reached physiological maturity/dry down color; Days since planting calculated
Lodging	≈R8	Visual estimate of incidence of lodging severity	0-100%; Estimated % of plants lodged in plot; 100% = all plants in plot were lodged
Plant Height	≈R8	Average plant height: from soil surface to growing tip (at senescence / after leaf shed)	Recorded the average height of all plants in plot (stand) in centimeters (cm); One value for each plot; If plot was lodged, a representative group of plants was held up to obtain a measurement
Final Population (Stand Count)	≈R8	Number of plants in a representative 1 meter section of one row per plot	Number of plants in 1 meter; Did not sample a section where plants were removed during previous sampling
Plant Morphology(number of seeds and pods)	≈R8 (prior to harvest)	Number of pods and seeds from 5 plants collected from each plot	Recorded the number of pods and seeds present on 5 representative plants collected from each plot

Trait	Evaluation Timing	Description	Scale
Shattering	≈R8 (prior to harvest)	Visual estimate of pod shattering	0-100%; Estimated % of shattered pods for each plot; 100% = all pods shattered
Yield	≈R8	Weight of grain harvested from each plot	Recorded the weight in grams of grain harvested from each plot
100 seed weight	≈R8	Weight of 100 representative seeds from bulk yield sample in grams	Recorded the weight in grams for 100 representative seeds taken from the bulk yield sample

Analysis of variance was conducted across field sites (combined-site analysis) for agronomic data using a mixed model (SAS Institute Inc., 2009). Entry was considered a fixed effect and location, block within location, and location-by-entry were designated as random effects. Significant differences were declared at the 95% confidence level. The significance of an overall treatment effect was estimated using an F-test. Comparisons were made between DAS-81419-2 soybean and the control (Maverick) using t-tests.

Due to the large number of comparisons made in this study, multiplicity was an issue. Multiplicity is an issue when a large number of comparisons are made in a single study to look for unexpected effects. Under these conditions, the probability of falsely declaring differences based on comparison-wise P-values is very high ($1\text{-}0.95^{\text{number of comparisons}}$). In this study, there was one comparison per endpoint (14 analyzed observation types for agronomics), resulting in 14 comparisons made in the combined-site agronomic analysis. Therefore, the probability of declaring one or more false differences based on unadjusted P-values was >51% for agronomics ($1\text{-}0.95^{14}$).

One method to account for multiplicity is to adjust P-values to control the experiment-wise error rate, but when many comparisons are made in a study, the power for detecting specific effects can be reduced significantly. An alternative with much greater power is to adjust P-values to control the probability that each declared difference is significant. This can be accomplished using False Discovery Rate (FDR) control procedures (Benjamini and Hochberg, 1995); FDR methods are commonly applied in studies examining transgenic crops (Herman *et al.*, 2007; Coll *et al.*, 2008; Huls *et al.*, 2008; Jacobs *et al.*, 2008; Stein *et al.*, 2009; Herman *et al.*, 2010). Therefore, the P-values from the agronomics evaluations were each adjusted using the FDR method to improve discrimination of true differences among treatments from random effects (false positives). Differences were considered significant if the FDR-adjusted P-value was less than 0.05.

Agronomic Results

A statistical analysis of the agronomic data collected from the control (Maverick) and DAS-81419-2 entries was conducted. For each agronomic characteristic and entry, the least squares mean, standard error, and minimum and maximum sample values are reported.

Additionally, the minimum and maximum values from reference varieties across all sites (reference variety ranges) for each agronomic characteristic are provided for comparison. Each minimum and maximum value is an individual data point reported for a single test plot.

For the combined-site analysis (Table 19), no statistically significant differences were observed following False Discovery Rate (FDR) adjustment of P-values for all of the agronomic characteristics evaluated: early population, seedling vigor, days to flowering, disease incidence, insect damage, days to maturity, lodging, plant height, final population, number of pods per five plants, number of seeds per five plants (plant morphology), shattering, yield, and 100 seed weight. Unadjusted P-values were significant at the 0.05 level for paired t-tests for days to flowering and number of pods per five plants (plant morphology). For each significant unadjusted P-value, differences in mean values between DAS-81419-2 and control (Maverick) entries were negligible and the mean values of DAS-81419-2 were within the range observed for reference varieties included in the study. Results from this study demonstrate agronomic equivalence between event DAS-81419-2 and non-transgenic soybean.

Table 19. Summary of agronomic characteristics for the combined site analysis.

Agronomic Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max
Early Population - V2 (number of plants in a 1 m section of row)	0.267	17 ± 0 13 - 21	16 ± 0 11 - 21 (0.267, 0.386)	7 - 22
Seedling Vigor - V2 (0-100% scale, 0% = all plants are short with small thin leaves; 100% = all plants are tall with robust leaves)	0.074	86 ± 5 60 - 100	82 ± 5 50 - 100 (0.074, 0.137)	60 - 100
Days to Flowering - R1/R2 (days since planting)	**0.008**	42 ± 2 32 - 58	43 ± 2 33 - 58 (**0.008**, 0.053)	32 - 58
Disease Incidence - R6 (0-100% scale, 0% = no disease 100% = all plants diseased)	0.343	4 ± 3 0 - 30	5 ± 3 0 - 30 (0.343, 0.446)	0 - 50
Insect Damage - R6 (0-100% scale, 0% = no damage 100% = all plants damaged)	0.504	7 ± 3 0 - 30	6 ± 3 0 - 30 (0.504, 0.596)	0 - 30
Days to Maturity - R8 (days since planting)	0.957	114 ± 3 91 - 130	114 ± 3 93 - 133 (0.957, 0.957)	96 - 133
Lodging - R8 (0-100% scale, 0% = no lodging 100% = all plants lodged)	0.053	19 ± 6 0 - 90	7 ± 6 0 - 30 (0.053, 0.137)	0 - 35
Plant Height - R8 (Representative plant height in cm)	0.066	108 ± 4 80 - 135	103 ± 4 71 - 126 (0.066, 0.137)	63 - 129
Final Population - R8 (number of plants in a 1 m section of row)	0.742	14 ± 1 7 - 22	14 ± 1 8 - 22 (0.742, 0.804)	7 - 17
Number of Pods - R8 (number of pods on 5 plants)	**0.006**	331 ± 10 203 - 476	304 ± 10 172 - 408 (**0.006**, 0.053)	153 - 808
Number of Seeds - R8 (number of seeds from 5 plants)	0.059	783 ± 38 491 - 1154	728 ± 38 318 - 1031 (0.059, 0.137)	313 - 1244

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

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

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

Table 19 (Cont). Summary of agronomic characteristics for the combined site analysis.

Agronomic Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control (Maverick) Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max
Shattering - R8 (0-100% scale, 0% = no shattering 100% = all pods shattered)	NA	NA 0 - 0	NA 0 - 0	0 - 0
Yield - R8 (bushels per acre)	0.092	37 ± 4 5 - 55	35 ± 4 6 - 54 (0.092, 0.149)	7 - 62
100 Seed Weight - R8 (grams)	0.051	14 ± 1 9.76 - 17.61	13 ± 1 10.83 - 16.65 (0.051, 0.137)	11.69 - 25.82

Abbreviations: NA (Not Available) = analysis not performed, insufficent variation in response variable to enable ANOVA.

[a] Unit of measure was not converted prior to analysis except for yield. Unit of measure for yield was converted from grams per plot to bushels per acre prior to analysis using the formula: $(X \text{ g}/125 \text{ ft}^2) \times (43560 \text{ ft}^2/\text{A}) \times (1 \text{ kg}/1000 \text{ g}) \times (1 \text{ bu.}/27.2155 \text{ kg})$, where X is the individual sample value.

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

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

Conclusions

Agronomic characteristics of DAS-81419-2 soybean were evaluated in field trials in 2011. DAS-81419-2 agronomic characteristics were statistically indistinguishable from the control or were within the reference variety ranges for non-transgenic soybean. No unintended agronomic effects were observed for DAS-81419-2 soybean. Results from this study demonstrate agronomic equivalence between event DAS-81419-2 and non-transgenic soybean.

8.2. Ecological Evaluations

The DAS-81419-2 soybean field trials were monitored and observed by personnel familiar with soybean cultivation practices (breeders, field station managers, field agronomists, field associates). The personnel conducting the field tests visually monitored the incidence of plant disease and pests on DAS-81419-2 soybeans compared with the conventional soybean varieties, including Maverick and six reference lines, in the same trials. Disease and insect damage was rated on a numerical scale of 0-100%, with 0% representing no damage due to disease incidence or insect pests. The insect and disease pressure observed in these trials was typical of the growing locations. Table 20 summarizes the insects and diseases that caused the insect damage and disease incidence at each field site. In all cases, the insects and diseases observed were found on both test and control plots. Table 21 summarizes the results obtained from the field trials conducted in 2011 at ten sites. There were no statistically significant differences between DAS-81419-2 soybean and the non-transgenic control Maverick in susceptibility to and interactions with diseases and insects. It should be noted that efficacy evaluation of DAS-81419-2 soybean was not the objective of this study (refer to Section 9.1.1 for efficacy evaluation of DAS-81419-2 soybean). 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 soybean. The results do not indicate an ecological impact of DAS-81419-2 soybean on non-target insects or disease.

Table 20. Insect and disease present by site.

Site	Insects Present	Diseases Present
110000IA1	Not Recorded[a]	None Present
110000IA2	Grasshoppers	Bacterial Pustule
110000IL1	Green Cloverworms, Bean Leaf Beetles, Grasshoppers	None Present
110000IL2	Not Recorded[a]	Not Recorded[a]
110000IN	Bean Leaf Beetles	None Present
110000MO1	None Present	Not Recorded[a]
110000MO2	None Present	None Present
110000NE1	Wooly Bear Caterpillars	Not Recorded[a]
110000NE2	Caterpillars	Mold, Mildew, Blight
110000PA	Loopers, Skippers	None Present

[a] Insects or Diseases were not recorded due to their presence on less than 30% of the site.

Table 21. Disease and insect incidence.

Agronomic Component (Units)[a]	Overall Treatment Effect (Pr > F)[b]	Control Mean ± SE Min - Max	DAS-81419-2 Mean ± SE Min - Max (P-value, Adj.P)[c]	Reference Variety Range Min - Max
Disease Incidence* - R6		4 ± 3	5 ± 3	
	0.343	0 - 30	0 - 30	0 - 50
			(0.343, 0.446)	
Insect Damage - R6**		7 ± 3	6 ± 3	
	0.504	0 - 30	0 - 30	0 - 30
			(0.504, 0.596)	

[a] Unit of measure was not converted prior to analysis.
[b]Overall treatment effect estimated using an F-test.
[c] P-value = Comparison to the control using t-tests; Adj. P = P-values adjusted using False Discovery Rate (FDR) procedure; P-values < 0.05 were considered significant.
*Disease incidence 0 – 100% scale. 0% = no disease; 100% = all plants diseased.
**Insect damage 0 – 100% scale. 0% = no damage; 100% = all plants damaged.

8.3. Germination and Dormancy Evaluations

The germination of DAS-81419-2 soybean seed compared with the non-transgenic control (Maverick) under warm and cold conditions was evaluated to determine any impact on seed dormancy characteristics. The cold and warm germination tests consisted of four replications of each entry with 100 seeds per replication in a completely randomized design.

Cold Germination Methods: The seeds were covered with approximately 1/8 inch of a soil mixture and placed in an incubator set at 10°C for 7 days; the temperature was then raised to 25°C for an additional 7 days. After the seven-day incubation period at 25°C, the number of seedlings germinated was determined in accordance with the AOSA Seedling Evaluation guidelines (AOSA, 2010b) and documented. This method is adapted from the AOSA Seed Vigor Testing Handbook (AOSA, 2009).

Warm Germination Methods: Twenty-five soybean seeds per petri dish were saturated with water and positioned with four dishes placed closely together in a square to make one replication. The dishes were placed in an incubator set at 25°C and on day five, the number of seeds germinated in each replication was recorded. On day 8, all remaining seeds were scored as germinated (normal seedlings), abnormal, or hard seeds. 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).

Data were transformed using the arcsine of the square root of the decimal fraction of seeds germinated per replicate and then subjected to analysis of variance based on a completely randomized design (SAS Institute Inc., 2009).

Germination data and *P*-values for the significance of the effect of entry on germination are provided in Table 22. Germination of DAS-81419-2 soybean did not differ significantly ($\alpha = 0.05$) from that of the non-transgenic, near-isogenic control Maverick under cold ($P = 0.5372$) and warm ($P = 0.6129$) conditions. The results indicate that seed dormancy characteristics have not been changed in DAS-81419-2 soybean.

Table 22. Germination percentage for DAS-81419-2 and non-transgenic control soybean.

		Soybean Germination (%)					
Temperature	**Entry**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**	**Mean (±SE)**	***P***
Cold	DAS-81419-2	88	93	90	91	90.5 (±1.0)	0.5372
Cold	Control	90	91	90	88	89.8 (±0.6)	
Warm	DAS-81419-2	99	98*	100	99	99.0 (±0.4)	0.6129
Warm	Control	98*	100	100	99	99.3 (±0.5)	

*One hard seed was found in these replicates.

8.4. Summary of Agronomic Characteristics

The agronomic study evaluating plant growth characteristics throughout the growing season demonstrated the equivalence of DAS-81419-2 soybean with conventional non-transgenic soybean. Agronomic and phenotypic characteristics, including response to ecological stressors as indicated by susceptibility to disease and insect pressure, were not different between DAS-81419-2 soybean and conventional soybean grown across diverse environments. In addition, no differences were detected in germination and dormancy of seed from DAS-81419-2 compared with control soybean. The data support the conclusion that agronomic, disease, and pest characteristics of DAS-81419-2 soybean are not significantly different from that of conventional soybean, and there is no indication that DAS-81419-2 soybean will pose an increased plant pest risk.

9. Impact on Environment and Agronomic Practices

This section describes the potential impact of DAS-81419-2 soybean on non-target organisms including threatened and endangered species and an assessment on the impact of introducing DAS-81419-2 soybean on the current agronomic practices for soybean in the U.S. and insect resistance management. This section also provides an evaluation of weediness potential and gene flow.

9.1. Potential Impact on Environment

9.1.1. Efficacy against Target Pests

The efficacy of DAS-81419-2 soybean against lepidopteran pests was evaluated under field conditions and through laboratory bioassay of field collected foliage at the Dow AgroSciences Southern U.S. Research Center located near Greenville, Mississippi. DAS-81419-2 soybean was compared directly with the non-transgenic soybean control Maverick. T4 seed (four generations post-transformation) bearing DAS-81419-2 were planted for field evaluation. Bioassay of excised leaves with soybean looper (*Chrysodeixis includens,* formerly *Pseudoplusia includens*), velvetbean caterpillar (*Anticarsia gemmatalis*), fall armyworm (*Spodoptera frugiperda*), and tobacco budword (*Heliothis virescens*) was used to measure the efficacy. In addition, the number of natural *C. includens* larvae and associated levels of leaf damage were observed in the DAS-81419-2 and 'Maverick' plots. Excellent efficacy against soybean looper, velvetbean caterpillar, fall armyworm, and tobacco budworm was demonstrated.

Trial Design: Plots were planted in randomized complete block with 4 replicates of each entry replicated one time per block. Each replicate plot consisted of 4 rows, 20 ft (6.1 m) in length, spaced 40 inches (1.02 m) apart. There were 160 seeds planted per row, spaced 1.5 inches (3.8 cm) apart within the row. Two additional rows of 'Maverick' were planted between the experimental plots and four rows of 'Maverick' were planted adjacent to the outermost experimental plots to attract natural insect pest populations.

Dates of Efficacy Evaluations: Leaves for the first bioassay were collected 15 July 2011, when the soybean plants were in the R2 growth stage. Leaves for the second bioassay were collected from the same plants on 12 August 2011, when the soybean plants were in the R5 growth stage. Field populations of *C. includens* were sampled on 8, 15, 22, and 29 August 2011.

Bioassay of Excised Leaves at Growth Stage R2: Fully-expanded, mainstem trifoliate leaves, showing no signs of damage or discoloration and located four nodes below the meristem, were excised for the feeding bioassay. A single trifoliate leaf was excised from each of 15 plants per replicate; 4 trifoliates per replicate were used for the bioassay of *C. includens* (formerly *P. includens*), 4 trifoliates per replicate were used for the bioassay of *S. frugiperda*, 4 trifoliates per replicate were used for the bioassay of *H. virescens*, and 3 trifoliates per replicate were used for the bioassay of *A. gemmatalis*.

The two side leaflets of each trifoliate were excised and placed in separate, labeled petri plates containing a thin layer of agar. Two second instars of *C. includens*, *A. gemmatalis*, *S. frugiperda* or *H. virescens* were placed on each leaflet. For *C. includens*, *S. frugiperda*, and *H. virescens*, 16 larvae per replicate (64 larvae in total) were exposed to leaf tissue from event DAS-81419-2 and 'Maverick'. For *A. gemmatalis*, 12 larvae per replicate (48 larvae in total) were exposed to leaf

tissue from event DAS-81419-2 and 'Maverick'. All insect larvae were provided by the Dow AgroSciences Insectary in Indianapolis, Indiana. The petri plates holding the infested leaflets were held at 25° C and 40% RH. After 2 days, larvae were determined to be dead (no movement when stimulated with a sharp probe), moribund (larva responds to stimulus but unable to right itself if placed on side), stunted (smaller in size than larvae held on 'Maverick' leaves), or alive (normal in size and response to stimulus). Also, the percentage of each leaflet consumed was estimated visually. If no feeding damage was evident, 0% consumption was recorded. If only "pinholes" in the leaf tissue were evident, 0.5% consumption was recorded. For greater amounts of consumption, a photographic guide was used to aid in estimating the percentage consumed (Rice, 2002). Larval condition and leaf consumption were observed again 4 days after larvae had been placed on leaflets.

Bioassay of Excised Leaves at Growth Stage R5: The feeding bioassay procedure at R5 was the same as the procedure used at R2 with the exception that for *S. frugiperda*, and *H. virescens*, all three leaflets were excised from each trifoliate and a single, second instar was placed on each leaflet. This resulted in 12 larvae per replicate (48 larvae in total) of *S. frugiperda* and *H. virescens* being exposed to leaflets from DAS-81419-2 and 'Maverick'.

Field Sampling of Natural *Chrysodeixis includens* Infestations: Insect counts were initiated when the natural *C. includens* (formerly *P. includens*) population began to cause damage in 'Maverick' plots, and continued weekly while *C. includens* larvae were present. The center two rows of each plot were sampled. A 3 ft × 3 ft (91 cm × 91 cm) white cloth was placed at a randomly selected location between the center two rows. The plants in the section of row adjacent to one edge of the sheet were bent over the cloth and shaken 15 times to dislodge any insects present. This process was repeated for the row on the opposite edge of the cloth. Larvae were counted by species and size: larvae < 0.25 in (< 6 mm) in length were counted as small larvae and larvae ≥ 0.25 in (≥ 6 mm) in length were counted as large larvae. All insects were removed from the cloth before taking the next measurement. The cloth was moved to a second randomly selected location between the two center rows and the sampling process was repeated, resulting in two subsamples per plot at each sampling date. On the last sample date, percent defoliation for 20 plants per replicate was estimated visually on the trifoliate at the fifth mainstem node below the meristem with the aid of a photographic guide (Rice, 2002).

Data Analysis: Given the binomial nature of the insect mortality data (alive and healthy vs. dead, moribund or stunted), the data were arranged as a 2×2 contingency table (as shown below) and subjected to Pearson's chi-square test. The calculated chi-square values and significance levels (*P* values) of these chi-square values are presented in Table 23.

	DAS-81419-2	'Maverick'	
Total number of live, healthy larvae	*a*	*b*	Row 1 total
Total number of dead, moribund, or stunted larvae	*c*	*d*	Row 2 total
	Column 1 total	Column 2 total	Grand total

All other results were subjected to analysis of variance to determine if significant treatment or replicate effects existed. There were no significant replication effects for any variable measured. Bartlett's test was used to test for the equality of treatment variances. Where variances were unequal, the $ln(x+1)$ transformation was applied. If the $ln(x+1)$ transformation did not equalize treatment variances, the square root$(x+0.5)$ transformation was applied. If the square root$(x+0.5)$ transformation did not equalize treatment variances, the arcsine square root transformation was applied. The calculated *F* values and significance levels (*P* values) of these *F* values are presented in Table 23.

Results

Bioassays of field-grown foliage of soybean event DAS-81419-2 demonstrated excellent control of *C. includens*, *A. gemmatalis*, *S. frugiperda*, and *H. virescens* at both the R2 and R5 plant development stages (Table 23). Likewise, field evaluations indicated excellent control of *C. includens.*

Table 23. Efficacy of DAS-81419-2 soybean compared to 'Maverick'.

	DAS-81419-2	'Maverick'	Test	χ^2 or *F* value	*P*
Excised leaf bioassay at R2, 15 July 2011					
P. includens, total number of live 2nd instars, 4d*	0	64	χ^2	*128.0*	*<0.0001*
P. includens, average percent leaf consumption, 4d	0.50	46.28	*F*	*32.91*	*0.0105*
A. gemmatalis, total number of live 2nd instars, 4d**	0	48	χ^2	*96.00*	*<0.0001*
A. gemmatalis, average percent leaf consumption, 4d†	0.60	26.66	*F*	*278.5*	*0.0005*
S. frugiperda, total number of live 2nd instars, 4d*	0	64	χ^2	*128.0*	*<0.0001*
S. frugiperda, average percent leaf consumption, 4d†	1.55	27.68	*F*	*164.0*	*0.0010*
H. virescens, total number of live 2nd instars, 4d*	0	64	χ^2	*128.0*	*<0.0001*
H. virescens, average percent leaf consumption, 4d‡	0.52	31.73	--	--	--
Excised leaf bioassay at R5, 12 August 2011					
P. includens, total number of live 2nd instars, 4d*	0	63	χ^2	*127.0*	*<0.0001*
P. includens, average percent leaf consumption, 4d	0.50	32.20	*F*	*600.5*	*0.0001*
A. gemmatalis, total number of live 2nd instars, 4d**	0	44	χ^2	*81.23*	*<0.0001*
A. gemmatalis, average percent leaf consumption, 4d	0.50	20.00	*F*	*466.6*	*0.0002*
S. frugiperda, total number of live 2nd instars, 4d**	0	47	χ^2	*92.08*	*<0.0001*
S. frugiperda, average percent leaf consumption, 4d†	1.00	11.72	*F*	*1519*	*<0.0001*
H. virescens, total number of live 2nd instars, 4d**	0	45	χ^2	*84.71*	*<0.0001*
H. virescens, average percent leaf consumption, 4d†	0.57	15.98	*F*	*534.5*	*0.0002*
Sampling of natural infestation					
P. includens, average number of small larvae, 8 Aug	0.00	0.00	*F*	*0.000*	*1.0000*
P. includens, average number of large larvae, 8 Aug	0.00	2.25	*F*	*4.765*	*0.1170*
P. includens, average number of small larvae, 15 Aug	0.00	2.75	*F*	*5.418*	*0.1024*
P. includens, average number of large larvae, 15 Aug	0.00	3.50	*F*	*29.40*	*0.0123*
P. includens, average number of small larvae, 22 Aug	0.25	5.25	*F*	*75.00*	*0.0032*
P. includens, average number of large larvae, 22 Aug	0.00	17.50	*F*	*193.4*	*0.0008*
P. includens, average number of small larvae, 29 Aug	0.00	0.00	*F*	*0.000*	*1.0000*
P. includens, average number of large larvae, 29 Aug†	0.25	4.00	*F*	*7.784*	*0.0684*
Average percent defoliation, 29 Aug	0.00	2.50	*F*	*75.00*	*0.0320*

* 16 larvae tested per replicate, 64 larvae tested in total.
** 12 larvae tested per replicate, 48 larvae tested in total.
† Data were $ln(x+1)$ transformed to equalize treatment variances prior to Analysis of Variance, untransformed means are presented in the table.
‡ Data transformations were unable to equalize treatment variances.
C. includens (formerly *P. includens).*

In conclusion, laboratory bioassay of field grown event DAS-81419-2 soybean foliage, and evaluation of field infestations, demonstrate the efficacy of this event against four lepidopteran pests of soybean.

9.1.2. Potential Impact on Non-Target Organisms

A risk assessment was conducted to characterize the potential for adverse effects of cultivating DAS-81419-2 soybean, expressing Cry1Ac and Cry1F proteins, on non-target organisms (NTOs). The assessment focused on non-target arthropods associated with soybean agroecosystems with special emphasis on beneficial taxa and threatened or endangered species. Assessment factors included: established safety data for Cry proteins including data supporting previous deregulations for corn and cotton products expressing the Cry1Ac and Cry1F proteins, the expression levels of the Cry1Ac and Cry1F proteins in DAS-81419-2 soybean (Section 6), the environmental fate of the Cry1Ac and Cry1F proteins (Section 9.1.4), feeding tests of Cry1Ac and Cry1F proteins to representative NTOs, results from a field survey of non-target arthropods associated with DAS-81419-2, and information from key databases for threatened and endangered species. Results from the assessment indicate that DAS-81419-2 is unlikely to have adverse effects on non-target organisms, including threatened or endangered species, under normal agricultural practices.

Regulatory guidelines for risk assessment of genetically modified crops expressing plant-incorporated protectants (PIPs) were developed by the U.S. EPA (EPA, 1998), and followed by Scientific Advisory Panel (SAP) meetings (EPA, 2001c, 2001b, 2004), and publications that provide refinements applicable to Bt PIPs (EPA, 2007; Wolt *et al.*, 2010; Carstens *et al.*, 2011). The assessment for DAS-81419-2 focused on non-target arthropods associated with soybean agroecosystems using a step-wise process that included problem formulation, hazard identification and characterization, exposure assessment, and risk characterization. Problem formulation accounted for the familiarity of mode of action for Cry proteins (Aronson and Shai, 2001; Rang *et al.*, 2004; Lee *et al.*, 2006; Gouffon *et al.*, 2011; Liu *et al.*, 2011), the narrow spectrum of activity for Cry proteins (McClintock *et al.*, 1995; Romeis *et al.*, 2006; Marvier *et al.*, 2007; Duan *et al.*, 2008; Wolfenbarger *et al.*, 2008; Duan *et al.*, 2009), the demonstrated history of safe use for Bt cops (Naranjo, 2005; Sanvido *et al.*, 2007) and the agronomic equivalence of DAS-81419-2 soybean to conventional soybean (Section 8). The hazard characterization was informed by existing safety data for the Cry1Ac and Cry1F proteins, spectrum of activity data that were produced to support the deregulation and registration of Cry1Ac- and Cry1F-expressing WideStrike® cotton events DAS-21023-5 (also described as 3006-210-23) and DAS-24236-5 (also described as 281-24-236) (EPA, 2005), and lack of synergy between these two proteins (EPA, 2005). The exposure characterization was based on the expression analyses for Cry1Ac and Cry1F in DAS-81419-2 soybean (Section 6) and environmental fate of Cry proteins (Section 9.1.4). Special consideration for threatened or endangered species is addressed in Section 9.1.3.

Laboratory studies used Cry1Ac and Cry1F proteins that have been demonstrated to be equivalent with the Cry1Ac and Cry1F proteins produced by DAS-81419-2 (Section 6). Laboratory dose levels were based on Cry1Ac and Cry1F protein expression levels in WideStrike® cotton events DAS-21023-5 and DAS-24236-5, and therefore for the present assessment, the margins of exposure (MOE) for representative NTOs were recalculated based on high-end exposure estimates (HEEEs) (EPA, 2009) derived from Cry1Ac and Cry1F protein expression in DAS-81419-2. Based on the level of concern (LOC) of 50% mortality at 10× the estimated environmental concentration (EEC) (EPA, 2007), and HEEEs for Cry1Ac and Cry1F in DAS-81419-2 cropping systems, the resulting MOE values support the conclusion of

negligible risk to NTOs representing diverse taxonomic groups and exposure scenarios (Table 24).

Table 24. Estimated margins of exposure (MOE) to non-target organisms based on high-end exposure estimates (HEEE) for Cry1Ac and Cry1F proteins produced in DAS-81419-2.

Organism	Protein[1]	HEEE[2]	NOEC[3]	MOE[4]
Mouse	Cry1Ac	28.33 ng/mg	> 700 mg/kg	> 100
(CD-1)	Cry1F	63.32 ng/mg	> 600 mg/kg	> 100
Earthworm[5]	Cry1Ac	0.0910 mg/kg soil	107 mg/kg soil	> 100
(*Eisenia fetida*)	Cry1F	0.345 mg/kg soil	247 mg/kg soil	> 100
Collembola[5]	Cry1Ac	0.0910 mg/kg soil	> 22.6 mg/kg diet	> 100
(*Folsomia candida*)	Cry1F	0.345 mg/kg soil	> 702 mg/kg diet	> 100
Lady beetle	Cry1Ac	4.76 ng/mg fw	> 20.5 ng/mg fw	> 4
(*Hippodamia convergens*)	Cry1F	10.6 ng/mg fw	> 273 ng/mg fw	> 25

[1] Microbe-derived Cry1Ac and Cry1F proteins used in laboratory studies have been demonstrated to be equivalent with the Cry1Ac and Cry1F proteins expressed in DAS-81419-2 (Section 6).

[2] High-End Exposure Estimate (HEEE) represents the 90% upper bound of the reported expression; HEEE = mean expression concentration + ($t_{0.1,\ \text{upper tail},\ n-1}$ × std. dev.) / $n^{1/2}$; HEEE values based on Cry1Ac and Cry1F expression level determined for DAS-81419-2 plant tissue relevant to the potential exposure route for the organism of interest; values for earthworm and Collembola are expressed as estimated environmental concentrations for soil (soil EECs) based on the HEEEs for forage tissue at the R3 growth stage; values for mice and lady beetles were based on leaf expression at the V5 growth stage.

[3] No Observed Effect Concentration (NOEC).

[4] Margin of Exposure (MOE) was calculated based on the ratio of the NOEC to HEEE.

[5] The MOE values for earthworm and Collembola were calculated using the following parameter assumptions: 175,000 soybean plants/acre, soybean plant dry weight of 71.2 g/plant, soil bulk density of 1500 kg/cubic meter, soil volume in a one-acre area with a depth of 0.15 m, and Cry1Ac and Cry1F soil EEC values of 6.65 and 25.22 ng/mg dw, respectively, for forage tissue at the R3 growth stage.

The estimated MOEs ranged from approximately 4 to greater than 100. Where the MOE for lady beetles (>4×) in the Cry1Ac test was not demonstrated to be greater than 10× NOEC, the potential risk was examined in the context of the known spectrum of activity of Cry1Ac being restricted to the order Lepidoptera, an additional high-dose laboratory study of the Cry1Ac protein against lady beetles (Li *et al.*, 2011) as well as data for coccinellids monitored in DAS-81419-2 field trials that demonstrated no adverse effects on lady beetle performance and abundance, respectively. Given no detrimental effects were observed in independent laboratory and field studies, risk to lady beetles was concluded to be negligible.

A lack of adverse effects following exposure to Cry1Ac and/or Cry1F proteins has also been shown for collembolans (*Folsomia candida* and *Xenylla grisea*) (Sims and Martin, 1997), the minute pirate bug (*Orius albidipennis*) (González-Zamora *et al.*, 2007), green lacewing (*Chrysopa carnea*) (Rodrigo-Simón *et al.*, 2006), hymenopteran parasitoids (*Nasonia vitripennis*), honeybees (*Apis mellifera*), and bobwhite quail. Agreement between laboratory and field observations for transgenic Bt proteins (Duan *et al.*, 2009) has been supported through

meta-analyses of NTO data at the order- (Naranjo, 2009) and species-level (Duan *et al.*, 2008). The meta-analysis for honeybees conducted by Duan *et al.* (2008) included six studies incorporating Cry1Ac or Cry1F (truncated or full length protein), suggesting that negative effects on pollinators are unlikely given exposure to the Cry1Ac and Cry1F proteins expressed in DAS-81419-2.

To confirm previous laboratory findings and to address any uncertainties in the risk assessment focused on taxonomic groups that were most likely to be exposed to the Cry proteins produced by DAS-81419-2 soybean in field settings, the potential for adverse effects of DAS-81419-2 on non-target arthropod populations was assessed in a US field study. Field study details are provided in Appendix 8. Assessment of population abundance and composition was conducted for several taxonomic groups, including: Oribatida (soil mites), Araneae (foraging and web spiders), Collembola (springtails), Hemiptera (e.g. *Orius* spp.), Coleoptera (e.g. ground beetles, lady beetles), Neuroptera (e.g. *Chrysopa* spp.), Lepidoptera, Diptera, and Hymenoptera (e.g. parasitoid wasps). Consistent across study site locations and sampling methods, arthropod populations associated with DAS-81419-2 were similar to those of non-transgenic soybean. Field monitoring confirmed the results of laboratory studies examining direct exposure to Cry1Ac and Cry1F proteins, and demonstrated no adverse effects of DAS-81419-2 cultivation on non-target arthropod communities. Field trial results for DAS-81419-2 contribute to a series of studies demonstrating no adverse effects of Bt crops on NTOs across a diversity of receiving environments (Pilcher *et al.*, 1997; Lozzia *et al.*, 1998; Bhatti *et al.*, 2005; Bitzer *et al.*, 2005; Daly and Buntin, 2005; Dively, 2005; Head *et al.*, 2005; Lopez *et al.*, 2005; Naranjo, 2005; Pilcher *et al.*, 2005; Torres and Ruberson, 2005; Whitehouse *et al.*, 2005).

The Cry1Ac and Cry1F proteins expressed in DAS-81419-2 have been previously demonstrated to have no adverse effects on fish (rainbow trout) or aquatic invertebrates (e.g. *Daphnia magna*). Risk posed to aquatic invertebrates (including the aquatic Lepidoptera) through DAS-81419-2 cultivation was concluded to be negligible due to a low likelihood of exposure (Carstens *et al.*, 2011) and no verified instances of sensitive species. Therefore, the existing studies in *Daphnia* and rainbow trout were considered to be adequate in supporting the conclusion that DAS-81419-2 deployment poses little risk to aquatic organisms.

9.1.3. Potential Impact on Threatened and Endangered Species

Given the narrow spectrum of activity observed for Cry proteins (McClintock *et al.*, 1995; Romeis *et al.*, 2006; Marvier *et al.*, 2007; Wolfenbarger *et al.*, 2008; Duan *et al.*, 2009) consideration for threatened and endangered (T/E) species in the assessment for DAS-81419-2 focused on taxa within the order Lepidoptera.

Proximity relationships between potential growing sites where DAS-81419-2 could be planted and locations of T/E lepidopteran species were determined from the information contained in the FIFRA Endangered Species Task Force (FESTF) databases. Ecology and life history information for species identified as being present in soybean-growing counties was accessed from the US Fish and Wildlife Service and NatureServe Explorer databases (http://www.natureserve.org/explorer/).

Four lepidopteran butterfly species (Karner blue, *Lycaeides melissa samuelis*; Mitchell's satyr, *Neonympha mitchellii mitchellii*; Saint Francis' satyr, *Neonympha mitchellii francisci*; and

Uncompahgre fritillary, *Boloria acrocnema*) were indicated to co-occur with soybean use sites in 66 counties located in 13 states. Ecology and life history information demonstrated that habitat requirements for larvae and adults of three of the identified species (Mitchell's satyr, Saint Francis' satyr, and Uncompahgre fritillary) do not overlap with commercial soybean acreage, and are therefore not expected to be impacted by DAS-81419-2 cultivation. There is a possible overlap between the geographic range for Karner blue butterfly and soybean use sites; however, the potential for direct exposure to the Cry proteins produced by DAS-81419-2 soybean is negligible. Karner blue larvae feed only on lupines (*Lupinus* spp.) and soybean is neither identified as a larval food source, nor is soybean open-pollinated so DAS-81419-2 soybean pollen is highly unlikely to be deposited on lupine leaves. Furthermore, soybean is not identified as a nectaring source for Karner blue adults. Therefore, the risk posed to the Karner blue butterfly was considered to be negligible. In agreement with previous T/E species assessments for Cry1-containing corn (Herculex®) and cotton (WideStrike®) products, the assessment for DAS-81419-2 supports a conclusion of negligible risk to T/E species.

9.1.4. Environmental Fate of Introduced Proteins and Impact on Soil-dwelling Organisms

Proteins are not known to persist in the environment due to the ubiquitous nature of proteases in microbes. The Cry1Ac protein has been used in sprayable Bt formulations for over half a century and is present in both WideStrike® cotton event DAS-21023-5 (also described as 3006-210-23) and Bollgard® cotton (event MON 531, as well as a discontinued corn event: DBT418Cry1Ac corn) (Mendelsohn *et al.*, 2003; Sanahuja *et al.*, 2011). The Cry1F protein has also been used in sprayable Bt formulations and is present in WideStrike® cotton event DAS-24236-5 (also described as 281-24-236), as well as, in Herculex® and SmartStax® corn (event TC1507) (Mendelsohn *et al.*, 2003; Sanahuja *et al.*, 2011). Laboratory experiments have shown that both Cry1Ac and Cry1F are quickly inactivated, and field studies have shown no accumulation of the proteins as a result of continuous planting of crops containing these proteins (Head *et al.*, 2002; Herman *et al.*, 2002; Shan *et al.*, 2008). It has further been suggested that the Cry proteins in general are unlikely to represent a significant risk in the soil environment (Icoz and Stotzky, 2008).

9.1.5. Weediness Potential

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

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

® Bollgard and SmartStax are registered trademarks of Monsanto Technology LLC.

The introduction of insecticidal proteins Cry1Ac and Cry1F into soybean does not alter the weediness characteristics of soybean. Agronomic properties of DAS-81419-2 soybean related to weediness, such as emergence (Section 8.1), seedling vigor (Section 8.1), response to environmental stressors (Section 8.2), and germination (Section 8.3), have been shown to be equivalent to conventional soybean. These findings support the conclusion that DAS-81419-2 soybean does not have increased weediness potential compared to conventional soybean.

9.1.6. Gene Flow Assessment

Vertical Gene Flow

Soybean is considered to be a self-pollinated species, although natural crossing can occur at low rates (OECD, 2000). The level of cross pollination can vary as a function of growing conditions, spatial arrangement of plants, and genotype. Cross pollination levels ranging from 0.09% up to 3.6% in adjacent rows have been reported (Beard and Knowles, 1971; Ahrent and Caviness, 1994), but outcrossing frequency rapidly declines with distance. Cross pollination rates have been reported to be <0.1% at distances of >5 m (Caviness, 1966; Ray *et al.*, 2003). Cultivated soybeans can cross only with members of its subgenus *Soja*. Wild soybean species of the subgenus *Soja* are native to Korea, Japan, Taiwan, northeastern China, and areas of the former USSR, but do not exist naturally in the United States (OECD, 2000). Therefore, there is no potential for gene flow from DAS-81419-2 soybeans to wild soybean relatives in the United States.

Horizontal Gene Flow

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Conner *et al.*, 2003). Even if such a transfer were to take place, transfer of the *cry1Ac (synpro), cry1Fv3* and *pat* genes from DAS-81419-2 soybean would not present a human health or plant pest risk, based on the safety data presented in this petition.

9.2. Current Agronomic Practices for Soybean and Potential Impact

9.2.1. Soybean Production

Large scale soybean production in the U.S. began in the early 1920s (USDA ERS, 2012). According to USDA NASS (2012), approximately 1.5 million acres were planted to soybean in 1924. Over the following two decades soybean acreage expanded rapidly reaching 15 million acres. Since 1997 more than 70 million acres were planted to soybean every year with an exception in 2007 when an increase in corn acreage limited soybean cropland. This increase in soybean acreage is largely due to ideal planting conditions, increased crop rotation, rising yield, and lower production costs (USDA ERS, 2012). Average soybean yield per acre varied from 33.9 bushels to 44 bushels between 1997 and 2011 with the lowest yield recorded in 2003 and the highest in 2009. Total annual soybean production during the same time period ranged from approximately 2.45 billion bushels in 2003 to 3.36 billion bushels in 2009. Farm value of U.S. soybean production ranged from $12.21 billion in 1999 to $37.55 billion in 2010 (USDA NASS, 2012). An average of 1.6 billion bushels of soybean were crushed annually in the last ten years to produce soybean meal for livestock and soybean oil for consumption and industrial products (Soy Stats, 2012).

In the U.S. the majority of soybeans are grown in the north central states with the remaining crop primarily grown in the southeastern and Mississippi-delta regions. About 80% of the planted soybean acres and 80% of the harvested soybean acres are concentrated in 11 states, including Iowa, Illinois, Minnesota, Indiana, Missouri, Nebraska, Ohio, South Dakota, North Dakota, Kansas and Arkansas, where growers achieve higher yields at lower per-acre production cost (USDA ERS, 2012). Soybean yield in the north central region in 2011 ranged from 27 bushels per acre in Kansas to 53.5 bushels per acre in Nebraska (USDA NASS, 2012). Soybean yield is largely dependent on variety selection, environmental conditions and agronomic decisions. Variety selection is critical in achieving high yield while environmental conditions and agronomic decisions such as weeds and disease management and fertilizer application have a significant impact on genetic yield potential. In 2011, seed, chemicals and fertilizers contributed to 70% of the operating cost (USDA ERS, 2012). In the southern U.S. states higher fertilizer and chemical insecticide applications per acre soybean planted contributed to higher operating cost (Soy Stats, 2012).

U.S. is the world's leading soybean exporter. In 2011, U.S. exported 1.275 billion bushels of soybean which accounted for 42% of the U.S. production and 37% of the world's soybean trade (Soy Stats, 2012; USDA ERS, 2012). U.S. soybean and soybean product exports exceeded $21.5 billion in 2011 with soybean accounting for 82%, soybean meal 13% and soybean oil 6% (Soy Stats, 2012). China was the largest export market for U.S. soybean with purchases exceeding $10.4 billion. Mexico was the second largest market for U.S. soybean with purchases of nearly $1.6 billion. Other significant export markets for U.S. soybean are Japan, Indonesia and Taiwan. Canada was the largest export market for U.S. soybean meal in 2011 with purchases reaching $375 million, Mexico was second with $369 million followed by Venezuela at $260 million. The top three export markets for U.S. soybean oil were Morocco, Mexico and China with combined purchases exceeding $659 million (Soy Stats, 2012).

Soybean seed for commercial planting is produced throughout most of the U.S. soybean growing regions. Soybean varieties are developed and adapted to certain geographical zones and are separated into ten maturity groups (OECD, 2000). Seed production for these maturity groups is grown in respective geographical zone for each maturity group.

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 seed 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 so handled as 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.

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 (7 CFR §201.72). Federal regulation 7 CFR §201.76 specifies minimum land, isolation, field, and seed standards required for soybean Foundation, Registered and Certified seed. To produce all classes of certified seed, the land requirement is that the crop shall not be grown on land where soybeans were grown the previous year unless the preceding soybean crop was planted with a class of certified seed of the same variety or unless the preceding soybean crop and the variety being planted have an identifiable character difference. Isolation requirement varies somewhat among states and seed producers. Some states require an isolation distance of five feet while other states and seed producers require 10 feet of isolation distance. To qualify as Certified seed, only one plant of another variety in 200 soybean plants and 0.5% of seed of other varieties or off-types are permitted (7 CFR §201.76). To qualify as Registered seed, only one plant of another variety in 500 soybean plants and 0.2% of seed of other variety or off-types are permitted. To be certified as Foundation seed, only one plant of another variety in 1,000 soybean plants and 0.1% of seed of other variety or off-types are permitted.

DAS-81419-2 does not differ from conventional soybean in agronomic characteristics (Section 8) and thus is not expected to impact U.S. soybean grain or seed production.

9.2.2. Weeds in Soybean and Weed Management

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

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

competition could reduce soybean yields by the time weeds reached 6, 6, and >12 inches in height in 7.5, 15, and 30 inch rows, respectively.

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

In 2006, USDA NASS surveyed 19 states (Arkansas, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Michigan, Minnesota, Mississippi, Missouri, Nebraska, North Carolina, North Dakota, Ohio, South Dakota, Tennessee, Virginia, and Wisconsin; about 96% of the total U.S. soybean acreage) and found that 98% of the planted soybean acreage was treated with herbicides, an indication of the intensive weed management that is used in U.S. soybeans (USDA NASS, 2007). In 2006, 97.5% of the planted soybean acres received an average of 2.2 herbicide applications to control weeds (USDA ERS ARMS, 2006). Many herbicides are registered for pre-plant, pre-emergent and/or post-emergent application to selectively control most weed species commonly found in soybeans. In general, soybeans sometimes receive a soil applied or burndown herbicide prior to planting or at plant, but almost always receive a post-emergence herbicide application. In 2006, the most widely used herbicide in soybeans was glyphosate, driven by the high adoption of glyphosate tolerant soybeans (USDA NASS, 2007). Soybeans received an average of 1.7 applications of glyphosate on 96% of the acres planted in 2006. Herbicides such as 2,4-D (10% of acres), chlorimuron (4% of acres), and trifluralin (2% of acres) were applied on significantly fewer acres. All other herbicides applied in soybeans that year comprised 3% or less of the total acres planted. Integrated Weed Management (IWM) programs advocate the use of a combination of preventive, cultural, mechanical and chemical tools to keep weed pressure below threshold levels that reduce yield and profits (Knezevic, 2010).

DAS-81419-2 soybean expresses the phosphinothricin acetyltransferase (PAT) protein and therefore it is tolerant to the herbicide glufosinate. With the exception of glufosinate tolerance, no impact is expected from the deregulation of DAS-81419-2 soybean on current weed management practices.

DAS-81419-2 soybean expresses the phosphinothricin acetyltransferase (PAT) protein and therefore it is tolerant to the herbicide glufosinate. As glufosinate tolerance in soybeans is currently available today to U.S. growers, no impact is expected from the deregulation of DAS-81419-2 soybean on current weed management practices.

9.2.3. Crop Rotation and Tillage Practices

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

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

Given the data presented in this petition demonstrating no difference between DAS-81419-2 and conventional soybean in agronomic characteristics (Section 8), no impact is expected from the deregulation of DAS-81419-2 soybean on current crop rotation and tillage practices.

9.2.4. Management of Insect Pests

Agricultural production of soybean is a relatively recent development; soybean did not become a major export crop in North America until the 1970s (Smith, 1994). Insect injury has not traditionally resulted in economic losses in most North American growing regions. However, in recent years, losses have been severe in some southern regions of the United States (Catchot, 2011). Economic thresholds, which are the level of insect pressure where the benefits of treatment cover the cost of that treatment, have traditionally been based on $4-6 per bushel soybeans. However, recent prices of $12-15 per bushel have significantly lowered economic thresholds associated with many soybean pests, expanding the area and number of insecticide treatments applied to soybeans (USDA ERS, 2011). Increases in pest insect populations in soybean have also been observed; increases are attributed by some to global warming or insect adaptation to the soybean plant (Way, 1994). Lower economic thresholds coupled with higher insect populations favor more aggressive pest management strategies (Pedigo, 1996).

Insect injury can negatively impact yield, plant maturity, and seed quality. Insect injury is most often defined by the plant parts that are injured: roots, stems, foliage, or pods. Root- and stem-feeding insect groups typically remain undetected until damage has occurred. Although leaf-feeding insects are the most diverse group, pod-feeding insects are generally considered to be proportionately more important, because high-value soybean reproductive parts are affected.

Many soybean foliar feeders are caterpillars (Lepidoptera larvae) in the family Noctuidae. These include the soybean looper, *Chrysodeixis includens* formerly *Pseudoplusia includens* Walker, velvetbean caterpillar, *Anticarsia gemmatalis* Hübner and green cloverworm, *Hypena scabra* (Fab.). These species commonly occur sympatrically and procedures are being developed for making management decisions that acknowledge combined effects of these pests.

A major effect of foliar feeding is reduced light interception by the soybean canopy; which ultimately decreases yield (Higley, 1994). Soybean plants can withstand up to 35% leaf loss pre-bloom without lowering yield (de Freitas Bueno *et al.*, 2011). However, defoliation during the flowering and pod-filling stages poses a greater threat to yield because the plant has less time to compensate for injury. From pod-set to maturity, the plant can tolerate only a 20% defoliation

level before yield is affected (de Freitas Bueno *et al.*, 2011). The tolerance of soybeans for defoliation varies somewhat depending on cultivar (de Freitas Bueno *et al.*, 2011).

In addition to the plant tissue attacked, soybean responses to injury are influenced by the mode of feeding. For example, yield loss incurred from pod-feeding stink bugs, such as the southern green stinkbug *Nezara viridula* (L.) (Hemiptera: Pentatomidae), is linear in relation to the amount of plant tissue damage caused by feeding. In contrast, for seedcorn maggot, *Delia platura* (Meigen) (Diptera: Anthomyiidae), larval feeding on roots reduces yield only when injury levels are relatively high (Higley, 1994). Environmental factors also impact the response of soybean to insect injury; yield reductions from insect injury are more severe under conditions associated with water stress.

Although soybean is attacked by many pests, only a few pose an economic threat. Damage is concentrated in certain production regions (Way, 1994). Eight insect species are responsible for most insect damage in U.S. soybean production: velvetbean caterpillar, soybean looper, green cloverworm, Mexican bean beetle, *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae), southern green stinkbug, bean leaf beetle, *Cerotoma trifurcata* Forster (Coleoptera: Coccinellidae), southern green podworm/corn earworm, *Helicoverpa zea* (Boddie) (formerly *Heliothis zea*) (Lepidoptera: Noctuidae), and green stink bug (Higley and Boethel, 1994). Soybean insect pest populations and extent of soybean damage varies annually and regionally due to differences in climatic and weather conditions, species distributions and environmental tolerances and production practices (Way, 1994).

Generally, soybean insect pest problems are less severe in the Midwest states of the U.S. than in other soybean producing areas (Way, 1994). Green cloverworm is the only known lepidopteran pest that occurs frequently in the Midwest; cutworms, *Agrostis ipsilon* (Hufnagel) and *Peridroma saucia* (Hübner) (Noctuidae) and Painted Lady, *Vanessa cardui* L. (Nymphalidae) have also been reported but are less commonly economically important. Economic insect problems increased in the 1990s (Hammond, 2012) with greater increases in the north-central U.S. around 2000 (McWilliams *et al.*, 1999). Generally, insect challenges still remain below economic threshold levels for the U.S. Midwest.

Several insect pests have been reported in the northeastern United States and Southern Canada in numbers high enough to cause economic losses in soybean (Penn State Extension, 2012). Defoliators include: green cloverworm, Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae), Mexican bean beetle, bean leaf beetle, and grasshoppers, *Melanoplus spp.* (Orthoptera: Acrididae). Sap-sucking insects include: soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), potato leafhopper, *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae) and stink bugs. Stink bugs in particular will feed on both foliage and pods. Seedcorn maggots feed on seeds.

Insect pressure is generally greatest in the southern U.S. states bordering the Gulf of Mexico and the Atlantic Ocean. Four of the major soybean insect pests in the Southeast region are lepidopteran insect pests. Velvetbean caterpillar and soybean looper infestations are greatest in the southeastern states because of their close proximity to the tropics. The warm climate allows these pests to overwinter and facilitates multiple generations per year.

Stink bugs (*Nezara viridula, Acrosternum hilare, Euschistus servus*) are responsible for most infestations and greatest economic losses in the southeastern United States (McPherson *et al.*, 1997; Guillebeau *et al.*, 2008). Lepidopteran insects, primarily soybean looper, velvetbean caterpillar, corn earworm (soybean podworm), and lesser cornstalk borer, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae) are responsible for most of the remaining damage (McPherson *et al.*, 1997; Guillebeau *et al.*, 2008). Velvetbean caterpillar and soybean looper are considered the most damaging defoliating insects in the South (Way, 1994; Hammond, 2012). Insecticides are used on approximately 50% of the soybean acreage in Georgia for lepidopteran pests with velvetbean caterpillar being the most targeted pest (Gianessi *et al.*, 2002). Approximately 40% of the soybean acreage in Louisiana is treated with insecticides for lepidopteran pests, with soybean looper being the main target (Gianessi *et al.*, 2002).

Although insecticides provide effective and economical control or suppression of soybean insect pests that reach economic thresholds, Integrated Pest Management (IPM) programs that incorporate preventive pest management with insecticidal control have proven to be highly effective in managing pest problems (Pedigo, 1996; Kogan, 1998). Effective management of insects in soybean requires consideration of agronomic, economic and biological factors. For example, preventive pest management practices are most important where pest problems are anticipated. Agricultural practices can be adjusted to adversely affect pest species or aid beneficial species. Similarly, variety selection, crop rotation, tillage, planting dates, and adjacent crops all impact the potential for pest outbreaks and the success of natural enemies in suppressing pest populations.

As it is not possible to eliminate all economic losses due to insects, IPM programs are implemented to minimize these losses. IPM practice includes monitoring fields for insect growth stage, insect development and population density, and occasionally natural enemy development and population density. Management decisions for insect populations in individual fields are based on economic injury level, which is defined as the lowest population density of each insect likely to cause economic damage. The economic injury level usually changes during the growing season, with later stages of development warranting action at a lower level of defoliation. IPM programs integrate chemical control and biological control, cultural control and plant resistance to minimize insecticide resistance and reduce dependence on insecticides (Pedigo, 1996). Effective IPM requires intense insect scouting and is justified in high value crops or where insect pressure is common. However, the majority of soybean fields are not scouted because damage is infrequent. Failure to scout risks allowing damage that greatly exceeds the economic threshold to occur unnoticed; discovery may occur too late for subsequent insecticide applications to be effective.

The growing problem of soybean insect pest pressure is evidenced by increasing insecticide treatments in recent years (USDA NASS, 2007). According to USDA-NASS statistics, about 16% of the U.S. soybean acreage in 2006 received an insecticide treatment, with chlorpyrifos (6%), esfenvalerate (5%), and lambda-cyhalothrin (3%); these accounted for nearly all treated acres (USDA NASS, 2007). Each of these insecticides controls a similar broad spectrum of insect pests including lepidopteran and non-lepidopteran pests. USDA-NASS (2007) also reported that approximately 15% of the soybean acreage in the Midwest region and approximately 25% of the soybean acreage in the Southeast and Eastern Coastal regions of the

U.S. received an insecticide treatment in 2006 with some states in the Southeast requiring treatments on up to 75% of their acreage (USDA NASS, 2007)

Chemical insecticides can have limited efficacy in controlling lepidopteran infestations in soybean. Narrow application windows, the emergence of insecticide resistance, and public pressure for reduced pesticide use limit the desirability of this approach to pest management (Thomas and Boethel, 1994). Soybean looper has developed extensive insecticide resistance (Thomas and Boethel, 1994); resistance to pyrethroids is widespread across the southern U.S. (Felland *et al.*, 1990; Leonard *et al.*, 1990). Insecticides remain effective against velvetbean caterpillar. However, infestations can quickly reach damaging levels and cause economic loss if insecticides are not applied promptly.

Bt crops (expressing at least one of several insecticidal proteins isolated from *Bacillus thuringiensis*) are effective in targeting specific insect pests, easy to adopt, and cost effective; Increasing adoption rates of Bt corn and cotton since 1996 have coincided with increased yields (USDA ERS, 2006). Pesticide use has also decreased since the introduction of these insect-resistant crops, with one study showing an 8% reduction in insecticide use for adopters of Bt corn than non-adopters (USDA ERS, 2006). Importantly, Bt crops have also be credited with increasing yields, even when economic thresholds have not been reached in either Bt- or non-Bt-fields due to regional insect population reductions (Hutchison *et al.*, 2010).

Like Bt corn and cotton, DAS-81419-2 soybean offers an efficient and environmentally sound alternative to chemical insecticides for controlling lepidopteran soybean pests. Genetically modified crops are now an important component of many IPM programs, providing effective management of lepidopteran pests with reduced insecticide use (Perlak *et al.*, 2001; Pilcher and Rice, 2003). Additional benefits from DAS-81419-2 soybean include a reduced need for scouting, preservation of beneficial insect populations, increased convenience and greater performance consistency.

9.2.5. Insect Resistance Management

DAS-81419-2 soybean expresses two insecticidal proteins, Cry1Ac and Cry1F, originally from the naturally occurring soil bacterium *Bacillus thuringiensis*. Data presented in this petition demonstrate that Cry1Ac and Cry1F are expressed in the tissues of the field-grown DAS-81419-2 soybean throughout the growing season. DAS-81419-2 soybean provides an effective pest management tool while reducing or replacing current insecticide applications to control lepidopteran pests in regions where these insect pests cause significant plant damage and yield loss.

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA), are subject to regulation by the U.S. Environmental Protection Agency (EPA). Pesticides produced in planta, known as plant-incorporated protectants (PIPs), are also subject to regulation by the EPA under FIFRA. To prevent or delay the onset of insect resistance to Bt proteins expressed in transgenic crops, EPA has mandated specific insect resistance management (IRM) programs to protect the benefits of Bt crops and sustainable long-term use of Bt crops (EPA, 2001a). EPA requires that an IRM plan is based on the characteristics of the crop, the biology and sensitivity of the target insect pests, and the cropping systems in which the product will be deployed. A core element of an IRM program is the use of

refuges, which are non-Bt host plants of the target insects that support susceptible target insects and reduce selection pressure for resistance. Susceptible insects produced from the refuge may mate with any resistant insects that survive in the Bt crop producing heterozygote insects that may be susceptible to the Bt crop (EPA, 2001a). Given that the registration DAS is currently seeking from EPA for planting of DAS-81419-2 soybean in the U.S. will be limited in acreage for breeding and seed increase purposes to support product development, DAS has proposed that planting of a refuge is not necessary and an IRM program for DAS-81419-2 soybean is not needed. The proposal is based on previous registration decisions, impact on the natural refuge for key target pests of Bt cotton, durability of DAS-81419-2 soybean, and target pest biology. A summary of the analyses is presented below. Detailed analyses will be provided to EPA in connection with the FIFRA Section 3 seed increase registration application.

In previous registration decisions, EPA indicated that cultivation of crops containing PIPs for seed production on up to 20,000 acres per county and 250,000 acres total does not require planting of a refuge (for example, Herculex® I corn, EPA registration number 68467-2 as amended on September 29th, 2010). Similarly, on July 27th 2012, EPA issued registrations under FIFRA Section 3 that permit cultivation of each Bt cotton, Cry1Ac Insect Protected cotton seed (EPA registration number 68467-17) or Cry1F Insect Protected cotton seed (EPA registration number 68467-18), on up to a total of 20,000 acres per county and up to a combined total of 30,000 acres per year without planting of a refuge for breeding, agronomic testing and seed production purposes.

In the U.S., the majority of the soybeans are grown in the north central states and the remaining in the southeastern and Mississippi delta regions. In the southern and southeastern U.S., soybean is a component of the *Helicoverpa zea* (bollworm) natural refuge for Bt cotton (Jackson *et al.*, 2008) and DAS-81419-2 soybean is expected to cause reduced survival of *H. zea* larvae feeding on it relative to non-Bt soybean. EPA has oversight of insect resistance management for Bt crops in the U.S. Through the terms of registration for DAS-81419-2 soybean, EPA will impose resistance management requirements to promote the durability of Bt traits in cotton, corn, and soybean.

DAS-81419-2 soybean expresses two insecticidal proteins whose modes of action differ with respect to receptor binding. For example Cry1Ac and Cry1F have been shown to bind to different receptors in the midgut of the target soybean insect pest tobacco budworm *H. virescens* (Jurat-Fuentes and Adang, 2001). Cry1Ac binds to at least three sets of receptors while Cry1F binds to at least two, only one of which also binds Cry1Ac. The major receptor for Cry1Ac is not recognized by Cry1F (Jurat-Fuentes and Adang, 2006). Such incomplete shared binding is expected to lead to incomplete cross-resistance when resistance is mediated by receptor changes. Bt gene pyramiding offered by DAS-81419-2 soybean provides additional protection against the development of insect resistance and offers greater durability than Bt crops carrying a single Bt trait.

In addition to *H. zea*, other lepidopteran pests of soybean that are sensitive to the Cry1F and/or Cry1Ac Bt proteins, such as soybean looper (*Chrysodeixis includens,* formerly *Pseudoplusia includens*), velvetbean caterpillar (*Anticarsia gemmatalis*), and fall armyworm (*Spodoptera frugiperda*), are polyphagous, feeding on numerous wild and cultivated plants (Sparks, 1979; Waters and Barfield, 1989; Jost and Pitre, 2002). With other Bt crops grown for breeding and

seed increase purposes, plantings of up to 20,000 acres per county are permitted by EPA without a refuge because with these limits the selection pressure for resistance in sensitive insect species is negligible. The natural refuge of non-Bt crops and wild hosts for these polyphagous species that are pests of soybean further reduces selection pressure for resistance.

The information presented here indicates that planting of DAS-81419-2 soybean under limited acreage for breeding and seed increase purposes in the U.S. will have very little effect on resistance development in *H. zea* to Bt cotton. Furthermore, the level of selection pressure on other lepidopteran pests of soybean will be negligible under the proposed acreage limits. We therefore anticipate that EPA will not require a structured refuge for DAS-81419-2 soybean under the conditions of use. If DAS chooses to cultivate DAS-81419-2 soybean for commercial purposes in the U.S. in the future, DAS will apply for a FIFRA Section 3 commercial use registration with EPA and will develop a specific IRM program in connection with the commercial use registration.

Under the terms of the EPA's registrations for commercial cultivation of Bt cotton and Bt corn, DAS is required to monitor for resistance in several lepidopteran pests of cotton that are also pests of soybean. This monitoring will continue after the introduction of DAS-81419-2 soybean.

9.3. Summary of Impact on Environment and Agronomic Practices

The efficacy study demonstrates that Cry1Ac and Cry1F expressed in DAS-81419-2 soybean provide protection against target lepidopteran pests. There are no new phenotypic characteristics in DAS-81419-2 soybean to indicate it is any different from conventional soybean in weediness potential, and like conventional soybean, the risk of gene flow from DAS-81419-2 soybean to wild relatives in the U.S. is negligible.

No significant impact is expected on current crop management practices, non-target or endangered species, or crop rotation from the introduction of DAS-81419-2 soybean. DAS-81419-2 offers an efficient and environmentally sound alternative to chemical insecticides. In addition, DAS-81419-2 offers greater durability and therefore improved insect resistance management. The availability of DAS-81419-2 is expected to reduce the need for scouting, preserve beneficial insect population, and provide increased convenience and greater performance consistency.

10. Adverse Consequences of Introduction

Field and laboratory testing of DAS-81419-2 soybean demonstrated that the transgenic soybean is substantially equivalent to non-transgenic conventional soybean apart from the intended change of insect resistance. DAS knows of no study results or other observations associated with DAS-81419-2 soybean that indicate there would be anticipated to be adverse consequences from introduction.

11. Appendices

Appendix 1. Materials and Methods for Molecular Characterization of DAS-81419-2 Soybean

Appendix 2. Materials, Methods and Results for Characterization of Cry1Ac Protein

Appendix 3. Materials, Methods and Results for Characterization of Cry1F Protein

Appendix 4. Materials, Methods and Results for Characterization of PAT Protein

Appendix 5. Materials and Methods for Cry1Ac, Cry1F and PAT Protein Expression Analysis

Appendix 6. Analytical Methods for Compositional Analysis

Appendix 7. Literature Ranges for Compositional Analysis

Appendix 8. Non-Target Arthropod Field Study

Appendix 9. USDA Notifications for DAS-81419-2 Soybean

Appendix 10. Stewardship of Insect-Resistant DAS-81419-2 Soybean

Appendix 11. References

Appendix 1. Materials and Methods for Molecular Characterization of DAS-81419-2 Soybean

DAS-81419-2 Soybean Material

Transgenic soybean seeds from five distinct generations of soybean containing event DAS-81419-2 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 assay according to the manufacturer's instructions (Envirologix Inc.). Each plant was given a "+" or "-" for the presence or absence of the PAT protein.

Control Soybean Material

Seeds from the non-transgenic soybean line Maverick were planted in the greenhouse. The Maverick seeds had a genetic background representative of the transgenic seeds but did not contain the *cry1Ac(synpro)*, *cry1Fv3,* or *pat* genes.

Reference Materials

DNA of the plasmid pDAB9582 was added to samples of the non-transgenic control genomic DNA at a ratio approximately equivalent to 1 copy of the transgene per soybean genome with a soybean genome size of ~1.1×10^9 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 pDAB9582. 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 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 individually ground in liquid nitrogen followed by addition of CTAB extraction buffer (~5:1 ratio milliliter CTAB extraction buffer: gram leaf tissue) and RNase-A (>10 µL) (Qiagen). After approximately 1 hour of incubation at ~65°C with gentle shaking, samples were spun down and the supernatants were extracted with equal volumes of chloroform:octanol = 24:1 (Sigma). DNA was precipitated by mixing the supernatants with equal volumes of CTAB precipitation buffer. The precipitated DNA was dissolved in high salt TE buffer followed by precipitation with isopropyl alcohol. The precipitated DNA was rinsed with 70% ethanol, air-dried, and dissolved in appropriate volume of 1 × TE buffer (pH 8.0). The DNA was quantified with 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 soybean leaf tissue was digested with restriction enzymes by combining approximately 10 μg of genomic DNA with approximately 5-10 units of the selected restriction enzyme per μg of DNA in the corresponding reaction buffer. Each sample was incubated at 37°C overnight 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. Materials, Methods and Results for Characterization of Cry1Ac Protein

Materials and Methods

Test Substance/Test System
The test substance was the Cry1Ac protein expressed in and extracted from grain of transgenic soybean event DAS-81419-2: Source ID: YX10MX500029.0001 (T4 generation). The seeds were planted, grown, and harvested under DAS protocol number 110000. After harvest, the grain was frozen, lyophilized, ground, and stored at -20°C. The presence of Cry1Ac protein in the soybean grain was confirmed by a commercially available lateral flow assay kit from EnviroLogix Inc., as described below.

Control Substances
The control substance used in this study was a non-transgenic soybean grain grown from seed (*Glycine max* cv Maverick). Grain of the Maverick soybean line (Source ID: YX10MX030001.0002 – T4 generation) were planted, grown, harvested, and processed under the same conditions as the transgenic plants described above. The absence of Cry1Ac protein in the non-transgenic soybean tissue was confirmed by a commercially available lateral flow assay kit as described below.

Recombinant Cry1Ac microbial protein, (TSN102591, Lot #: 1757-66), has a molecular weight of ~130 kDa and a concentration of 144 μg/mg. The microbial preparation was produced and purified from recombinant *Pseudomonas fluorescens* at Dow AgroSciences and an aliquot of the purified sample was sent to the Test Substance Coordinator located in Indianapolis, IN for dispensation.

Reference Substances
The commercially available (non-GLP) reference substances used in this study are listed in the following table:

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	MH161385	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	NA165380 & NE170914	SDS-PAGE & glycosylation assay	ThermoFisher
Unstained Molecular Weight Markers	Novex Sharp unstained protein standards	1143231	SDS-PAGE	Invitrogen: Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 10, and 3.5 kDa

Reference Substance	Product Name	Lot Number	Assay	Reference
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	1095889, 1141762, & 1022458	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

Lateral Flow Strip Assay

The soybean grain tissues of the transgenic event DAS-81419-2 and non-transgenic Maverick were planted, grown, and harvested under protocol 110000 in 2011. After harvest, the samples were shipped frozen, ground, and stored at –20°C until use. To confirm the presence/absence of the Cry1Ac protein in the ground grain, approximately 250-mg samples of the grain were weighed out in 2-mL microfuge tubes and tested by the lateral flow strip assay as described by Envirologix Inc. Briefly, the soluble proteins were extracted by adding 1.5 mL of EB2 extraction buffer and grinding in a Geno-Grinder for 3 minutes at 500 strokes per minute. The test strips were added to the tubes and incubated at room temperature in the samples for 10 minutes to develop. After the assay was complete, the strips were removed and allowed to air dry and the results were recorded.

SDS-PAGE and Western Blot

SDS-PAGE and western blot analysis of the crude protein extracts prepared from DAS-81419-2 soybean grain and non-transgenic Maverick soybean grain 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 ~15 mg of the ground soybean grain in 1.5 mL PBS/Triton/ascorbic acid buffer for 3 minutes in a 2 mL micro-centrifuge tube. The supernatants were clarified by briefly centrifuging the samples at >20,000×g (4°C), and 250 µL of each extract was mixed with 250 µL of 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), 40 µL of each supernatant was loaded directly on the gel. The reference standard, microbe-derived Cry1Ac (TSN102591), and control standard, BSA (ThermoScientific), 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 ThermoScientific 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) with a Criterion transfer cell (Bio-Rad) for 60 minutes under a constant voltage of 100V. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was probed with a Cry1Ac specific polyclonal rabbit antibody (α-Cry1Ac PAb, NB1434-63, 1.46 mg/mL) for 60 minutes (1:2500 dilution) at room temperature. A 1:5000 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.

Purification of Soybean-Derived Cry1Ac from Event DAS-81419-2

The Cry1Ac protein was extracted from the ground soybean grain with a modified RIPA buffer (phosphate buffered saline containing an additional 0.1 M NaCl, 12.5 mM EDTA, 1% Tween-20,

0.1% SDS, and 0.5% sodium deoxycholate) by weighing ~25 grams of ground grain into a chilled Waring blender cup and adding ~175 mL of buffer and 150 µL of protease inhibitor cocktail mix. The tissue was blended on high speed four times in 20 second pulses and the solution was filtered through 2 layers of pre-wetted miracloth (Calbiochem) and clarified by centrifugation at 38,000×g for 30 minutes. The supernatant was removed and filtered through 2 layers of miracloth and another 150 µL of protease inhibitor cocktail was added to the extract and held on ice.

The Cry1Ac protein was purified from the supernatant by immuno-precipitation using monoclonal antibodies (cell line #158E6, lot #: 200.687-3-5 (6 mg/mL) or cell line #158E7 (5.8 mg/ml)) cross-linked to Thermo Scientific's Protein A/G Agarose resin at 1.0 µg of antibody per mL of resin. For each 15 mL of clarified supernatant, 100 µL (100 µg of antibody) of coupled resin was added and allowed to incubate on a rotating mixer for 30 minutes at 4°C. The resin was recovered by centrifugation at 500×g for 5 minutes at 4°C and then resuspended in an additional 15 mL of extract for a total of 30 mL of extract applied to each resin preparation. The resin was then washed with 15 mL of extraction buffer for 15 minutes at 4ºC, followed by two washes with 15 mL of wash buffer (PBS, 0.7 M NaCl, 25 mM EDTA, 25 mM galactose, 1% Tween 20). After the final wash, spin, and decant, the resin was transferred to a 1.5 mL Eppendorf tube with 1 mL of wash buffer and centrifuged for 2 minutes at 500×g at 4°C to pellet the resin. The supernatant was carefully aspirated and the pellet was washed an additional two times with 1 mL of PBS, 5 mM EDTA. The pelleted resin was resuspended in 1 mL of PBS, 5 mM EDTA and 500 µL of the slurry was removed and centrifuged at 21000×g for 1 minute to pellet the resin. After the supernatant was removed, the resin was stored at -80°C for later use. From the remaining 500 µL of slurry, the resin was pelleted as described above and the bound proteins were eluted by incubating the resin for 5 minutes in 80 µL of 2% SDS at room temperature. The resin was pelleted with a brief centrifugation and 75 µL of supernatant was removed and the elution procedure was repeated for a total of 150 µL of eluted protein. The extracted resin, eluted protein, and other fractions were all stored at -80°C for future analysis.

Detection of Post-Translational Glycosylation

The immunoaffinity-purified, soybean-derived Cry1Ac protein was mixed with Laemmli sample buffer (37.5 µL + 12.5 µL of 4x LSB) and heated at 95°C for 10 minutes. The microbe-derived Cry1Ac, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted with 2x LSB to the approximate concentration of the purified soybean-derived Cry1Ac protein. After mixing the proteins with Laemmli sample buffer, the proteins were heated at ~95°C for 10 minutes and centrifuged at 20,000×g for 2 minutes to obtain a clarified supernatant. The resulting supernatants were applied directly to a Bio-Rad Mini-Protean TGX gel and electrophoresed at 100V for ~90 minutes. After electrophoresis, the gel was cut in half and one half was stained with GelCode Blue stain 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 remaining half of the 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 15 minutes. Finally, the gel was immersed in the

reduction solution for 5 minutes, and rinsed with 3% acetic acid. The glycoproteins (with a detection limit as low as 0.625 ng per band) 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.

MALDI-TOF MS and LC/MS Peptide Mass Fingerprinting and Sequence Analysis of Soybean- and Microbe-Derived Cry1Ac

Sample preparation and deposition

Tryptic (in-gel digest), and Chymotryptic (in-gel digest) peptides were purified using Millipore ZipTip C18 as per manufacturer's procedure. Purified peptides were eluted sequentially with aqueous 10%, 25%, 50%, and 100% ACN (supplemented 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.

MALDI-TOF MS

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 (S/N AK011030605H). Mass calibration was performed with a mass standards kit for calibration of AB SCIEX TOF/TOF instruments. The plate wide calibration model was used for MS calibration.

MALDI-TOF MS, Electrospray Ionization-Liquid Chromatography Mass Spectrometry (ESI-LC/MS), glycoprotein analysis, and N-terminal and internal protein sequence analysis of the microbe-derived Cry1Ac (TSN102591) was conducted previously at Dow AgroSciences.

Results

Lateral Flow Strip Assay

The presence of the Cry1Ac protein in the ground grain of DAS-81419-2 soybean was confirmed using commercially prepared lateral flow strips from EnviroLogix Inc. The strips, capable of detecting one transgenic seed in 400, easily discriminated between transgenic and non-transgenic tissue. The extracts prepared from DAS-81419-2 soybean tested positive for Cry1Ac protein and the non-transgenic extracts of Maverick did not contain detectable immunoreactive protein (Figure 40). This result was also confirmed by the western blot analysis using polyclonal antibodies specific to the Cry1Ac protein (Figure 41).

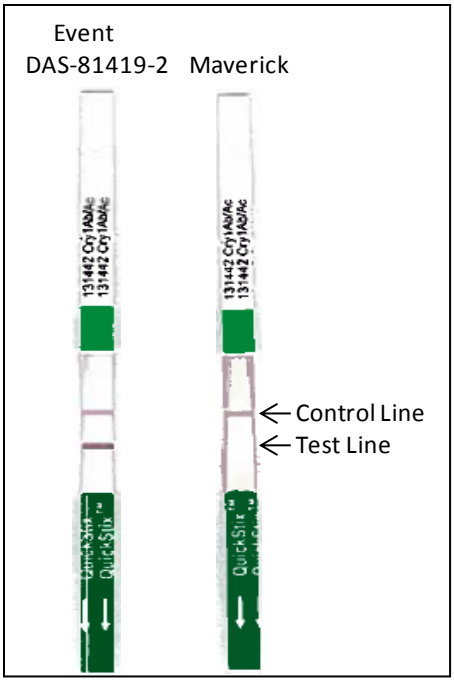


Figure 40. Lateral flow strip assay for the Cry1Ac protein expression in Event DAS-81419-2 and Maverick grain extracts.

Western Blot Analysis of Transgenic Grain Extracts

The microbe-derived Cry1Ac protein showed a positive signal of the expected size (the full-length Cry1Ac protein is ~130 kDa) by polyclonal antibody western blot analysis (Figure 41). This was also observed in the DAS-81419-2 transgenic soybean grain extract whereas the non-transgenic Maverick extract did not contain any immunoreactive proteins. In the Cry1Ac western blot analysis, some immunoreactive proteins of truncated Cry1Ac were observed in the microbe-derived standard; however no alternate size Cry1Ac proteins (aggregates or degradation products) were observed in the transgenic samples (Figure 41). These results add to the evidence that the protein expressed in soybean is not glycosylated or post-translationally modified which would add to or subtract from the overall protein molecular weight.

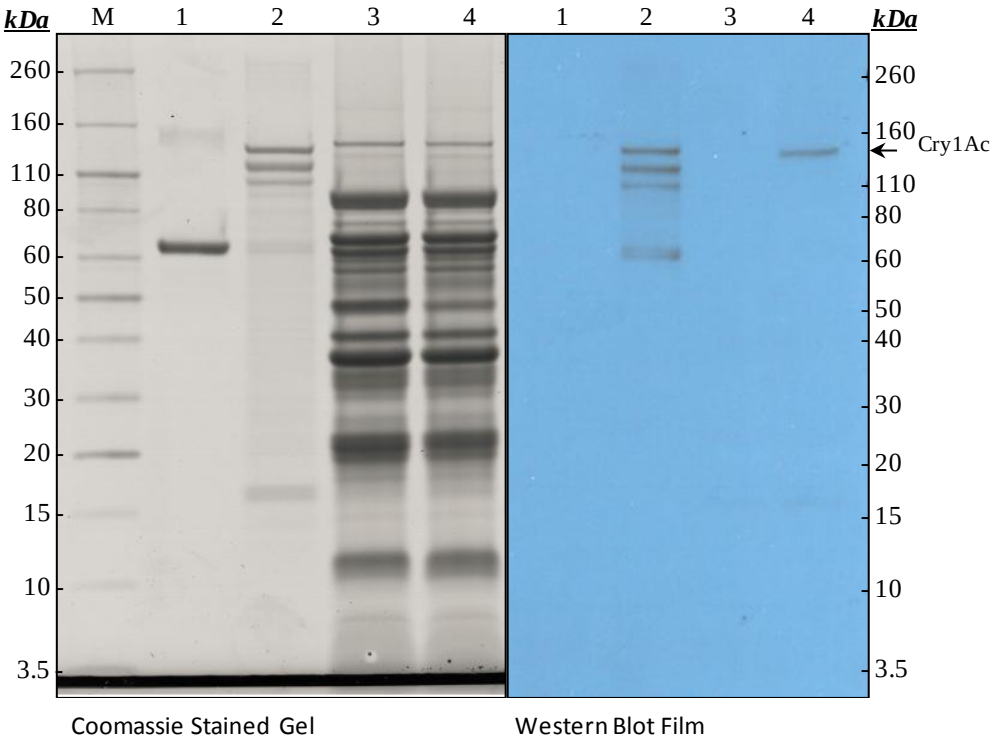


Figure 41. Western blot analysis of microbe- and soybean-derived Cry1Ac proteins.

Lane	*Sample*	*Amount Loaded*
M	Novex unstained MW markers	10 µL
1 (gel)	Bovine serum albumin	1.3 µg
1 (blot)	Bovine serum albumin	2.5 ng
2 (gel)	Cry1Ac protein standard (TSN102591)	0.72 µg
2 (blot)	Cry1Ac protein standard (TSN102591)	1.9 ng
3	Maverick crude (non-transgenic) grain extract	40 µL
4	DAS-81419-2 crude (transgenic) grain extract	40 µL

Purification of the Cry1Ac Protein from Transgenic Event DAS-81419-2 Grain Extracts
Immunoaffinity precipitation was conducted on an aqueous extract of ~25 grams of ground DAS-81419-2 soybean grain. The protein that bound to the monoclonal antibody columns was eluted from the column and examined by SDS-PAGE which demonstrated that the final concentrated fractions contained the intact Cry1Ac protein at an approximate molecular weight of ~130 kDa and fragments that contain the active core toxin (Figure 42). Once isolated, the soybean-derived Cry1Ac was compared with the microbe-derived protein.

SDS-PAGE Analysis of the Immuno-purified Cry1Ac
In the toxicology-lot preparation of *P. fluorescens*-produced Cry1Ac (TSN102591), the major protein bands, as visualized on Coomassie stained SDS-PAGE gels, were approximately 130, 110, and 90 kDa. As expected, the corresponding soybean-derived Cry1Ac protein was identical in size to the full-length microbe-expressed protein (Figure 42). Predictably, the plant purified fractions contained a minor amount of impurities in addition to the full-length and truncated Cry1Ac core protein. The co-eluted proteins were likely retained on the column 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 immuno-adsorbents (Holroyde *et al.*, 1976; Kennedy and Barnes, 1983; Williams *et al.*, 2006) as well as antibody leaching from the column (Goldberg *et al.*, 1991).

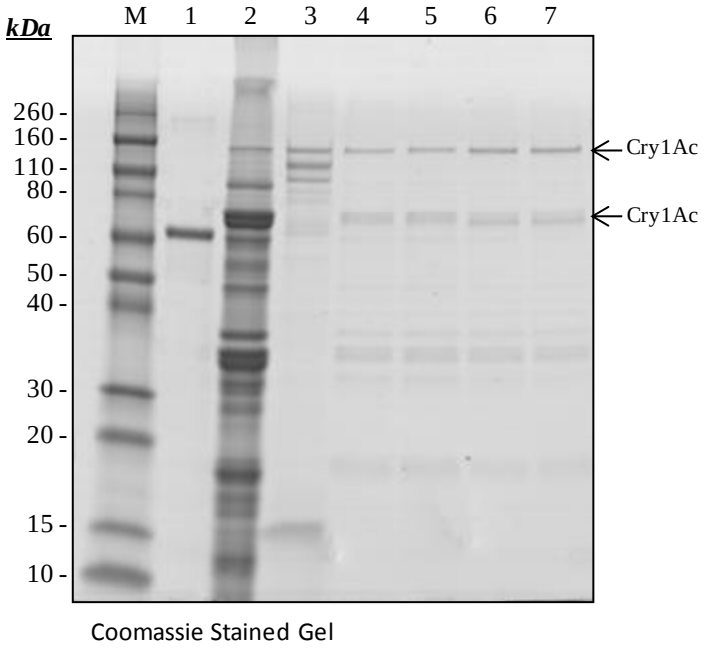


Figure 42. SDS-PAGE analysis of immunoaffinity-purified soybean-derived Cry1Ac.

Lane	*Sample*	*Amount Loaded*
M	Novex prestained MW markers	10 µL
1	Bovine serum albumin	~1 µg
2	DAS-81419-2 crude grain extract	3 µL
3	Cry1Ac protein standard (TSN102591)	~1 µg
4	Soybean-derived Cry1Ac (DAS-81419-2)	20 µL
5	Soybean-derived Cry1Ac (DAS-81419-2)	20 µL
6	Soybean-derived Cry1Ac (DAS-81419-2)	20 µL
7	Soybean-derived Cry1Ac (DAS-81419-2)	20 µL

Detection of Glycosylation

Potential protein glycosylation of soybean-derived Cry1Ac was assessed with a GelCode Glycoprotein Staining Kit from ThermoScientific. The immunoaffinity-purified Cry1Ac protein was electrophoresed simultaneously with a set of control and reference protein standards. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation, and non-glycoproteins, microbe-derived Cry1Ac, soybean trypsin inhibitor, and bovine serum albumin, were employed as negative controls. The results showed that both the soybean- and microbe-derived Cry1Ac proteins had no detectable covalently linked carbohydrates (Figure 43).

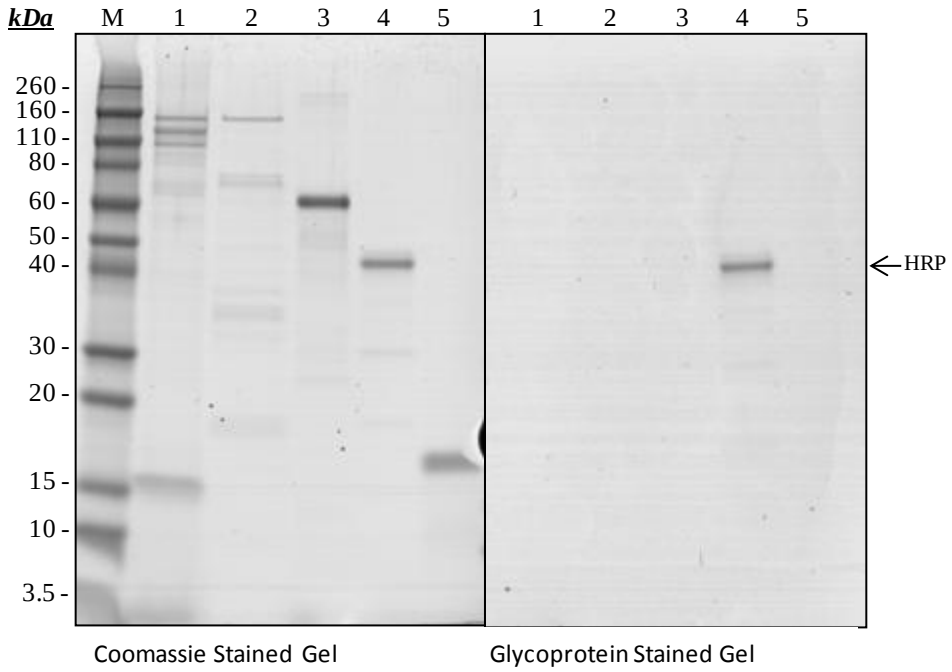


Figure 43. SDS-PAGE analysis of microbe- and soybean-derived Cry1Ac protein stained with GelCode Blue total protein and glycoprotein stains.

Lane	*Sample*	*Amount Loaded*
M	Novex prestained MW markers	10 µL
1	Cry1Ac protein standard (TSN102591)	~1 µg
2	Soybean-derived Cry1Ac (DAS-81419-2)	20 µL
3	Bovine serum albumin (- control)	0.5 µg
4	Horseradish peroxidase (+ control)	0.5 µg
5	Soybean trypsin inhibitor (- control)	0.5 µg

MALDI-TOF MS and MS/MS Peptide Mass Fingerprinting and Sequence Analysis

The Cry1Ac protein derived from DAS-81419-2 soybean tissue was separated by SDS-PAGE (Figure 42) and the respective Cry1Ac bands were excised and subjected to in-gel digestion by trypsin and chymotrypsin. 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 expected masses based on trypsin or chymotrypsin cleavage sites within the sequence of the soybean-derived Cry1Ac protein. Figure 44 and Figure 45 illustrates the theoretical cleavage which was generated *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The Cry1Ac protein, once denatured, is readily digested by endoproteases to yield numerous peptides that are able to be detected using mass spectrometry.

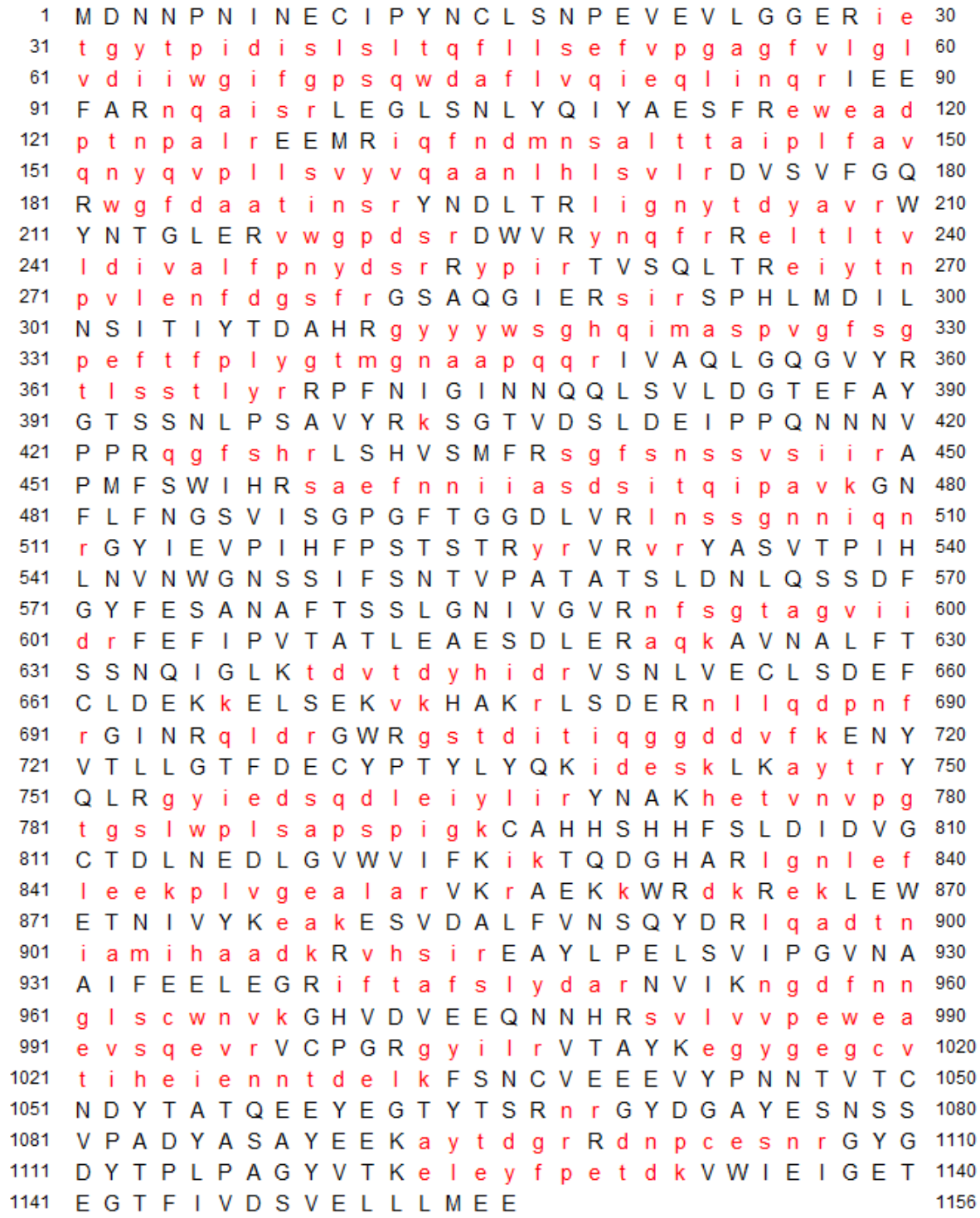


Figure 44. Theoretical cleavage of the Cry1Ac protein with trypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC.

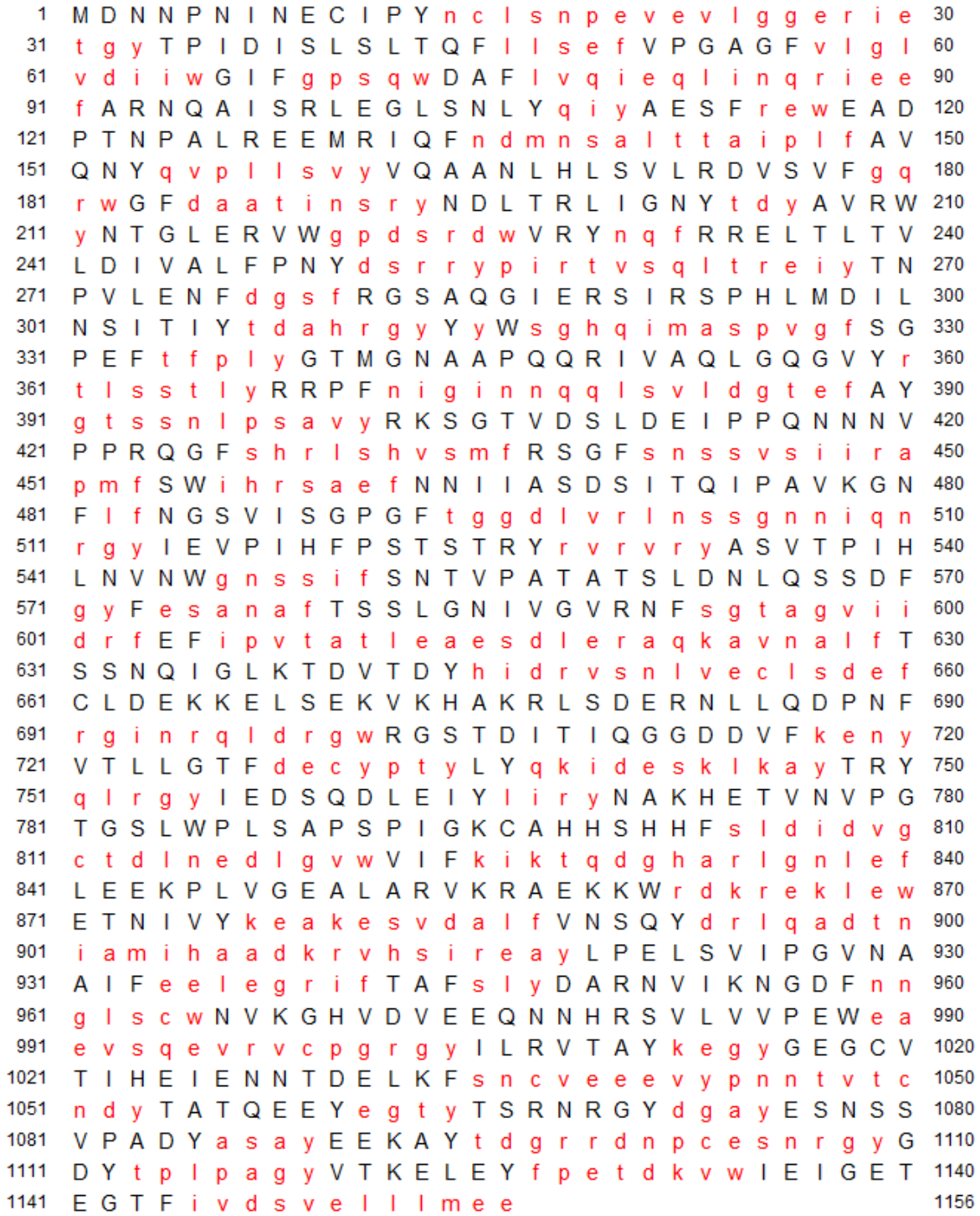


Figure 45. Theoretical cleavage of the Cry1Ac protein with chymotrypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC.

In the endoprotease digests of the transgenic-soybean-derived Cry1Ac protein, the peptide sequence coverage from peptide mass fingerprint (PMF) data was extensive at 86.1%. Of the 86.1% sequence coverage from PMF data, 83.2% was confirmed by tandem mass spectrometry sequencing. The detected peptide fragments covered nearly the entire protein sequence with only a few peptide fragments undetected (Figure 46 - Figure 48). This analysis confirmed the soybean-derived protein amino acid sequence matched that of the microbe-derived Cry1Ac protein near the N- and C-terminus as well as a major portion of the internal sequence. In the MS spectra, there were unidentified peptides detected in the enzyme digest preparations (data not shown). Many factors contribute to the formation of these unidentified peptides, such as over digestion (which results in non-specific cleavage), self-digestion products of trypsin and chymotrypsin, as well as random breakage of peptides during ionization. Unidentified peptides do not indicate the protein is different from the predicted amino acid sequence. The results of these analyses indicate that the amino acid sequence of the soybean-derived Cry1Ac protein was equivalent to the *P. fluorescens*-expressed protein previously characterized.

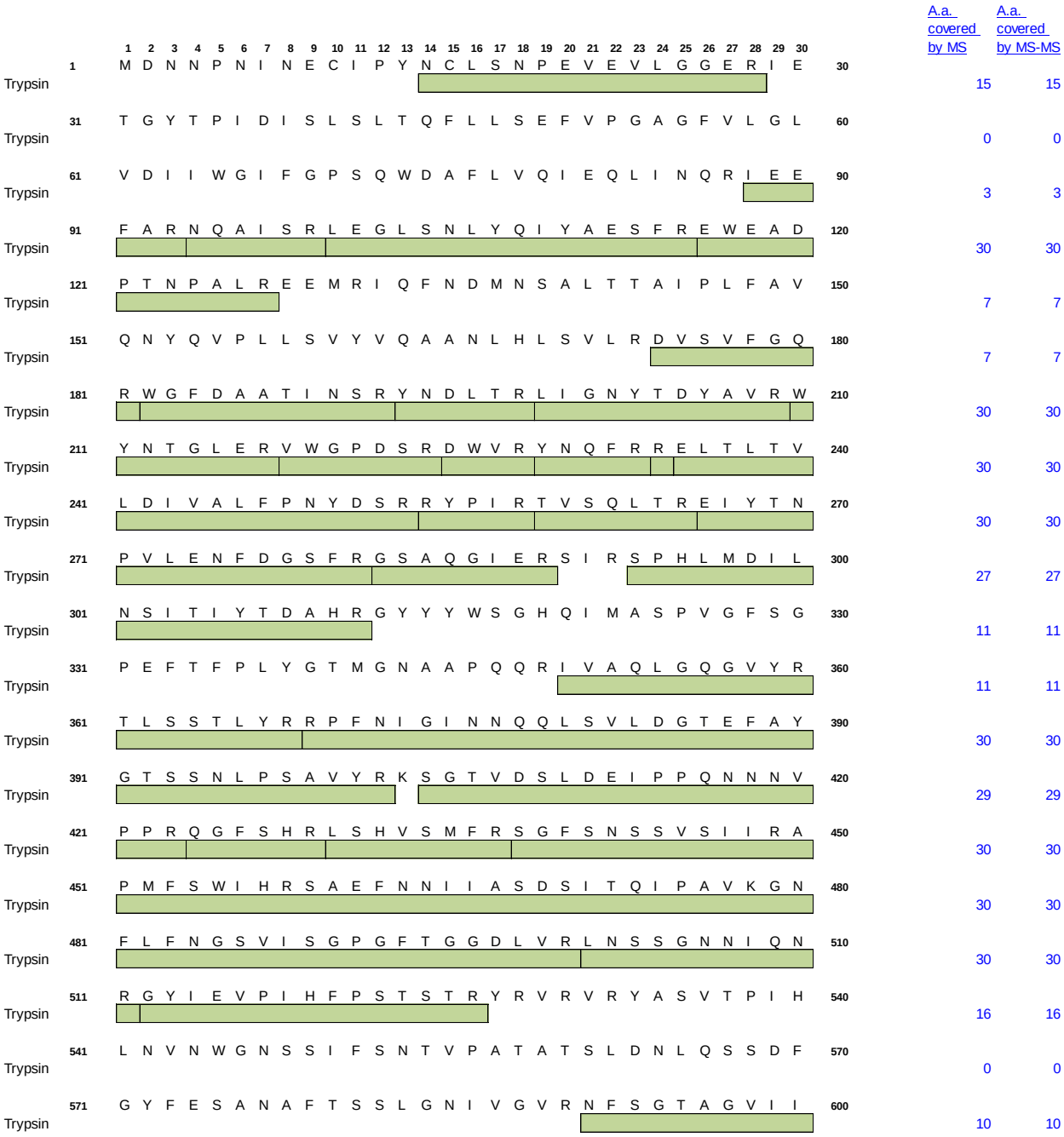


Figure 46. Tryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 67.9% with PMF data and 67.9% by tandem MS.

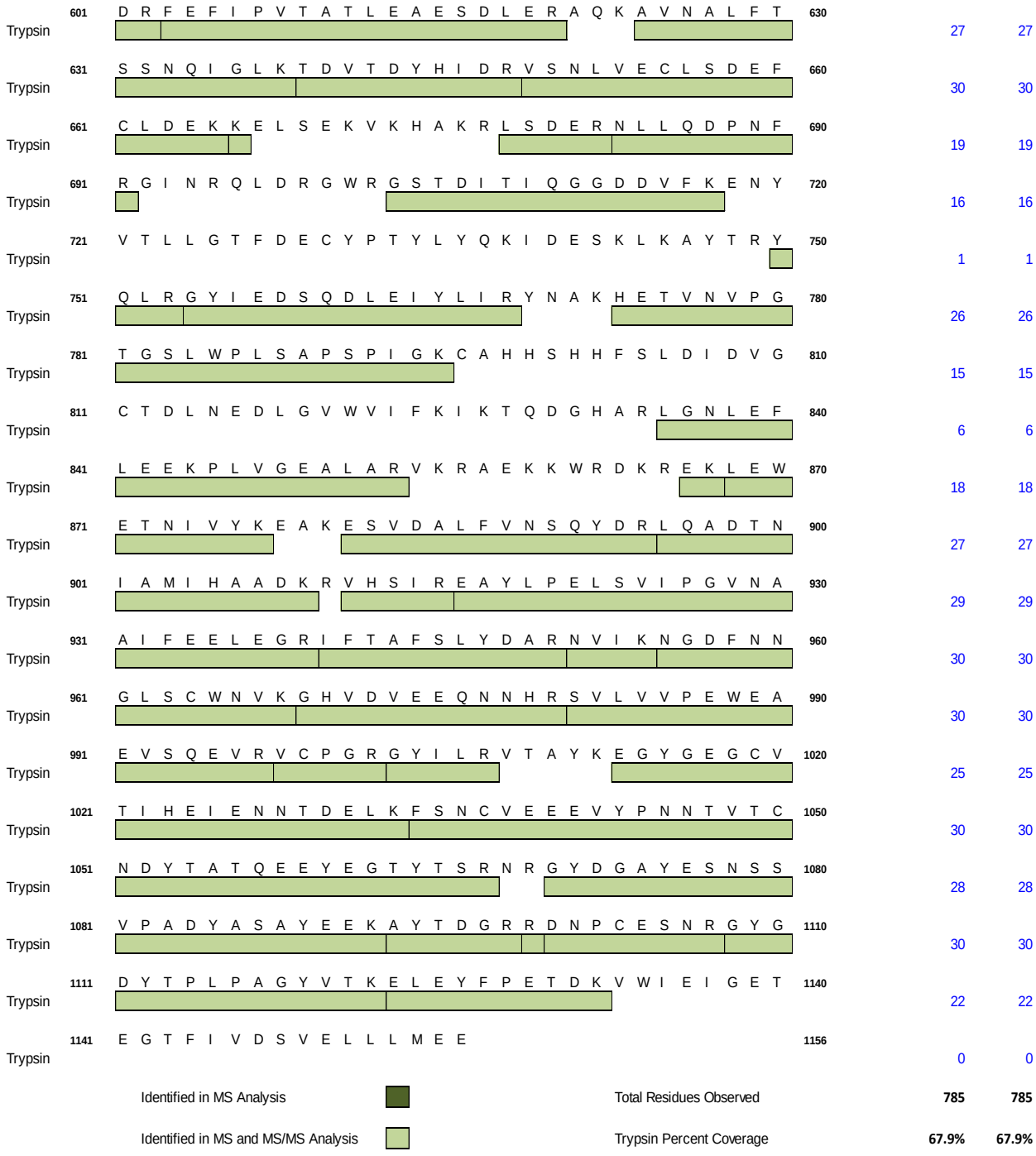


Figure 46 (Cont). Tryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

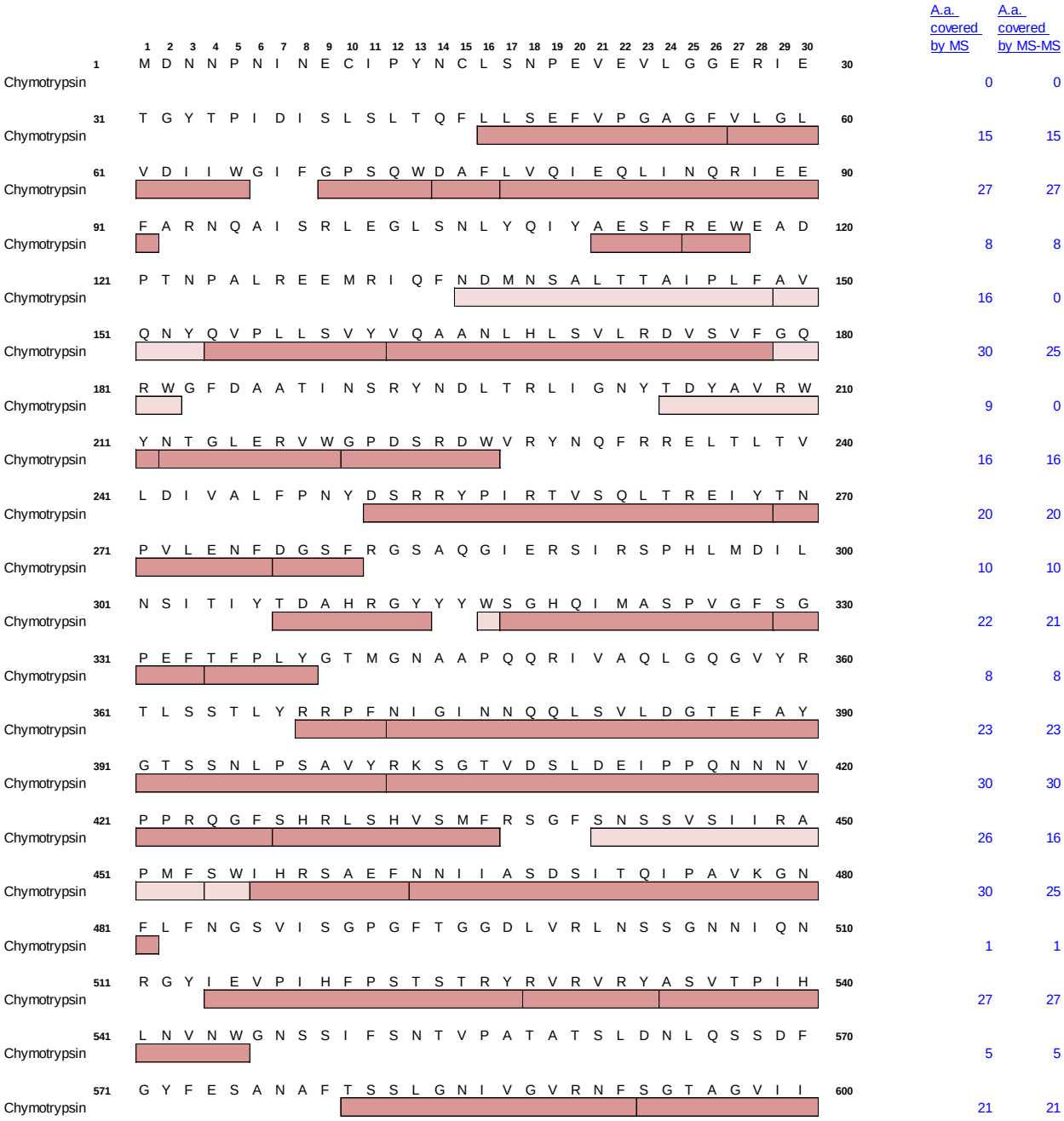


Figure 47. Chymotryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 53.5% with PMF data and 49.0% by tandem MS.

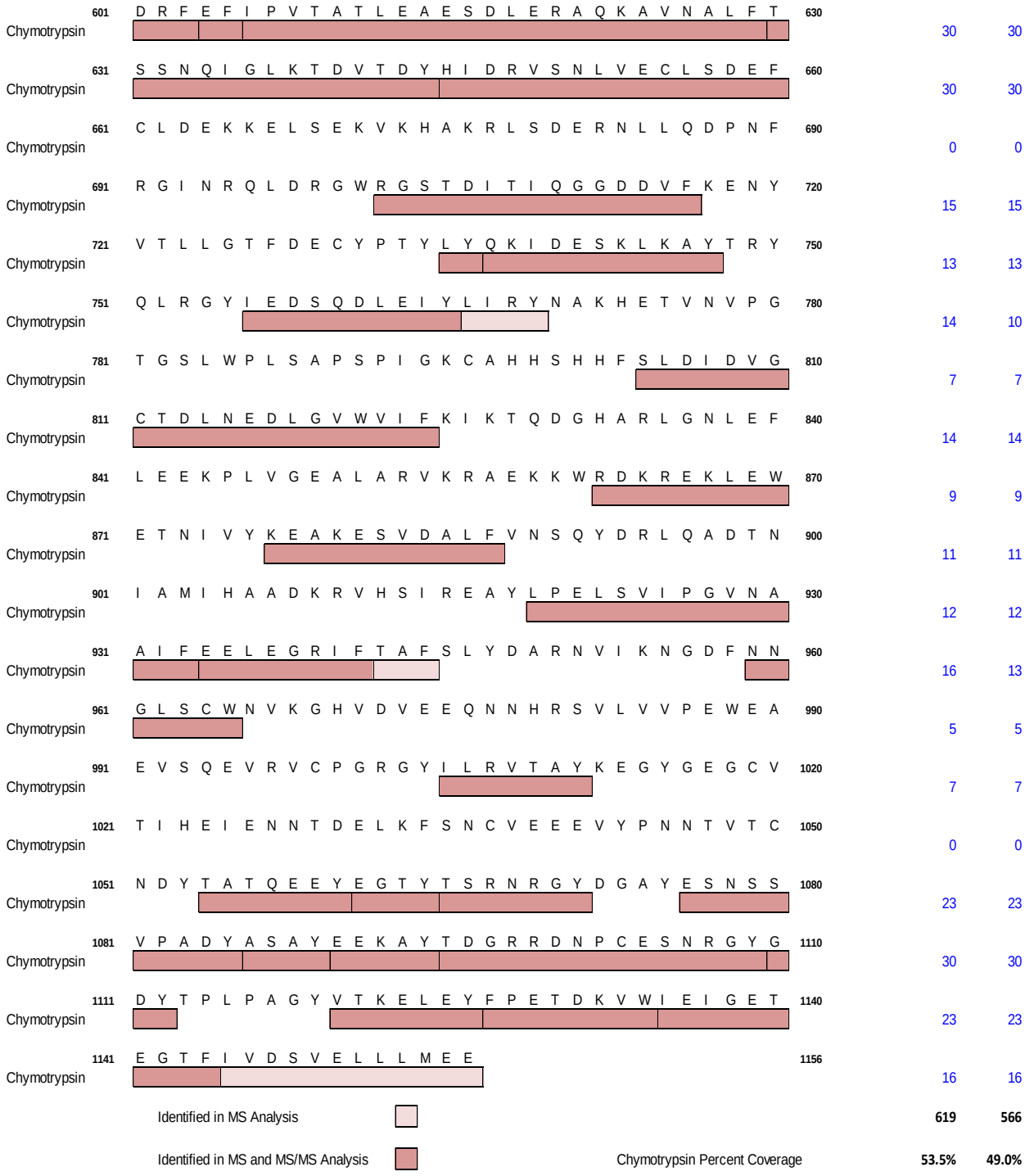


Figure 47 (Cont). Chymotryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

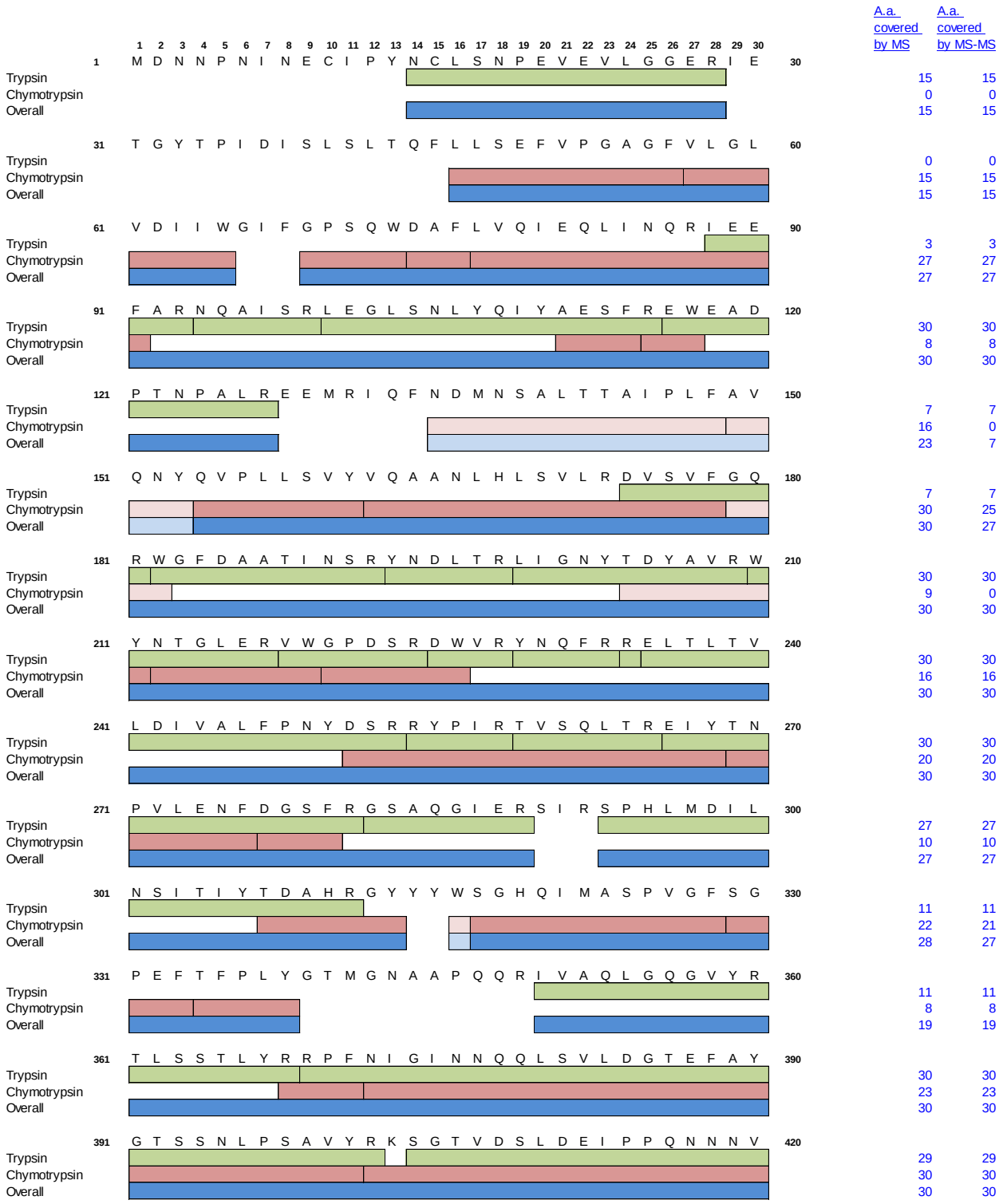


Figure 48. Overall sequence coverage of trypsin and chymotrypsin digests for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Overall sequence coverage was 86.1% with PMF data and 83.2% by tandem MS.

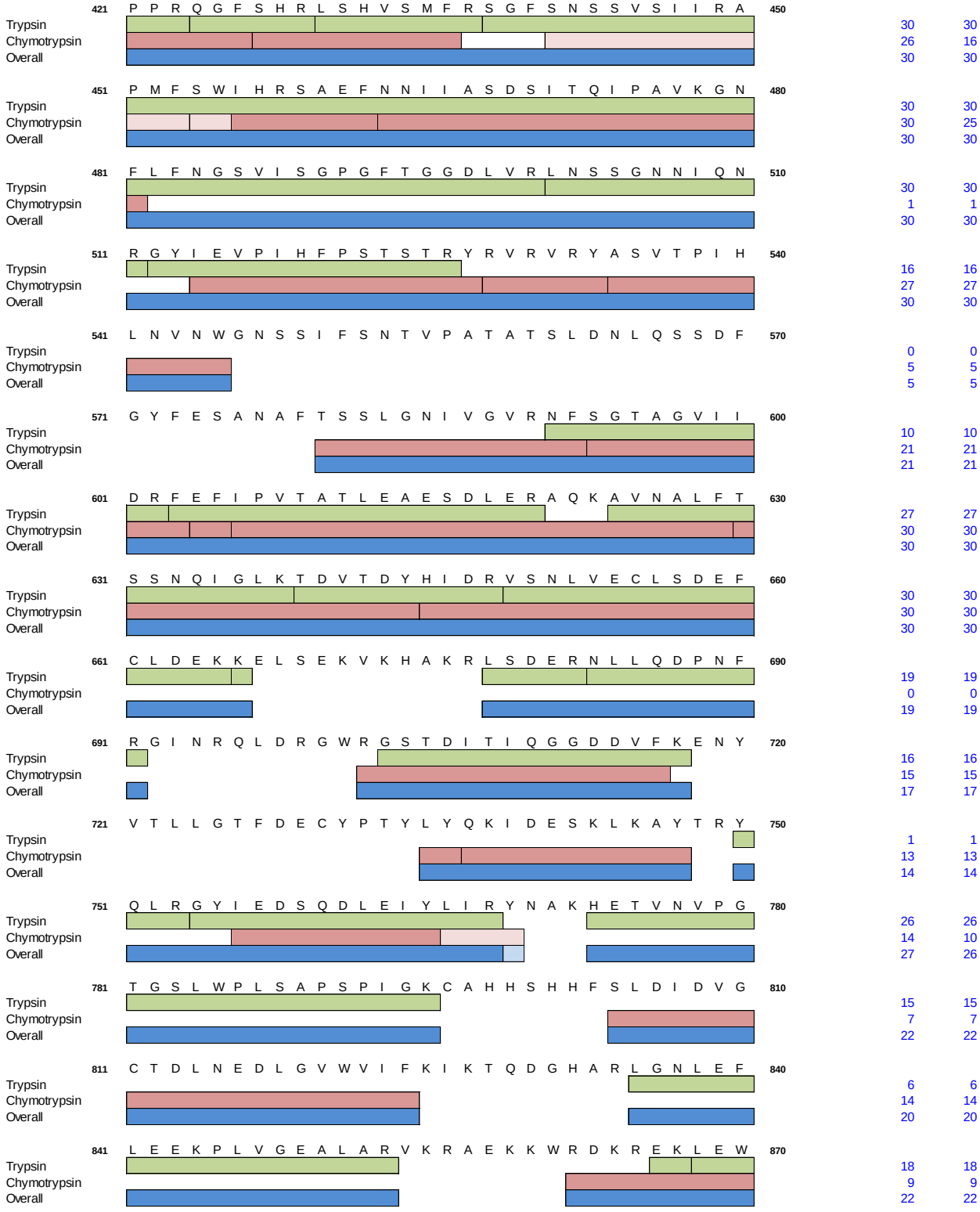


Figure 48 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

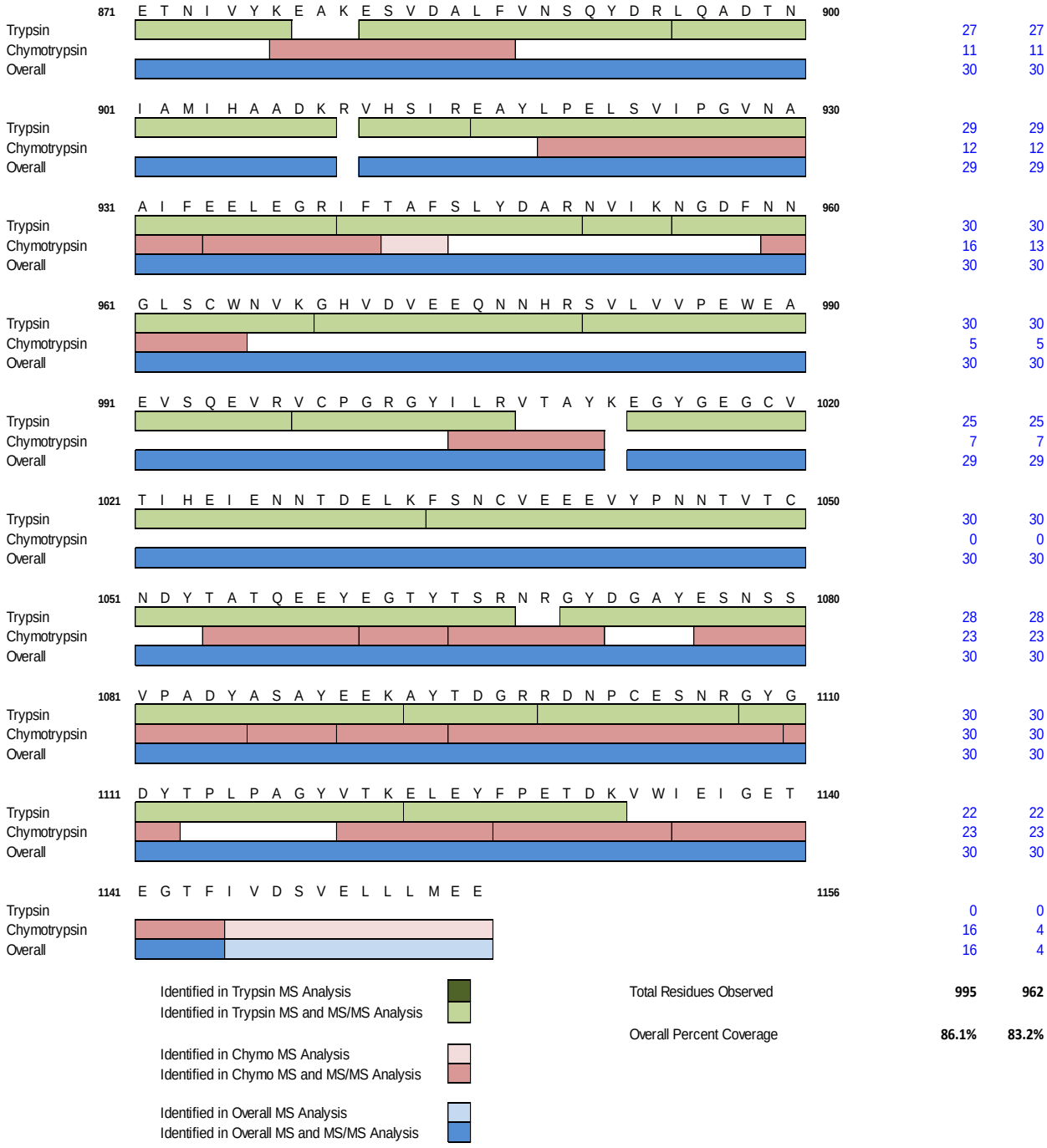


Figure 48 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

Conclusions

It was demonstrated that the biochemical identity of *P. fluorescens*-produced Cry1Ac protein was equivalent to the protein purified from grain of soybean event DAS-81419-2. Both the soybean- and microbe-derived Cry1Ac proteins had an apparent molecular weight of ~130 kDa and were immunoreactive to Cry1Ac protein-specific antibodies in both lateral flow strip and Western blot assays. The amino acid sequences were confirmed by enzymatic peptide mass fingerprinting by MALDI-TOF MS and verified by MS/MS. In addition, the lack of glycosylation of the soybean-derived Cry1Ac protein provided additional evidence that the Cry1Ac protein produced by *P. fluorescens* and transgenic soybean were essentially equivalent molecules.

Appendix 3. Materials, Methods and Results for Characterization of Cry1F Protein

Materials and Methods

Test Substance/Test System
The test substance was the Cry1F protein expressed and extracted from grain of transgenic soybean event DAS-81419-2: Source ID: YX10MX500029.0001 (T4 generation). The seeds were planted, grown, and harvested under DAS protocol number 110000. After harvest, the grain was frozen, lyophilized, ground, and stored at -20°C. The presence of Cry1F protein in the soybean grain was confirmed by a commercially available lateral flow assay kit from EnviroLogix Inc., as described below.

Control Substances
The control substance used in this study was a non-transgenic soybean grain grown from seed (*Glycine max* cv Maverick***)***. Grain of the Maverick soybean line (Source ID: YX10MX030001.0002) were planted, grown, harvested, and processed under the same conditions as the transgenic plants described above. The absence of Cry1F protein in the non-transgenic soybean tissue was confirmed by a commercially available lateral flow assay kit as described below.

Recombinant Cry1F microbial protein, (TSN103748, Lot #: 020404), has a molecular weight of ~130 kDa and a concentration of 233 µg/mg. The microbial preparation was produced and purified from recombinant *Pseudomonas fluorescens* at Dow AgroSciences and the purified Cry1F protein was sent to the Test Substance Coordinator located in Indianapolis.

Reference Substances
The commercially available (non-GLP) reference substances used in this study are listed in the following table:

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	MH161385	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	NA165380 and NE170914	SDS-PAGE & glycosylation assay	ThermoFisher
Unstained Molecular Weight Markers	Novex Sharp unstained protein standards	1143231	SDS-PAGE	Invitrogen: Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 10, and 3.5 kDa

Reference Substance	Product Name	Lot Number	Assay	Reference
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	1095889 & 1022458	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

Lateral Flow Test Strip Assay

The soybean grain tissues of the transgenic event and non-transgenic Maverick were planted, grown and harvested under protocol 110000 in 2011. The samples after harvest were shipped frozen, ground, and stored at -20°C until use. To confirm the presence/absence of the Cry1F protein in the ground grain, approximately 250-mg samples of the grain were weighed out in 2-mL micro-centrifuge tubes and tested by the lateral flow test strip assay as described by Envirologix Inc. Briefly, the soluble proteins were extracted by adding 1.5 mL of EB2 extraction buffer and grinding in a Geno-Grinder for 3 minutes at 500 strokes per minute at room temperature. The test strips were added to the tubes and developed for 10 minutes. After the assay was complete, the strips were removed and allowed to air dry and the results were recorded.

SDS-PAGE and Western Blot

SDS-PAGE and western blot analysis of the crude protein extracts prepared from the transgenic DAS-81419-2 soybean grain and non-transgenic Maverick soybean grain 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 ~15 mg of the ground soybean grain in 1.5 mL PBS/Triton/ascorbic acid buffer for 3 minutes in a 2 mL micro-centrifuge tube. The supernatants were clarified by briefly centrifuging the samples at >20,000×g (4°C), and 250 µL of each extract was mixed with 250 µL of 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), 40 µL of each supernatant was loaded directly on the gel. The reference standard, microbe-derived Cry1F (TSN103748), and control standard, BSA (ThermoScientific), 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 ThermoScientific 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) with a Criterion transfer cell (Bio-Rad) for 60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was probed with a Cry1F specific polyclonal rabbit antibody (α-Cry1F PAb, Lot#: 200.310-4-63, 6.48 mg/mL) for 60 minutes (1:5000 dilution) at room temperature. A 1:5000 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.

Purification of Soybean-Derived Cry1F from Event DAS-81419-2
The Cry1F protein was extracted from the ground soybean grain with a modified RIPA buffer [phosphate buffered saline containing an additional 0.1 M NaCl, 12.5 mM EDTA, 1% Tween-20, 0.1% SDS, and 0.5% sodium deoxycholate] by weighing ~25 grams of ground grain into a chilled Waring blender cup and adding ~175 mL of buffer and 150 µL of a broad spectrum protease inhibitor cocktail mix. The tissue was blended on high speed four times in 20 second pulses and the solution was filtered through 2 layers of pre-wetted miracloth (Calbiochem) and clarified by centrifugation at 38,000×g for 30 minutes. The supernatant was removed and filtered through 2 layers of miracloth and another 150 µL of protease inhibitor cocktail was added to the extract and held on ice.

The Cry1F protein was purified from the supernatant by immuno-precipitation using monoclonal antibodies (cell lines #96A22.2 and 96A19) cross-linked to Thermo Scientific's Protein A/G Agarose resin at 1.0 µg of antibody per mL of resin. For each 15 mL of clarified supernatant, 100 µL (100 µg of antibody) of coupled resin was added and allowed to incubate on a rotating mixer for 30 minutes at 4°C. The resin was recovered by centrifugation at 500×g for 5 minutes at 4°C and then resuspended in an additional 15 mL of extract for a total of 30 mL of extract applied to each resin preparation. The resin was then washed with 15 mL of extraction buffer for 15 minutes at 4°C, followed by two washes with 15 mL of wash buffer (PBS, 0.7 M NaCl, 25 mM EDTA, 25 mM galactose, 1% Tween 20). After the final wash, spin, and decant, the resin was transferred to a 1.5 mL Eppendorf tube with 1 mL of wash buffer and centrifuged for 2 minutes at 500×g at 4°C to pellet the resin. The supernatant was carefully aspirated and the pellet was washed an additional two times with 1 mL of PBS, 5 mM EDTA. The pelleted resin was resuspended in 1 mL of PBS, 5 mM EDTA and 500 µL of the slurry was removed and subjected to centrifugation at 21,000×g for 1 minute to pellet the resin. After the supernatant was removed the resin was stored at -80°C. From the remaining 500 µL of slurry, the resin was pelleted as described above and the bound proteins were eluted by incubating the resin for 5 minutes in 80 µL of 2% SDS at room temperature. The resin was pelleted with a brief centrifugation and 75 µL of supernatant was removed and the elution procedure was repeated for a total of 150 µL of eluted protein. The extracted resin, eluted protein and other fractions were all stored at -80°C.

Detection of Post-Translational Glycosylation
The immunoaffinity-purified, soybean-derived Cry1F protein was mixed with Laemmli sample buffer (37.5 µL + 12.5 µL of 4x LSB) and heated at 95 °C for 10 minutes. The microbe-derived Cry1F, soybean trypsin inhibitor, bovine serum albumin, and horseradish peroxidase were diluted with 2x LSB to the approximate concentration of the purified soybean-derived Cry1F protein. After mixing the proteins with Laemmli sample buffer, the proteins were heated at ~95 °C for 10 minutes and centrifuged at 20000×g for 2 minutes to obtain a clarified supernatant. The resulting supernatants were applied directly to a Bio-Rad Mini-Protean TGX gel and electrophoresed at 100V for ~90 minutes. After electrophoresis, the gel was cut in half and one half was stained with GelCode Blue stain 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 remaining half of the 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 15 minutes. Finally, the gel was immersed in the reduction solution for 5 minutes, and rinsed with 3% acetic acid. The glycoproteins (with a detection limit as low as 0.625 ng per band) 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.

MALDI-TOF MS and LC/MS Peptide Mass Fingerprinting and Sequence Analysis of Soybean- and Microbe-Derived Cry1F

Sample preparation and deposition

Tryptic (in-gel digest), and Chymotryptic (in-gel digest) peptides were purified using Millipore ZipTip C18 as per manufacturer's procedure. Purified peptides were eluted sequentially with aqueous 10%, 25%, 50%, and 100% ACN (supplemented 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.

MALDI-TOF MS

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 (S/N AK011030605H). Mass calibration was performed with a mass standards kit for calibration of AB SCIEX TOF/TOF instruments. The plate wide calibration model was used for MS calibration.
MALDI-TOF MS, Electrospray Ionization-Liquid Chromatography Mass Spectrometry (ESI-LC/MS), glycoprotein analysis, and N-terminal and internal protein sequence analysis of the microbe-derived Cry1F (TSN103748) was conducted previously by (Gao *et al.*, 2006).

Results

Lateral Flow Test Strip Assay
The presence of the Cry1F protein in the ground grain of event DAS-81419-2 was confirmed using commercially prepared lateral flow test strips from EnviroLogix Inc. The strips, capable of detecting one transgenic seed in 400, easily discriminated between transgenic and non-transgenic tissue. The transgenic extracts were positive for Cry1F protein and the non-transgenic extracts of Maverick did not contain detectable immunoreactive protein (Figure 49). This result was also confirmed by the western blot analysis using polyclonal antibodies specific to the Cry1F protein (Figure 50).

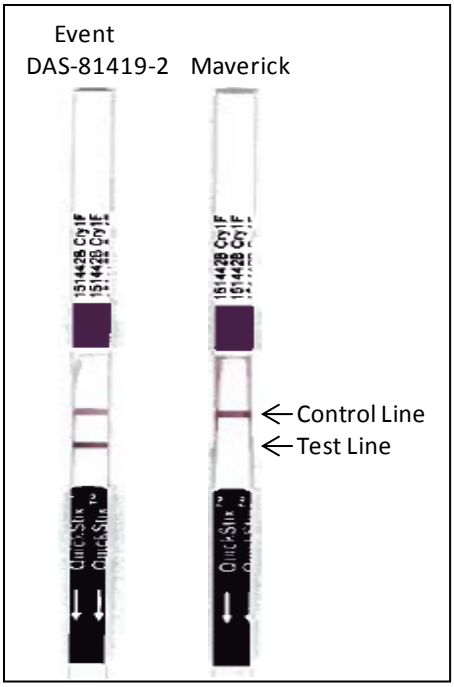


Figure 49. Lateral flow strip assay for the Cry1F protein expression in Event DAS-81419-2 and Maverick grain extracts.

Western Blot Analysis of Transgenic Grain Extracts

The microbe-derived Cry1F protein showed a positive signal of the expected size (the full-length Cry1F protein is ~130 kDa) by polyclonal antibody western blot analysis (Figure 50). This was also observed in the DAS-81419-2 transgenic soybean grain extract whereas the non-transgenic Maverick extracts did not contain any immunoreactive proteins. In the Cry1F western blot analysis, some immunoreactive proteins of truncated Cry1F were observed in the microbe-derived standard; however no alternate size Cry1F proteins (aggregates or degradation products) were observed in the transgenic samples (Figure 50). These results add to the evidence that the protein expressed in soybean is not glycosylated or post-translationally modified which would add to or subtract from the overall protein molecular weight.

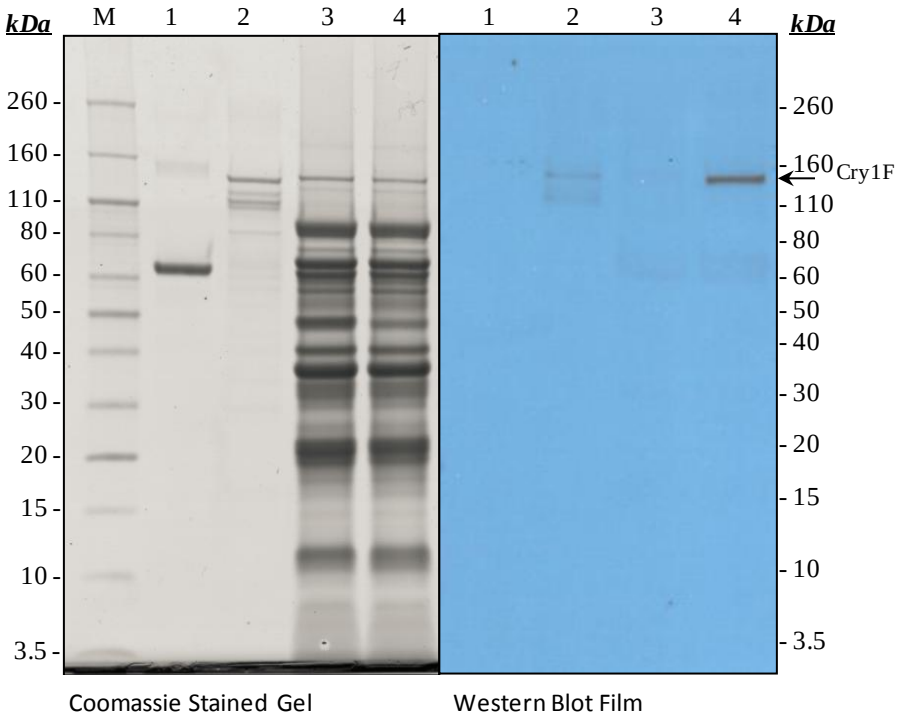


Figure 50. Western blot analysis of microbe- and soybean-derived Cry1F proteins.

Lane	*Sample*	*Amount Loaded*
M	Novex unstained MW markers	10 µL
1 (gel)	Bovine serum albumin	1.3 µg
1 (blot)	Bovine serum albumin	2.5 ng
2 (gel)	Cry1F protein standard (TSN103748)	0.47 µg
2 (blot)	Cry1F protein standard (TSN103748)	1.2 ng
3	Maverick crude (non-transgenic) grain extract	40 µL
4	DAS-81419-2 crude (transgenic) grain extract	40 µL

Purification of the Cry1F Protein from Transgenic Event DAS-81419-2 Grain Extracts
Immunoaffinity precipitation was conducted on an aqueous extract of ~25 grams of ground DAS-81419-2 transgenic grain. The protein that bound to the monoclonal antibody columns was examined by SDS-PAGE which demonstrated that the final concentrated fractions contained the intact Cry1F protein at an approximate molecular weight of ~130 kDa and fragments that contain the active core toxin (Figure 51). Once isolated, the soybean-derived Cry1F was then compared with the microbe-derived protein.

SDS-PAGE Analysis of the Immuno-purified Cry1F
In the toxicology-lot preparation of *P. fluorescens*-produced Cry1F (TSN103748), the major protein bands, as visualized on Coomassie stained SDS-PAGE gels, were approximately 130, 110, and 90 kDa. As expected, the corresponding soybean-derived Cry1F protein was identical in size to the full-length microbe-expressed proteins (Figure 51). Predictably, the plant purified fractions contained a minor amount of impurities in addition to the full-length and truncated Cry1F core protein. The co-eluted proteins were likely retained on the column 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).

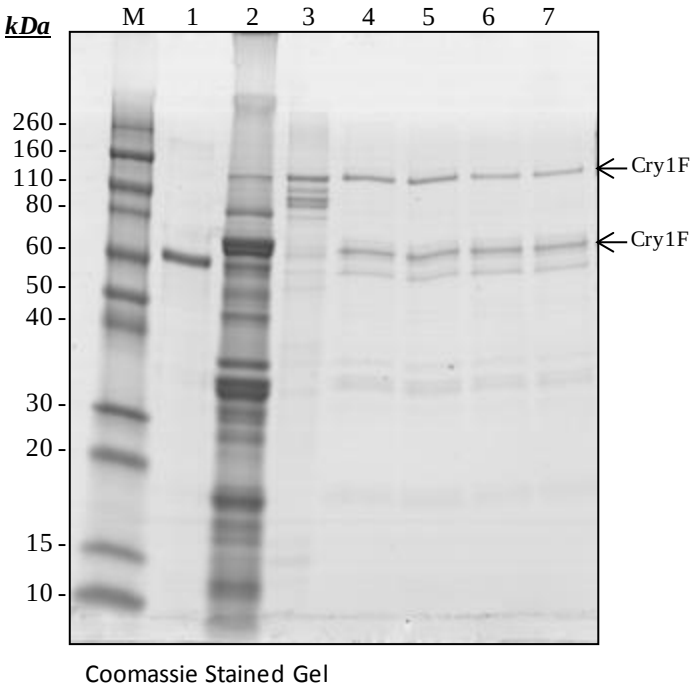


Figure 51. SDS-PAGE analysis of immunoaffinity-purified soybean-derived Cry1F.

Lane	*Sample*	*Amount Loaded*
M	Novex prestained MW markers	10 µL
1	Bovine serum albumin	~1 µg
2	DAS-81419-2 crude grain extract	3 µL
3	Cry1F protein standard (TSN103748)	0.25 µg
4	Soybean-derived Cry1F (DAS-81419-2)	20 µL
5	Soybean-derived Cry1F (DAS-81419-2)	20 µL
6	Soybean-derived Cry1F (DAS-81419-2)	20 µL
7	Soybean-derived Cry1F (DAS-81419-2)	20 µL

Detection of Glycosylation

Potential protein glycosylation of soybean-derived Cry1F was assessed with a GelCode Glycoprotein Staining Kit from ThermoScientific. The immunoaffinity-purified Cry1F protein was electrophoresed simultaneously with a set of control and reference protein standards. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation, and non-glycoproteins, microbe-derived Cry1F, soybean trypsin inhibitor, and bovine serum albumin, were employed as negative controls. The results showed that both the soybean- and microbe-derived Cry1F proteins had no detectable covalently linked carbohydrates (Figure 52).

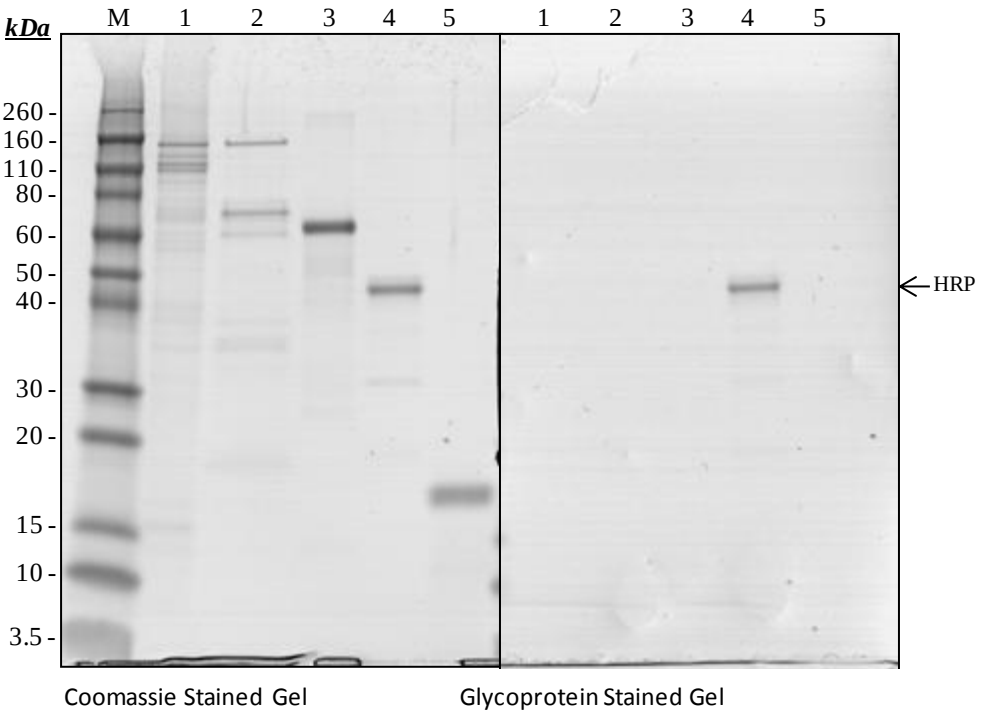


Figure 52. SDS-PAGE analysis of microbe- and soybean-derived Cry1F protein stained with GelCode Blue total protein and glycoprotein stains.

Lane	*Sample*	*Amount Loaded*
M	Novex prestained MW markers	10 µL
1	Cry1F protein standard (TSN103748)	~1 µg
2	Soybean-derived Cry1F (DAS-81419-2)	20 µL
3	Bovine serum albumin (- control)	0.5 µg
4	Horseradish peroxidase (+ control)	0.5 µg
5	Soybean trypsin inhibitor (- control)	0.5 µg

MALDI-TOF MS and MS/MS Peptide Mass Fingerprinting and Sequence Analysis
The Cry1F protein derived from DAS-81419-2 soybean tissue was separated by SDS-PAGE (Figure 51) and the respective bands were excised and subjected to in-gel digestion by trypsin and chymotrypsin. 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 chymotrypsin cleavage sites in the sequence of the soybean-derived Cry1F protein. Figure 53 and Figure 54 illustrate the theoretical cleavage of the Cry1F protein which was generated *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The Cry1F protein, once denatured, is readily digested by endoproteases to yield numerous peptides that are able to be detected using mass spectrometry.

1	M	E	N	N	I	Q	N	Q	C	V	P	Y	N	C	L	N	N	P	E	V	E	I	L	N	E	E	R	s	t	g	30
31	r	L	P	L	D	I	S	L	S	L	T	R	f	l	l	s	e	f	v	p	g	v	g	v	a	f	g	l	f	d	60
61	l	i	w	g	f	i	t	p	s	d	w	s	l	f	l	l	q	i	e	q	l	i	e	q	r	I	E	T	L	E	90
91	R	n	r	A	I	T	T	L	R	g	l	a	d	s	y	e	i	y	i	e	a	l	r	E	W	E	A	N	P	N	120
121	N	A	Q	L	R	e	d	v	r	I	R	f	a	n	t	d	d	a	l	i	t	a	i	n	n	f	t	l	t	s	150
151	f	e	i	p	l	l	s	v	y	v	q	a	a	n	l	h	l	s	l	l	r	D	A	V	S	F	G	Q	G	W	180
181	G	L	D	I	A	T	V	N	N	H	Y	N	R	l	i	n	l	i	h	r	Y	T	K	h	c	l	d	t	y	n	210
211	q	g	l	e	n	l	r	G	T	N	T	R	q	w	a	r	F	N	Q	F	R	r	D	L	T	L	T	V	L	D	240
241	I	V	A	L	F	P	N	Y	D	V	R	t	y	p	i	q	t	s	s	q	l	t	r	E	I	Y	T	S	S	V	270
271	I	E	D	S	P	V	S	A	N	I	P	N	G	F	N	R	a	e	f	g	v	r	p	p	h	l	m	d	f	m	300
301	n	s	l	f	v	t	a	e	t	v	r	S	Q	T	V	W	G	G	H	L	V	S	S	R	n	t	a	g	n	r	330
331	I	N	F	P	S	Y	G	V	F	N	P	G	G	A	I	W	I	A	D	E	D	P	R	P	F	Y	R	t	l	s	360
361	d	p	v	f	v	r	G	G	F	G	N	P	H	Y	V	L	G	L	R	g	v	a	f	q	q	t	g	t	n	h	390
391	t	r	T	F	R	n	s	g	t	i	d	s	l	d	e	i	p	p	q	d	n	s	g	a	p	w	n	d	y	s	420
421	h	v	l	n	h	v	t	f	v	r	W	P	G	E	I	S	G	S	D	S	W	R	a	p	m	f	s	w	t	h	450
451	r	S	A	T	P	T	N	T	I	D	P	E	R	i	t	q	i	p	l	v	k	A	H	T	L	Q	S	G	T	T	480
481	V	V	R	g	p	g	f	t	g	g	d	i	l	r	R	t	s	g	g	p	f	a	y	t	i	v	n	i	n	g	510
511	q	l	p	q	r	Y	R	a	r	I	R	y	a	s	t	t	n	l	r	I	Y	V	T	V	A	G	E	R	i	f	540
541	a	g	q	f	n	k	T	M	D	T	G	D	P	L	T	F	Q	S	F	S	Y	A	T	I	N	T	A	F	T	F	570
571	P	M	S	Q	S	S	F	T	V	G	A	D	T	F	S	S	G	N	E	V	Y	I	D	R	f	e	l	i	p	v	600
601	t	a	t	l	e	a	e	s	d	l	e	r	A	Q	K	a	v	n	a	l	f	t	s	s	n	q	i	g	l	k	630
631	T	D	V	T	D	Y	H	I	D	R	v	s	n	l	v	e	c	l	s	d	e	f	c	l	d	e	k	K	e	l	660
661	s	e	k	V	K	h	a	k	R	l	s	d	e	r	N	L	L	Q	D	P	N	F	R	g	i	n	r	Q	L	D	690
691	R	g	w	r	G	S	T	D	I	T	I	Q	G	G	D	D	V	F	K	e	n	y	v	t	l	l	g	t	f	d	720
721	e	c	y	p	t	y	l	y	q	k	I	D	E	S	K	l	k	A	Y	T	R	y	q	l	r	G	Y	I	E	D	750
751	S	Q	D	L	E	I	Y	L	I	R	y	n	a	k	H	E	T	V	N	V	P	G	T	G	S	L	W	P	L	S	780
781	A	P	S	P	I	G	K	c	a	h	h	s	h	h	f	s	l	d	i	d	v	g	c	t	d	l	n	e	d	l	810
811	g	v	w	v	i	f	k	I	K	t	q	d	g	h	a	r	L	G	N	L	E	F	L	E	E	K	P	L	V	G	840
841	E	A	L	A	R	v	k	R	a	e	k	K	w	r	D	K	r	E	K	l	e	w	e	t	n	i	v	y	k	E	870
871	A	K	e	s	v	d	a	l	f	v	n	s	q	y	d	r	L	Q	A	D	T	N	I	A	M	I	H	A	A	D	900
901	K	r	V	H	S	I	R	e	a	y	l	p	e	l	s	v	i	p	g	v	n	a	a	i	f	e	e	l	e	g	930
931	r	I	F	T	A	F	S	L	Y	D	A	R	n	v	i	k	N	G	D	F	N	N	G	L	S	C	W	N	V	K	960
961	g	h	v	d	v	e	e	q	n	n	h	r	S	V	L	V	V	P	E	W	E	A	E	V	S	Q	E	V	R	v	990
991	c	p	g	r	G	Y	Y	I	L	R	v	t	a	y	k	E	G	Y	G	E	G	C	V	T	I	H	E	I	E	N	N	1020
1021	T	D	E	L	K	f	s	n	c	v	e	e	e	v	y	p	n	n	t	v	t	c	n	d	y	t	a	t	q	e	1050
1051	e	y	e	g	t	y	t	s	r	N	R	g	y	d	g	a	y	e	s	n	s	s	v	p	a	d	y	a	s	a	1080
1081	y	e	e	k	A	Y	T	D	G	R	r	D	N	P	C	E	S	N	R	g	y	g	d	y	t	p	l	p	a	g	1110
1111	y	v	t	k	E	L	E	Y	F	P	E	T	D	K	v	w	i	e	i	g	e	t	e	g	t	f	i	v	d	s	1140
1141	v	e	l	l	l	m	e	e																							1148

Figure 53. Theoretical cleavage of the Cry1F protein with trypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC.

1	M	E	N	N	I	Q	N	Q	C	V	P	Y	n	c	l	n	n	p	e	v	e	i	l	n	e	e	r	s	t	g	30
31	r	l	p	l	d	i	s	l	s	l	t	r	f	L	L	S	E	F	v	p	g	v	g	v	a	f	G	L	F	d	60
61	l	i	w	G	F	i	t	p	s	d	w	S	L	F	l	l	q	i	e	q	l	i	e	q	r	i	e	t	l	e	90
91	r	n	r	a	i	t	t	l	r	g	l	a	d	s	y	E	I	Y	i	e	a	l	r	e	w	E	A	N	P	N	120
121	N	A	Q	L	R	E	D	V	R	I	R	F	a	n	t	d	d	a	l	i	t	a	i	n	n	f	T	L	T	S	150
151	F	e	i	p	l	l	s	v	y	V	Q	A	A	N	L	H	L	S	L	L	R	D	A	V	S	F	g	q	g	w	180
181	G	L	D	I	A	T	V	N	N	H	Y	n	r	l	i	n	l	i	h	r	y	T	K	H	C	L	D	T	Y	n	210
211	q	g	l	e	n	l	r	g	t	n	t	r	q	w	A	R	F	n	q	f	R	R	D	L	T	L	T	V	L	D	240
241	I	V	A	L	F	P	N	Y	d	v	r	t	y	p	i	q	t	s	s	q	l	t	r	e	i	y	T	S	S	V	270
271	I	E	D	S	P	V	S	A	N	I	P	N	G	F	n	r	a	e	f	G	V	R	P	P	H	L	M	D	F	m	300
301	n	s	l	f	V	T	A	E	T	V	R	S	Q	T	V	W	g	g	h	l	v	s	s	r	n	t	a	g	n	r	330
331	i	n	f	p	s	y	G	V	F	n	p	g	g	a	i	w	I	A	D	E	D	P	R	P	F	y	R	T	L	S	360
361	D	P	V	F	v	r	g	g	f	G	N	P	H	Y	v	l	g	l	r	g	v	a	f	Q	Q	T	G	T	N	H	390
391	T	R	T	F	r	n	s	g	t	i	d	s	l	d	e	i	p	p	q	d	n	s	g	a	p	w	N	D	Y	s	420
421	h	v	l	n	h	v	t	f	V	R	W	P	G	E	I	S	G	S	D	S	W	r	a	p	m	f	S	W	t	h	450
451	r	s	a	t	p	t	n	t	i	d	p	e	r	i	t	q	i	p	l	v	k	a	h	t	l	q	s	g	t	t	480
481	v	v	r	g	p	g	f	T	G	G	D	I	L	R	R	T	S	G	G	P	F	a	y	T	I	V	N	I	N	G	510
511	Q	L	P	Q	R	Y	r	a	r	i	r	y	A	S	T	T	N	L	R	I	Y	v	t	v	a	g	e	r	i	f	540
541	A	G	Q	F	n	k	t	m	d	t	g	d	p	l	t	f	Q	S	F	s	y	A	T	I	N	T	A	F	t	f	570
571	p	m	s	q	s	s	f	T	V	G	A	D	T	F	s	s	g	n	e	v	y	I	D	R	F	e	l	i	p	v	600
601	t	a	t	l	e	a	e	s	d	l	e	r	a	q	k	a	v	n	a	l	f	T	S	S	N	Q	I	G	L	K	630
631	T	D	V	T	D	Y	h	i	d	r	v	s	n	l	v	e	c	l	s	d	e	f	C	L	D	E	K	K	E	L	660
661	S	E	K	V	K	H	A	K	R	L	S	D	E	R	N	L	L	Q	D	P	N	F	r	g	i	n	r	q	l	d	690
691	r	g	w	R	G	S	T	D	I	T	I	Q	G	G	D	D	V	F	k	e	n	y	V	T	L	L	G	T	F	d	720
721	e	c	y	p	t	y	L	Y	q	k	i	d	e	s	k	l	k	a	y	T	R	Y	q	l	r	g	y	I	E	D	750
751	S	Q	D	L	E	I	Y	l	i	r	y	N	A	K	H	E	T	V	N	V	P	G	T	G	S	L	W	P	L	S	780
781	A	P	S	P	I	G	K	C	A	H	H	S	H	H	F	s	l	d	i	d	v	g	c	t	d	l	n	e	d	l	810
811	g	v	w	V	I	F	k	i	k	t	q	d	g	h	a	r	l	g	n	l	e	f	L	E	E	K	P	L	V	G	840
841	E	A	L	A	R	V	K	R	A	E	K	K	W	r	d	k	r	e	k	l	e	w	E	T	N	I	V	Y	k	e	870
871	a	k	e	s	v	d	a	l	f	V	N	S	Q	Y	d	r	l	q	a	d	t	n	i	a	m	i	h	a	a	d	900
901	k	r	v	h	s	i	r	e	a	y	L	P	E	L	S	V	I	P	G	V	N	A	A	I	F	e	e	l	e	g	930
931	r	i	f	T	A	F	s	l	y	D	A	R	N	V	I	K	N	G	D	F	n	n	g	l	s	c	w	N	V	K	960
961	G	H	V	D	V	E	E	Q	N	N	H	R	S	V	L	V	V	P	E	W	e	a	e	v	s	q	e	v	r	v	990
991	c	p	g	r	g	y	I	L	R	V	T	A	Y	k	e	g	y	G	E	G	C	V	T	I	H	E	I	E	N	N	1020
1021	T	D	E	L	K	F	s	n	c	v	e	e	e	v	y	p	n	n	t	v	t	c	n	d	y	T	A	T	Q	E	1050
1051	E	Y	e	g	t	y	T	S	R	N	R	G	Y	d	g	a	y	E	S	N	S	S	V	P	A	D	Y	a	s	a	1080
1081	y	E	E	K	A	Y	t	d	g	r	r	d	n	p	c	e	s	n	r	g	y	G	D	Y	t	p	l	p	a	g	1110
1111	y	V	T	K	E	L	E	Y	f	p	e	t	d	k	v	w	I	E	I	G	E	T	E	G	T	F	i	v	d	s	1140
1141	v	e	l	l	l	m	e	e																							1148

Figure 54. Theoretical cleavage of the Cry1F protein with chymotrypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC.

In the endoprotease digests of the transgenic-soybean-derived Cry1F protein, the peptide sequence coverage from peptide mass fingerprint (PMF) data was extensive at 81.7%. Of the 81.7% 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 55, Figure 56 and Figure 57). This analysis confirmed the soybean-derived protein amino acid sequence matched that of the microbe-derived Cry1F protein near the N- and C-terminus as well as a major portion of the internal sequence (Gao *et al.*, 2006). In the MS spectra, there were unidentified peptides detected in the enzyme digest preparations (data not shown). Many factors contribute to the formation of these unidentified peptides, such as over digestion (which results in non-specific cleavage), self-digestion products of trypsin and chymotrypsin, as well as random breakage of peptides during ionization. Unidentified peptides do not indicate the protein is different from the predicted amino acid sequence. The results of these analyses indicate that the amino acid sequence of the soybean-derived Cry1F protein was equivalent to the *P. fluorescens*-expressed protein previously characterized (Gao *et al.*, 2006).

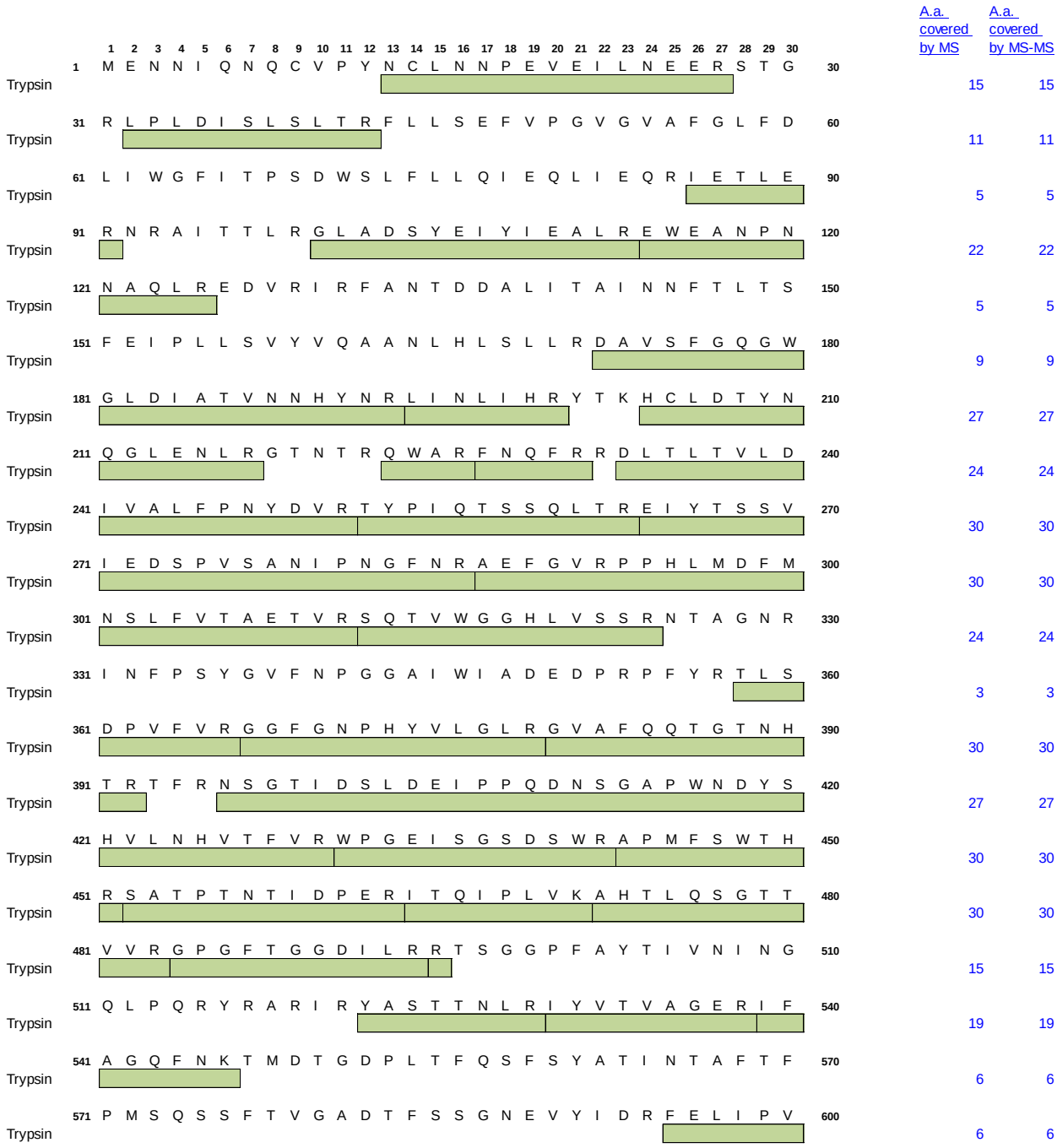


Figure 55. Tryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI-TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 65.1% with PMF data and 65.1% by tandem MS.

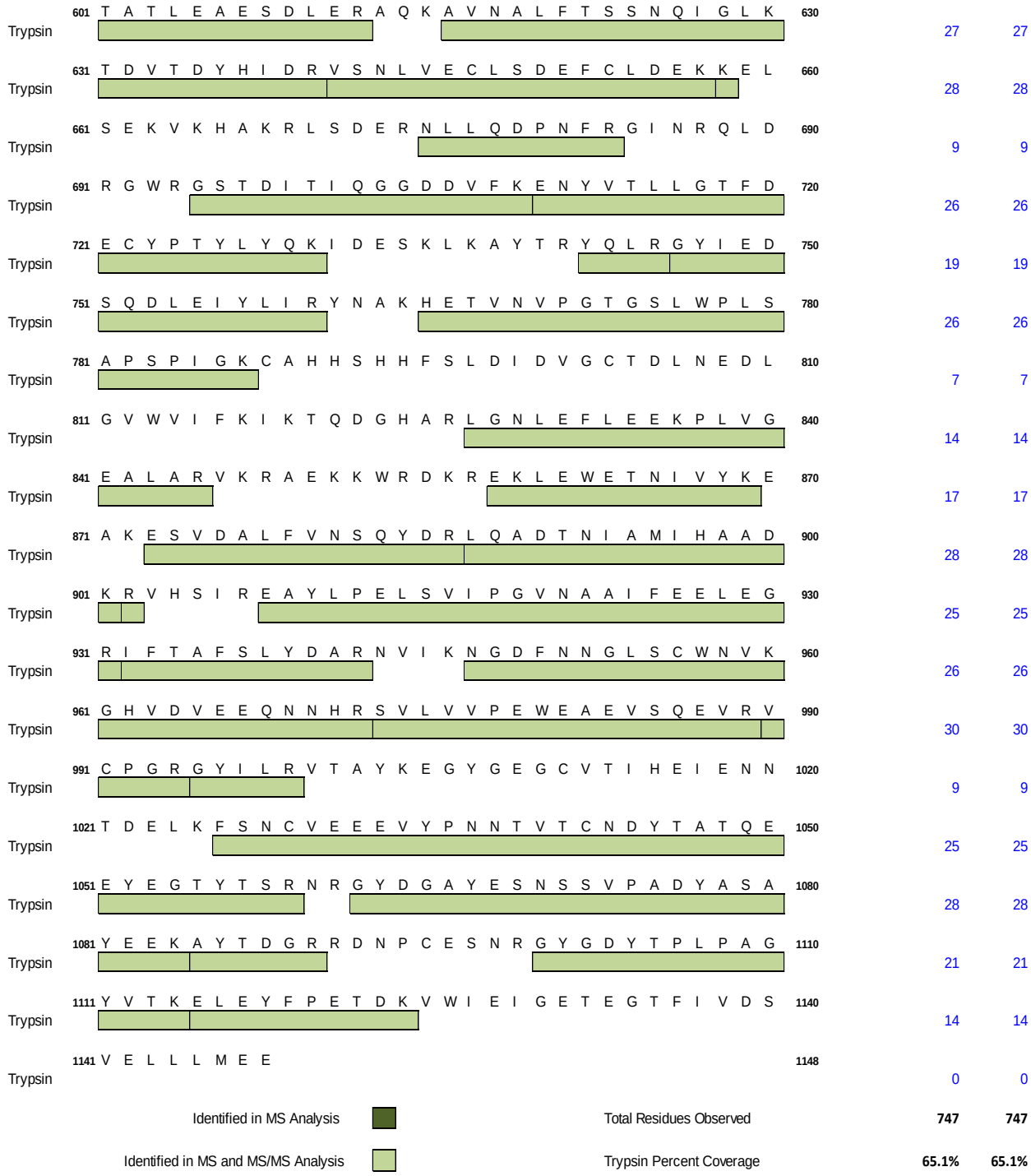


Figure 55 (Cont). Tryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI-TOF/TOF.

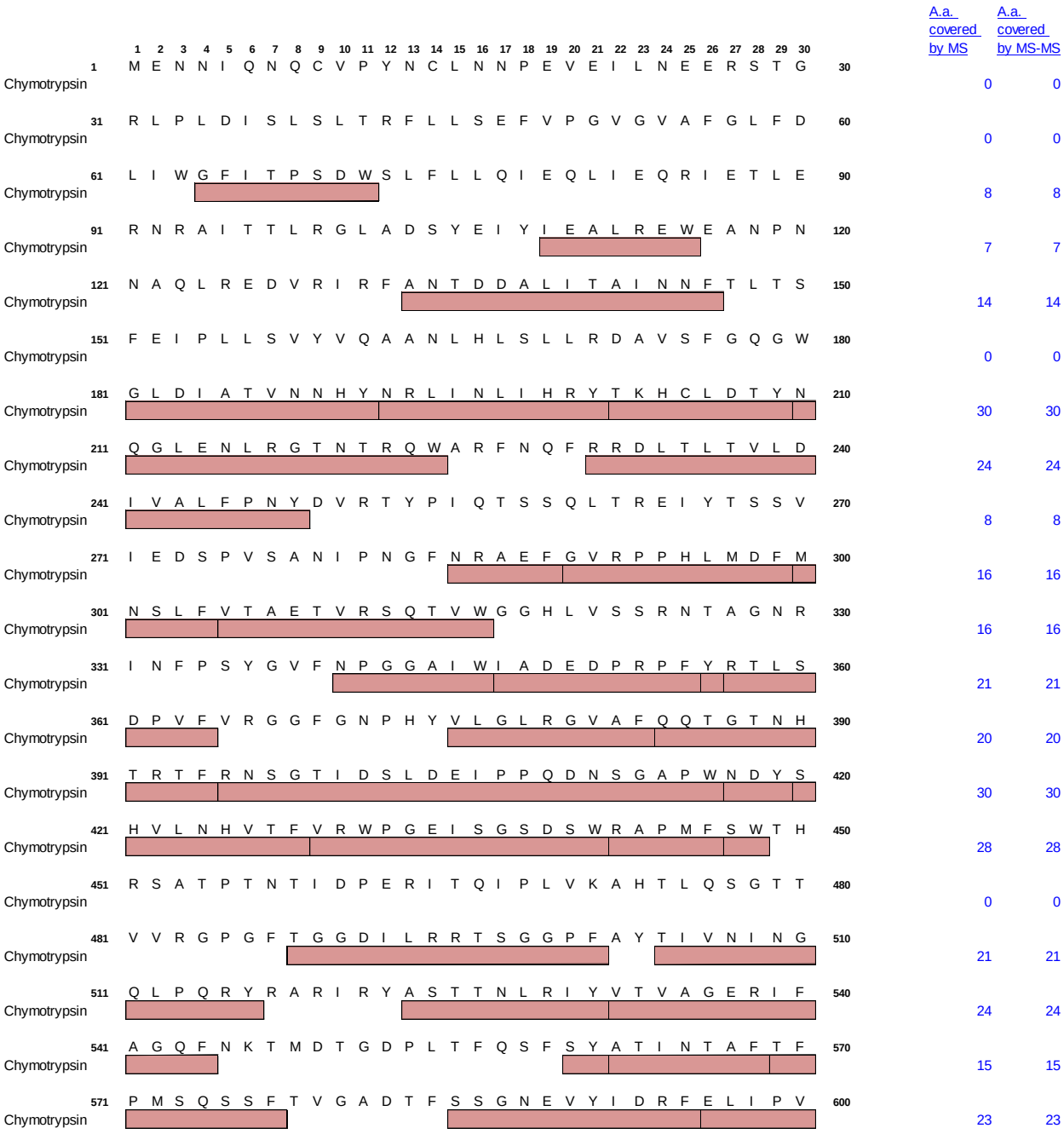


Figure 56. Chymotryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 53.8% with PMF data and 52.4% by tandem MS.

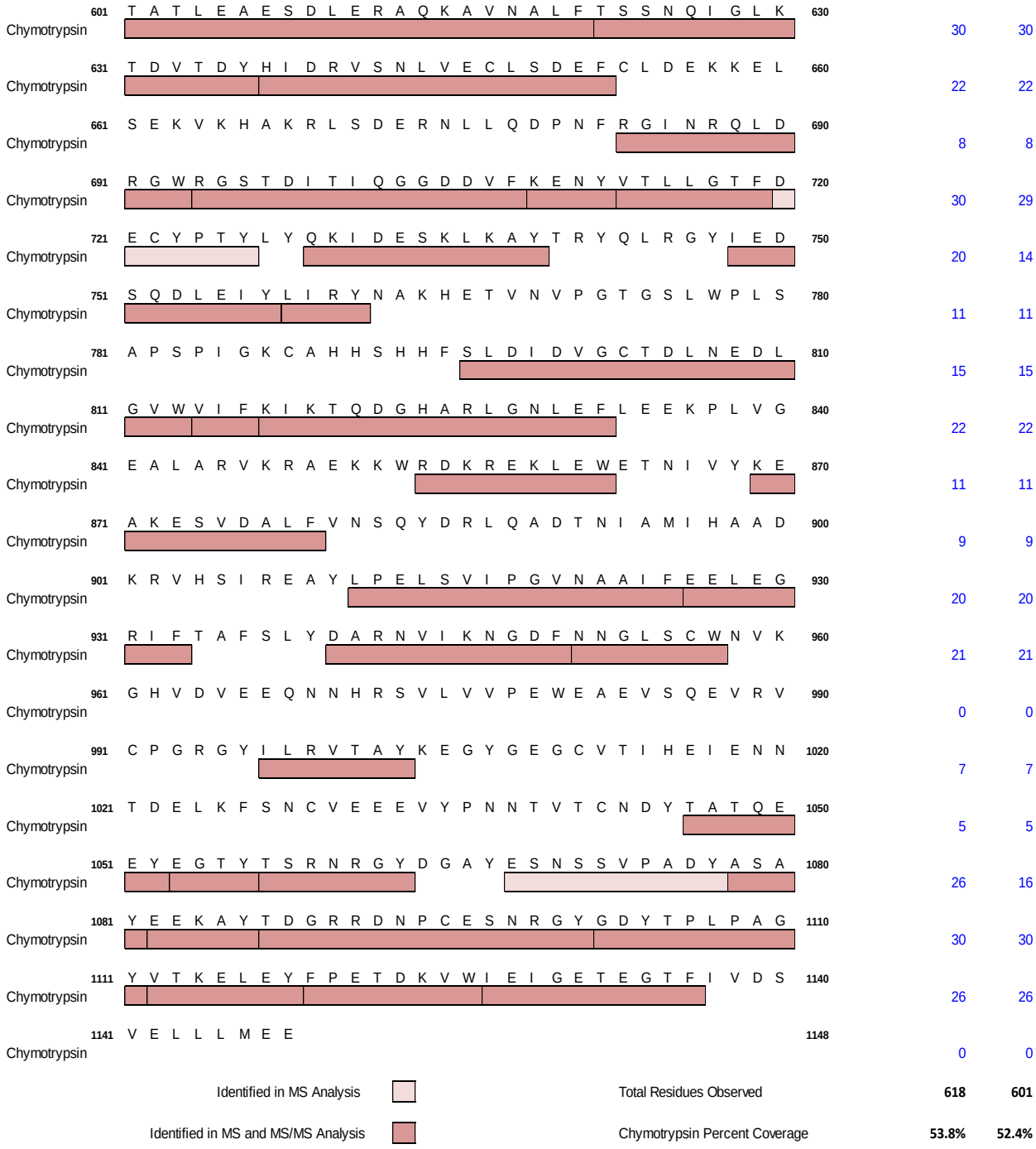


Figure 56 (Cont). Chymotryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

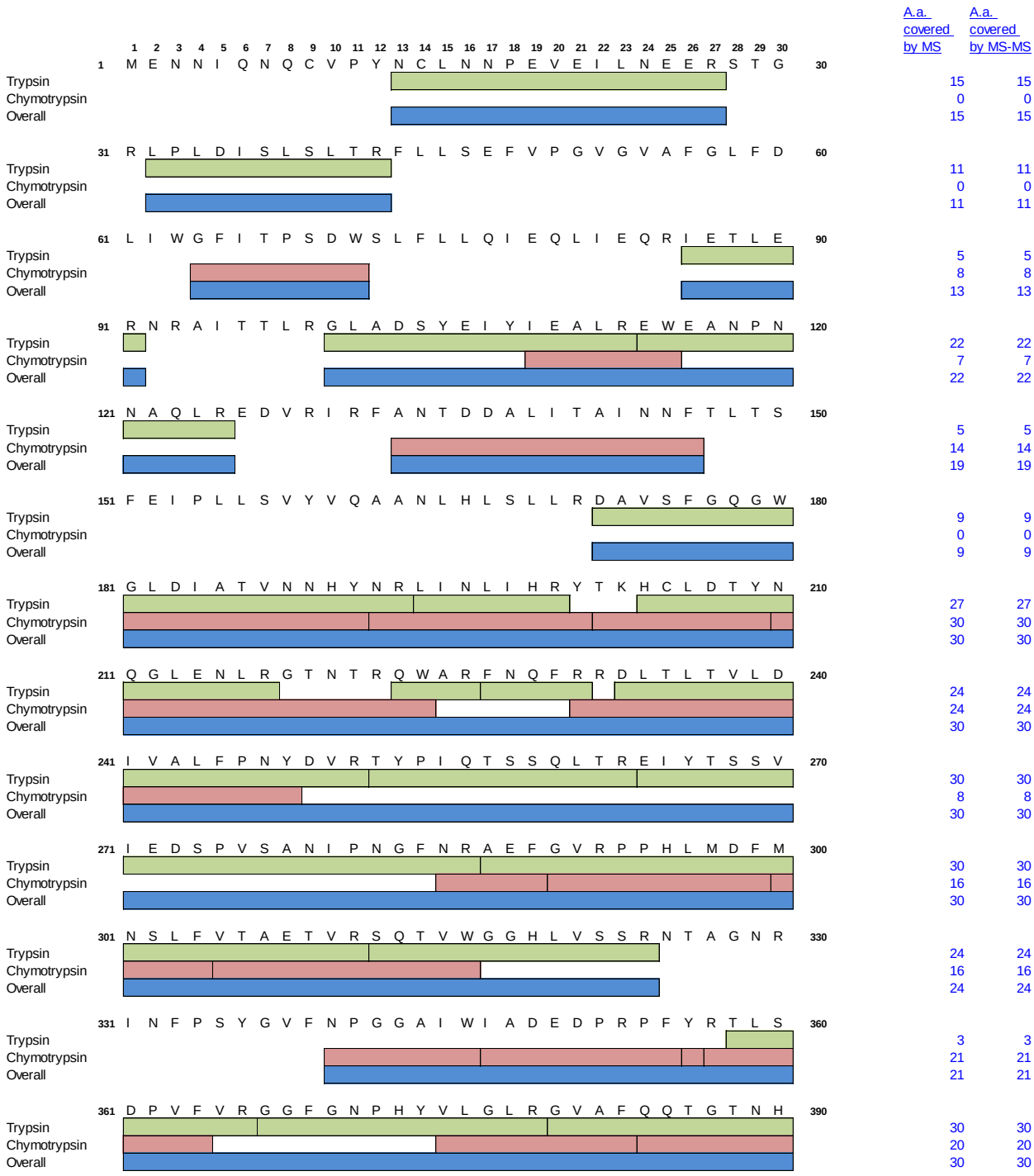


Figure 57. Overall sequence coverage of trypsin and chymotrypsin digests for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Overall sequence coverage was 81.7% with PMF data and 81.7% by tandem MS.

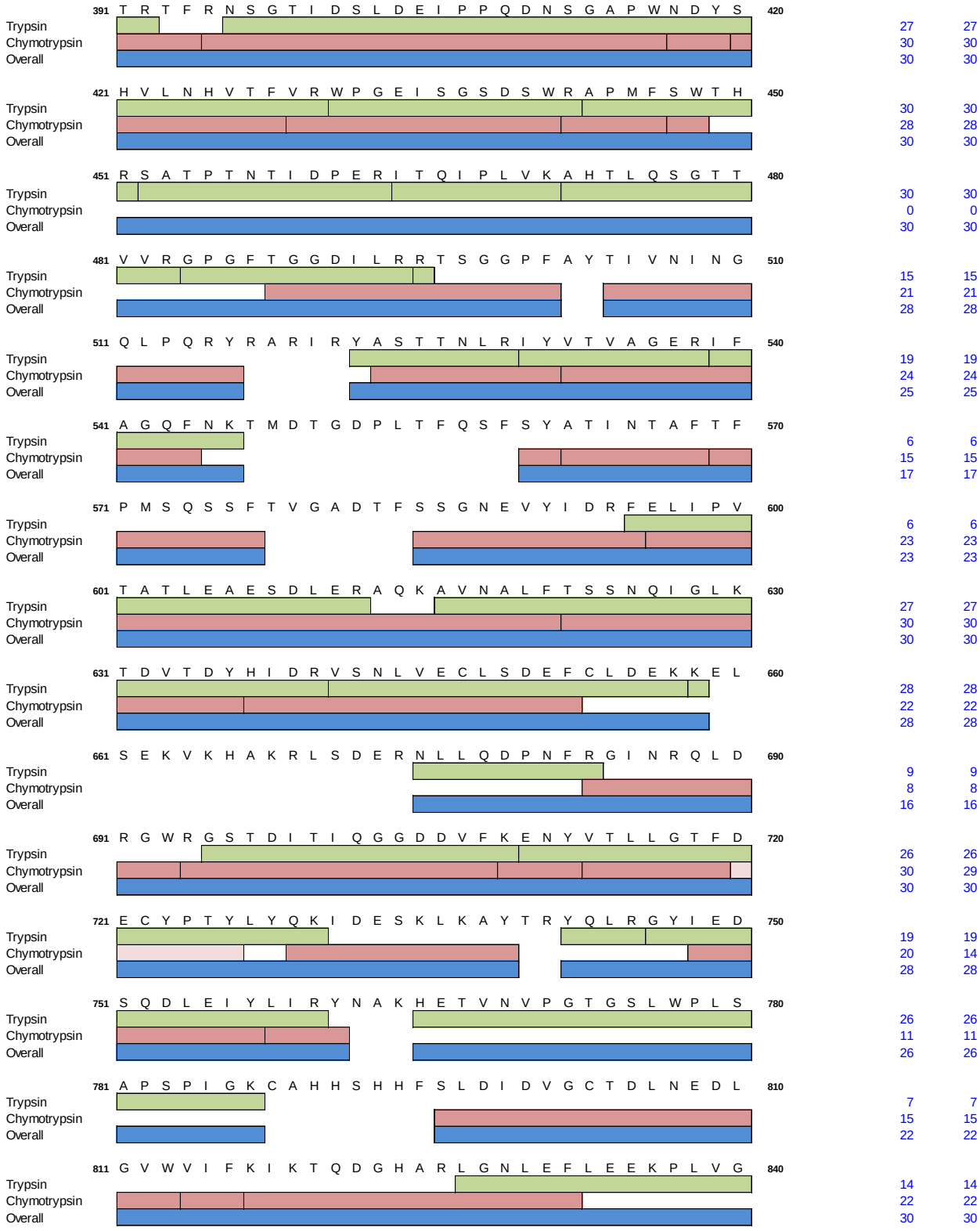


Figure 57 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

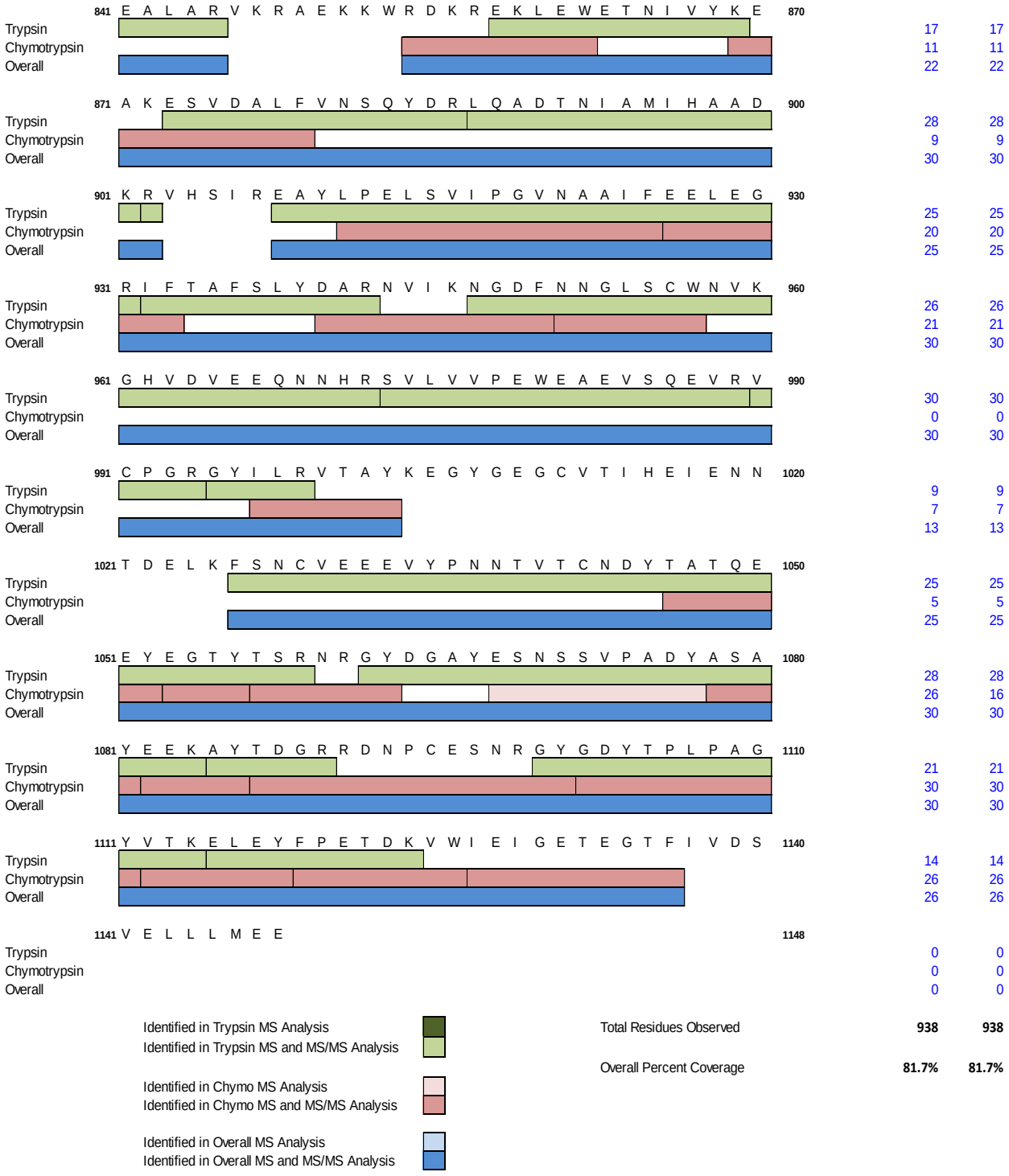


Figure 57 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

Conclusions

It was demonstrated that the biochemical identity of *P. fluorescens*-produced Cry1F protein was equivalent to the protein purified from grain of soybean event DAS-81419-2. Both the soybean- and microbe-derived Cry1F proteins had an apparent molecular weight of ~130 kDa and were immunoreactive to Cry1F protein-specific antibodies in both lateral flow strip and western blot assays. The amino acid sequences were confirmed by enzymatic peptide mass fingerprinting by MALDI-TOF MS and verified by MS/MS. In addition, the lack of glycosylation of the soybean-derived Cry1F protein provided additional evidence that the Cry1F protein produced by *P. fluorescens* and transgenic soybean were essentially equivalent.

Appendix 4. Materials, Methods and Results for Characterization of PAT Protein

Materials and Methods

Test Substance/Test System
The test substance was the PAT protein expressed and extracted from tissues grown from the T5 seeds of transgenic soybean event DAS-81419-2: Source ID: YTR11ETR760037 (TSN303074). The seeds were planted in the greenhouise and 3 to 7 week old leaf tissue was harvested, frozen, lyophilized, ground, stored at -20°C to vent the dry ice, and stored at -80°C. The presence of PAT protein in the soybean tissue was confirmed by a commercially available lateral flow assay kit from EnviroLogix Inc., as described below.

Control Substances
The control substance used in this study was a non-transgenic soybean plant extract (*Glycine max* cv Maverick*).* Seeds of the Maverick soybean line (Source ID: YX11AX090001) were planted, grown, harvested and processed under the same conditions as the transgenic plants described above. The absence of PAT protein in the non-transgenic soybean tissue was confirmed by a commercially available lateral flow assay kit as described below.

Recombinant PAT microbial protein, (TSN031116-0001, Lot #: 55238-1A), has a molecular weight of ~21 kDa and a concentration of 810 µg/mL. An enzymatically active microbial preparation was produced in recombinant *E. coli* and based on confirmation of the gene sequence in the *E. coli* expression system (Madduri and Snodderley, 2007), the *E. coli*-derived PAT protein sequence is identical to the proteins expressed in deregulated transgenic corn, cotton, soybean, canola, event DAS-81419-2, and the native organism *S. viridochromogenes* (Wohlleben *et al.*, 1988; AgrEvo, 1994; Northrup King, 1995; AgrEvo, 1996, 1997; Dow AgroSciences, 2003c, 2003b, 2003a). The DAS-81419-2 and *E. coli*-derived PAT protein sequences are also identical to the PAT protein sequence described in the OECD consensus document (OECD, 1999) and in other publications (Herouet *et al.*, 2005).

Reference Substances
The commercially available (non-GLP) reference substances used in this study are listed in the following table:

Reference Substance	Product Name	Lot Number	Assay	Reference
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	1095889	SDS-PAGE and Western blot	Invitrogen: Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa
Unstained Molecular Weight Markers	Novex Sharp protein standards	1030454	SDS-PAGE	Invitrogen: Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa

Lateral Flow Strip Assay

The soybean leaf tissues of the transgenic and non-transgenic events were harvested fresh as described above and were frozen, lyophilized, ground, and stored at approximately –80°C until use. To confirm the presence/absence of the PAT protein in the pooled tissues, approximately 16 mg of the lyophilized tissues (Event DAS-81419-2 and Maverick) were weighed into 2.0-mL microfuge tubes and tested as described by EnviroLogix Inc. Briefly, the soluble proteins were extracted by adding 2 metal beads to the extraction buffer (0.25 mL EB2 buffer supplied by the manufacturer) and grinding in a Spex Geno-Grinder for 3 minutes at 500 strokes per minute. The samples were subjected to centrifugation for 5 minutes at 20,000×g and the resulting supernatants were transferred to a fresh tube and the strips were developed according to the manufacturer's instructions.

SDS-PAGE and Western Blot

SDS-PAGE analysis of the transgenic (DAS-81419-2) and non-transgenic Maverick soybean extracts was performed with Bio-Rad Criterion gels fitted in a Criterion Cell gel module with MES running buffer. Extracts were prepared by grinding ~43 mg of tissue for 3 minutes in a Geno-Grinder with steel ball bearings in ~ 1 mL of PBST based buffer (Table 25). The supernatants were clarified by centrifuging for 5 minutes at 20,000×g, and then 120 µL of each extract was mixed with 30 µL of 5x Laemmli sample buffer (LSB, 2% SDS, 50 mM Tris pH 6.8, 0.2 mg/mL bromophenol blue, 50% (w/w) glycerol containing 10% freshly added 2-mercaptoethanol). Samples were heated for 5 minutes at ~95°C, and after a brief centrifugation, 20 µL of the supernatant was loaded directly on the gel. The reference standard, microbe-derived PAT (TSN031116-0001) was diluted with Bio-Rad 2x LSB containing 5% β-mercaptoethanol and processed as described earlier. The electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes using MES running buffer from Bio-Rad. 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 to obtain a permanent record of the image. The remaining half of the gel was electro-blotted to a nitrocellulose membrane from Bio-Rad with a Criterion trans-blot electrophoretic transfer cell for ~60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was probed with a PAT specific polyclonal rabbit antibody (Lot #: D2976-27, 1.1 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase from Pierce was used as the secondary (detection) antibody. Pierce ECL chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to Thermo Scientific CL-XPosure detection film for various time points and subsequently developed with a Radiation Services film developer.

Table 25. Soybean-derived PAT extraction buffer composition.

Ingredient	Amount
Phosphate Buffered Saline with 0.5% Tween[a] 20, pH 7.4	5.924 mL
0.5 M EDTA	60 µL
Protease inhibitor cocktail	6.0 µL
β-mercaptoethanol	10 µL

Note: the extraction buffer was prepared immediately before use.

Results

Lateral Flow Strip Assay

The presence of the PAT protein in the pooled T5 leaf tissue of DAS-81419-2 was confirmed using a commercially prepared lateral flow test strip from EnviroLogix Inc. The assay easily discriminated between transgenic and non-transgenic plants as the non-transgenic extracts of Maverick did not contain detectable amounts of immunoreactive protein (Figure 58).

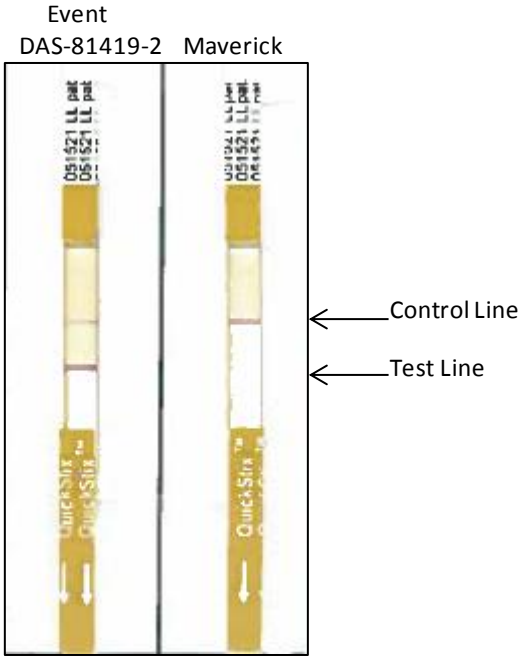


Figure 58. Lateral flow strip assay for PAT protein expression in Event DAS-81419-2 and Maverick leaf extracts.

* Note: Event DAS-81419-2 is positive for the PAT protein

SDS-PAGE and Western Blot Analysis

In the toxicology-lot preparation of *E. coli*-produced PAT protein (TSN031116-0001), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, was approximately 21 kDa (Figure 59). As expected, the corresponding soybean-derived PAT protein was visualized by immunospecific polyclonal antibodies at an identical size to the microbe-expressed proteins. In the PAT Western blot analysis, no immunoreactive proteins, consistent with the PAT protein,

were observed in the control Maverick extract; however a non-specific band was detected at ~40 kDa and was detected in both the transgenic and non-transgenic extracts. In addition the polyclonal antibody did detect a small amount of a protein dimer in the microbe-derived PAT protein preparation. These results add to the evidence that the PAT protein expressed in soybean is not post-translationally modified (the PAT enzyme it does not contain N-glycosylation sites (Herouet *et al.*, 2005) or processed which would have added to or subtracted from the overall protein molecular weight.

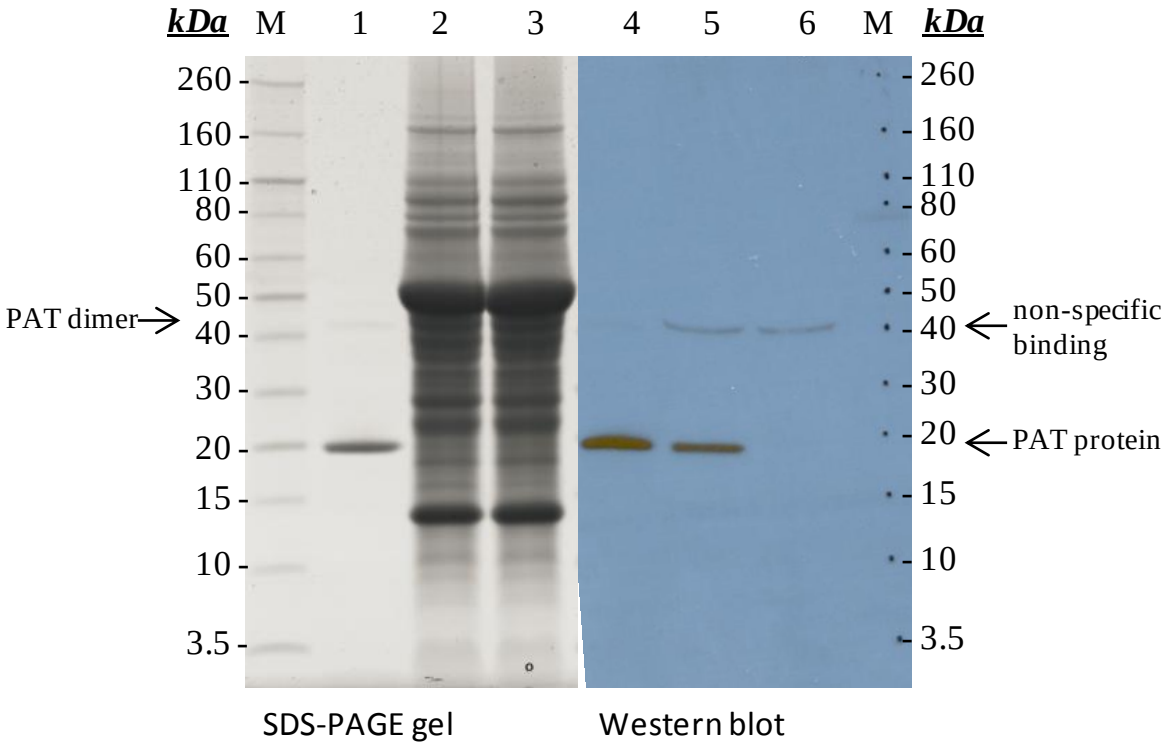


Figure 59. SDS-PAGE and Western blot analysis of microbe-derived PAT, transgenic soybean Event DAS-81419-2, and non-transgenic Maverick extracts.

Lane	*Sample*	*Amount Loaded*
M	Novex unstained MW Markers	10 µL
1	PAT Protein Standard (TSN031116-0001)	~1 µg
2	Transgenic Soybean - Event DAS-81419-2	20 µL
3	Non-transgenic Soybean – Maverick	20 µL
4	PAT Protein Standard (TSN031116-0001)	~5 ng
5	Transgenic Soybean - Event DAS-81419-2	20 µL
6	Non-transgenic Soybean – Maverick	20 µL
M	Novex Prestained MW Markers*	10 µL

*Note: The molecular weight markers were manually transferred to the film after development.

Conclusion

The results of this study demonstrated that both the transgenic soybean-plant extract and the microbe-derived PAT toxicological lot contained the intact, full-length PAT protein. This was confirmed by SDS-PAGE molecular-weight approximation, Western blot analysis, and immunoreactivity using a commercially available lateral flow strip assay. Together, these biochemical tests indicate that the plant- and microbe-derived PAT proteins are substantially equivalent and consistent with the published data on PAT.

Appendix 5. Materials and Methods for Cry1Ac, Cry1F and PAT Protein Expression Analysis

Experimental Design

This study used the same plots that were used for agronomic performance and nutrient composition studies. The experimental design included ten field sites located in Iowa (2 sites), Illinois (2 sites), Indiana, Missouri (2 sites), Nebraska (2 sites), and Pennsylvania. Each trial site included DAS-81419-2 soybean, the non-transgenic near-isogenic control (Maverick), and three non-transgenic reference lines. At each of the ten sites, all entries were arranged in a randomized complete block design with four blocks. Across all sites, each control and DAS-81419-2 was represented by a total of 40 plots (10 sites, 4 replicate plots per entry at each site). Three of the six reference lines were included at each site by randomizing across sites in a balanced incomplete-block design. Each of the six reference lines was assigned to five sites; therefore, each reference line was represented by a total of 20 plots across sites (5 sites per reference line, 4 replicate plots per entry at each site). At each site, four replicate plots of each entry were established, with each plot consisting of four 25 ft (7.62 m) rows. Soybean seeds were planted at a seeding rate of approximately 125 seeds per row with seed spacing within each row of approximately 2.4 inches (6 cm). Each soybean plot was bordered by two rows of a non-transgenic soybean cultivar of similar maturity. The entire trial site was surrounded by a minimum of four rows (10 ft or 3.0 m) of a non-transgenic soybean cultivar of similar maturity. Appropriate insect, weed, and disease control practices were applied to produce an agronomically acceptable crop.

Sample Collection

Plant tissue samples were collected as described below and shipped to Dow AgroSciences Regulatory Sciences and Government Affairs laboratories where they were maintained frozen until use. Samples of soybean tissues were prepared for expression analysis by coarse grinding, lyophilizing and/or fine-grinding with a Geno/Grinder (Certiprep, Metuchen, New Jersey).

Leaf (V5 and V10-12)
One leaf sample per plot, each sample containing 8 trifoliate sets of leaves collected from separate plants, was collected for each test and control entry. Each leaf sample was the youngest set of fully expanded trifoliate leaves.

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

Forage (R3)
One forage sample (representing 3 plants) per plot each consisting of the aerial portion (no roots) of 3 whole plants was collected from each test and control entry.

Grain (R8 – Maturity)
One individual sample was collected from each plot of each test and control entry. Each sample contained approximately 500-grams of grain.

Determination of Cry1Ac Protein Concentration

Soybean tissue samples were analyzed using Dow AgroSciences method 110674. In this method, the soluble, extractable Cry1Ac protein was quantified using an enzyme-linked immunosorbent assay (ELISA) kit from Romer Labs, Inc.

The Cry1Ac protein was extracted from lyophilized, ground soybean tissues with a phosphate buffered saline solution containing 0.05% Triton X-100 with 1 mg/mL sodium ascorbate (PBS/Triton/AA). The extract was centrifuged, and then the aqueous supernatant was collected and subjected to trypsin digestion to convert full length Cry1Ac to the truncated Cry1Ac core toxin. After truncation by trypsin, the reaction was stopped by the protease inhibitor phenylmethanesulfonyl fluoride (PMSF). The digested extract was diluted and assayed using a specific Cry1Ac ELISA kit. A sequential sandwich ELISA format was applied in this assay. An aliquot of the diluted sample was incubated in the wells of a plate pre-coated with an immobilized monoclonal anti-Cry1Ac antibody, and then the unbound samples are removed from the plate by washing with phosphate buffered saline solution containing 0.05% Tween 20 (PBST). An excess amount of enzyme-conjugated monoclonal antibody specific to the Cry1Ac protein was then added to the wells for incubation. These antibodies bind with Cry1Ac protein in the wells and form a "sandwich" with Cry1Ac protein bound between the soluble and immobilized antibodies. At the end of an incubation period, the unbound reagents are removed from the plate by washing with PBST. The presence of Cry1Ac was detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate, generating a colored product. Since the Cry1Ac is bound in the antibody sandwich, the level of color development is proportional to the concentration of Cry1Ac in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm was measured either using a Grifols Triturus Automated Immunoassay Analyzer with a 620 nm background subtraction or using a microplate reader with a 650 nm background subtraction. A calibration curve was estimated from 7 standard concentrations using a quadratic regression equation with a coefficient of determination $\geq$ 0.990.

Determination of Cry1F Protein in Soybean Tissue Samples

Soybean tissue samples were analyzed using Dow AgroSciences method 110675. In this method, the soluble, extractable Cry1F protein was quantified using an enzyme-linked immunosorbent assay (ELISA) kit from Romer Labs, Inc.

The Cry1F protein was extracted from lyophilized, ground soybean tissues with a phosphate buffered saline solution containing 0.05% Triton X-100 with 1 mg/mL sodium ascorbate (PBS/Triton/AA). The extract was centrifuged; the aqueous supernatant was collected, diluted, and assayed using a specific Cry1F ELISA kit. A sandwich ELISA format was applied in this assay. An aliquot of the diluted sample was incubated in the wells of a plate pre-coated with an immobilized monoclonal anti-Cry1F antibody coated plate along with an enzyme-conjugated monoclonal antibody specific to the Cry1F protein. These antibodies bind with Cry1F protein in the wells and form a "sandwich" with Cry1F protein bound between the soluble and immobilized antibodies. At the end of an incubation period, the unbound reagents are removed from the plate by washing with PBST. The presence of Cry1F was detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate, generating a colored product. Since the Cry1F is

bound in the antibody sandwich, the level of color development is proportional to the concentration of Cry1F in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm was measured either using a Grifols Triturus Automated Immunoassay Analyzer with a 620 nm background subtraction or using a microplate reader with a 650 nm background subtraction. A calibration curve was estimated from 7 standard concentrations using a quadratic regression equation.

Determination of PAT Protein in Soybean Tissue Samples

Soybean tissue samples were analyzed using Dow AgroSciences method GRM 08.05. In this method, the soluble, extractable PAT protein was quantified using an enzyme-linked immunosorbent assay (ELISA) kit from EnviroLogix, Inc.

The PAT protein was extracted from lyophilized, ground soybean samples with a phosphate buffered saline solution containing 0.05% Tween 20 and 1% polyvinylpyrrolidone (PBST/PVP). The extract was centrifuged; the aqueous supernatant was collected, diluted and assayed using a specific PAT ELISA kit. An aliquot of the diluted sample was incubated with enzyme-conjugated anti-PAT protein monoclonal antibody in the wells of an anti-PAT polyclonal antibody coated plate in a sandwich ELISA format. Both antibodies in the sandwich pair capture the PAT protein in the sample. At the end of the incubation period, the unbound reagents were removed from the plate by washing with PBST. The presence of PAT was detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate, generating a colored product. Since the PAT is bound in the antibody sandwich, the level of color development is proportional to the concentration of PAT in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm was measured either using a Grifols Triturus Automated Immunoassay Analyzer with a 620 nm background subtraction or using a microplate reader with a 650 nm background subtraction. A calibration curve was estimated from the 7 standard concentrations using a quadratic regression equation.

Appendix 6. Analytical Methods for Compositional Analysis

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

Amino Acid Composition (TALC/TPLC)
Total aspartic acid (including asparagine)
Total threonine
Total serine
Total glutamic acid (including glutamine)
Total proline
Total glycine
Total alanine
Total valine
Total isoleucine
Total leucine
Total tyrosine
Total phenylalanine
Total histidine
Total lysine
Total arginine
Total tryptophan
Sulfur-containing amino acids: Total methionine
Total cystine (including cysteine)

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 are 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 are derivatized with fluorenylmethyl chloroformate (FMOC) before injection.

Reference Standards:

Component	Manufacture	Lot No.	Purity (%)
L-Alanine	Sigma-Aldrich	1440397	99.9
L-Arginine Monohydrochloride	Sigma-Aldrich	1361811	100.0
L-Aspartic Acid	Sigma-Aldrich	BCBB9274	100.6
L-Cystine	Sigma-Aldrich	1418036 1451329	99.9 100
L-Glutamic Acid	Sigma-Aldrich	1423805	100.2
Glycine	Sigma-Aldrich	1119375	100.0
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	BCBB1348	99.9
L-Isoleucine	Sigma-Aldrich	1423806	100.0

Component	Manufacture	Lot No.	Purity (%)
L-Leucine	Sigma-Aldrich	BCBB1733	98.6
L-Lysine Monohydrochloride	Sigma-Aldrich	1362380	100.2
L-Methionine	Sigma-Aldrich	1423807	99.9
L-Phenylalanine	Sigma-Aldrich	BCBB9200	100
L-Profile	Sigma-Aldrich	1414414	99.7
L-Serine	Sigma-Aldrich	1336081	99.9
L-Threonine	Sigma-Aldrich	1402329	100.0
L-Tryptophan	Sigma-Aldrich	BCBB1284	99.8
L-Tyrosine	Sigma-Aldrich	BCBB5393	99.5
L-Valine	Sigma-Aldrich	1352709	100.0

Ash (ASHM)
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.

Beta Carotene (BCLC)
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.

Reference Standard:
Sigma-Aldrich, Beta Carotene, 99.0%, Lot No. 021M1304V

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

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

Fat by Acid Hydrolysis (FAAH)
The samples were hydrolyzed with hydrochloric acid. The fat was extracted using ether and hexane. The extracts were dried down and filtered through a sodium sulfate column.
The remaining extracts were then evaporated, dried, and weighed.

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.

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.

Reference Standards:

Manufacturer	Lot No.	Component	Weight (%)		Purity (%)
			S27-V	S28-V	
Nu-Chek Prep GLC Reference Standard Covance 1 Covance 2	S27-V S28-V	Methyl Octanoate	3.0	1.25	99.7
		Methyl Decanoate	3.25	1.25	99.6
		Methyl Laurate	3.25	1.25	99.8
		Methyl Myristate	3.25	1.25	99.8
		Methyl Myristoleate	1.0	1.25	99.5
		Methyl Pentadecanoate	1.0	1.25	99.6
		Methyl Pentadecenoate	1.0	1.25	99.4
		Methyl Palmitate	10.0	15.0	99.8
		Methyl Palmitoleate	3.0	1.25	99.7
		Methyl Heptadecanoate	1.0	1.25	99.6
		Methyl 10-Heptadecenoate	1.0	1.25	99.5
		Methyl Stearate	7.0	11.0	99.8
		Methyl Oleate	10.0	15.0	99.8
		Methyl Linoleate	10.0	15.0	99.8
		Methyl Gamma Linolenate	1.0	1.25	99.4
		Methyl Linolenate	3.0	1.25	99.5
		Methyl Arachidate	2.0	1.25	99.8
		Methyl 11-Eicosenoate	2.0	1.25	99.6
		Methyl 11-14 Eicosadienoate	1.0	1.25	99.5
		Methyl 11-14-17	1.0	1.25	99.5
		Methyl Arachidonate	1.0	1.25	99.4
		Methyl Behenate	1.0	1.5	99.8

Folic acid (FOAN)
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.

Reference Standard:
USP, Folic acid, 98.9%, Lot No. Q0G151

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

Inorganic Ventures Reference Standards and Limits of Quantitation:

Mineral	Lot No.	Concentration (µg/mL)
Calcium	E2-MEB393070MCA, E2-MEB393072	200, 1000
Copper	E2-MEB393070MCA, E2-MEB393071MCA	2.00, 10.00
Iron	E2-MEB393070MCA, E2-MEB393073	10.0, 50.0
Magnesium	E2-MEB393070MCA, E2-MEB393071MCA	50.0, 250
Manganese	E2-MEB393070MCA, E2-MEB393071MCA	2.0, 10.0
Phosphorus	E2-MEB393070MCA, E2-MEB393072	200, 1000
Potassium	E2-MEB393070MCA, E2-MEB393072	200, 1000
Sodium	E2-MEB393070MCA, E2-MEB393072	200, 1000
Zinc	E2-MEB393070MCA, E2-MEB393071MCA	10.0, 50.0

Isoflavones (ASOF)
The samples were extracted at approximately 65°C with a 80/20 methanol:water solution and the extract was saponified with dilute NaOH solution. The extract was then acidified, filtered, and diluted. The samples were analyzed on a high-performance liquid chromatography system with ultraviolet spectrophotometric detection and was compared against an external standard curve.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Daidzein	LC Labs	DA-121	99.7
Glycitein	LC Labs	ARH-114	99.8
Genistein	LC Labs	CH-148	99.7
Daidzin	LC Labs	ARF-114	99.7
Glycitin	LC Labs	ARG-113	99.6
Genistin	LC Labs	ARE-109	99.4

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

Moisture (M100)

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

Neutral Detergent Fiber (NDFA)
The ANKOM2000 Fiber Analyzer automated the process of the removal of protein, carbohydrate, and ash. Fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin and insoluble protein fraction were left in the filter bag and determined gravimetrically.

Niacin (NIAP)
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.

Reference Standard:
USP, Niacin, 99.8%, Lot No. I0E295

Pantothenic Acid (PANN)
The samples were diluted with water or treated with an enzyme mixture to liberate the pantothenic acid from coenzyme A and the pH was adjusted to remove interferences. The amount of pantothenic acid was determined by comparing the growth response of the samples, using the bacteria *Lactobacillus plantarum*, with the growth response of a calcium pantothenate standard. This growth response was measured turbidimetrically.

Reference Standard:
USP, Calcium Pantothenate, 99.0%, Lot No. O1H081

Phytic Acid (PHYT)
The samples were extracted using hydrochloric acid and sonication, purified using a silica based anion exchange column, concentrated and injected onto a high-performance liquid chromatography (HPLC) system with a refractive index detector.

Reference Standard:
Sigma-Aldrich, Phytic Acid Sodium Salt Hydrate, 93.5%, Lot No. BCBF5728V

Protein (PGEN)
The BUCHI Automated Digester was used to automate the following process. 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. The percent nitrogen was alculated and converted to equivalent protein using the factor 6.25.

Selenium by Inductively Coupled Plasma-Mass Spectrometry (SEMS)
The samples were closed-vessel microwave digested with nitric acid (HNO_3) and water. After digestion, the solution was 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.

Reference Standard:
SPEX, Selenium, 1000 mg/L, Lot No. 16-177SE

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

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
D-(+)-Raffinose pentahydrate	Sigma-Aldrich	019K1156	99.6
Stachyose hydrate	Sigma-Aldrich	049K3800	98

Total Tocopherols (TTLC)
The samples were saponified to break down any fat and release vitamin E. The saponified mixtures were extracted with an organic solvent, dried down and brought to a suitable volume in hexane. The samples were then quantitated by high-performance liquid chromatography using a silica column.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Alpha Tocopherol	USP	N0F068	98.9
rac-beta-Tocopherol	Matreya LLC	23260	>99
Gamma Tocopherol	ACROS	A0083534	99.3
(+)-δ-Tocopherol (Delta)	Sigma-Aldrich	090M1916V	92

Total Dietary Fiber (TDF)

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 were calculated using protein and ash values and the weighed residue fractions.

Trypsin Inhibitor (TRIP)
The samples were ground and defatted with petroleum ether. A sample of matrix was extracted with 0.01N sodium hydroxide. Varying aliquots of the sample suspensions were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride. The samples were allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was halted by the addition of acetic acid. The absorbance was determined at 410 nm against a sample blank. Trypsin inhibitor activity was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DLarginine~p~nitroanilide hydrochloride.

Thiamine Hydrochloride (BIDE)
The samples were autoclaved under weak acid conditions to extract the thiamine. The resulting solutions were incubated with a buffered enzyme solution to release any bound thiamine. The solutions were purified on a cation-exchange column. Aliquots were reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard.

Reference Standard:
USP, Thiamine Hydrochloride, 99.8%, Lot No. O1F236

Vitamin B_2 (Riboflavin) (B2FV)

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.

Reference Standard:
USP, Riboflavin, 99.7%, Lot No. N1J079

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.

Reference Standard:
USP, Pyridoxine hydrochloride, 99.8%, Lot No. Q0G409

Vitamin C (VCF)
The vitamin C in the samples were extracted, oxidized, and mixed with o-phenylenediamine to produce a fluorophor having an activation maximum at approximately 350 nm and a fluorescence maximum at 430 nm. Fluorescence was proportional to concentration. Development of the fluorescence compound with the vitamin was prevented by forming a boric acid-dehydroascorbic acid complex prior to addition of the o-phenylenediamine solution. Any remaining fluorescence was due to extraneous material and served as the blank.

Reference Standard:
USP, Ascorbic Acid, 99.9%, Lot No. Q1G135

Appendix 7. Literature Ranges for Compositional Analysis

Matrix	Category	Analyte	Units	OECD[1]		ILSI[2]		Literature		Literature Citations	
				Min	Max	Min	Max	Min	Max	Min	Max
Forage	Fiber	ADF	% DW	32	38	NR	NR	22.72	59.03	Harrigan et al. 2007	Berman et al. 2010
Forage	Fiber	NDF	% DW	34	40	NR	NR	19.61	73.05	Lundry et al. 2008	Berman et al. 2010
Forage	Proximate	Ash	% DW	8.8	10.5	6.718	10.782	4.68	9.24	Harrigan et al. 2007	Lundry et al. 2008
Forage	Proximate	Carbohydrates	% DW	NR	NR	59.8	74.7	60.61	80.18	Berman et al. 2009	Berman et al. 2010
Forage	Proximate	Moisture	% FW	74	79	73.5	81.6	32.05	84.60	Berman et al. 2009	Berman et al. 2010
Forage	Proximate	Crude Protein	% DW	11.2	17.3	14.38	24.71	11.77	24.29	Berman et al. 2010	Lundry et al. 2008
Forage	Proximate	Total Fat	% DW	NR	NR	1.302	5.132	1.01	9.87	Berman et al. 2010	Lundry et al. 2008
Forage	Mineral	Calcium	mg/100g dry wt.	NR	NR	NR	NR	NR	NR	NR	NR
Forage	Mineral	Phosphorus	mg/100g dry wt.	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Fiber	ADF	% DW	9.0	11.1	7.81	18.61	9.22	26.26	Lundry et al. 2008	Lundry et al. 2008
Grain	Fiber	NDF	% DW	10.0	14.9	8.53	21.25	10.79	23.90	Lundry et al. 2008	Lundry et al. 2008
Grain	Proximate	Ash	% DW	4.5	6.4	3.885	6.994	4.29	6.44	Padgette et al. 1996	Harrigan et al. 2007
Grain	Proximate	Carbohydrates	% DW	31.7	31.8	29.6	50.2	29.3	44.35	Padgette et al. 1996	Harrigan et al. 2007
Grain	Proximate	Moisture	% FW	NR	NR	4.7	34.4	4.71	14.30	Harrigan et al. 2007	Taylor et al. 1999
Grain	Proximate	Crude Protein	% DW	32	43.6	33.19	45.48	32.29	48.4	Berman et al. 2011	Hartwig and Kilen 1991
Grain	Proximate	Total Fat	% DW	15.5	24.7	8.104	23.562	14.10	23.67	Padgette et al. 1996	Berman et al. 2010
Grain	Mineral	Calcium	mg/100g dry wt.	NR	NR	116.55	307.10	258	510	Iskander 1987	Bilyeu et al. 2008
Grain	Mineral	Copper	mg/100g dry wt.	NR	NR	NR	NR	0.632	1.092	Bilyeu et al. 2008	Bilyeu et al. 2008
Grain	Mineral	Iron	mg/100g dry wt.	NR	NR	5.536	10.954	3.734	6.624	Bilyeu et al. 2008	Bilyeu et al. 2008
Grain	Mineral	Magnesium	mg/100g dry wt.	NR	NR	219.40	312.84	261	280	Iskander 1987	Bilyeu et al. 2008
Grain	Mineral	Manganese	mg/100g dry wt.	NR	NR	NR	NR	2.52	3.876	Iskander 1987	Bilyeu et al. 2008
Grain	Mineral	Phosphorus	mg/100g dry wt.	NR	NR	506.74	935.24	770	790	Bilyeu et al. 2008	Bilyeu et al. 2008
Grain	Mineral	Potassium	mg/100g dry wt.	NR	NR	1868.01	2316.14	1910	2510	Iskander 1987	Bilyeu et al. 2008
Grain	Mineral	Selenium	ppb DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Mineral	Sodium	mg/100g dry wt.	NR	NR	NR	NR	4.05	30	Iskander 1987	Bilyeu et al. 2008
Grain	Mineral	Zinc	mg/100g dry wt.	NR	NR	NR	NR	4.98	7.578	Iskander 1987	Bilyeu et al. 2008
Grain	Amino Acid	Alanine	% total amino acid	NR	NR	4.23	4.74	4.16	4.54	Berman et al. 2011	Harrigan et al. 2007
Grain	Amino Acid	Arginine	% total amino acid	NR	NR	6.70	8.41	6.41	7.60	Berman et al. 2010	Lundry et al. 2008
Grain	Amino Acid	Aspartic Acid	% total amino acid	NR	NR	11.39	12.04	11.37	12.68	Taylor et al. 1999	Berman et al. 2009
Grain	Amino Acid	Cystine	% total amino acid	NR	NR	1.02	1.87	1.40	1.75	Berman et al. 2010	Berman et al. 2011
Grain	Amino Acid	Glutamic Acid	% total amino acid	NR	NR	17.71	19.24	18.25	20.48	Harrigan et al. 2007	Berman et al. 2010
Grain	Amino Acid	Glycine	% total amino acid	NR	NR	4.19	4.61	4.24	4.62	Berman et al. 2010	Berman et al. 2011
Grain	Amino Acid	Histidine	% total amino acid	NR	NR	2.49	2.85	2.65	2.89	Lundry et al. 2008	Berman et al. 2011
Grain	Amino Acid	Isoleucine	% total amino acid	NR	NR	4.13	5.11	4.51	4.62	Berman et al. 2009	Taylor et al. 1999
Grain	Amino Acid	Leucine	% total amino acid	NR	NR	7.62	8.29	7.46	7.88	Berman et al. 2010	Lundry et al. 2008

Matrix	Category	Analyte	Units	OECD[1]		ILSI[2]		Literature		Literature Citations	
				Min	Max	Min	Max	Min	Max	Min	Max
Grain	Amino Acid	Lysine	% total amino acid	NR	NR	6.29	7.16	6.23	7.38	Berman et al. 2011	Berman et al. 2010
Grain	Amino Acid	Methionine	% total amino acid	NR	NR	1.18	1.71	1.31	1.47	Lundry et al. 2008	Lundry et al. 2008
Grain	Amino Acid	Phenylalanine	% total amino acid	NR	NR	4.91	5.44	4.95	5.44	Berman et al. 2010	Berman et al. 2011
Grain	Amino Acid	Proline	% total amino acid	NR	NR	4.75	5.62	4.96	5.23	Berman et al. 2010	Taylor et al. 1999
Grain	Amino Acid	Serine	% total amino acid	NR	NR	3.25	6.04	5.03	5.47	Berman et al. 2010	Lundry et al. 2008
Grain	Amino Acid	Threonine	% total amino acid	NR	NR	3.15	4.24	3.92	4.08	Berman et al. 2009	Berman et al. 2011
Grain	Amino Acid	Tryptophan	% total amino acid	NR	NR	0.95	1.29	0.97	1.49	Lundry et al. 2008	Padgette et al. 1996
Grain	Amino Acid	Tyrosine	% total amino acid	NR	NR	2.83	3.72	2.62	3.67	Berman et al. 2010	Padgette et al. 1996
Grain	Amino Acid	Valine	% total amino acid	NR	NR	4.28	5.57	4.69	4.93	Padgette et al. 1996	Berman et al. 2011
Grain	Fatty Acid	8:0 Caprylic	% total fatty acid	NR	NR	0.148	0.148	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	10:0 Capric	% total fatty acid	NR	NR	ND	ND	ND	0.27	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	12:0 Lauric	% total fatty acid	NR	NR	0.082	0.132	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	14:0 Myristic	% total fatty acid	NR	NR	0.071	0.238	ND	0.11	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	14:1 Myristoleic	% total fatty acid	NR	NR	0.121	0.125	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	15:0 Pentadecanoic	% total fatty acid	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	15:1 Pentadecenoic	% total fatty acid	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	16:0 Palmitic	% total fatty acid	NR	NR	9.55	15.77	1.40	12.63	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	16:1 Palmitoleic	% total fatty acid	NR	NR	0.086	0.194	ND	0.14	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	17:0 Heptadecanoic	% total fatty acid	NR	NR	0.085	0.146	ND	0.13	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	17:1 Heptadecenoic	% total fatty acid	NR	NR	0.073	0.087	ND	0.064	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	18:0 Stearic	% total fatty acid	NR	NR	2.70	5.88	0.50	5.63	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	18:1 Oleic	% total fatty acid	NR	NR	14.3	32.2	2.60	45.68	Harrigan et al. 2007	Berman et al. 2010
Grain	Fatty Acid	18:2 Linoleic	% total fatty acid	NR	NR	42.3	58.8	7.58	57.72	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	18:3 Linolenic	% total fatty acid	NR	NR	3.00	12.52	1.27	9.90	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	18:3 gamma Linolenic	% total fatty acid	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	20:0 Arachidic	% total fatty acid	NR	NR	0.163	0.482	0.038	0.57	Harrigan et al. 2007	Berman et al. 2009
Grain	Fatty Acid	20:1 Eicosenoic	% total fatty acid	NR	NR	0.140	0.350	0.024	0.35	Harrigan et al. 2007	Berman et al. 2010
Grain	Fatty Acid	20:2 Eicosadienoic	% total fatty acid	NR	NR	0.077	0.245	ND	0.065	Harrigan et al. 2007	Berman et al. 2010
Grain	Fatty Acid	20:3 Eicosatrienoic	% total fatty acid	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	20:4 Arachidonic	% total fatty acid	NR	NR	ND	ND	ND	ND	Harrigan et al. 2007	Harrigan et al. 2007
Grain	Fatty Acid	22:0 Behenic	% total fatty acid	NR	NR	0.277	0.595	0.043	0.65	Harrigan et al. 2007	Berman et al. 2009
Grain	Vitamin	Alpha Tocopherol (Vitamin E)	mg/kg DW	NR	NR	1.934	61.693	10.8	84.9	Berman et al. 2010	Zhou et al. 2011
Grain	Vitamin	Beta Tocopherol	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Delta Tocopherol	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Gamma Tocopherol	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Total Tocopherol	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR

Matrix	Category	Analyte	Units	OECD[1]		ILSI[2]		Literature		Literature Citations	
				Min	Max	Min	Max	Min	Max	Min	Max
Grain	Vitamin	Vitamin A (Beta Carotene)	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Vitamin B1 (Thiamine HCl)	mg/kg DW	NR	NR	1.01	2.54	NR	NR	NR	NR
Grain	Vitamin	Vitamin B2 (Riboflavin)	mg/kg DW	NR	NR	1.90	3.21	NR	NR	NR	NR
Grain	Vitamin	Vitamin B3 (Niacin)	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Vitamin B5 (Pantothenic Acid)	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Vitamin B6 (Pyridoxine HCl)	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Vitamin	Vitamin B9 (Folic Acid)	mg/kg DW	NR	NR	2.386	4.709	NR	NR	NR	NR
Grain	Vitamin	Vitamin C (Ascorbic acid)	mg/kg DW	NR	NR	NR	NR	NR	NR	NR	NR
Grain	Bioactive	Total Daidzein Equivalent	mcg/g DW	NR	NR	60.0	2453.5	25	2100.00	McCann et al. 2005	Berman et al. 2011
Grain	Bioactive	Total Genistein Equivalent	mcg/g DW	NR	NR	144.3	2837.2	28	2600.70	McCann et al. 2005	Harrigan et al. 2007
Grain	Bioactive	Total Glycitein Equivalent	mcg/g DW	NR	NR	15.3	310.0	32	349.19	Berman et al. 2011	Harrigan et al. 2007
Grain	Bioactive	Lectin	HU/mg Protein DW	37	323	NR	NR	37	323	Kakade et al. 1972	Kakade et al. 1972
Grain	Bioactive	Phytic Acid	% DW	NR	NR	0.634	1.960	0.41	2.68	Lundry et al. 2008	Berman et al. 2010
Grain	Bioactive	Raffinose	% DW	NR	NR	0.212	0.661	0.22	1.85	Harrigan et al. 2007	Berman et al. 2011
Grain	Bioactive	Stachyose	% DW	NR	NR	1.21	3.50	1.52	6.65	Harrigan et al. 2007	Berman et al. 2011
Grain	Bioactive	Trypsin Inhibitor	TIU/mg DW	NR	NR	19.59	118.68	18.14	75.5	Berman et al. 2009	McCann et al. 2005

ND = Not Detected, NR = Not Reported

[1] (OECD, 2001) Consensus document on compositional considerations for new varieties of soybean: Key food and feed nutrients and anti-nutrients. ENV/JM/MONO(2001)15. 30. www.oecd.org/dataoecd/15/60/46815135.pdf.

[2] International Life Sciences Institute, Crop Composition Database v4.2 (accessed January 10 and May 16, 2012)

Appendix 8. Non-Target Arthropod Field Study

A non-target arthropod field study with DAS-81419-2 soybean, a near-isogenic non-transgenic control (Maverick) and four non-transgenic reference lines (DSR 3590; IL 3505; Porter 75148; Williams 82) was conducted in 2011 at two sites in the U.S. located in Richland, Iowa and York, Nebraska. Each trial site included six entries, including one entry of DAS-81419-2, one control entry (Maverick), and four entries of non-transgenic reference varieties. At each site, entries were arranged in a randomized complete block design with four blocks. Across sites, each control, reference, and DAS-81419-2 entry was represented by a total of eight plots (2 sites, 4 replicate plots per entry at each site). Each study plot (observational unit) measured approximately 335 m^2 (3600 ft^2; 24 rows wide, 60 ft length). Soybean seeds were planted at a seeding rate of approximately 300 seeds per row with seed spacing within each row of approximately 2.4 inches (6 cm; 5 seeds per row foot). Study plots were separated by four rows of a non-transgenic soybean cultivar of similar maturity. The entire trial site was surrounded by a minimum of four rows (3.0 m; 10 ft) of a non-transgenic soybean cultivar of similar maturity. Therefore, the entire trial area formed a continuous field of soybean. Appropriate weed and disease control practices were applied to produce an agronomically acceptable crop. Arthropod pest management, including sprayed insecticides and miticides, was excluded from study.

Arthropod abundance was monitored using three sampling methods: pitfall trapping, sticky card trapping, and vertical beat sheet sampling. Sample timings included vegetative and reproductive soybean stages. Arthropods collected during the study included representatives from multiple orders and diverse ecological roles, including phytophagous, predatory, parasitic, and saprophagous modes of feeding. Assessment was conducted for several taxonomic groups, including: Oribatida (soil mites), Araneae (foraging and web spiders), Collembola (springtails), Hemiptera (e.g. *Orius* spp.), Coleoptera (e.g. ground beetles, lady beetles), Neuroptera (e.g. *Chrysopa* spp.), Lepidoptera, Diptera, and Hymenoptera (e.g. parasitoid wasps). A step-wise approach to statistical analysis was used, where community-level data were first analyzed using redundancy analysis (RDA) and Monte Carlo simulations (Dively, 2005) to test for differences between arthropod populations associated with DAS-81419-2 and the near-isogenic control. Redundancy analysis results were used to construct principal response curves (PRCs) to visualize population patterns. When defined criteria were met, a second step assessment included univariate analysis of variance (ANOVA) and application of the false discovery rate (FDR) procedure (Benjamini and Hochberg, 1995; Curran-Everett, 2000). Results of both the PRC and ANOVA analyses confirmed no unintended effects of DAS-81419-2 soybean on any of the arthropod groups monitored. Consistent across locations and sampling methods, arthropod populations associated with DAS-81419-2 soybean were similar to those of non-transgenic soybean. Field monitoring confirmed the results of established laboratory studies examining the Cry1Ac and Cry1F proteins, and demonstrated no adverse effects of DAS-81419-2 cultivation on non-target arthropod communities.

Appendix 9. USDA Notifications for DAS-81419-2 Soybean

USDA Notification Number	Notification Authorization Date	Notification Expiration Date	States	Number of Trials	Trial Status[1]
12-088-113n	4/23/2012	4/23/2013	AL, AR, GA, IA, IL, IN, MD, MO, NE	24	pending
12-072-105n	4/2/2012	4/2/2013	IA, IL, MO, NE, PA, IN	8	pending
12-060-107n	4/1/2012	4/1/2013	IA, IL, MO, NE, PA, IN	12	pending
11-305-101n	11/30/2011	11/30/2012	PR	1	pending
11-216-106n	9/9/2011	9/9/2012	PR	8	pending
11-088-112n	5/6/2011	5/6/2012	IA, IN, MO	4	pending
11-088-101n	5/9/2011	5/9/2012	IA, NE	2	pending
11-087-115n	4/21/2011	4/21/2012	AR, GA, IA, IL, IN, MD, MI, MO, MS, NE, PR	26	pending
11-087-113n	4/15/2011	4/15/2012	IA, IL, IN, MO, NE, PA	10	pending
10-243-105n	9/30/2010	9/30/2011	PR	3	submitted
10-077-106n	4/14/2010	4/14/2011	IN	1	submitted
09-259-107n	9/30/2009	9/30/2010	PR	1	submitted

[1]Pending reports as of July 28, 2012 to be submitted within six months of the notification expiration date.

Appendix 10. Stewardship of Insect-Resistant DAS-81419-2 Soybean

Dow AgroSciences is committed to promoting the responsible use and stewardship of its products throughout the product life cycle. DAS has a long-standing commitment to product stewardship and is a founding member of Excellence through Stewardship. In addition, DAS has voluntarily established a Biotechnology Quality Management System (BQMS) which has been verified by third-party audits as conforming to the USDA APHIS BQMS Program Audit Standard. Consistent with its existing stewardship programs and quality management systems, DAS will implement comprehensive stewardship measures for insect-resistant DAS-81419-2 soybean. These programs and practices include rigorous field compliance, quality assurance, quality control, and auditing.

DAS has submitted an application for a FIFRA Section 3 seed increase registration with EPA to support future breeding and seed increase activities in the United States. Under a Section 3 seed increase registration, commercial sale of DAS-81419-2 soybean in the U.S. is prohibited. If DAS chooses to cultivate DAS-81419-2 soybean for commercial purposes in the U.S. in the future, DAS will apply for a FIFRA Section 3 commercial use registration with EPA.

As with all products, DAS is committed to the rigorous product stewardship of DAS-81419-2 soybean. This includes having processes in place to restrict and steward the use of DAS-81419-2 soybean based on the registered uses in the U.S. All breeding or seed multiplication work will be done directly by DAS or its licensees under a contract. As long as DAS-81419-2 soybean is cultivated in the U.S. under the Section 3 seed increase registration, the limitations on the EPA registration, combined with contractual production of the seed, will serve to effectively limit use of DAS-81419-2 soybean and product stewardship requirements will be implemented accordingly. In the future, should DAS choose to register DAS-81419-2 soybean for commercial production in the U.S., product stewardship measures would be expanded to reflect commercial grower use and insect resistance management requirements.

Before commercially launching DAS-81419-2 soybean in any country, DAS will obtain regulatory approval from key soybean import countries with functioning regulatory systems to assure regulatory compliance, promote product launch stewardship, and support the flow of international trade. DAS is in the process of making submissions to the regulatory authorities of trade partners for import approval and/or cultivation approval. DAS also commits to industry practices to maintain plant product integrity and seed purity of DAS-81419-2 soybean. A detection method will be made available to soybean producers, processors and buyers before commercializing DAS-81419-2 soybean in any country.

Appendix 11. References

Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Barker, R.F., Thompson, D.V., 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene 36, 289-300.

AgrEvo, 1994. Petition for determination of nonregulated status: Glufosinate resistant corn transformation events T14 and T25. http://www.aphis.usda.gov/brs/aphisdocs/94_35701p.pdf

AgrEvo, 1996. Petition for determination of nonregulated status: Glufosinate resistant soybean transformation events. http://www.aphis.usda.gov/brs/aphisdocs/96_06801p.pdf

AgrEvo, 1997. Petition for determination of nonregulated status: Glufosinate tolerant canola transformation event T45. http://www.aphis.usda.gov/brs/aphisdocs/97_20501p.pdf.

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

AOSA, 2009. Seed vigor testing handbook contribution no. 32. In: Baalbaki, R.J., Marcos-Filho, J., Elias, S.G., McDonald, M. (Eds.).

AOSA, 2010a. AOSA rules for testing seeds volume 1. Principles and procedures. Association of Official Seed Analysts Inc.

AOSA, 2010b. AOSA rules for testing seeds volume 4. Seeding evaluation. Association of Official Seed Analysts Inc.

AOSCA, 2012. Seed certification. http://www.aosca.org/seed%20certification.htm.

Aronson, A.I., Shai, Y., 2001. Why Bacillus thuringiensis insecticidal toxins are so effective: Unique features of their mode of action. FEMS Microbiology Letters 195, 1-8.

Arumuganathan, K., Earle, E., 1991. Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter 9, 208-218.

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

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

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

Berman, K.H., Harrigan, G.G., Nemeth, M.A., Oliveira, W.S., Berger, G.U., Tagliaferro, F.S., 2011. Compositional equivalence of insect-protected glyphosate-tolerant soybean MON 87701 × MON 89788 to conventional soybean extends across different world regions and multiple growing seasons. Journal of Agricultural and Food Chemistry 59, 11643-11651.

Berman, K.H., Harrigan, G.G., Riordan, S.G., Nemeth, M.A., Hanson, C., Smith, M., Sorbet, R., Zhu, E., Ridley, W.P., 2009. Compositions of seed, forage, and processed fractions from insect-protected soybean MON 87701 are equivalent to those of conventional soybean. Journal of Agricultural and Food Chemistry 57, 11360-11369.

Berman, K.H., Harrigan, G.G., Riordan, S.G., Nemeth, M.A., Hanson, C., Smith, M., Sorbet, R., Zhu, E., Ridley, W.P., 2010. Compositions of forage and seed from second-generation glyphosate-tolerant soybean MON 89788 and insect-protected soybean MON 87701 from brazil are equivalent to those of conventional soybean (*Glycine max*). Journal of Agricultural and Food Chemistry 58, 6270-6276.

Bhatti, M.A., Duan, J.J., Head, G.P., Jiang, C., McKee, M.J., Nickson, T.E., Pilcher, C.L., Pilcher, C.D., 2005. Field evaluation of the impact of corn rootworm (coleoptera: Chrysomelidae)–protected *Bt* corn on foliage-dwelling arthropods. Environmental Entomology 34, 1336 - 1345.

Bilyeu, K.D., Zeng, P., Coello, P., Zhang, Z.J., Krishnan, H.B., Bailey, A., Beuselinck, P.R., Polacco, J.C., 2008. Quantitative conversion of phytate to inorganic phosphorus in soybean seeds expressing a bacterial phytase. Plant Physiology 146, 468-477.

Bitzer, R.J., Rice, M.E., Pilcher, C.D., Pilcher, C.L., Lam, W.-k.f., 2005. Biodiversity and community structure of epedaphic and euedaphic springtails (collembola) in transgenic rootworm *Bt* corn. Environmental Entomology 34, 1346 - 1376.

Bradford, K.J., 2006. Methods to maintain genetic purity of seed stocks. ANR Publication 8189, 5.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., Zhuang, M., Gill, S.S., Soberon, M., 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochimica et Biophysica Acta 1667, 38-46.

Cardineau, G.A., Stelman, S.J., Narva, K.E., 2001. Plant-optimized genes encoding pesticidal toxins. 6,218,188 B1.

Carstens, K., Anderson, J., Bachman, P., De Schrijver, A., Dively, G., Federici, B., Hamer, M., Gielkens, M., Jensen, P., Lamp, W., Rauschen, S., Ridley, G., Romeis, J., Waggoner, A., 2011. Genetically modified crops and aquatic ecosystems: Considerations for environmental risk assessment and non-target organism testing. Transgenic Research, 1-30.

CAST, 2009. Sustainability of U.S. Soybean production: Conventional, transgenic, and organic production systems. http://www.cast-science.org/publications/.

Catchot, A., 2011. Soybean insect pest problems in the mid-south, what to watch for in 2011.

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

Coates, B.S., Sumerford, D.V., Hellmich, R.L., Lewis, L.C., 2005. Sequence variation in the cadherin gene of *Ostrinia nubilalis*: A tool for field monitoring. Insect Biochemistry and Molecular Biology 35, 129-139.

Coll, A., Nadal, A., Palaudelmàs, M., Messeguer, J., Melé, E., Puigdomènech, P., Pla, M., 2008. Lack of repeatable differential expression patterns between MON810 and comparable commercial varieties of maize. Plant Molecular Biology 68, 105-117.

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

Curran-Everett, D., 2000. Multiple comparisons: Philosophies and illustrations. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 279, R1-R8.

Dalley, C.D., Renner, K.A., Kells, J.J., 2002. Weed competition in Roundup Ready soybeans and corn. http://msue.anr.msu.edu/.

Daly, T., Buntin, G.D., 2005. Effect of *Bacillus thuringiensis* transgenic corn for lepidopteran control on nontarget arthropods. Environmental Entomology 34, 1292 - 1301.

de Freitas Bueno, R.C.O., de Freitas Bueno, A., Moscardi, F., Postali Parra, J.R., Hoffmann-Campo, C.B., 2011. Lepidopteran larva consumption of soybean foliage: Basis for

developingmultiple-species economic thresholds for pestmanagement decisions. Pest Management Science 67, 170 - 174.

Dively, G.P., 2005. Impact of transgenic vip3a × cry1ab lepidopteran-resistant field corn on the nontarget arthropod community. Environmental Entomology 34, 1267-1291.

Dow AgroSciences, 2000. Petition for determination of non-regulated status B.t. Cry1F insect-resistant glufosinate-tolerant maize line 1507. http://www.aphis.usda.gov/brs/aphisdocs/00_13601p.pdf

Dow AgroSciences, 2003a. Petition for determination of non-regulated status B.t. Cry1Ac insect-resistant cotton event 3006-210-23. http://www.aphis.usda.gov/brs/aphisdocs/03_03602p.pdf.

Dow AgroSciences, 2003b. Petition for determination of non-regulated status B.t. Cry1F insect-resistant cotton event 281-24-236. http://www.aphis.usda.gov/brs/aphisdocs/03_03601p.pdf.

Dow AgroSciences, 2003c. Petition for the determination of nonregulated status Bt Cry34/35Ab1 insect-resistant, herbicide-tolerant corn: Corn line 59122. . http://www.aphis.usda.gov/brs/aphisdocs/03_35301p.pdf.

Duan, J.J., Lundgren, J.G., Naranjo, S., Marvier, M., 2009. Extrapolating non-target risk of *Bt* crops from laboratory to field. Biology Letters 6, 74-77.

Duan, J.J., Marvier, M., Huesing, J., Dively, G., Huang, Z.Y., 2008. A meta-analysis of effects of Bt crops on honey bees (hymenoptera: Apidae). PLoS ONE 3, e1415.

Duke, S.O., 1996. Herbicide-resistant crops: Agricultural, environmental, economic, regulatory, and technical aspects. CRC Press, Inc., Boca Raton.

EPA, 1997. Phosphinothricin acetyltransferase and the genetic matetial necessary for its production in all plants; exemption from the requirement of a tolerance on all raw agricultural commodities. Federal Register 62 (70): 17717-17720. http://www.gpo.gov/fdsys/pkg/FR-1997-04-11/html/97-9373.htm.

EPA, 1998. Guidelines for ecological risk assessment. EPA/630/R-95/002F. www.epa.gov/raf/publications/pdfs/ECOTXTBX.PDF.

EPA, 2001a. Biopesticides registration action document: *Bacillus thuringiensis* (*Bt*) plant-incorporated protectants. http://www.epa.gov/opp00001/biopesticides/pips/bt_brad.htm.

EPA, 2001b. SAP Report No. 2000-07 FIFRA scientific advisory panel meeting, October 18 - 20, 2000; "issues pertaining to the Bt plant pesticides risk and benefit assessments", section c. Environmental assessment. 95.

EPA, 2001c. SAP Report No. 2000-07 FIFRA scientific advisory panel meeting, October 18 - 20, 2000; final report: "Sets of scientific issues being considered by the environmental protection agency regarding: Bt plant-pesticides risk and benefit assessments".

EPA, 2004. SAP Report No. 2004-05 FIFRA scientific advisory panel meeting, June 8 - 10, 2004; product characterization, human health risk, ecological risk, and insect resistance management for *Bacillus thuringiensis* (Bt) cotton products. 84.

EPA, 2005. Biopesticide registration action document - *Bacillus thuringiensis* Cry1F (synpro) and Cry1Ac (synpro) construct 281/3006 insecticidal crystal proteins as expressed in cotton. http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006512.pdf.

EPA, 2007. White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on non-target arthropods for regulatory risk assessments.

EPA, 2009. Terms of environment: Glossary, abbreviations, and acronyms. http://ofmpub.epa.gov/sor_internet/registry/termreg/searchandretrieve/termsandacronyms/search.do.

EPA, 2011. *Bacillus thuringiensis*: Preliminary work plan and summary document for registration review. http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2011-0705-0002.

Evans, S.L., 2004. Producing proteins derived from genetically modified organisms for toxicology and environmental fate assessment of biopesticides. In: Parekh, S.R. (Ed.), The GMO Handbook: Genetically modified animals, microbes, and plants in biotechnology. Humana Press, Inc., Totowa, NJ, pp. 53-83.

Felland, C.M., Pitre, H.N., Luttrell, R.G., Hamer, J.L., 1990. Resistance to pyrethroid insecticides in soybean looper (lepidoptera: Noctuidae) in Mississippi. Journal of Economic Entomology 83, 35 - 40.

Fling, M.E., Kopf, J., Richards, C., 1985. Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3"(9)-*0*-nucleotidyltransferase. Nucleic Acids Research 13, 7095-7106.

Gahan, L.J., Gould, F., Heckel, D.G., 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857-860.

Gao, Y., Fencil, K.J., Xu, X., Schwedler, D.A., Gilbert, J.R., Herman, R.A., 2006. Purification and characterization of a chimeric Cry1F δ-endotoxin expressed in transgenic cotton plants. Journal of Agricultural and Food Chemistry 54, 829-835.

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

Gianessi, L.P., 2009. The benefits of insecticide use: Soybeans. CropLife Foundation. Washington, DC.

Gianessi, L.P., Silvers, C.S., Sankula, S., Carpenter, J.E., 2002. Plant biotechnology: Current and potential impact for improving pest management in U.S. Agriculture - an analysis of 40 case studies. National Center for Food & Agricultural Policy.

Gilroy, T.E., Wilcox, E.R., 1992. Hybrid *Bacillus thuringiensis* gene, plasmid and transformed *Pseudomonas fluorescens*. 5,128,130.

Givens, W.A., Shaw, D.R., Kruger, G.R., Johnson, W.G., Weller, S.C., Young, B.G., Wilson, R.G., Owen, M.D.K., Jordan, D., 2009. Survey of tillage trends following the adoption of glyphosate-resistant crops. Weed Technology 23, 150-155.

Goldberg, M., Knudsen, K.L., Platt, D., Kohen, F., Bayer, E.A., Wilchek, M., 1991. Specific interchain crosslinking of antibodies using bismaleimides. Repression of ligand leakage in immunoaffinity chromatography. Bioconjugate Chemistry 2, 275-280.

González-Zamora, J.E., Camúñez, S., Avilla, C., 2007. Effects of *Bacillus thuringiensis* Cry toxins on developmental and reproductive characteristics of the predator *Orius albidipennis* (hemiptera: Anthocoridae) under laboratory conditions. Environmental Entomology 36, 1246 - 1253.

Gouffon, C., Van Vliet, A., Van Rie, J., Jansens, S., Jurat-Fuentes, J.L., 2011. Binding sites for Bacillus thuringiensis cry2ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or vip3a toxin. Applied and Environmental Microbiology 77, 3182-3188.

Guillebeau, P., Hinkle, N., Roberts, P., 2008. Summary of losses from insect damage and cost of control in Georgia 2006. University of Georgia.

Hammond, R.B., 2012. Soybean insect ipm. In: Radcliffe, E.B., Hutchison, W.D., Cancelado, R.E. (Eds.), Radcliffe's ipm world textbook. University of Minnesota, St. Paul, MN.

Harrigan, G.G., Ridley, W.P., Riordan, S.G., Nemeth, M.A., Sorbet, R., Trujillo, W.A., Breeze, M.L., Schneider, R.W., 2007. Chemical composition of glyphosate-tolerant soybean 40-3-2 grown in europe remains equivalent with that of conventional soybean (*Glycine max* L.). Journal of Agricultural and Food Chemistry 55, 6160-6168.

Hartwig, E.E., Kilen, T.C., 1991. Yield and composition of soybean seed from parents with different protein, similar yield. Crop Science 31, 290-292.

Hartzler, B., Pringnitz, B., 2005. Early-season weed competition. Department of Agronomy, Iowa State University, IA. http://www.ipm.iastate.edu/ipm/icm/2000/5-8-2000/earlyweed.html.

Head, G., Moar, W., Eubanks, M., Freeman, B., Ruberson, J., Hagerty, A., Turnipseed, S., 2005. A multiyear, large-scale comparison of arthropod populations on commercially managed *Bt* and non-*Bt* cotton fields. Environmental Entomology 34, 1257 - 1266.

Head, G., Surber, J.B., Watson, J.A., Martin, J.W., Duan, J.J., 2002. No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. Environmental Entomology 31, 30-36.

Herman, R.A., Phillips, A.M., Lepping, M.D., Fast, B.J., Sabbatini, J., 2010. Compositional safety of event DAS-40278-9 (AAD-1) herbicide-tolerant maize. GM Crops 1, 294-311.

Herman, R.A., Scherer, P.N., Young, D.L., Mihaliak, C.A., Meade, T., Woodsworth, A.T., Stockhoff, B.A., Narva, K.E., 2002. Binary insecticidal crystal protein from *Bacillus thuringiensis*, strain PS149B1: Effects of individual protein components and mixtures in laboratory bioassays. Journal of Economic Entomology 95, 635-639.

Herman, R.A., Storer, N.P., Phillips, A.M., Prochaska, L.M., Windels, P., 2007. Compositional assessment of event DAS-59122-7 maize using substantial equivalence. Regulatory Toxicology and Pharmacology 47, 37-47.

Herouet, C., Esdaile, D.J., Mallyon, B.A., Debruyne, E., Schulz, A., Currier, T., Hendrickx, K., Klis, R.-J.v.d., Rouan, D., 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the pat and bar sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory Toxicology and Pharmacology 41, 134-149.

Higley, L.G., 1994. Insect injury to soybean. In: Higley, L.G., Boethel, D.J. (Eds.), Handbook of soybean insect pests. Entomological Society of America, Lanham, MD, pp. 11-13.

Holroyde, M.J., Chesher, J.M.E., Trayer, I.P., Walker, D.G., 1976. Studies on use of sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-d-glucopyranose for large-scale purification of hepatic glucokinase. Biochemical Journal 153, 351-361.

Hood, E.E., Helmer, G.L., Fraley, R.T., Chilton, M.D., 1986. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. Journal of Bacteriology 168, 1291-1301.

Huls, T.J., Erickson, G.E., Klopfenstein, T.J., Luebbe, M.K., Vander Pol, K.J., Rice, D.W., Smith, B., Hinds, M., Owens, F., Liebergesell, M., 2008. Effect of feeding DAS-59122-7 corn grain and nontransgenic corn grain to individually fed finishing steers. The Professional Animal Scientist 24, 572-577.

Hutchison, W.D., Burkness, E.C., Mitchell, P.D., Moon, R.D., Leslie, T.W., Fleischer, S.J., Abrahamson, M., Hamilton, K.L., Steffey, K.L., Gray, M.E., Hellmich, R.L., Kaster, L.V., Hunt, T.E., Wright, R.J., Pecinovsky, K., Rabaey, T.L., Flood, B.R., Raun, E.S., 2010. Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. Science 330, 222 - 225.

Icoz, I., Stotzky, G., 2008. Fate and effects of insect-resistant Bt crops in soil ecosystems. Soil Biology and Biochemistry 40, 559-586.

ILSI, 2010. International Life Sciences Institute crop composition database version 4.2. www.cropcomposition.org.

Iskander, F.Y., 1987. Maturation stage and mineral content in soybeans. Food Chemistry 24, 29-35.

Jackson, R.E., Bradley, J.R., Van Duyn, J., Leonard, B.R., Allen, K.C., Luttrell, R.G., Ruberson, J., Adamczyk, J., Gore, J., Hardee, D.D., Voth, R., Sivasupramaniam, S., Mullins, J.W., Head, G., 2008. Regional assessment of *helicoverpa zea* populations on cotton and non-cotton crop hosts. Entomologia Experimentalis et Applicata 126.

Jacobs, C.M., Utterback, P.L., Parsons, C.M., Rice, D., Smith, B., Hinds, M., Liebergesell, M., Sauber, T., 2008. Performance of laying hens fed diets containing DAS-59122-7 maize grain compared with diets containing nontransgenic maize grain. Poultry Science 87, 475-479.

Jost, D.J., Pitre, H.N., 2002. Soybean looper (lepidoptera: Noctuidae) oviposition on cotton and soybean of different growth stages: Influence of olfactory stimuli. Journal of Economic Entomology 95, 286 - 293.

Jurat-Fuentes, J.L., Adang, M.J., 2001. Importance of Cry1 delta-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). Applied and Environmental Microbiology 67, 323-329.

Jurat-Fuentes, J.L., Adang, M.J., 2006. The *Heliothis virescens* cadherin protein expressed in *Drosophila* S2 cells functions as a receptor for *Bacillus thuringiensis* Cry1A but not Cry1Fa toxins. Biochemistry 45, 9688-9695.

Kakade, M.L., Simons, N.R., Liener, I.E., Lambert, J.W., 1972. Biochemical and nutritional assessment of different varieties of soybeans. Journal of Agricultural and Food Chemistry 20, 87-90.

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

Knezevic, S.Z., 2010. Use of herbicide tolerant crops as a component of an integrated weed management program. University of Nebraska-Lincoln Extension, Institute of Agriculture and Natural Resources. http://elkhorn.unl.edu/epublic/live/g1484/build/#concerns.

Kogan, M., 1998. Integrated pest management: Historical perspectives and contemporary developments. Annual Review of Entomology 43, 243 - 270.

Krausz, R.F., Young, B.G., Kapusta, G.E.O.R., Matthews, J.L., 2001. Influence of weed competition and herbicides on glyphosate-resistant soybean (*Glycine max*). Weed Technology 15, 530-534.

Kwiatkowski, R.W., Lyamichev, V., de Arruda, M., Neri, B., 1999. Clinical, genetic, and pharmacogenetic applications of the invader assay. Molecular Diagnosis 4, 353-364.

Lauer, J., 2007. Continuous corn, or rotate in 2008? The principle of crop rotation. Agronomy Advice 28, 426-451.

Lee, M.K., Miles, P., Chen, J.-S., 2006. Brush border membrane binding properties of Bacillus thuringiensis vip3a toxin to Heliothis virescens and helicoverpa zea midguts. Biochemical and Biophysical Research Communications 339, 1043-1047.

Leonard, B.R., Boethel, D.J., Sparks Jr., A.N., Layton, M.B., Mink, J.S., Pavloff, A.M., Burris, E., Graves, J.B., 1990. Variations in response of soybean looper (lepidoptera: Noctuidae) to selected insecticides in louisiana. Journal of Economic Entomology 83, 27 - 34.

Li, Y., Romeis, J., Wang, P., Peng, Y., Shelton, A.M., 2011. A comprehensive assessment of the effects of *Bt* cotton on *Coleomegilla maculata* demonstrates no detrimental effects by Cry1Ac and Cry2Ab. PLoS ONE 6, e22185.

Liu, J.-G., Yang, A.-Z., Shen, X.-H., Hua, B.-G., Shi, G.-L., 2011. Specific binding of activated vip3aa10 to *helicoverpa armigera* brush border membrane vesicles results in pore formation. Journal of Invertebrate Pathology 108, 92-97.

Lopez, M.D., Prasifka, J.R., Bruck, D.J., Lewis, L.C., 2005. Utility of ground beetle species in field tests of potential nontarget effects of Bt crops. Environmental Entomology 34, 1317 - 1324.

Loux, M., Stachler, J., Johnson, B., Nice, G., 2006. The benefits of preemergence herbicides in Roundup Ready soybean. www.btny.purdue.edu/weedscience/2008/BenefitsPRE.pdf.

Lozzia, G., Furlanis, C., Manachini, B., Rigamonti, L., 1998. Effects of Bt corn on *Rhopalosiphum padi* L. (Rhynchota Aphididae) and on its predator *Chrysoperla carnea* stephen (Neuroptera Chrysopidae). Bollettino di Zoologia agraria e di Bachicoltura 30, 153 - 164.

Lundry, D.R., Ridley, W.P., Meyer, J.J., Riordan, S.G., Nemeth, M.A., Trujillo, W.A., Breeze, M.L., Sorbet, R., 2008. Composition of grain, forage, and processed fractions from second-generation glyphosate-tolerant soybean, MON 89788, is equivalent to that of conventional soybean (*Glycine max* L.). Journal of Agricultural and Food Chemistry 56, 4611-4622.

Madduri, K.M., Snodderley, E.M., 2007. Expression of phosphinothricin N-acetyltransferase in *Escherichia coli* and *Pseudomonas fluorescens*: Influence of mRNA secondary structure, host, and other physiological conditions. Protein Expression and Purification 55, 352-360.

Marvier, M., McCreedy, C., Regetz, J., Kareiva, P., 2007. A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. Science 316, 1475-1477.

McCann, M.C., Liu, K., Trujillo, W.A., Dobert, R.C., 2005. Glyphosate-tolerant soybeans remain compositionally equivalent to conventional soybeans (*Glycine max* L.) during three years of field testing. Journal of Agricultural and Food Chemistry 53, 5331-5335.

McClintock, J.T., Schaffer, C.R., Sjoblad, R.D., 1995. A comparative review of the mammalian toxicity of Bacillus thuringiensis-based pesticides. Pesticide Science 45, 95-105.

McPherson, R.M., Hudson, R.D., Jones, D.C., 1997. Summary of losses from insect damage and costs of control in Georgia. http://www.bugwood.org/sl97/soybean.97.htm.

McWilliams, D.A., Berglund, D.R., Endres, G.J., 1999. Soybean growth and management quick guide. North Dakota State University.

Mendelsohn, M., Kough, J., Vaituzis, Z., Matthews, K., 2003. Are Bt crops safe? Nature Biotechnology 21, 1003-1009.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carrière, Y., Dennehy, T.J., Brown, J.K., Tabashnik, B.E.,

2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proceedings of the National Academy of Sciences 100, 5004-5009.

Naranjo, S.E., 2005. Long-term assessment of the effects of transgenic *Bt* cotton on the abundance of nontarget arthropod natural enemies. Environmental Entomology 34, 1193 - 1210.

Naranjo, S.E., 2009. Impacts of Bt crops on non-target invertebrates and insecticide use patterns CAB Reviews:Perspectives in Agriculture, Veterinary Science, Nutrition, and Natural Resources 4.

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

Northrup King, 1995. Petition for determination of non-regulated status for insect protected corn (*Zea mays* L.) expressing the Cry1A(b) gene from *Bacillus thuringiensis* var. *kurstaki*. http://www.aphis.usda.gov/brs/aphisdocs/95_19501p.pdf.

OECD, 1999. Consensus document on general information concerning the genes and their enzymes that confer tolerance to phosphinothricin herbicide. 11 (ENV/JM/MONO(99)13): 1-26. www.oecd.org/dataoecd/16/52/46815628.pdf.

OECD, 2000. Consensus document on the biology of *Glycine max* (L.). Merr. (soybean). ENV/JM/MONO(2000)9. http://www.oecd.org/dataoecd/16/56/46815668.pdf.

OECD, 2001. Consensus document on compositional considerations for new varieties of soybean: Key food and feed nutrients and anti-nutrients. ENV/JM/MONO(2001)15. 30. www.oecd.org/dataoecd/15/60/46815135.pdf.

OECD, 2004. OECD guidance for the designation of a unique identifier for transgenic plants. ENV/JM/MONO(2002)7 12. http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/env-jm-mono(2002)7.

Padgette, S.R., Taylor, N.B., Nida, D.L., Bailey, M.R., MacDonald, J., Holden, L.R., Fuchs, R.L., 1996. The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. The Journal of Nutrition 126, 702-716.

Pedersen, P., 2004. Soybean growth and development. http://extension.agron.iastate.edu/soybean/production_growthstages.html.

Pedigo, L.P., 1996. Pest management theory. Entomology and pest management. Prentice Hall, Upper Saddle River, NJ, pp. 279-300.

Penn State Extension, 2012. Soybean insect control. http://extension.psu.edu/agronomy-guide/pm/sec4/sec43a.

Perlak, F.J., Oppenhuizen, M., Gustafson, K., Voth, R., Sivasupramaniam, S., Heering, D., Carey, B., Ihrig, R.A., Roberts, J.K., 2001. Development and commercial use of Bollgard® cotton in the USA - early promises versus today's reality. The Plant Journal 27, 489 - 501.

Pilcher, C.D., Obrycki, J.J., Rice, M.E., Lewis, L.C., 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26, 446 - 454.

Pilcher, C.D., Rice, M.E., 2003. Economic analysis of planting dates to manage European corn borer (lepidoptera: Crambidae) with Bt corn. Journal of Economic Entomology 96, 941 - 949.

Pilcher, C.D., Rice, M.E., Obrycki, J.J., 2005. Impact of transgenic *Bacillus thuringiensis* corn and crop phenology on five nontarget arthropods. Environmental Entomology 34, 1302 - 1316.

Rang, C., Bergvingson, D., Bohorova, N., Hoisington, D., Frutos, R., 2004. Competition of *Bacillus thuringiensis* Cry1 toxins for midgut binding sites: A basis for the development and management of transgenic tropical maize resistant to several stemborers. Current Microbiology 49, 22-27.

Ray, J.D., Kilen, T.C., Abel, C.A., Paris, R.L., 2003. Soybean natural cross-pollination rates under field conditions. Environmental Biosafety Research 22, 133-138.

Rice, M., 2002. Estimatining soybean defoliation. http://www.ipm.iastate.edu/ipm/icm/2002/7-29-2002/soydefoliation.html.

Rodrigo-Simón, A., de Maagd, R.A., Avilla, C., Bakker, P.L., Molthoff, J., González-Zamora, J.E., Ferré, J., 2006. Lack of detrimental effects of *Bacillus thuringiensis* Cry toxins on the insect predator Chrysoperla carnea: A toxicological, histopathological, and biochemical analysis. Applied and Environmental Microbiology 72, 1595-1603.

Romeis, J., Meissle, M., Bigler, F., 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nature Biotechnology 24, 63-71.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P., 2011. *Bacillus thuringiensis*: A century of research, development and commercial applications. Plant Biotechnology Journal 9, 283-300.

Sanvido, O., Romeis, J., Bigler, F., 2007. Ecological impacts of genetically modified crops: Ten years of field research and commercial cultivation. Advances in Biochemical Engineering/Biotechnology 107, 235 - 278.

SAS Institute Inc., 2009. SAS/STAT(r) 9.2 User's Guide, second edition. SAS Institute Inc. Cary, NC.

Shan, G., Embrey, S.K., Herman, R.A., McCormick, R., 2008. Cry1F protein not detected in soil after three years of transgenic Bt corn (1507 corn) use. Environmental Entomology 37, 255-262.

Sims, S.R., Martin, J.W., 1997. Effect of the Bacillus thuringiensis insecticidal proteins Cry1A(b), Cry1A(c), cryiia, and cryiiia on folsomia candida and xenylla grisea (insecta: Collembola). Pedobiologia 41, 412 - 416.

Sleper, D.A., Nickell, C.D., Noel, G.R., Cary, T.R., Thomas, D.J., Clark, K.M., Rao Arelli, A.P., 1998. Registration of 'Maverick' soybean. Crop Science 38, 549-554a.

Smith, K., 1994. Status of soybean insect pests in the United States. In: Higley, L.G., Boethel, D.J. (Eds.), Handbook of soybean insect pests. Entomological Society of America, Lanham, MD, p. 3.

Soy Stats, 2012. Soy stats 2012. http://www.soystats.com/2012/Default-frames.htm.

Sparks, A.N., 1979. A review of the biology of the fall armyworm. The Florida Entomologist 62, 82 - 87.

Stalker, D.M., Thomas, C.M., Helinski, D.R., 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Molecular and General Genetics 181, 8-12.

Stein, H.H., Rice, D.W., Smith, B.L., Hinds, M.A., Sauber, T.E., Pedersen, C., Wulf, D.M., Peters, D.N., 2009. Evaluation of corn grain with the genetically modified input trait DAS-59122-7 fed to growing-finishing pigs. Journal of Animal Science 87, 1254-1260.

Strauch, E., Wohlleben, W., Pühler, A., 1988. Cloning of a phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* tü494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene 63, 65-74.

Taylor, N.B., Fuchs, R.L., MacDonald, J., Shariff, A.R., Padgette, S.R., 1999. Compositional analysis of glyphosate-tolerant soybeans treated with glyphosate. Journal of Agricultural and Food Chemistry 47, 4469-4473.

Thomas, J.D., Boethel, D.J., 1994. Synergism of insecticides in tests with resistant soybean looper larvae (lepidoptera: Noctuidae) in the laboratory and field. Journal of Economic Entomology 87, 1416-1422.

Torres, J.B., Ruberson, J.R., 2005. Canopy- and ground-dwelling predatory arthropods in commercial *Bt* and non-*Bt* cotton fields: Patterns and mechanisms. Environmental Entomology 34, 1242-1256.

USDA, 1996. Availability of determination of nonregulated status for soybeans genetically engineered for glufosinate herbicide tolerance. Federal Register 61 (160): 42581-42582. http://federalregister.gov/a/96-20921.

USDA, 2001. Availibility of determination of nonregulated status for corn genetically engineered for insect resistance and glufosinate herbicide tolerance. Federal Register 66 (157): 42624-42625. http://www.gpo.gov/fdsys/pkg/FR-2001-08-14/pdf/01-20307.pdf

USDA, 2004. Availability of determination of nonregulated status for cotton lines genetically engineered for insect resistance. Federal Register 69 (156): 50154-50155. http://www.gpo.gov/fdsys/pkg/FR-2004-08-13/pdf/04-18523.pdf.

USDA, 2005. Availability of determination of nonregulated status for genetically engineered corn (corn line DAS-59122-7). Federal Register 70 (194): 58663-58664. http://www.gpo.gov/fdsys/pkg/FR-2005-10-07/html/05-20194.htm.

USDA, 2006. The first decade of genetically engineered crops in the United States.

USDA ERS, 2011. Futures price forecast model: Estimates for the season-average price and counter-cyclical payment rate for soybeans http://www.ers.usda.gov/data-products/season-average-price-forecasts.aspx.

USDA ERS, 2012. Soybeans & oil crops. http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/background.aspx.

USDA ERS ARMS, USDA Economic Research Service - agricultural resource management survey. http://www.ers.usda.gov/Data/ARMS/CropOverview.htm.

USDA ERS ARMS, 2006. USDA Economic Research Service. Agricultural resource management survey. Crop production practices for soybeans: All survey states. http://www.ers.usda.gov/Data/ARMS/app/default.aspx?survey_abb=CROP.

USDA NASS, 2007. USDA National Agricultural Service. Agricultural chemical usage 2006 field crops summary. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1560.

USDA NASS, 2012. USDA National Agricultural Statistics Service: Quick stats. http://www.nass.usda.gov/Quick_Stats/.

Vadlamudi, R.K., Ji, T.H., Bulla, L.A., 1993. A specific binding protein from *Manduca sexta* for the insecticidal toxin of *Bacillus thuringiensis* subsp. *berliner*. Journal of Biological Chemistry 268, 12334-12340.

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

Waters, D.J., Barfield, C.S., 1989. Larval development and consumption by *Anticarsia gemmatalis* (lepidoptera: Noctuidae) fed various legume species. Environmental Entomology 18, 1006 - 1010.

Way, M.O., 1994. Status of soybean insect pests in the United States. In: Higley, L.G., Boethel, D.J. (Eds.), Handbook of soybean insect pests. Entomological Society of America, Lanham, MD, pp. 15-16.

Whitehouse, M.E.A., Wilson, L.J., Fitt, G.P., 2005. A comparison of arthropod communities in transgenic *Bt* and conventional cotton in Australia. Environmental Entomology 34, 1224-1241.

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

Wohlleben, W., Arnold, W., Broer, I., Hillemann, D., Strauch, E., Punier, A., 1988. Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* tü494 and its expression in *Nicotiana tabacum*. Gene 70, 25-37.

Wolfenbarger, L.L., Naranjo, S.E., Lundgren, J.G., Bitzer, R.J., Watrud, L.S., 2008. Bt crop effects on functional guilds of non-target arthropods: A meta-analysis. PLoS ONE 3, e2118.

Wolt, J.D., Keese, P., Raybould, A., Fitzpatrick, J.W., Burachik, M., Gray, A., Olin, S.S., Schiemann, J., Sears, M., Wu, F., 2010. Problem formulation in the environmental risk assessment for genetically modified plants. Transgenic Research 19, 425-436.

Yang, Y., Zhu, Y.C., Ottea, J., Husseneder, C., Leonard, B.R., Abel, C., Luttrell, R., Huang, F., 2011. Down regulation of a gene for cadherin, but not alkaline phosphatase, associated with Cry1Ab resistance in the sugarcane borer *Diatraea saccharalis*. PLoS ONE 6, e25783.

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

Zhou, J., Berman, K.H., Breeze, M.L., Nemeth, M.A., Oliveira, W.S., Braga, D.P.V., Berger, G.U., Harrigan, G.G., 2011. Compositional variability in conventional and glyphosate-tolerant soybean (Glycine max L.) varieties grown in different regions in brazil. Journal of Agricultural and Food Chemistry 59, 11652-11656.