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

OECD Unique Identifier: DAS-81419-2

DAS-81419-2 Soybean

Volume 1 of 5

Submitting Company:

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Submitted by:

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Glossary of Acronyms and Scientific Terms

ADF	Acid detergent fibre
APVMA	Australian Pesticides and Veterinary Medicines Authority
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*
CFIA	Canadian Food Inspection Agency
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-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	US Federal Food, Drug, and Cosmetic Act
FIFRA	US 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 fibre
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
PMRA	Pest Management Regulatory Agency
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
UTR	Untranslated region
w/v	Weight per volume

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1. GENERAL INFORMATION ON THE APPLICATION

1.1 The Applicant

This application is submitted by:

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

Mr Peter Dryden
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1.2 Executive Summary

Dow AgroSciences LLC (herein referred to as "DAS") is submitting an application to amend the Australia New Zealand Food Standards Code Standard 1.5.2 to approve the use of DAS-81419-2 Soybean; a new food produced using gene technology.

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

This submission contains sufficient supporting information to address the requirements identified by FSANZ to approve the use a new food produced using gene technology, as specified in section 3.5.1 of the FSANZ Application Handbook, 1st August, 2011. Only reports produced by Dow AgroSciences or The Dow Chemical Company are provided. All other citations are available; however, given that these are from published literature, they have not been copied. Any or all of these citations will be forwarded if requested.

The references provided in section 5.1 of this dossier are proprietary information which is owned by and has value to The Dow Chemical Company and their subsidiary companies. These reports may not be used or referenced by any other company or person without our express agreement. A list of the DAS studies relevant to the information parts in this application is indicated at the beginning of each section.

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.

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; MXB-7 (also described as 3006-210-23 or DAS-21023-5 expressing Cry1Ac) and MXB-9 (also described as 281-24-236 or DAS-24236-5 expressing Cry1F) (FSANZ 2005). 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 A2704-12 (also described as ACS-GMØØ5-3) (FSANZ 2004), Herculex® I corn DAS-01507-1 (also described as TC1507) (FSANZ 2003). MXB-7 (also described as 3006-210-23 or DAS-21023-5 expressing Cry1Ac) and MXB-9 (also described as 281-24-236 or DAS-24236-5 expressing Cry1F) comprising WideStrike® cotton (FSANZ 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.

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 (EPA 2011, Mendelsohn et al 2003, Sanahuja et al 2011). Both proteins are expressed in WideStrike® cotton which is authorized for cultivation in Australia, 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, the Unites States EPA established an exemption from the requirement of a tolerance for the plant-incorporated protectant Cry1Ac in all plants. The Australian Pesticides and Veterinary Medicines Authority have since determined that a Cry1Ac protein maximum residue limit for human food and animal feed is unnecessary (APVMA 2012). EPA also 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 mouse acute oral toxicity studies.

® WideStrike and Herculex are trademarks of Dow AgroSciences LLC.

® LibertyLink is a registered trademark of Bayer.

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* have been extensively assessed to establish the safety of the proteins. The assessments included acute oral toxicity in mice and protein digestibility in simulated gastric fluid. The proteins have a very low acute toxicity potential and are rapidly degraded in simulated gastric fluid. Bioinformatics 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 allergens 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 shares 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. Bioinformatics analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence. The PAT protein hydrolysed rapidly in simulated gastric fluid. There was no evidence of acute toxicity of the PAT protein in mice. 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 around the world.

Nutrient compositional analyses of forage and grain were conducted to compare the composition of DAS-81419-2 soybean with that of a non-transgenic soybean 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 demonstrate that

DAS-81419-2 soybean is substantially equivalent to conventional soybean. Endogenous allergen analyses indicate that the genetic modification used to generate DAS-81419-2 soybean did not alter the endogenous allergen content compared to the non-transgenic soybean.

Because DAS-81419-2 soybean is compositionally similar to conventional soybean, and Cry1Ac, Cry1F and PAT proteins have a history of safe use, no significant impact is expected on human or animal health via 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.

Dow AgroSciences is seeking an amendment of the Australia New Zealand Food Standards Code - Standard 1.5.2 Food Produced Using Gene Technology by inserting: “food derived from insect-resistant DAS-81419-2 soybean line”, into column 3 of the Schedule of Permitted Foods produced using Gene Technology, immediately after the last soybean entry.

STATUTORY DECLARATION – AUSTRALIA

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

STATUTORY DECLARATION

Statutory Declarations Act 1959

I, Carolina Ortiz Padilla, Regulatory Specialist ANZ, Dow AgroSciences Australia Ltd.

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

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

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

[redacted]

Declared at Frenchs Forest, NSW, Australia on 3rd of June 2013
Before me,

[redacted]

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

CHECKLIST FOR GENERAL REQUIREMENTS

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

General Requirements (3.1)

3.1.1 Form of application
☑ *Executive Summary*
☑ *Relevant sections of Part 3 identified*
☑ *Pages sequentially numbered*
☑ *Electronic + 2 hard copies*
☑ *Electronic and hard copies identical*
☑ *Hard copies capable of being laid flat*
☑ *All references provided*

☑ 3.1.2 Applicant details

☑ 3.1.3 Purpose of the application

☑ 3.1.4 Justification for the application

☑ 3.2.5 Information to support the application

☑ 3.1.6 Assessment procedure
☑ *General*
☐ *Major*
☐ *Minor*

☑ 3.1.7 Confidential Commercial Information
☐ *Confidential material separated in both electronic and hard copy*
☐ *Justification provided*

☑ 3.1.8 Exclusive Capturable Commercial Benefit

☑ 3.1.9 International and Other National standards

☑ 3.1.10 Statutory Declaration

☑ 3.1.11 Checklist/s provided with Application
☑ *3.1 Checklist*
☑ *Any other relevant checklists for Sections 3.2-3.7*

CHECKLIST FOR STANDARDS RELATED TO NEW FOODS

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

Foods Produced using Gene Technology (3.5.1)

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

Novel Foods (3.5.2)

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

C – Information on the safety of the novel food

(I) Plant or animal extracts

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

(II) Plant and animal extracts

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

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

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

1.3 Purpose of the application

Dow AgroSciences LLC (herein referred to as "DAS") has genetically engineered insect-resistant DAS-81419-2 soybean plant product. DAS-81419-2 soybean provides protection against several lepidopteran pests of soybean. DAS-81419-2 is the unique identifier of these plants, in accordance with the Organisation for Economic Co-operation and Development's (OECD) "Guidance for the Designation of a Unique Identifier for Transgenic Plants" (OECD, 2002).

This application to the Food Standards Australia New Zealand has been based on the submission generated for other overseas agencies. This dossier contains sufficient supporting information to address the requirements identified by FSANZ to approve the use a new food produced using gene technology, as per section 3.5.1 of the FSANZ Application Handbook, 1st August, 2011.

This application is a component of the Dow AgroSciences global approval process, especially for export destinations of soybean commodities and is consistent with Dow AgroSciences corporate policy of ensuring full regulatory compliance. As a result, Dow AgroSciences is seeking an amendment of the Australia New Zealand Food Standards Code -Standard 1.5.2 Food Produced Using Gene Technology by inserting: "food derived from insect-resistant DAS-81419-2 soybean line", into column 3 of the Schedule of Permitted Foods produced using Gene Technology, immediately after the last soybean entry.

1.4 Justification for application

1.4 a Costs and Benefits of the genetically modified food

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

The area planted to soybeans worldwide has expanded rapidly due to development of varieties suited to regional planting conditions, rising yields, low production costs and global demand (Dorff 2007). Approximately 32.4 million ha of soybeans were grown in Northern America in 2010 (FAOSTAT 2012). Other countries have seen dramatic increases in soybean production: Argentine soybean production increased from 8,637,500 ha in 2000 to 18,130,800 ha in 2010; Brazilian production increased from 13,640,000 ha to 23,327,300 ha in the same period (FAOSTAT 2012, USDA NASS 2012). Australia's soybean production was 31,300 ha in 2010, and a total of 1,872 tonnes of soybeans and 26,649 tonnes of soybean oil were imported to Australia in 2010 from around the world, herein including the Americas (FAOSTAT 2012).

Damage by defoliating insects 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 United States (Catchot 2011). Recent increases in pest insect populations in soybean have been observed and have been attributed by some to insect adaptation to the soybean plant or physiological effects on plants of anthropogenic tropospheric conditions (Hamilton et al 2005, Way 1994). Lower economic thresholds coupled with higher insect populations favour more aggressive pest management strategies (Pedigo 1996). Higher prices of soybeans have significantly lowered economic thresholds associated with many soybean pests, expanding the area and number of insecticide treatments (USDA ERS 2011).

Four Lepidopteran species are particularly problematic for soybean production in USA: soybean looper, velvetbean caterpillar, fall armyworm, and tobacco budworm; velvetbean caterpillar and soybean looper are the most damaging of these (Gianessi 2009, Way 1994). On average, about one-third of the soybean grown in the Southern U.S (Georgia, Louisiana, and North Carolina) have been treated with insecticides since 1991 and insecticides are used on approximately 50% of the soybean acreage in Georgia for lepidopteran pests (Gianessi et al 2002).
Argentine and Brazilian soybean defoliators include many of the same Lepidoptera species that are problematic in North America: soybean looper, velvetbean caterpillar, *Spodoptera* spp. and tobacco budworm (de Freitas Bueno et al 2011). Soybean production in tropical and subtropical regions is typically characterised by more consistent and relatively greater damage by these pests due in part to the endemic rather than migratory status of pest populations (Martinelli et al 2007, Sosa-Gómez 2004). In Australia, *helicoverpa spp*. (cotton bollworm and native budworm) are identified as major insect pests of soybean. Other defoliators of the same Lepidoptera species in Australia include cluster caterpillar, bean pod borer, and green and brown loopers (DAFF 2012).

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 & Boethel 1994). Soybean looper has developed extensive insecticide resistance (Thomas & 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).

Dow AgroSciences 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 & 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 & 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.

In summary, DAS-81419-2 soybean is an effective pest management tool for lepidopteran pests that could reduce or replace current insecticide applications in regions where these insect pests cause significant plant damage and yield loss.

(ii) Costs and benefits to the consumer

The information presented in this submission support the conclusion that food and feed derived from DAS-81419-2 soybean will be as safe and nutritious as those derived from conventional soybean. Because DAS-81419-2 soybean has been demonstrated to be compositionally similar to conventional soybean, and Cry1Ac, Cry1F and PAT proteins have a history of safe use, no significant impact is expected on human or animal health via commodity food and feed soybean products.

(iii) Costs and benefits to the government

The local cost implications are made up of DAS personnel time both locally and globally as well as the direct fees associated with the submission. There are few price or employment implications which are directly related to the FSANZ assessment of DAS-81419-2. The trade implications however are considerable since non-approval by FSANZ would impose a trade restriction on DAS-81419-2 and products derived from these lines.

1.4 b Potential impact on international trade

This application to the Food Standards Australia New Zealand has been based on the submission generated for other overseas agencies. It is a component of the Dow AgroSciences global approval process, especially for export destinations of soybean commodities and is consistent with Dow AgroSciences corporate policy of ensuring full regulatory compliance. It is a necessary component of the global approval process since without such food import approvals, the cultivation and marketing of DAS-81419-2 in the USA, Canada, Argentina and Brazil will be significantly hampered. DAS intends to submit dossiers to the regulatory authorities of trade partners for import clearance and production approval including, but not limited to, the USA, Canada, Mexico, Colombia, Brazil, Argentina, Japan, Korea, Taiwan, China, EU, South Africa and Philippines. The benefit and market share implication are difficult to quantify, however, freedom to operate in the marketplace is a market requirement and will have an impact on these factors.

2. TECHNICAL INFORMATION ON THE GM FOOD

2.1 Nature and identity of the genetically modified food

2.1 a Description of the GM organism

DAS-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 *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.

2.1 b GM organism identification

This transformed soybean is known as event DAS-81419-2. No commercial name has yet been identified.

2.1 c Food Identity

There is no intention to market food items containing soybean derived from DAS-81419-2 with specific brands or names.

2.1 d Products containing the food or food ingredients

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

- general description, including taxonomy, morphology, and the uses of soybean as a crop plant
- agronomic practices
- centres 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 (Gaska 2006, Pedersen 2004):

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

Soybeans are crushed to form two derivatives, meal and oil. The main product derived from soybean that is used in Australia is meal for animal feed. Soybean meal is particularly high in protein and is the preferred meal for pig and poultry productions systems. It is also used in the production of pet food. Domestic production of soybean in Australia (~30,000t) and New Zealand is supplemented by import of soybean-based products, predominantly meal, to meet the requirements by the animal industry. Soybean oil is also imported for table oil use or processed into margarines or mayonnaise and used by the food industry or the consumer.

2.2 History of Use of the Host and Donor Organisms

2.2 a Donor Organism

DAS-81419-2 soybean was generated by Agrobacterium-mediated transformation using plasmid pDAB9582 (Figure 3). 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 4). 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) (FDA 2004b) 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) (FDA 2004c) 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, USDA 2001, USDA 2004, USDA 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.

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 and Canada in 1972. Today there are more than 100 registered pesticide products containing *B. thuringiensis* as an active ingredient in North America (EPA 2011, Health Canada 2010). 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.

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 approved in Australia.

2.2 b Host Organism

Soybean (*Glycine max)* is a diploidized tetraploid (2n=40), in the family *Leguminosae,* subfamily *Papilionoideae,* tribe *Phaseoleae,* genus *Glycine* Willd, subgenus *Soja* (Moench). It is an erect, bushy herbaceous annual that can reach a height of 1.5m. Three types of growth habit can be found amongst soybean cultivars: determinate, semi-determinate and indeterminate. Determinate growth is characterized by the cessation of vegetative activity of the terminal bud when it becomes an inflorescence at both auxiliary and terminal racemes. Indeterminate genotypes continue vegetative activity throughout the flowering period. Semi-determinate types have indeterminate stems that terminate vegetative growth abruptly after the flowering period.

The primary leaves are unifoliate, opposite and ovate, the secondary leaves are trifoliolate and alternate, and compound leaves with four or more leaflets are occasionally present. The nodulated root system consists of a taproot from which emerges a lateral root system. The plans of most cultivars are covered with fine trichomes, but some glabrous types exist. The papilionaceous flowers consist of a tubular alyx of five sepals, a corolla of 5 petals, one pistil and nine fused stamens with a single separate posterior stamen. The pod is straight or slightly curved, varies in length from 2-7cm and consists of two halves of a single carpel which are joined by a dorsal and ventral suture. The shape of the seed, usually oval, can vary amongst cultivars from spherical to elongate and flattened.

The stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation and cross pollination is usually less than one percent. A soybean plant can produce as many as 400 pods, with 2 to 20 pods at a single node. Each pod contains 1-5 seeds. Neither the seed pod, nor the seed, has morphological characteristics that encourage animal transportation.

Soybean is primarily grown for the production of seed, has a multitude of uses in the food and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use. The United States, Brazil, Argentina and China produced 200 million metric tonnes of soybeans in 2005 which was 90% of the total global production.

(i) 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.

2.3 Nature of the Genetic Modification

Guttikonda S. 2012b. Molecular characterization of DAS-81419-2 soybean. Study ID 110813, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S, Richey K. 2012. Cloning and characterization of the DNA sequence for the insert and its flanking border regions of DAS-81419-2 soybean. Study ID 102126, Dow AgroSciences LLC, Indianapolis IN

Hoffman T, Shan G. 2012. Event Sorting and Selection Process for the Development of DAS-81419-2. Study ID 120687, Dow AgroSciences LLC, Indianapolis, IN

Mo J. 2012a. Molecular characterization of DAS-81419-2 soybean within a single segregating generation. Study ID 110814, Dow AgroSciences LLC, Indianapolis, IN

Zhuang M. 2012. Transformation information for plasmid pDAB9582. Study ID 110688, Dow AgroSciences LLC, Indianapolis, IN

2.3 a Transformation Method

Transgenic soybean (*Glycine max*) DAS-81419-2 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 (Zhuang 2012). *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 analysed 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 outlines DAS-81419-2 soybean development.

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

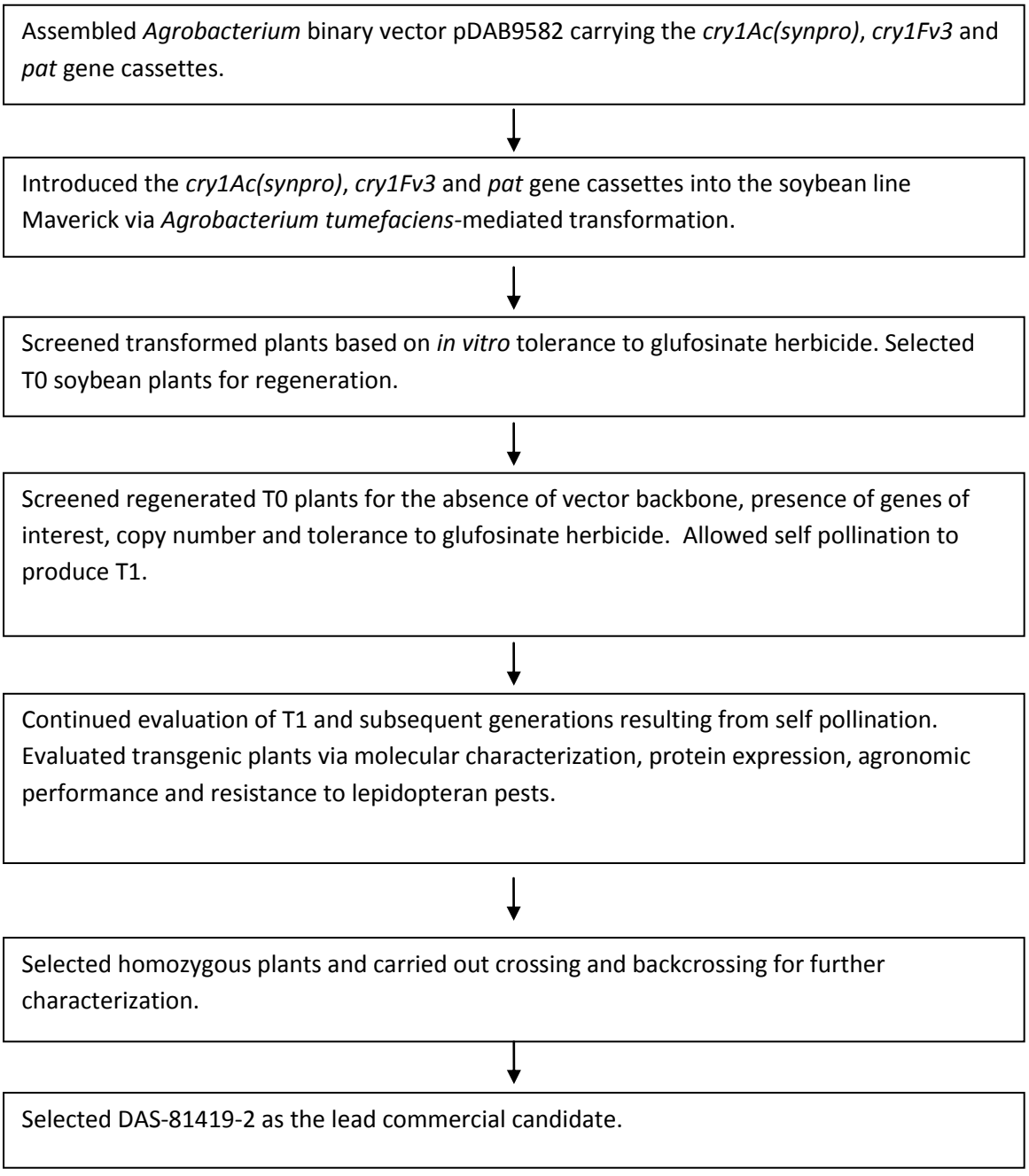
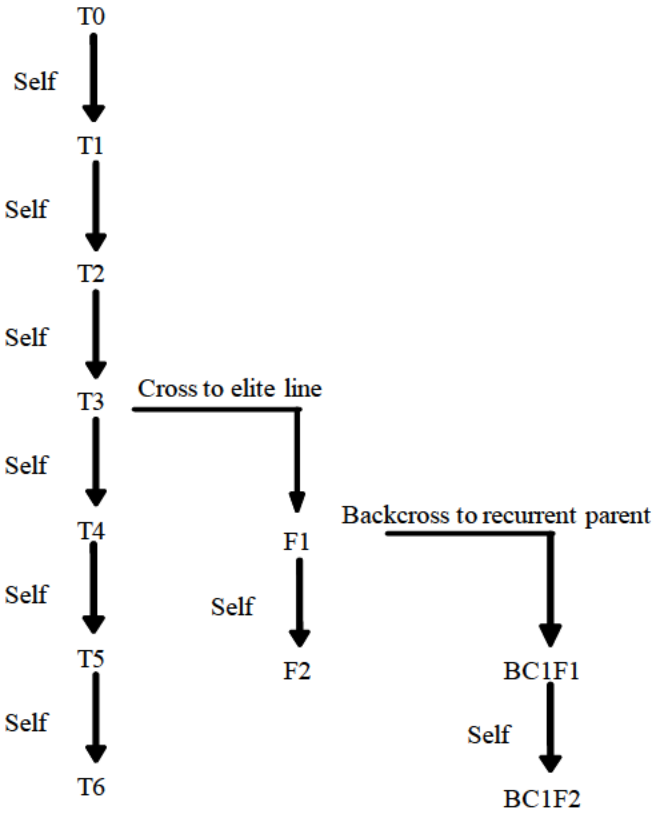


Figure 1. Event sorting and selection process for DAS-81419-2 soybean. (Hoffman & Shan 2012)



Analysis	DAS-81419-2 Soybean Generations	Control
Genetic Characterization	T1, T2, T3, T4, F2	Non-transgenic soybean Maverick
Segregation Analysis	F2, BC1F2	None
Protein Characterization	T4, T5	Non-transgenic soybean Maverick
Protein Expression	T4	Non-transgenic soybean Maverick
Agronomics	T4	Non-transgenic soybean Maverick
Germination/Dormancy	T4	Non-transgenic soybean Maverick
Composition	T4	Non-transgenic soybean Maverick
NTO	T4	Non-transgenic soybean Maverick
Efficacy	T4	Non-transgenic soybean Maverick

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

2.3 b Bacteria used for manipulation

A standard laboratory strain of *E.coli* was used for all vector manipulations and for amplication of the plasmid DNA (pDAB9582) that was used for the transformation.

2.3 c Gene Construct and Vectors

DAS-81419-2 soybean was generated by *Agrobacterium*-mediated transformation using plasmid pDAB9582 (Figure 3). 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 4). 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 (*berliner*). 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) (FSANZ 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.

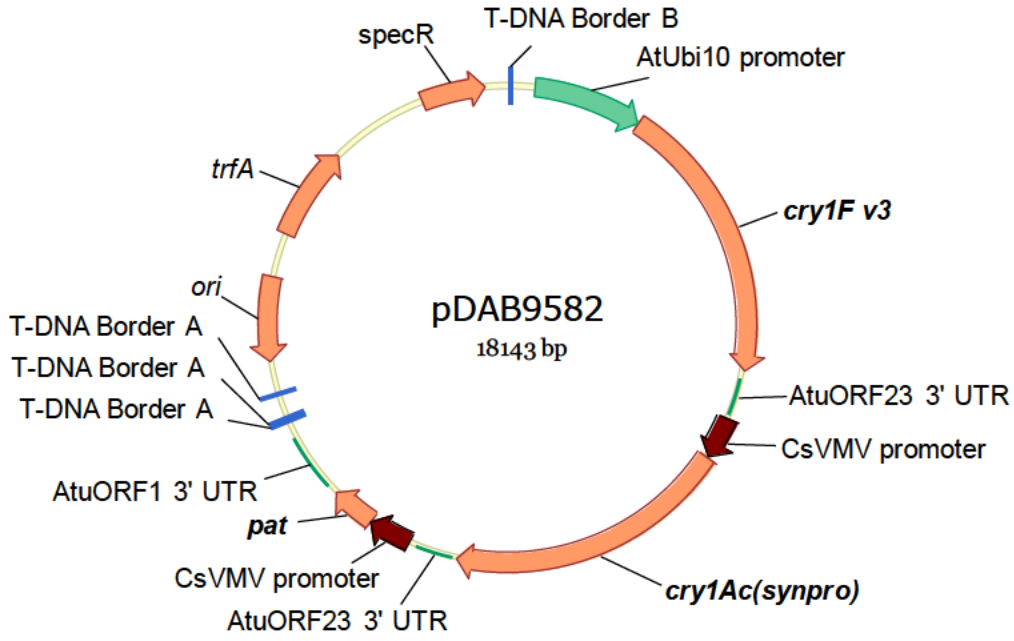


Figure 3. Plasmid map of pDAB9582.

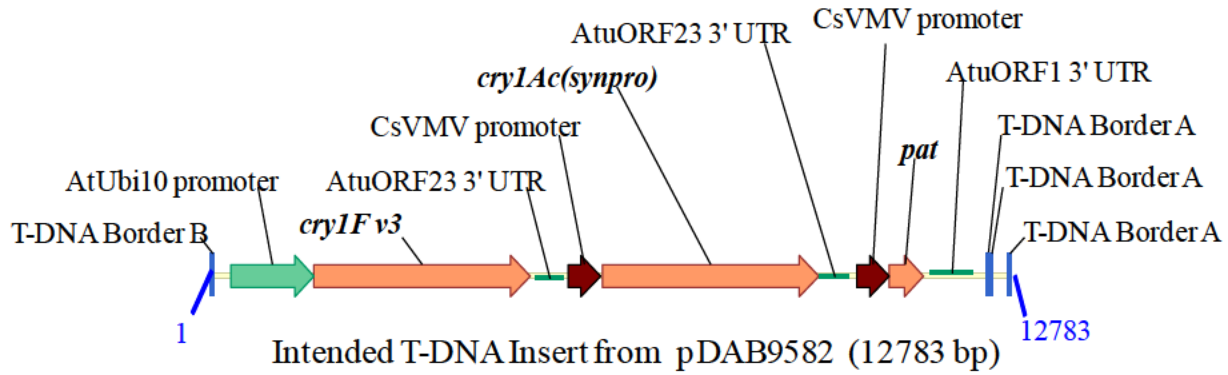


Figure 4. Diagram of intended T-DNA insert from plasmid pDAB9582.

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, Cardineau et al 2001, Gilroy & Wilcox 1992)
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

Feature Name	Feature Start	Feature Stop	Feature Length	Description
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
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)

Feature Name	Feature Start	Feature Stop	Feature Length	Description
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

2.3 d Molecular Characterization

Molecular characterization of DAS-81419-2 soybean was conducted by Southern blot analyses (Guttikonda 2012b) and DNA sequencing (Guttikonda & Richey 2012). 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 as determined by DNA sequencing 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

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. 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. 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. The inheritance of DAS-81419-2 soybean insert in segregating generations was investigated using event-

specific PCR and detection of the PAT protein expression (Mo 2012a). All results confirmed the predicted inheritance of the transgene in a single locus.

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) (Gao et al 2006, Schafer et al 2012b)
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

Feature Name	Feature Start	Feature Stop	Feature Length	Description
cry1Ac(synpro)	7630	11100	3471	*cry1Ac (synpro)* (synthetic version of the *cry1Ac* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD73) (Adang et al 1985, Dow AgroSciences 2003b, Gaska 2006)
Intervening sequence	11101	11133	33	Non-specific DNA sequences necessary for cloning
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

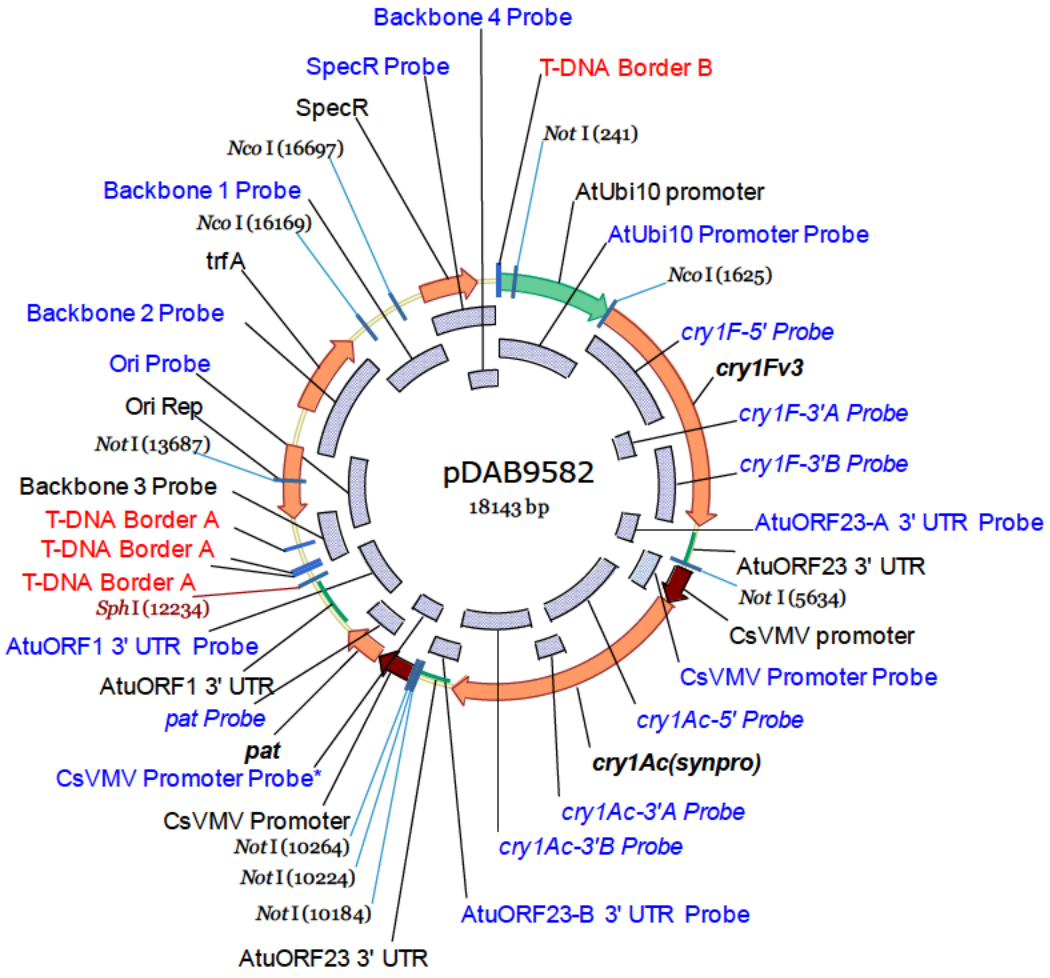


Figure 5. Location of the probes on pDAB9582 used in Southern blot analysis of DAS-81419-2 soybean.
*Note: * Since the CsVMV promoter element in the cry1Ac(synpro) and pat PTUs are 100% identical based on the nucleotide sequences, CsVMV Promoter probe generated from cry1Ac(synpro) PTU was used to hybridize 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	Figure 8A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>1737	~7500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 8B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1F-5′	*Nco*I	pDAB9582	2	14544	~14500	Figure 9A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 9B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1F-3′A	*Nco*I	pDAB9582	2	14544	~14500	Figure 10A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 10B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1F-3′B	*Nco*I	pDAB9582	2	14544	~14500	Figure 11A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 11B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
AtuORF23-A 3′ UTR	*Nco*I	pDAB9582	2	14544	~14500	Figure 12A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 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	Figure 13A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 13B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1Ac-5′	*Nco*I	pDAB9582	2	14544	~14500	Figure 14A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 14B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1Ac-3′A	*Nco*I	pDAB9582	2	14544	~14500	Figure 15A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759, >1737	~14500, ~7500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 15B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
cry1Ac-3′B	*Nco*I	pDAB9582	2	14544	~14500	Figure 16A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 16B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
AtuORF23-B 3′ UTR	*Nco*I	pDAB9582	2	14544	~14500	Figure 17A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 17B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>12346	~18100	
pat	*Nco*I	pDAB9582	2	14544	~14500	Figure 18A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
		Maverick	3	none	none	Figure 18B
		DAS-81419-2	4-18	>12346	~18100	
AtuORF1 3′ UTR	*Nco*I	pDAB9582	2	14544	~14500	Figure 19A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>10759	~14500	
	*Sph*I	pDAB9582	2	18143	~18100	Figure 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	Figure 20A
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, >353	~5400, ~6500	
cry1F-5′		pDAB9582	2	5393	~5400	Figure 20B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393	~5400	
cry1F-3′A		pDAB9582	2	5393, 4550	~5400, ~4600	Figure 21A
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
cry1F-3′B		pDAB9582	2	5393, 4550	~5400, ~4600	Figure 21B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
AtuORF23-A 3′ UTR		pDAB9582	2	5393, 4550	~5400, ~4600	Figure 22A
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
CsVMV Promoter	*Not*I/*Sph*I (Release *cry1Ac(synpro)* PTU)	pDAB9582	2	4550, 1970	~4600, ~2000	Figure 22B
		Maverick	3	none	none	
		DAS-81419-2	4-18	4550, 1970	~4600, ~2000	
cry1Ac-5′		pDAB9582	2	4550	~4600	Figure 23A
		Maverick	3	none	none	
		DAS-81419-2	4-18	4550	~4600	
cry1Ac-3′A		pDAB9582	2	5393, 4550	~5400, ~4600	Figure 24A
		Maverick	3	none	none	
		DAS-81419-2	4-18	>353, 4550, 5393	~6500, ~4600, ~5400	

Probe	Restriction Enzyme	Sample	Lane	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure Number
cry1Ac-3'B		pDAB9582	2	5393, 4550	~5400, ~4600	Figure 24B
		Maverick	3	none	none	
		DAS-81419-2	4-18	5393, 4550	~5400, ~4600	
AtuORF23-B 3' UTR		pDAB9582	2	5393, 4550	~5400, ~4600	Figure 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	Figure 25A
		Maverick	3	none	none	
		DAS-81419-2	4-18	1970	~2000	
AtuORF1 3' UTR		pDAB9582	2	1453, 1970,	~1500, ~2000	Figure 25B
		Maverick	3	none	none	
		DAS-81419-2	4-18	>150, 1970	~4000, ~2000	
Ori	*Sph*I	pDAB9582	2	18143	~18100	Figure 26A
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
SpecR	*Nco*I	pDAB9582	2	3071	~3100	Figure 26B
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
Backbone 1	*Nco*I	pDAB9582	2	14544, 3071, 528	~14500, ~3100, ~500	Figure 27A
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
Backbone 2	*Sph*I	pDAB9582	2	18143	~18100	Figure 27B
		Maverick	3	none	none	
		DAS-81419-2	4-18	none	none	
Backbone 3 + 4	*Nco*I	pDAB9582	2	14544, 3071	~14500, ~3100	Figure 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.

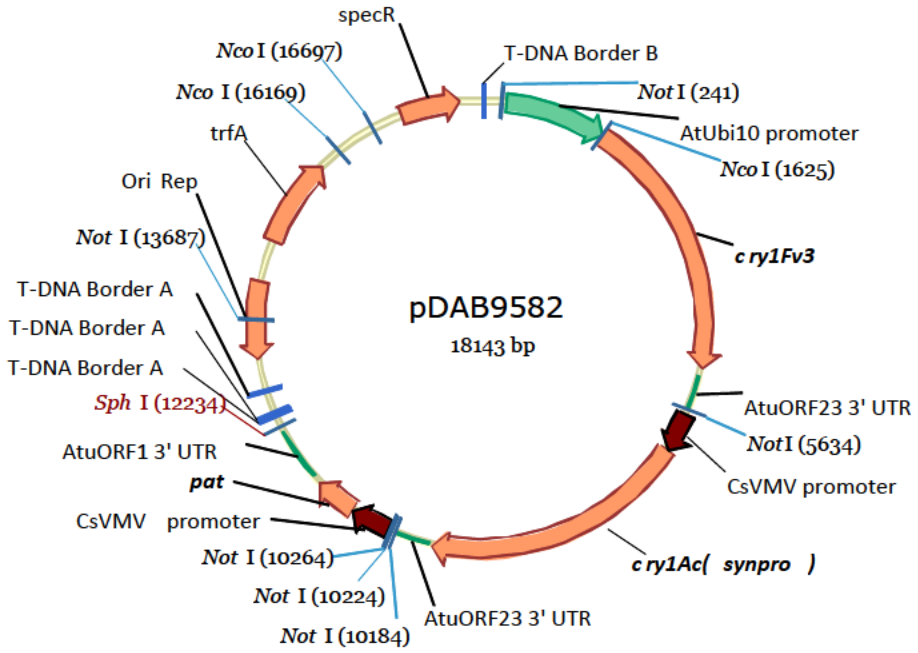
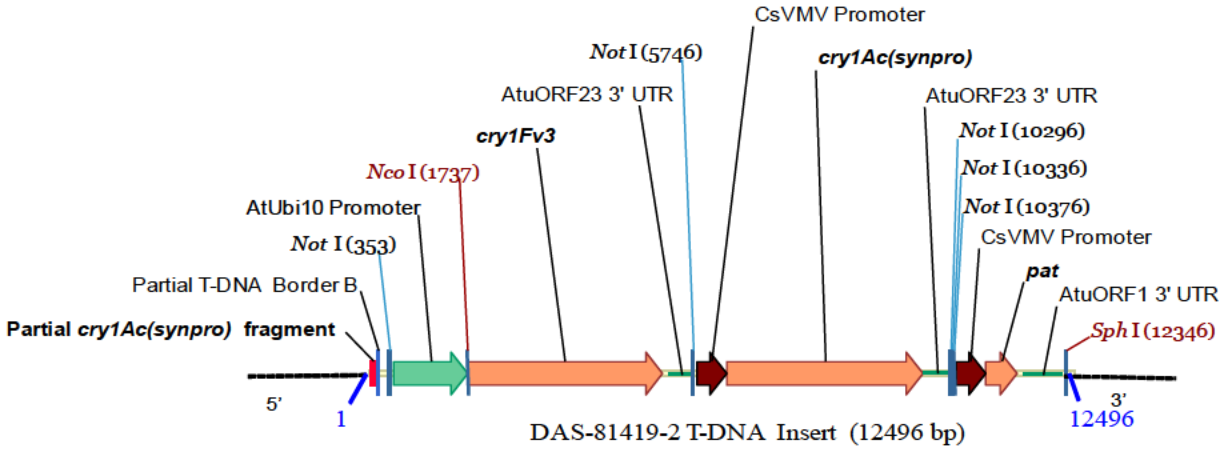


Figure 6. Plasmid map of pDAB9582 with selected restriction enzyme sites used for Southern analysis.



*Nco*I	>1737 bp	>10759 bp			
*Not*I/*Sph*I	>353 bp	5393 bp	4550 bp	1970 bp	>150bp
*Sph*I	>12346 bp				>150bp

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.

(i) Analysis of the Insert and Its Genetic Elements

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 to improve sensitivity), 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.

(ii) 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 (Guttikonda & Richey 2012), a 98-bp partial *cry1Ac(synpro)* fragment was also identified at the 5'end of the T-DNA insert in DAS-81419-2 soybean (Figire 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.

(iii) 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.

2.3 e Breeding Pedigree

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.

2.3 f Stability of the genetic changes

(i) Analysis of a Segregation 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 (Mo 2012a).

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

(ii) 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

(iii) 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.

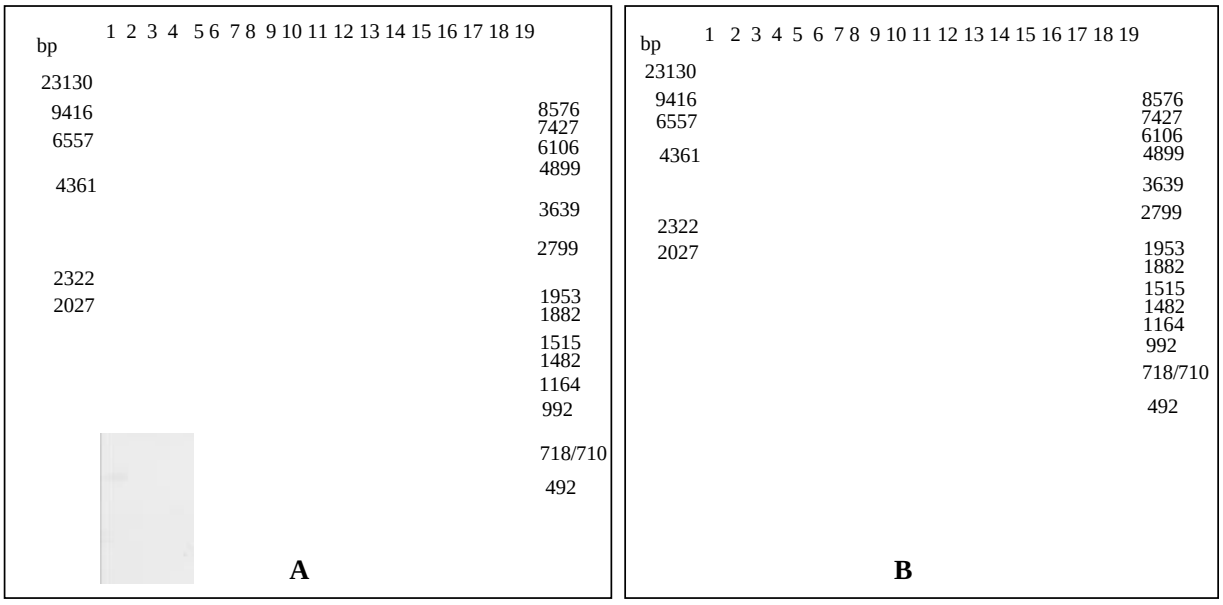


Figure 8. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtUbi10 promoter probe.

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		

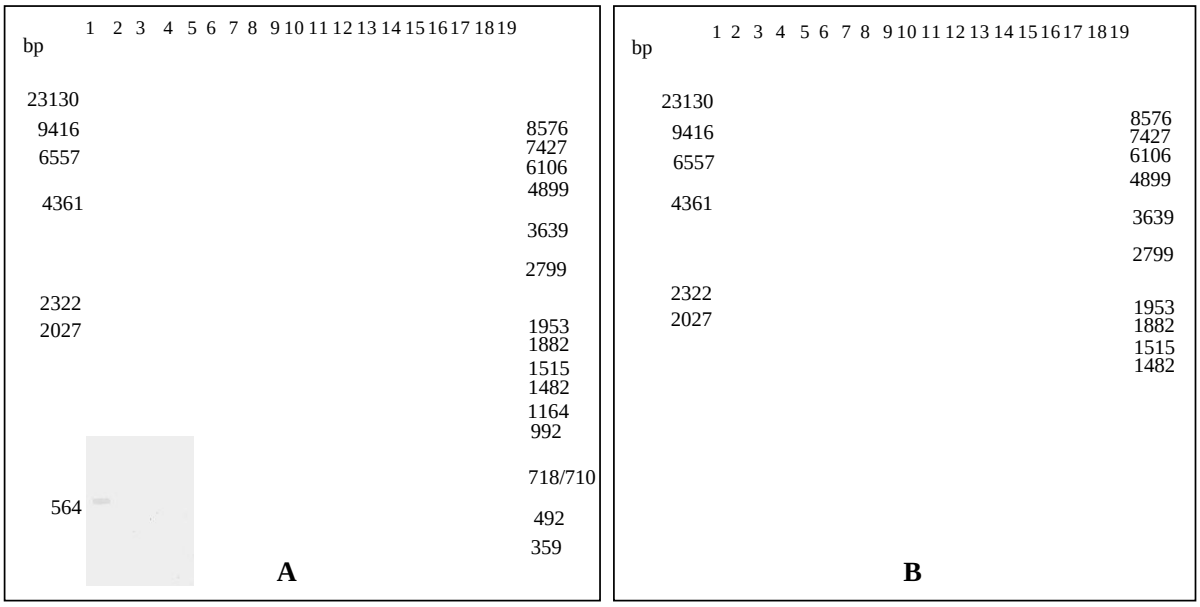


Figure 9. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1F-5'* probe.

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		

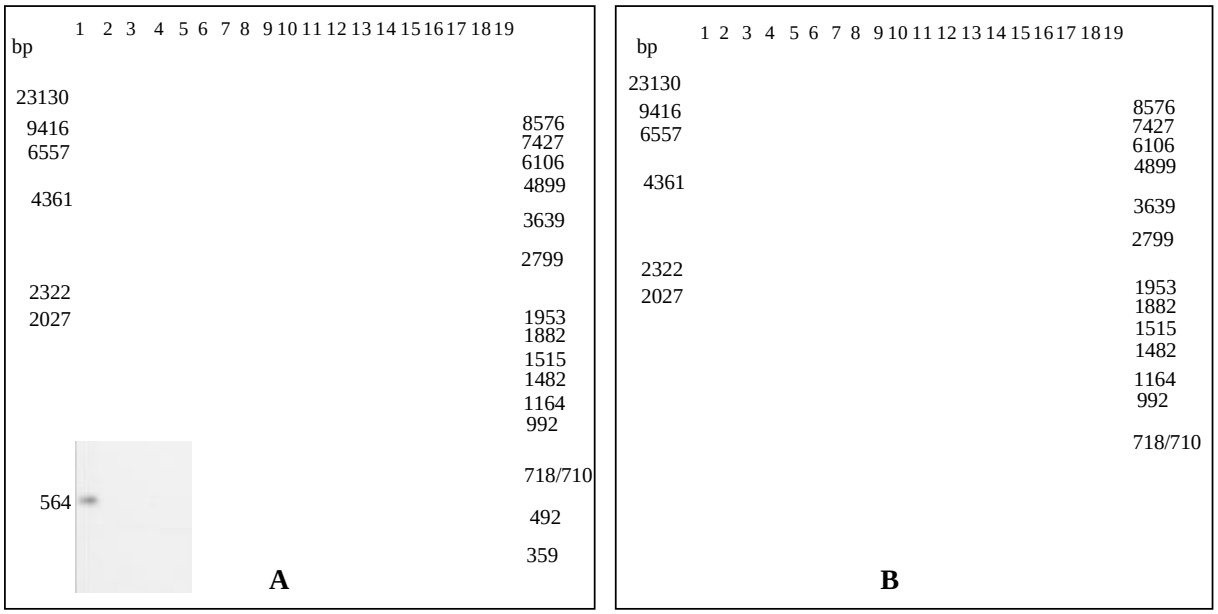


Figure 10. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1F-3'*A probe.

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		

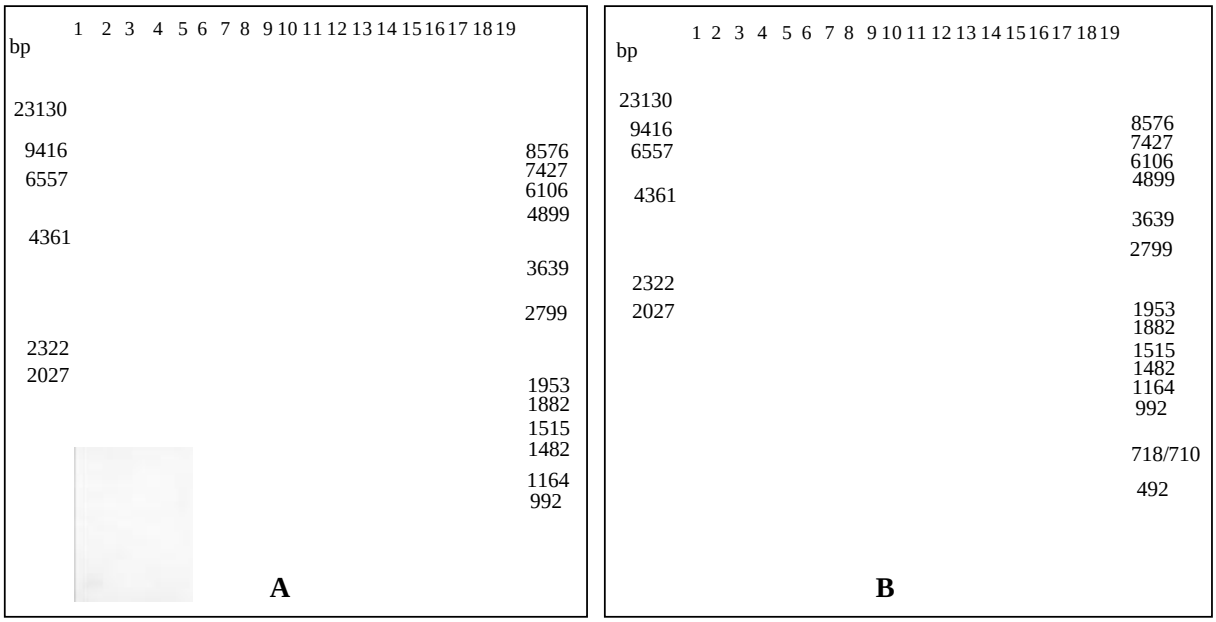


Figure 11. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1F-3'* B probe.

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		

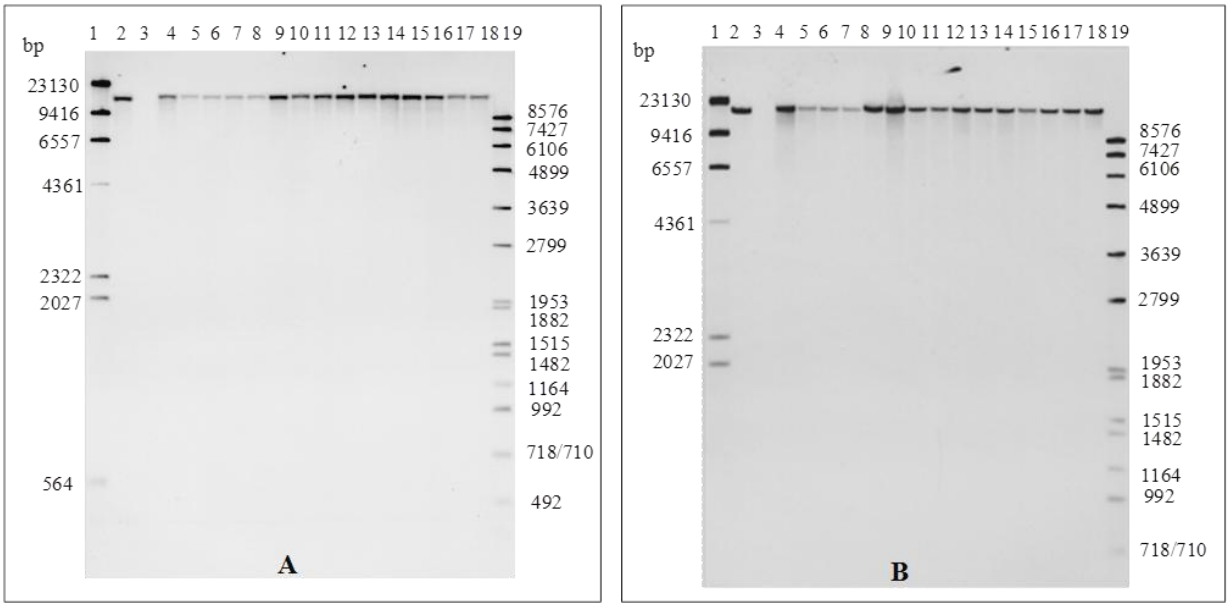


Figure 12. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtuORF23-A 3'UTR probe.

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		

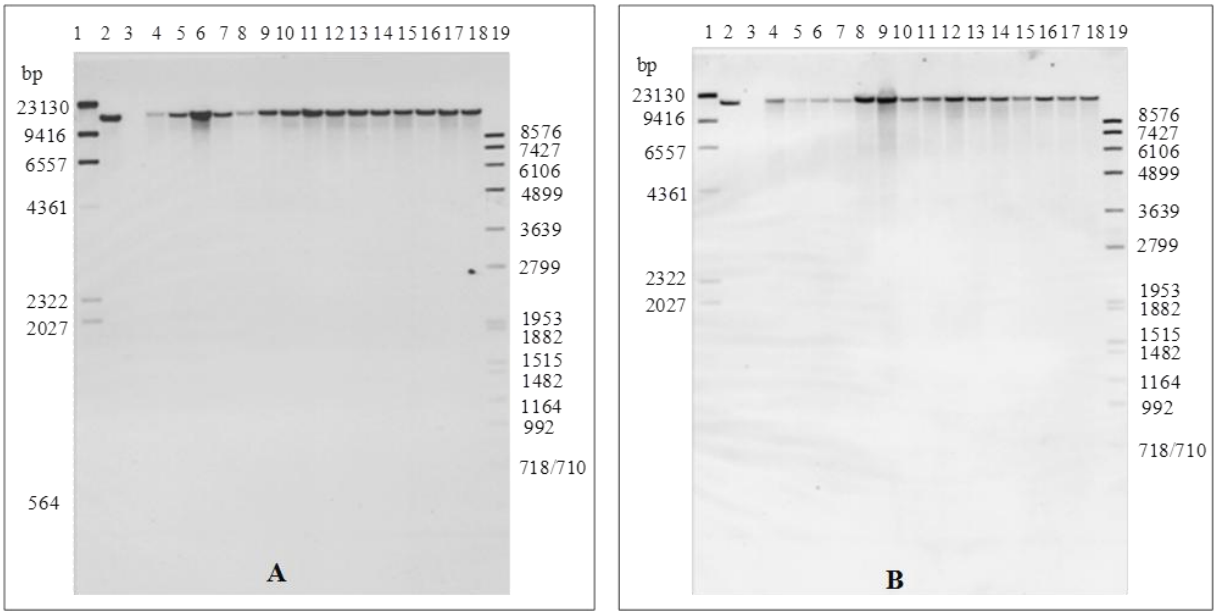


Figure 13. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); CsVMV promoter probe.

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		

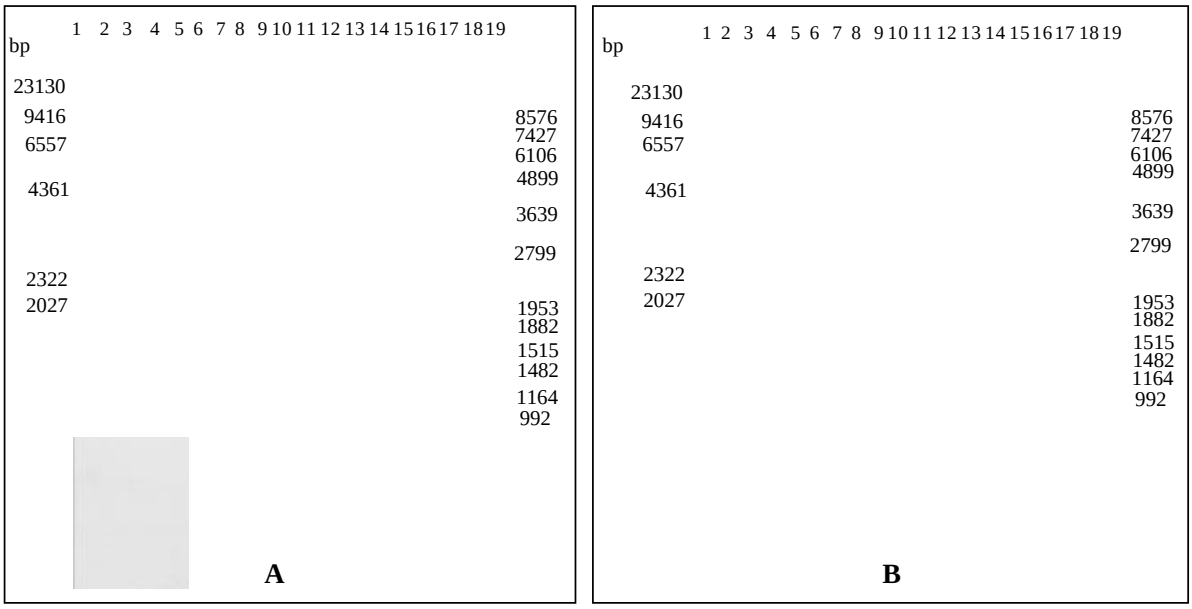


Figure 14. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1Ac*-5' probe.

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		

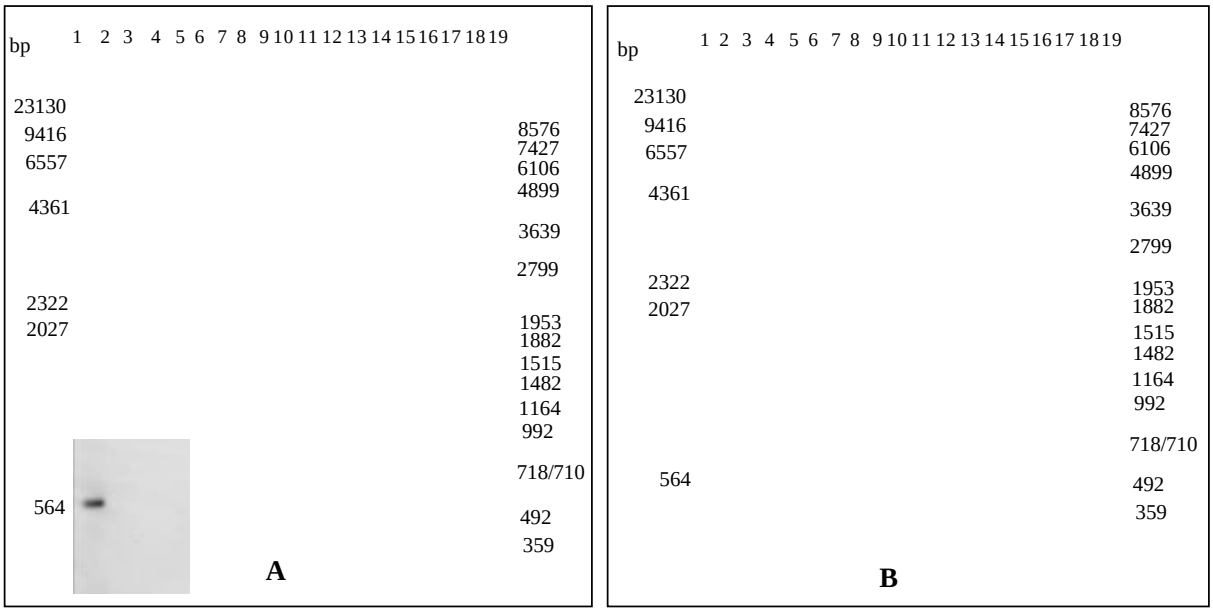


Figure 15. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1Ac*-3'A probe.

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		

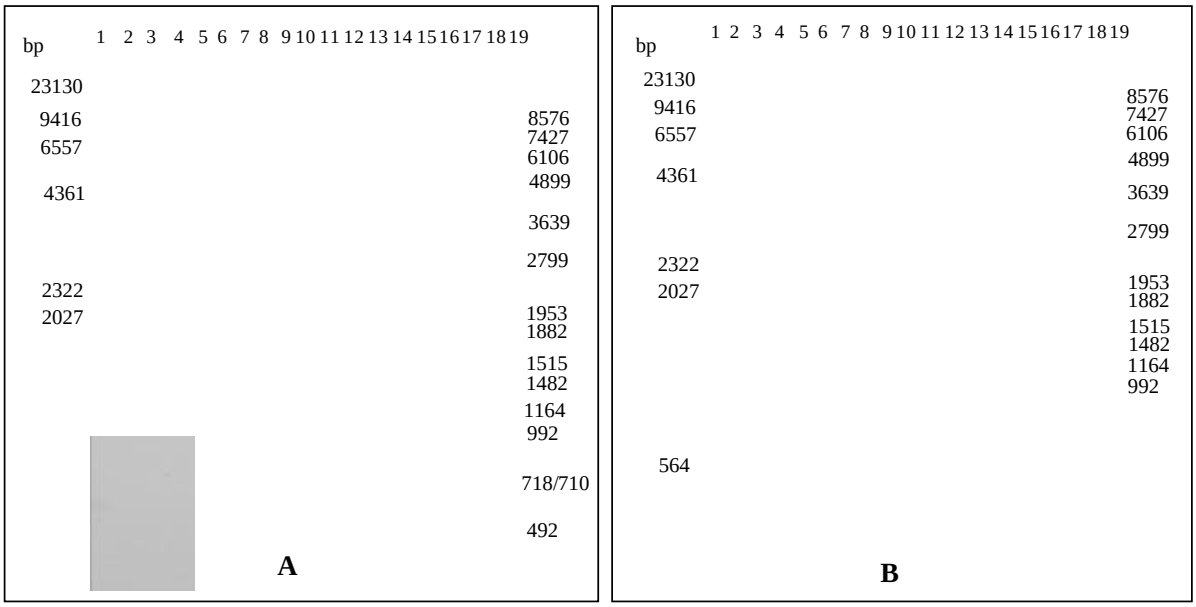


Figure 16. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *cry1Ac*-3'B probe.

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		

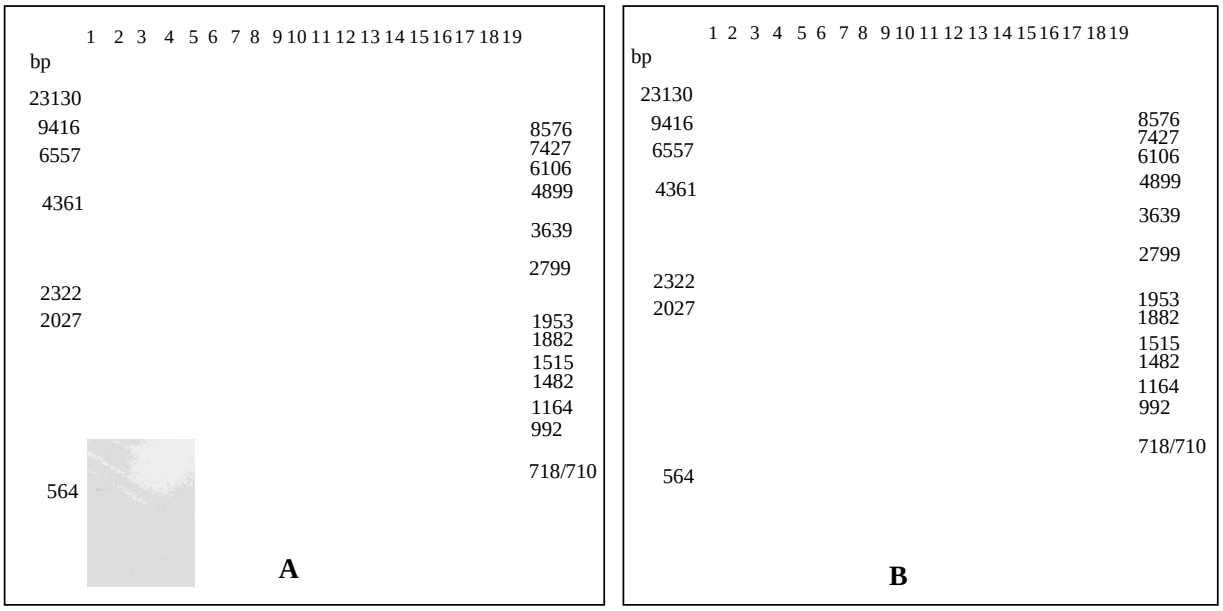


Figure 17. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtuORF23-B 3'UTR probe.

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		

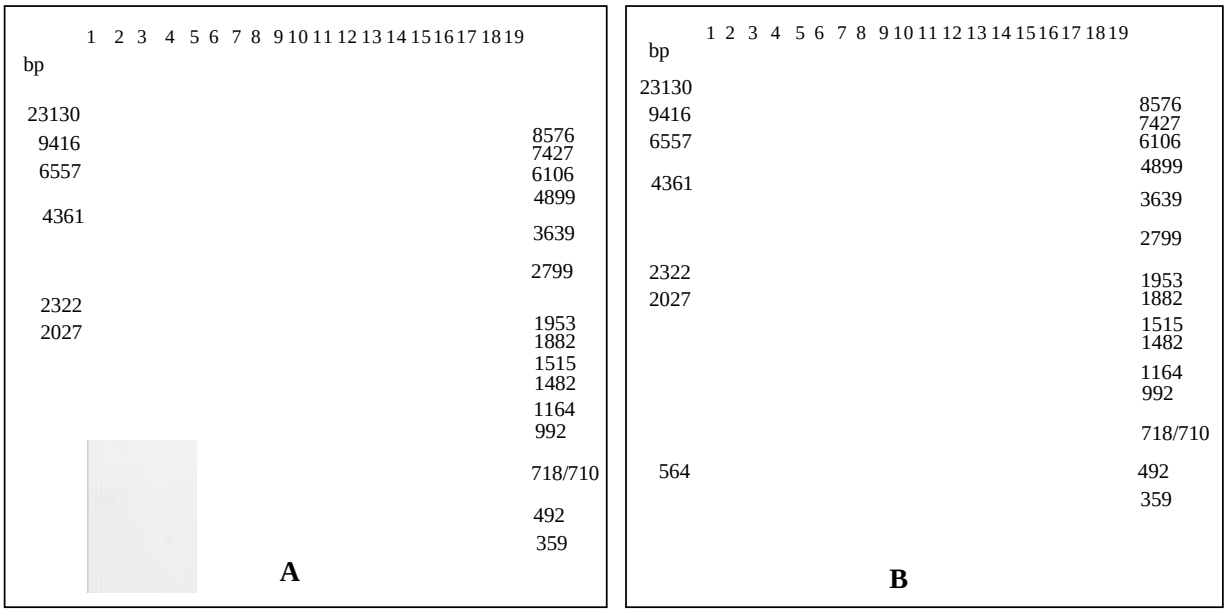


Figure 18. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); *pat* probe.

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		

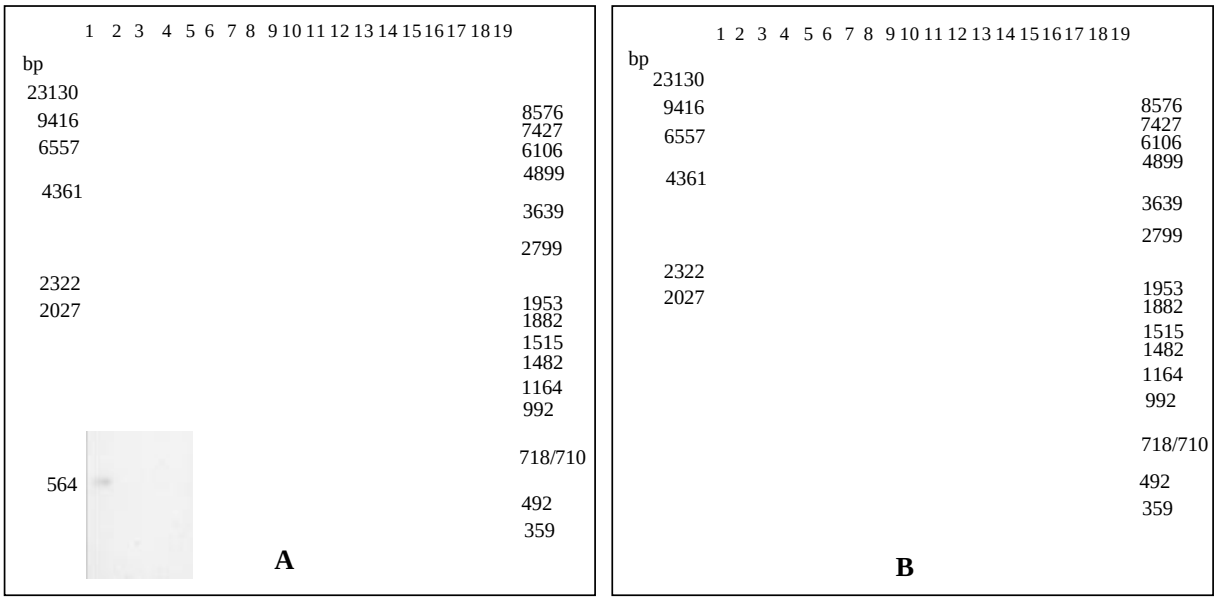


Figure 19. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I (A) and *Sph*I (B); AtuORF1 3' UTR probe.

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		

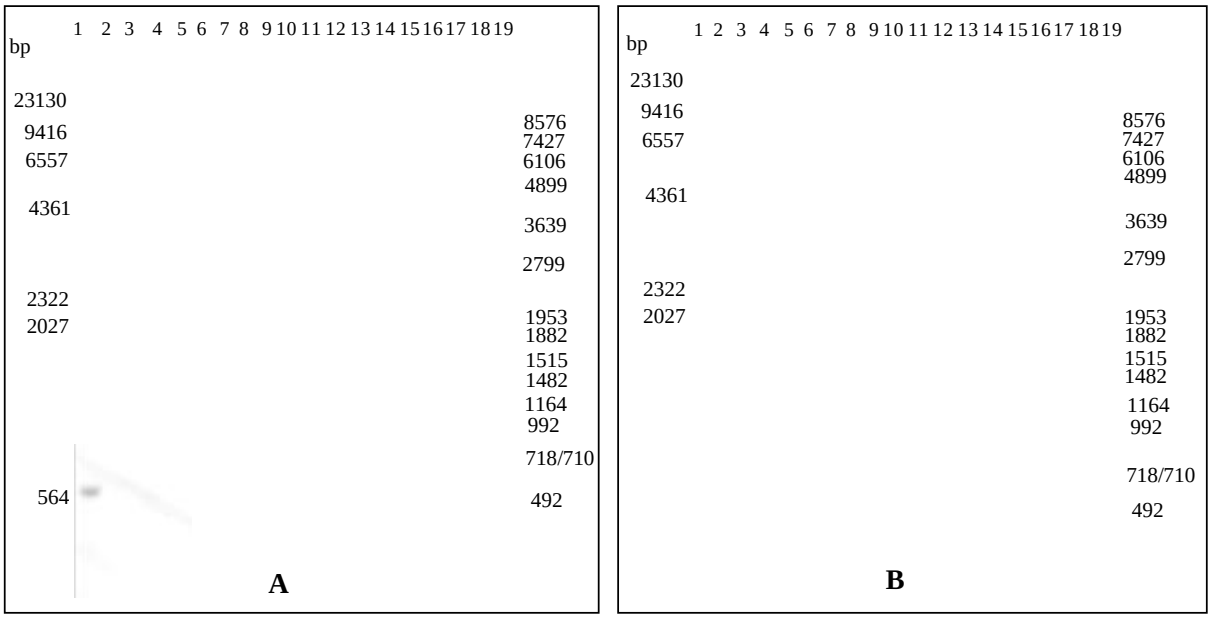


Figure 20. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) AtUbi10 promoter probe, (B) *cry1F-5'* probe.

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		

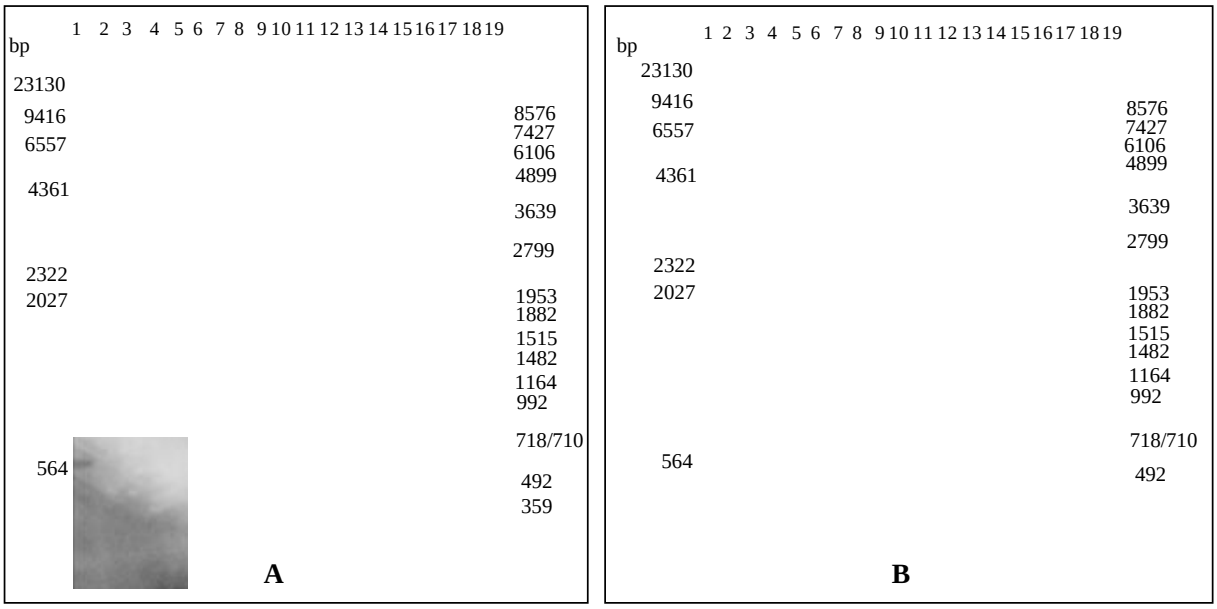


Figure 21. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *cry1F*-3'A probe, (B) *cry1F*-3'B probe.

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		

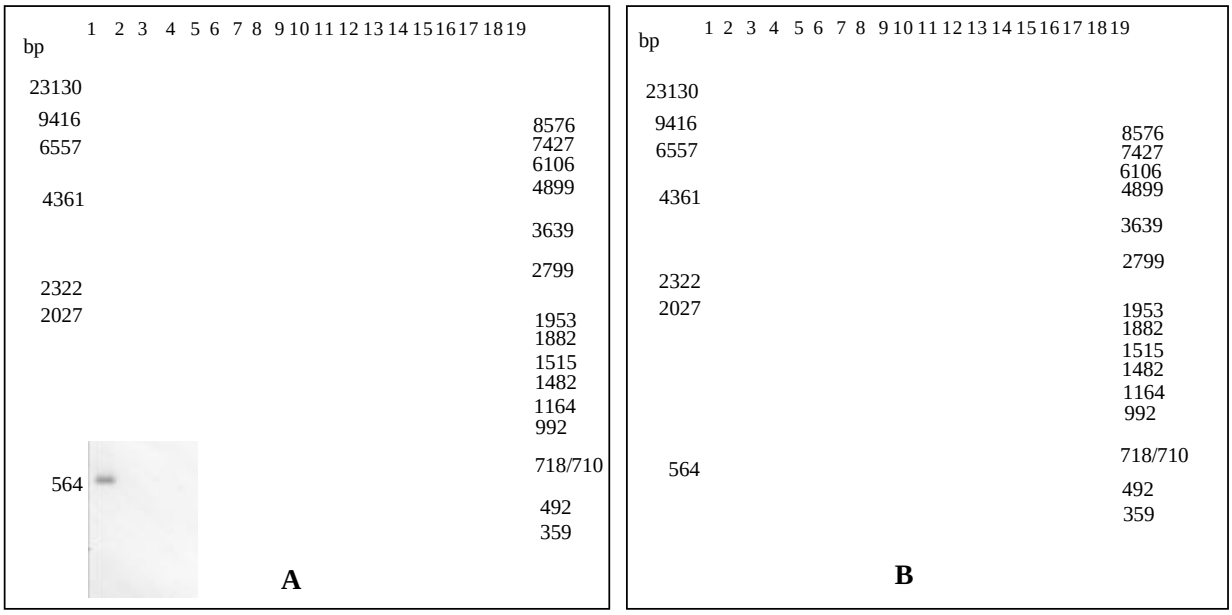


Figure 22. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) AtuORF23-A 3'UTR probe, (B) CsVMV promoter probe.

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		

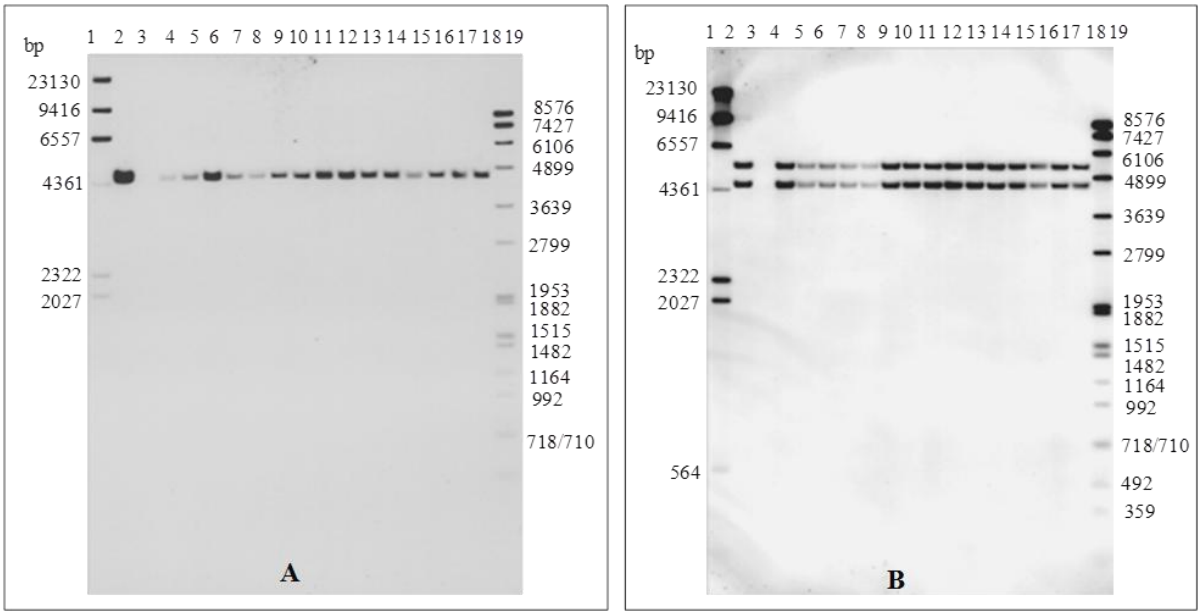


Figure 23. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *cry1Ac*-5' probe, (B) AtuORF23-B 3'UTR probe.

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		

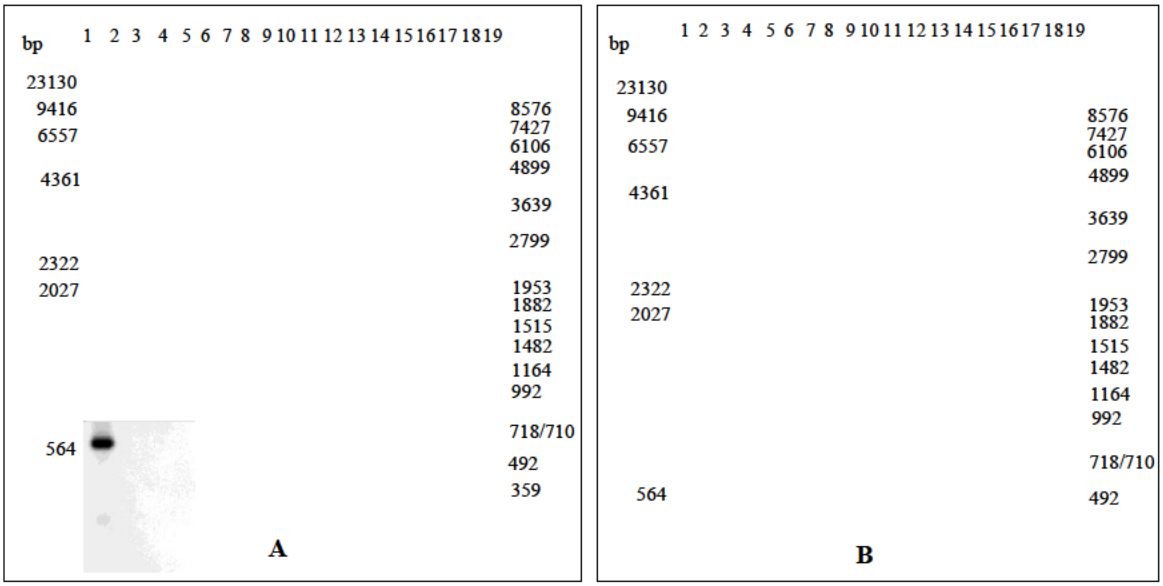


Figure 24. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *cry1Ac*-3'A probe, (B) *cry1Ac*-3'B probe.

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		

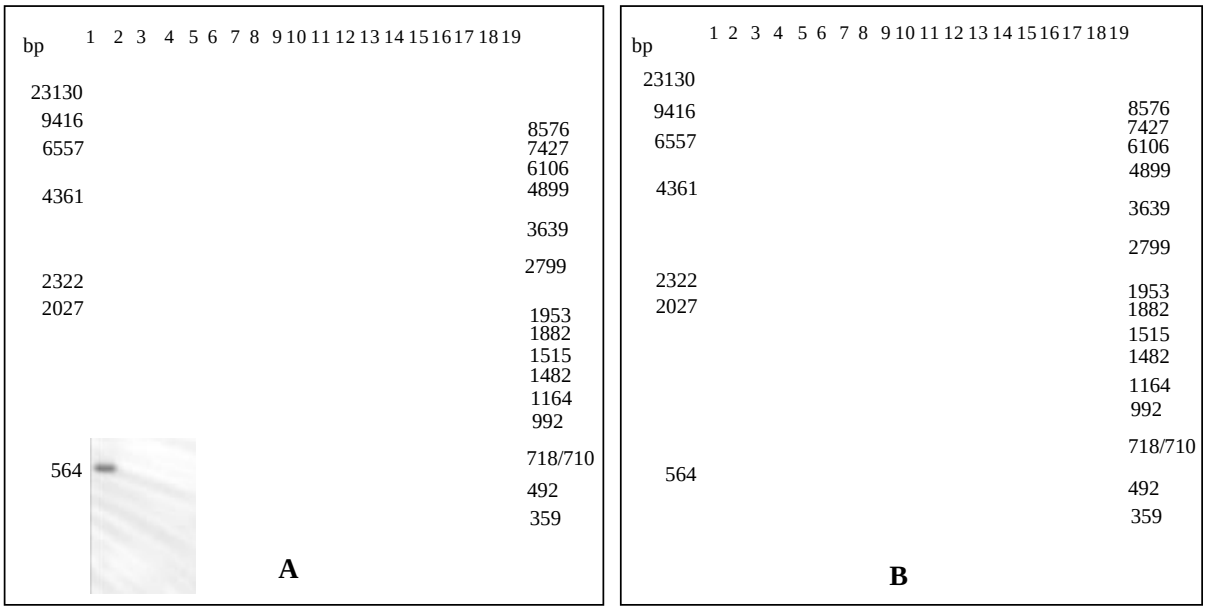


Figure 25. Southern blot analysis of DAS-81419-2 soybean digested with *Not*I/*Sph*I; (A) *pat* probe, (B) AtuORF1 3'UTR probe.

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		

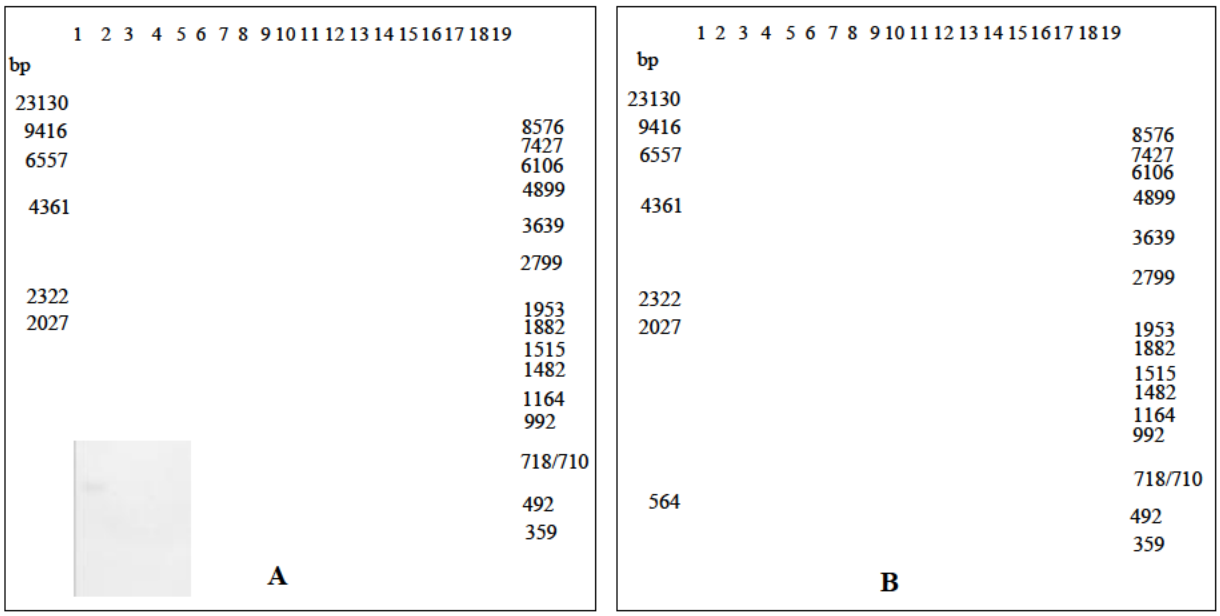


Figure 26. Southern blot analysis of DAS-81419-2 soybean digested with (A) *Sph*I; Ori probe, (B) *Nco*I; SpecR probe.

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		

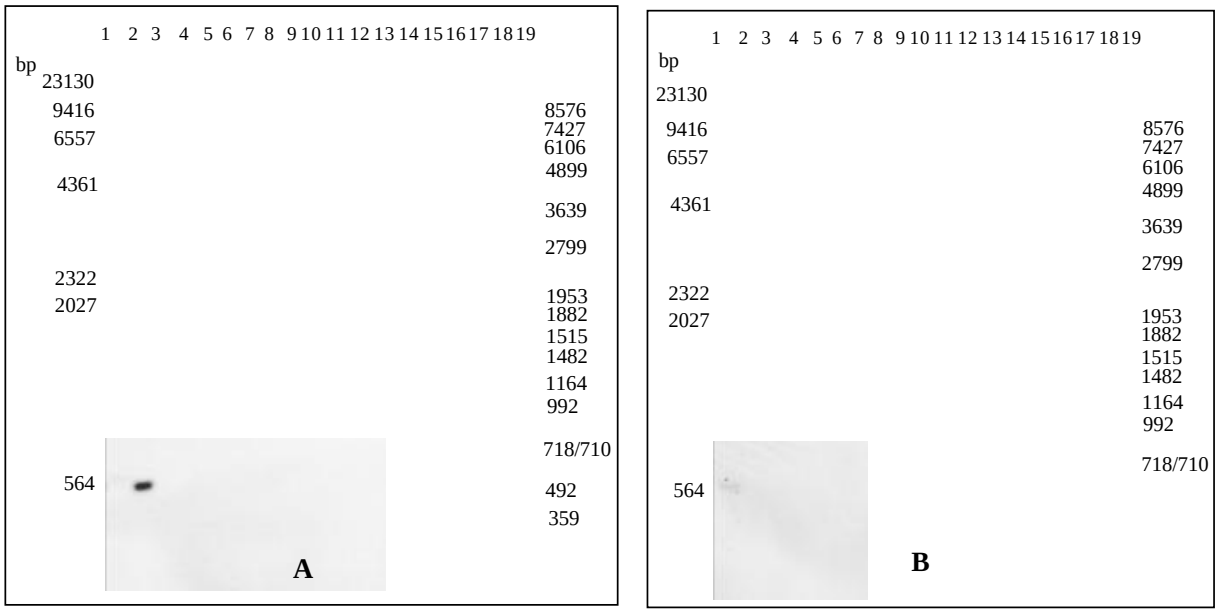


Figure 27. Southern blot analysis of DAS-81419-2 soybean digested with (A) *Nco*I; Backbone 1 probe, (B) *Sph*I; Backbone 2 probe.

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		

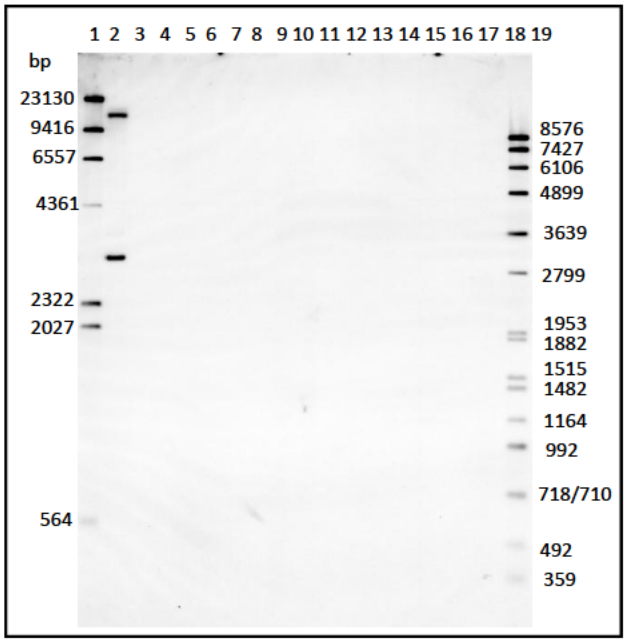


Figure 28. Southern blot analysis of DAS-81419-2 soybean digested with *Nco*I; Backbone 3 and 4 probes.

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		

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. 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 T-DNA insert displayed the expected Mendelian inheritance pattern for a single independent insert/locus in segregating generations (F2 and BC1F2).

(iv) 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 & 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^{o}C overnight for digestion. The digested DNA samples were precipitated with Quick-Precip (Edge BioSystems) and re-suspended to achieve the desired volume for gel loading. The DNA samples and molecular size markers were then electrophoresed through 0.8% agarose gels with 1× TBE buffer at 35-65 V for 18-22 hr to achieve fragment separation. The gels were stained with ethidium bromide and the DNA was visualized under UV light. A photographic record was made for each stained gel.

Southern Transfer

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

Hybridization

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

Detection

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

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

2.4 Analytical Method for Detection

Theoharis, N. (2012). Method Validation for the Determination of Cry1Ac Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA). Indianapolis, IN, Dow AgroSciences LLC. Study ID: 110674

Xu, L. (2012). Method Validation for the Determination of Cry1F Protein in Soybean Tissues Using an Enzyme-Linked Immunosorbent Assay (ELISA). Indianapolis, IN, Dow AgroSciences LLC. Study ID: 110675

2.4 a Determination of Cry1Ac Protein in Soybean Tissue Samples

Soybean tissue samples were analysed 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 (Theoharis 2012).

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 coloured product. Since the Cry1Ac is bound in the antibody sandwich, the level of colour development is proportional to the concentration of Cry1Ac in the sample (i.e., lower protein concentrations result in lower colour 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.

2.4 b Determination of Cry1F Protein in Soybean Tissue Samples

Soybean tissue samples were analysed 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 (Xu 2012).

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 coloured product. Since the Cry1F is bound in the antibody sandwich, the level of colour development is proportional to the concentration of Cry1F in the sample (i.e., lower protein concentrations result in lower colour 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.

2.4 c Determination of PAT Protein in Soybean Tissue Samples

Soybean tissue samples were analysed 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 coloured product. Since the PAT is bound in the antibody sandwich, the level of colour development is proportional to the concentration of PAT in the sample (i.e., lower protein concentrations result in lower colour 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.

3. SAFETY OF THE GENETICALLY MODIFIED FOOD

3.1 Antibiotic Resistance Marker Genes

As described in section 2.3 d of this dossier, Southern blot analyses confirmed that the transgenic insert in DAS-81419-2 soybean does not contain any vector backbone sequence from the plasmid pDAB9582. Therefore the spectinomycin resistance gene in plasmid pDAB9285 (Figure 3) was not inserted into the genome of DAS-81419-2 soybean.

3.1 a Clinical Relevance

Not applicable

3.1 b Therapeutic Efficacy

Not applicable

3.1 c Safety of the Gene Product

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

3.1 d End Use Viability (micro-organisms)

Not applicable

3.2 Characterization of the Novel Proteins

Brooks KJ, Andrus AK. 1999. Cry1F MICROBIAL PROTEIN (FL): ACUTE ORAL TOXICITY STUDY IN CD-1 MICE. Study ID 991178, Dow AgroSciences LLC, Indianapolis, IN

Brooks KJ, Yano BL. 2001. Cry1Ac-(SYNPRO) MICROBIAL PROTEIN: ACUTE ORAL TOXICITY STUDY IN CD-1 MICE. Study ID 011126, Dow AgroSciences LLC, Indianapolis, IN

Embrey S. 2012a. Certificate of Analysis for Full Length Cry1Ac (TSN102591) lyophilized protein standard. BIOT 01-5808, Dow AgroSciences LLC, Indianapolis, IN

Embrey S. 2012b. Certificate of Analysis for Full Length Cry1F (TSN103748) lyophilized protein standard. BIOT 02-7994, Dow AgroSciences LLC, Indianapolis, IN

Embrey SK, Schafer BW. 2009. Certificate of analysis of the test/reference/control substance: Phosphinothricin Acetyltransferase (PAT - TSN031116-0001). BIOT09-203839, Dow AgroSciences LLC, Indianapolis, IN

Gao Y, Gilbert JR, Ni W, Xu X. 2002a. Characterization of Cry1Ac(synpro) Delta-Endotoxin Derived from Recombinant Pseudomonas Fluorescens. Study ID GH-C 5508, Dow AgroSciences LLC, Indianapolis, IN

Gao Y, Ni W, Xu X. 2002b. Purification and Characterization of Cry1Ac Delta-Endotoxin from Transgenic Cotton Event 3006-210-23. GH-C 5548, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012a. Bioinformatics Evaluation of the Putative Reading Frames across the Whole T-DNA Insert and Junctions in DAS-81419-2 Soybean for Potential Protein Allergenicity and ToxicityStudy ID: 120934, Dow AgroSciences, LLC, Indianapolis, IN

Maldonado PM. 2012. Protein Expression of a Transformed Soybean Cultivar Containing Cry1Ac, Cry1F, and Phosphinothricin Acetyltransferase (PAT) - Event DAS-81419-2. Study ID 110000.02, Dow AgroSciences LLC, Indianapolis, IN

Schafer BW, Juba AN. 2012. Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-81419-2Study ID 120046, Dow AgroSciences LLC, Indianapolis, IN

Schafer BW, Oman TJ, Clement JM, Juba AN, Embrey SK. 2012a. Characterization of the Full Length Cry1Ac Protein Derived from Transgenic Soybean Event DAS-81419-2. Study ID 110840, Dow AgroSciences LLC, Indianapolis, IN

Schafer BW, Oman TJ, Clement JM, Juba AN, Embrey SK. 2012b. Characterization of the Full Length Cry1F Protein Derived from Transgenic Soybean Event DAS-81419-2. Study ID 110841, Dow AgroSciences LLC, Indianapolis, IN

3.2 a Biochemical function and phenotypic effect of novel protein

(i) Cry1Ac and Cry1F

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 (Coates et al 2005, Gahan et al 2001, Morin et al 2003, 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.

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 611 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
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Figure 29. Amino acid sequence of Cry1Ac protein.

Biochemical Characterisation 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 (Embrey 2012a, Gao et al 2002a, Schafer et al 2012a). 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 section 3.2 a(iii). 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, Gao et al 2002b).

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 603 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).

Biochemical Characterisation 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 (Embrey 2012b, Gao et al 2006, Schafer et al 2012b) . 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 3.2 a(iv). 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).

```
   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
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Figure 30. Amino acid sequence of Cry1F protein.

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 (refer to section 3.2 c *Site of Expression of Novel Substances* in this dossier).

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 (refer to section 3.2 c).

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.

(ii) PAT

Mode of Action of 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)).

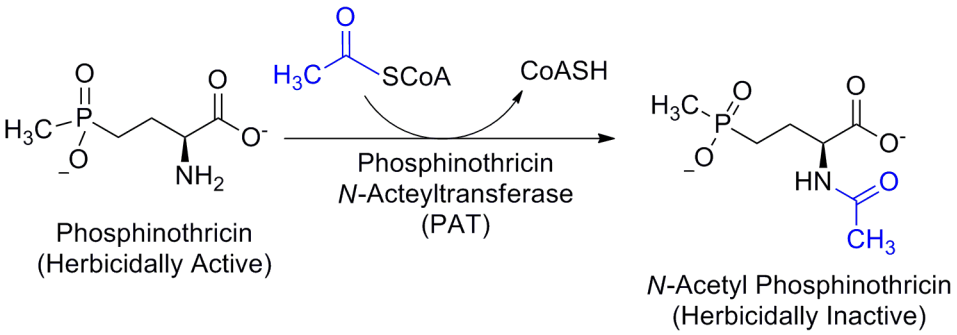

Figure 31. Mode of action of the PAT protein.

Identity of the PAT Protein

The phosphinothricin acetyltransferase (PAT) protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium (OECD 1999, Strauch et al 1988). 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.

Biochemical Characterisation 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 (Schafer & Juba 2012). 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 approved transgenic crops (FSANZ 2004, FSANZ 2005).

Summary of PAT Characterisation

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.

(iii) 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 (Embrey 2012a). 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
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 by (Gao et al 2002a). The report which contains the methods and results can be found in(Schafer et al 2012a).

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 33). This result was also confirmed by the western blot analysis using polyclonal antibodies specific to the Cry1Ac protein (Figure 34).

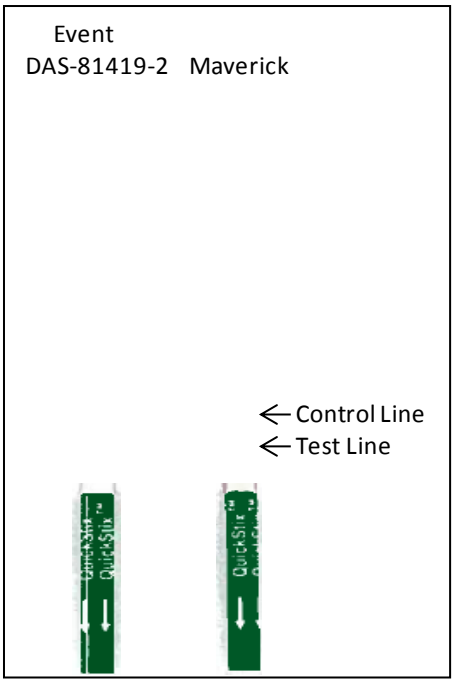

Figure 33. Lateral flow strip assay for the Cry1Ac protein expression in Event DAS-81419-2 and Maverick grain extracts.

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 34). 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 34). 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.

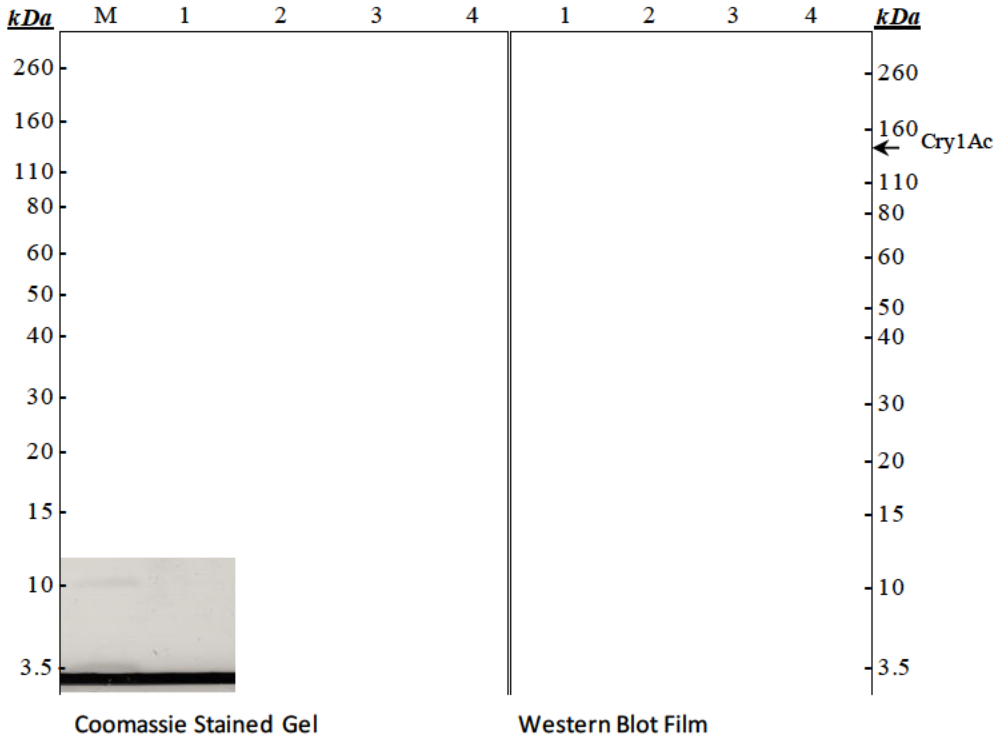

Figure 34. Western blot analysis of microbe- and soybean-derived Cry1Ac proteins.

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 35). 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 35). 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 & Barnes 1983, Williams et al 2006) as well as antibody leaching from the column (Goldberg et al 1991).

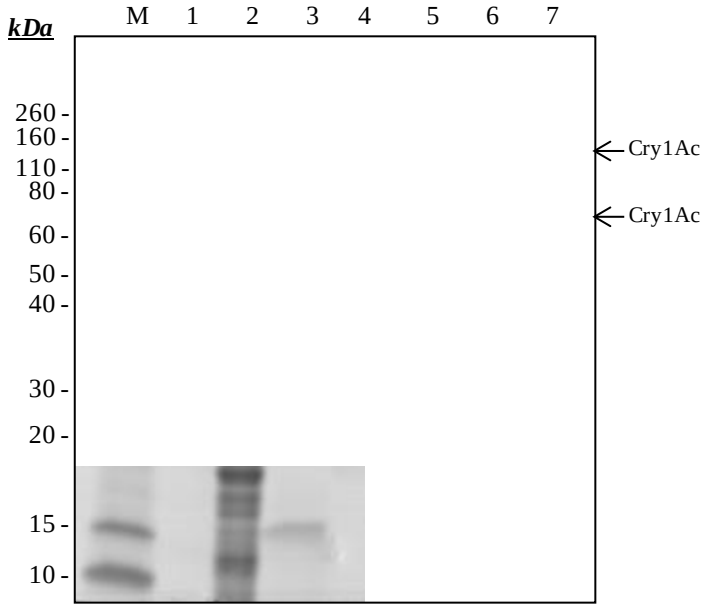

Figure 35. SDS-PAGE analysis of immunoaffinity-purified soybean-derived Cry1Ac.

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 36).

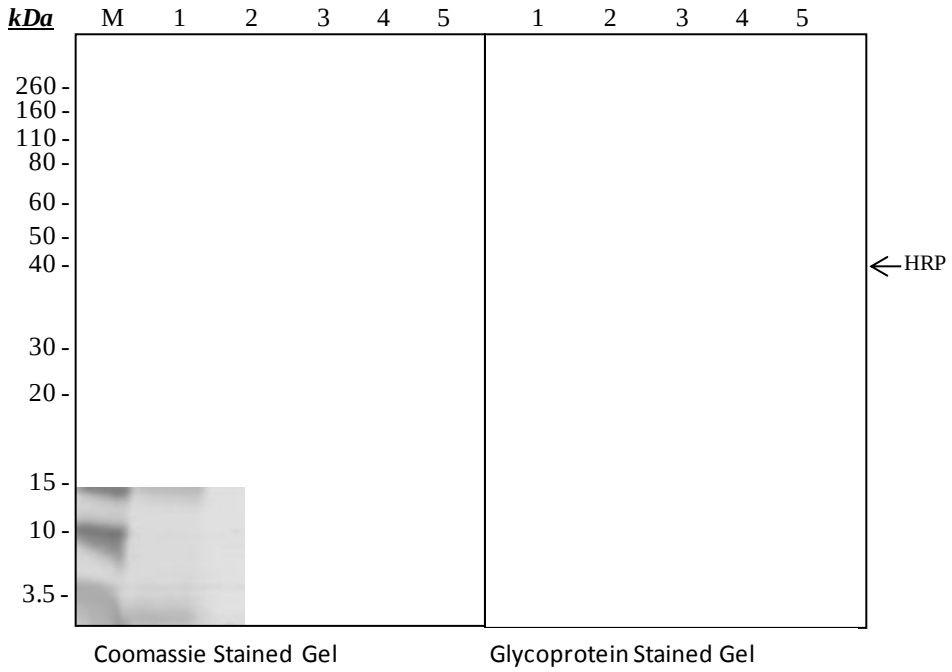

Figure 36. SDS-PAGE analysis of microbe- and soybean-derived Cry1Ac protein stained with GelCode Blue total protein and glycoprotein stains.

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 35) 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 (Schafer et al 2012a). 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 37 and Figure 38 illustrates the theoretical cleavage which was generated *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The theoretical and observed amino acid digest (and molecular weights) of the soybean-derived Cry1Ac protein is also described in (Schafer et al 2012a). The Cry1Ac protein, once denatured, is readily digested by endoproteases to yield numerous peptides that are able to be detected using mass spectrometry.

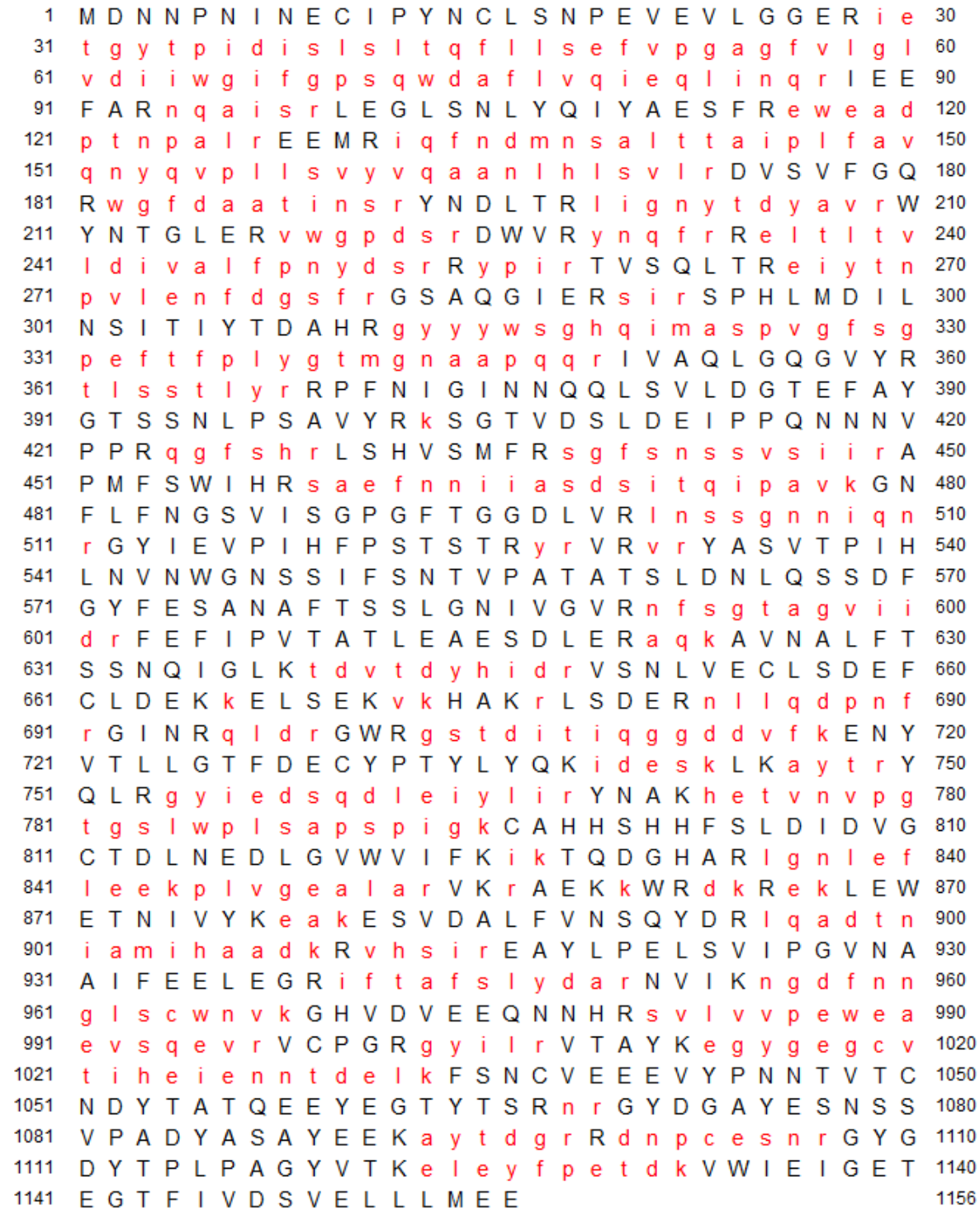

Figure 37. Theoretical cleavage of the Cry1Ac protein with trypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC.

1	M	D	N	N	P	N	I	N	E	C	I	P	Y	n	c	l	s	n	p	e	v	e	v	l	g	g	e	r	i	e	30
31	t	g	y	T	P	I	D	I	S	L	S	L	T	Q	F	l	l	s	e	f	V	P	G	A	G	F	v	l	g	l	60
61	v	d	i	i	w	G	I	F	g	p	s	q	w	D	A	F	l	v	q	i	e	q	l	i	n	q	r	i	e	e	90
91	f	A	R	N	Q	A	I	S	R	L	E	G	L	S	N	L	Y	q	i	y	A	E	S	F	r	e	w	E	A	D	120
121	P	T	N	P	A	L	R	E	E	M	R	I	Q	F	n	d	m	n	s	a	l	t	t	a	i	p	l	f	A	V	150
151	Q	N	Y	q	v	p	l	l	s	v	y	V	Q	A	A	N	L	H	L	S	V	L	R	D	V	S	V	F	g	q	180
181	r	w	G	F	d	a	a	t	i	n	s	r	y	N	D	L	T	R	L	I	G	N	Y	t	d	y	A	V	R	W	210
211	y	N	T	G	L	E	R	V	W	g	p	d	s	r	d	w	V	R	Y	n	q	f	R	R	E	L	T	L	T	V	240
241	L	D	I	V	A	L	F	P	N	Y	d	s	r	r	y	p	i	r	t	v	s	q	l	t	r	e	i	y	T	N	270
271	P	V	L	E	N	F	d	g	s	f	R	G	S	A	Q	G	I	E	R	S	I	R	S	P	H	L	M	D	I	L	300
301	N	S	I	T	I	Y	t	d	a	h	r	g	y	Y	y	W	s	g	h	q	i	m	a	s	p	v	g	f	S	G	330
331	P	E	F	t	f	p	l	y	G	T	M	G	N	A	A	P	Q	Q	R	I	V	A	Q	L	G	Q	G	V	Y	r	360
361	t	l	s	s	t	l	y	R	R	P	F	n	i	g	i	n	n	q	q	l	s	v	l	d	g	t	e	f	A	Y	390
391	g	t	s	s	n	l	p	s	a	v	y	R	K	S	G	T	V	D	S	L	D	E	I	P	P	Q	N	N	N	V	420
421	P	P	R	Q	G	F	s	h	r	l	s	h	v	s	m	f	R	S	G	F	s	n	s	s	v	s	i	i	r	a	450
451	p	m	f	S	W	i	h	r	s	a	e	f	N	N	I	I	A	S	D	S	I	T	Q	I	P	A	V	K	G	N	480
481	F	l	f	N	G	S	V	I	S	G	P	G	F	t	g	g	d	l	v	r	l	n	s	s	g	n	n	i	q	n	510
511	r	g	y	I	E	V	P	I	H	F	P	S	T	S	T	R	Y	r	v	r	v	r	y	A	S	V	T	P	I	H	540
541	L	N	V	N	W	g	n	s	s	i	f	S	N	T	V	P	A	T	A	T	S	L	D	N	L	Q	S	S	D	F	570
571	g	y	F	e	s	a	n	a	f	T	S	S	L	G	N	I	V	G	V	R	N	F	s	g	t	a	g	v	i	i	600
601	d	r	f	E	F	i	p	v	t	a	t	l	e	a	e	s	d	l	e	r	a	q	k	a	v	n	a	l	f	T	630
631	S	S	N	Q	I	G	L	K	T	D	V	T	D	Y	h	i	d	r	v	s	n	l	v	e	c	l	s	d	e	f	660
661	C	L	D	E	K	K	E	L	S	E	K	V	K	H	A	K	R	L	S	D	E	R	N	L	L	Q	D	P	N	F	690
691	r	g	i	n	r	q	l	d	r	g	w	R	G	S	T	D	I	T	I	Q	G	G	D	D	V	F	k	e	n	y	720
721	V	T	L	L	G	T	F	d	e	c	y	p	t	y	L	Y	q	k	i	d	e	s	k	l	k	a	y	T	R	Y	750
751	q	l	r	g	y	I	E	D	S	Q	D	L	E	I	Y	l	i	r	y	N	A	K	H	E	T	V	N	V	P	G	780
781	T	G	S	L	W	P	L	S	A	P	S	P	I	G	K	C	A	H	H	S	H	H	F	s	l	d	i	d	v	g	810
811	c	t	d	l	n	e	d	l	g	v	w	V	I	F	k	i	k	t	q	d	g	h	a	r	l	g	n	l	e	f	840
841	L	E	E	K	P	L	V	G	E	A	L	A	R	V	K	R	A	E	K	K	W	r	d	k	r	e	k	l	e	w	870
871	E	T	N	I	V	Y	k	e	a	k	e	s	v	d	a	l	f	V	N	S	Q	Y	d	r	l	q	a	d	t	n	900
901	i	a	m	i	h	a	a	d	k	r	v	h	s	i	r	e	a	y	L	P	E	L	S	V	I	P	G	V	N	A	930
931	A	I	F	e	e	l	e	g	r	i	f	T	A	F	s	l	y	D	A	R	N	V	I	K	N	G	D	F	n	n	960
961	g	l	s	c	w	N	V	K	G	H	V	D	V	E	E	Q	N	N	H	R	S	V	L	V	V	P	E	W	e	a	990
991	e	v	s	q	e	v	r	v	c	p	g	r	g	y	I	L	R	V	T	A	Y	k	e	g	y	G	E	G	C	V	1020
1021	T	I	H	E	I	E	N	N	T	D	E	L	K	F	s	n	c	v	e	e	e	v	y	p	n	n	t	v	t	c	1050
1051	n	d	y	T	A	T	Q	E	E	Y	e	g	t	y	T	S	R	N	R	G	Y	d	g	a	y	E	S	N	S	S	1080
1081	V	P	A	D	Y	a	s	a	y	E	E	K	A	Y	t	d	g	r	r	d	n	p	c	e	s	n	r	g	y	G	1110
1111	D	Y	t	p	l	p	a	g	y	V	T	K	E	L	E	Y	f	p	e	t	d	k	v	w	I	E	I	G	E	T	1140
1141	E	G	T	F	i	v	d	s	v	e	l	l	l	m	e	e															1156

Figure 38. 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 39 - Figure 41). This analysis confirmed the soybean-derived protein amino acid sequence matched that of the microbe-derived Cry1Ac (Gao et al 2002a) 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 (Embrey 2012a, Gao et al 2002a).

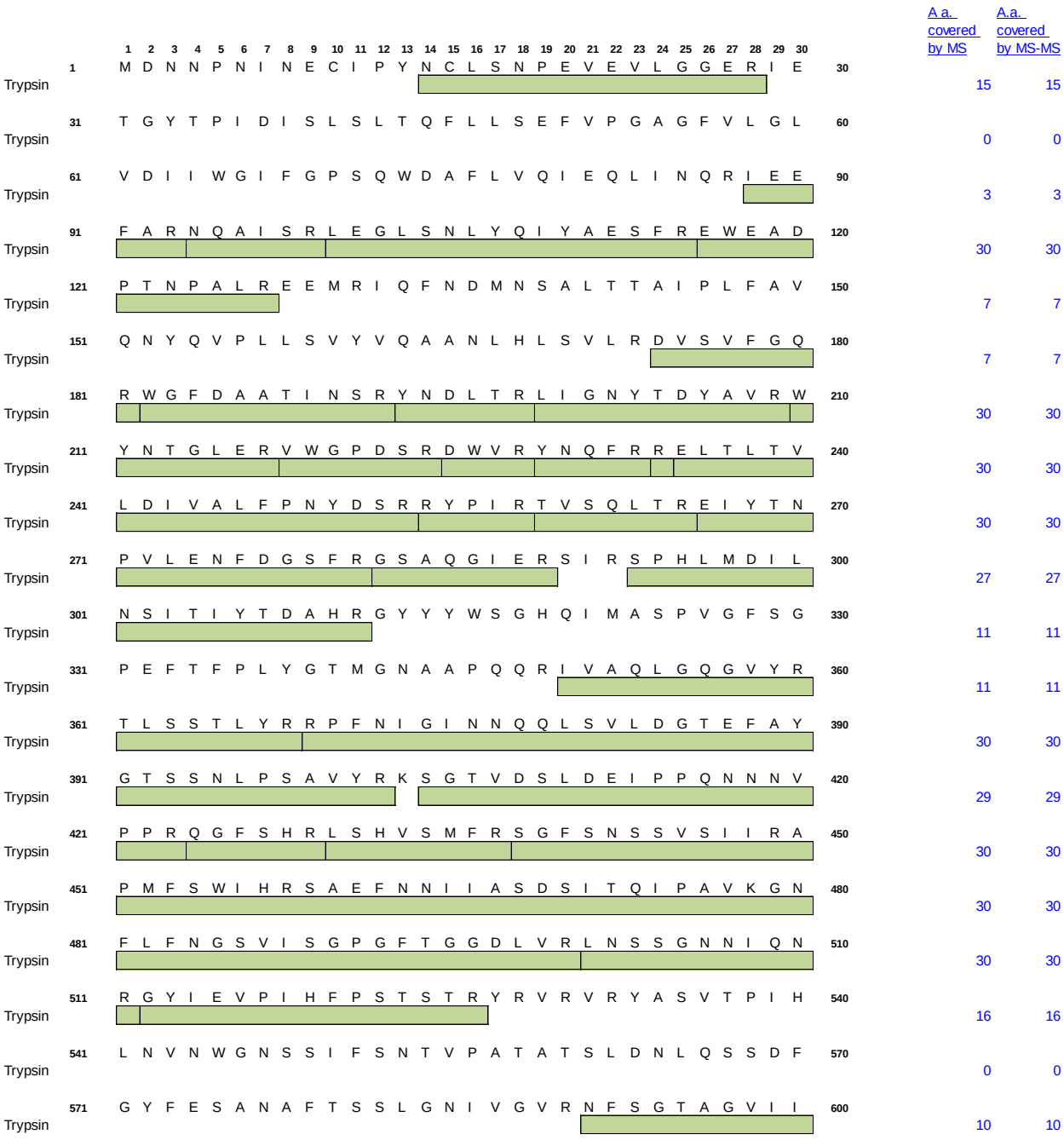

Figure 39. Tryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 67.9% with PMF data and 67.9% by tandem MS.

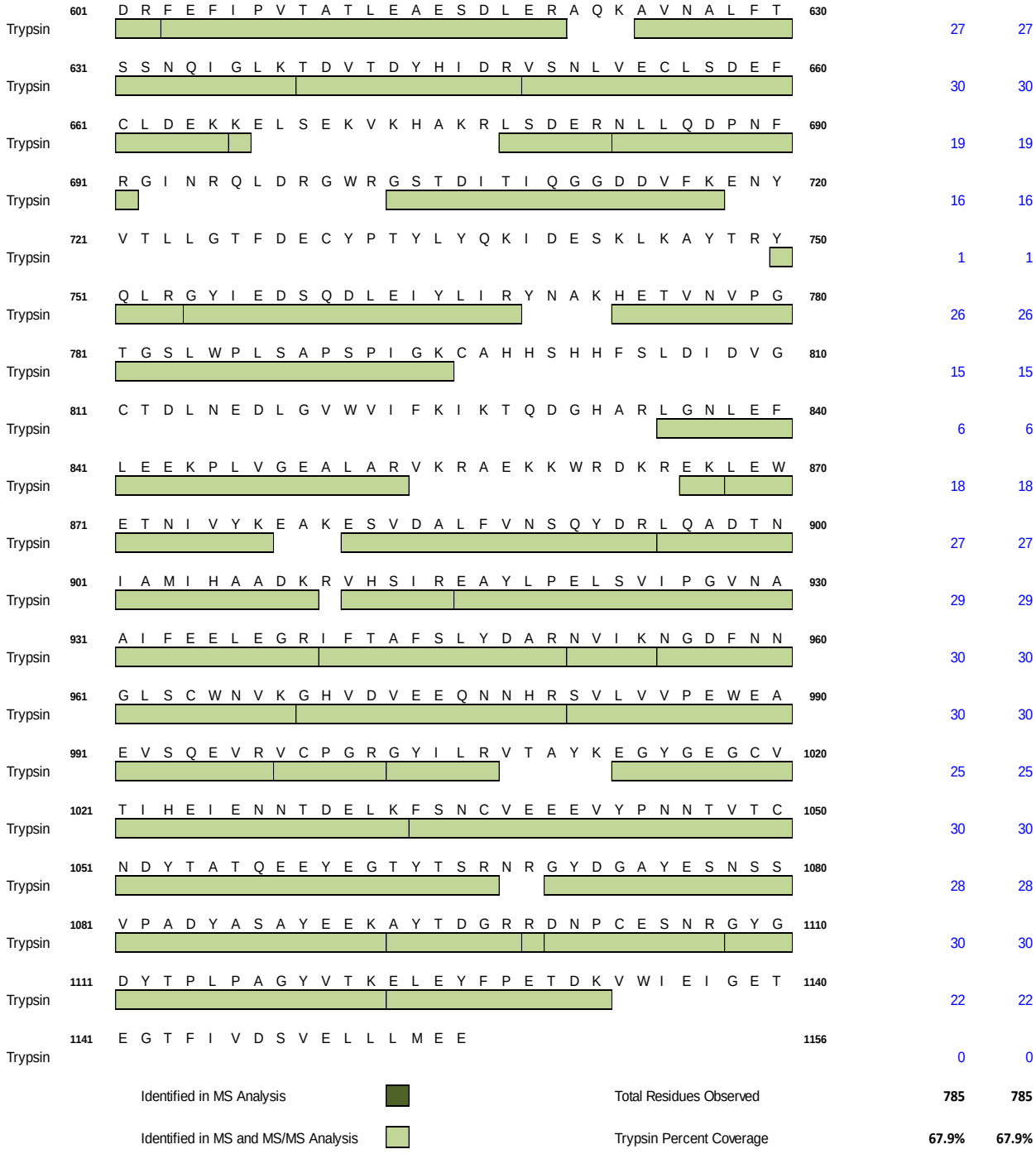

Figure 39 (Cont). Tryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

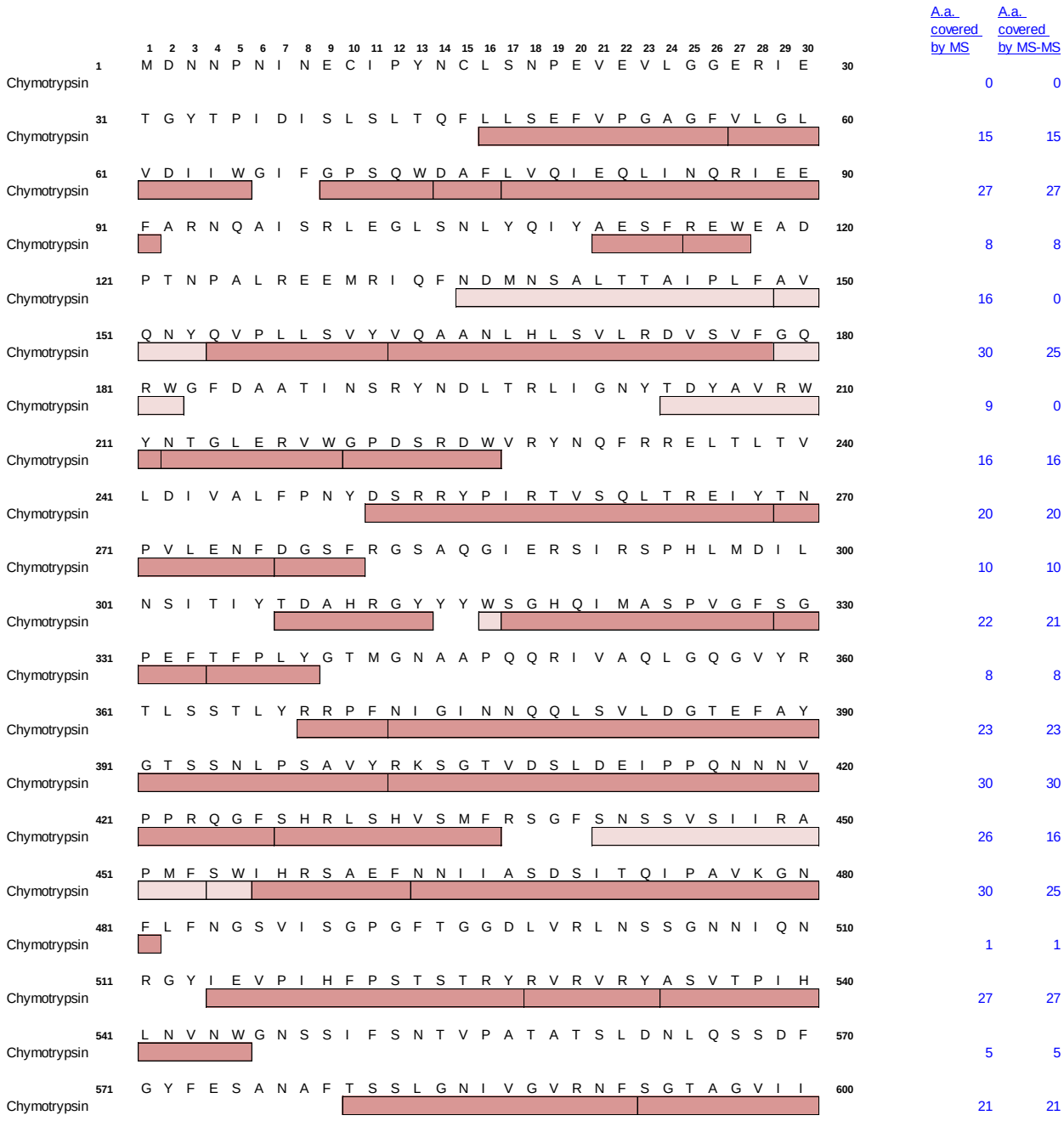

Figure 40. Chymotryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 53.5% with PMF data and 49.0% by tandem MS.

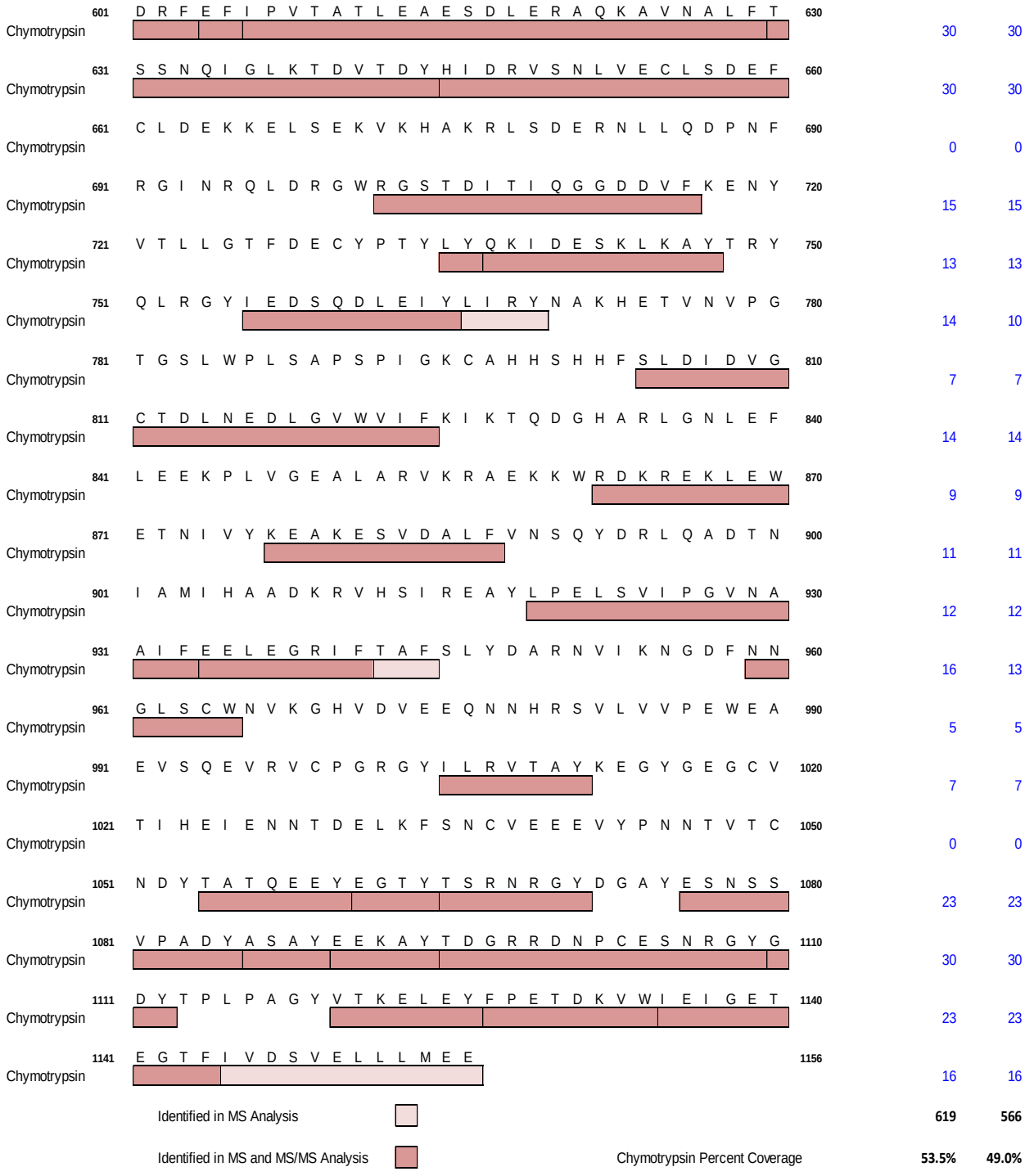

Figure 40 (Cont). Chymotryptic digest sequence coverage map for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

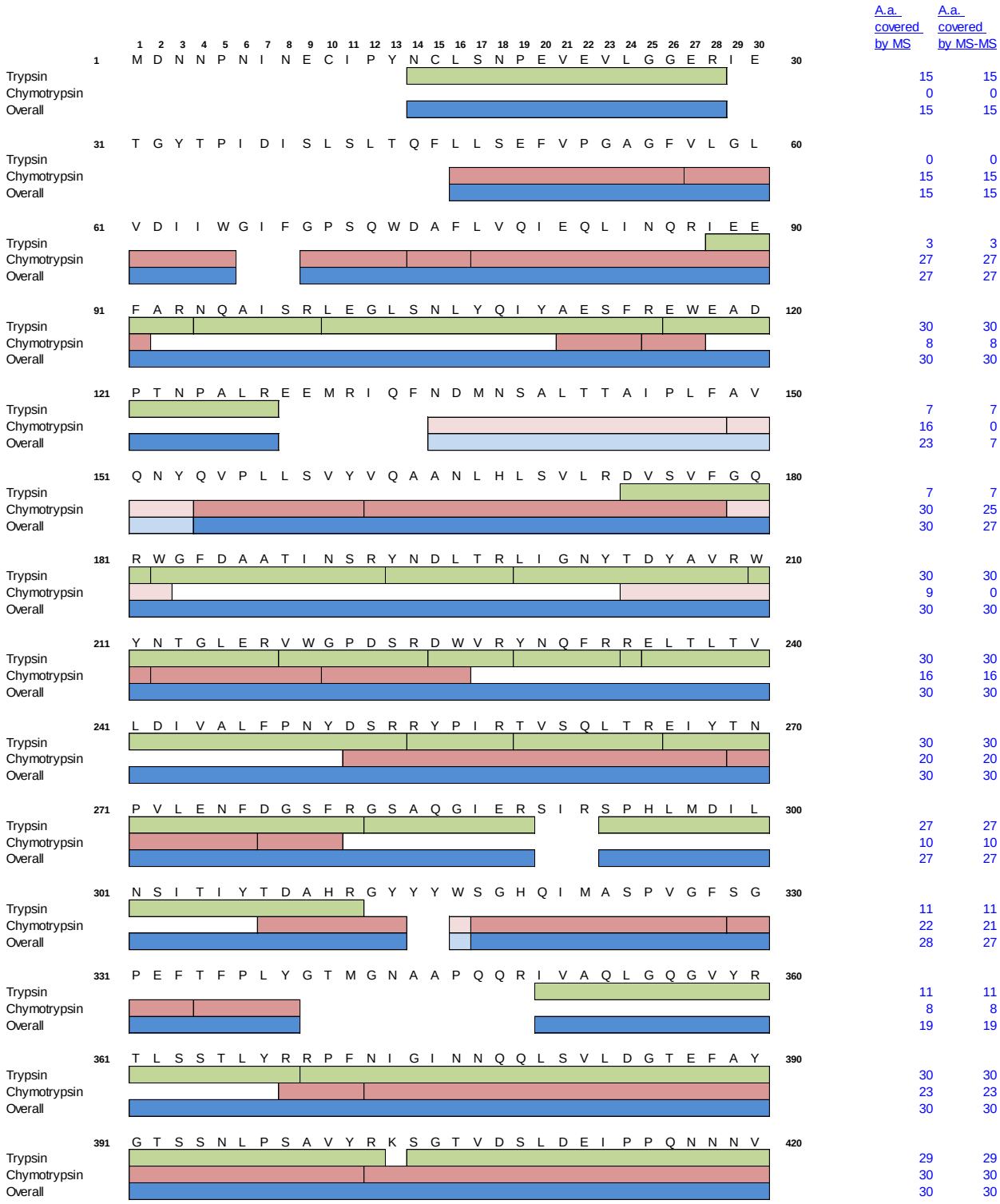

Figure 41. Overall sequence coverage of trypsin and chymotrypsin digests for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Overall sequence coverage was 86.1% with PMF data and 83.2% by tandem MS.

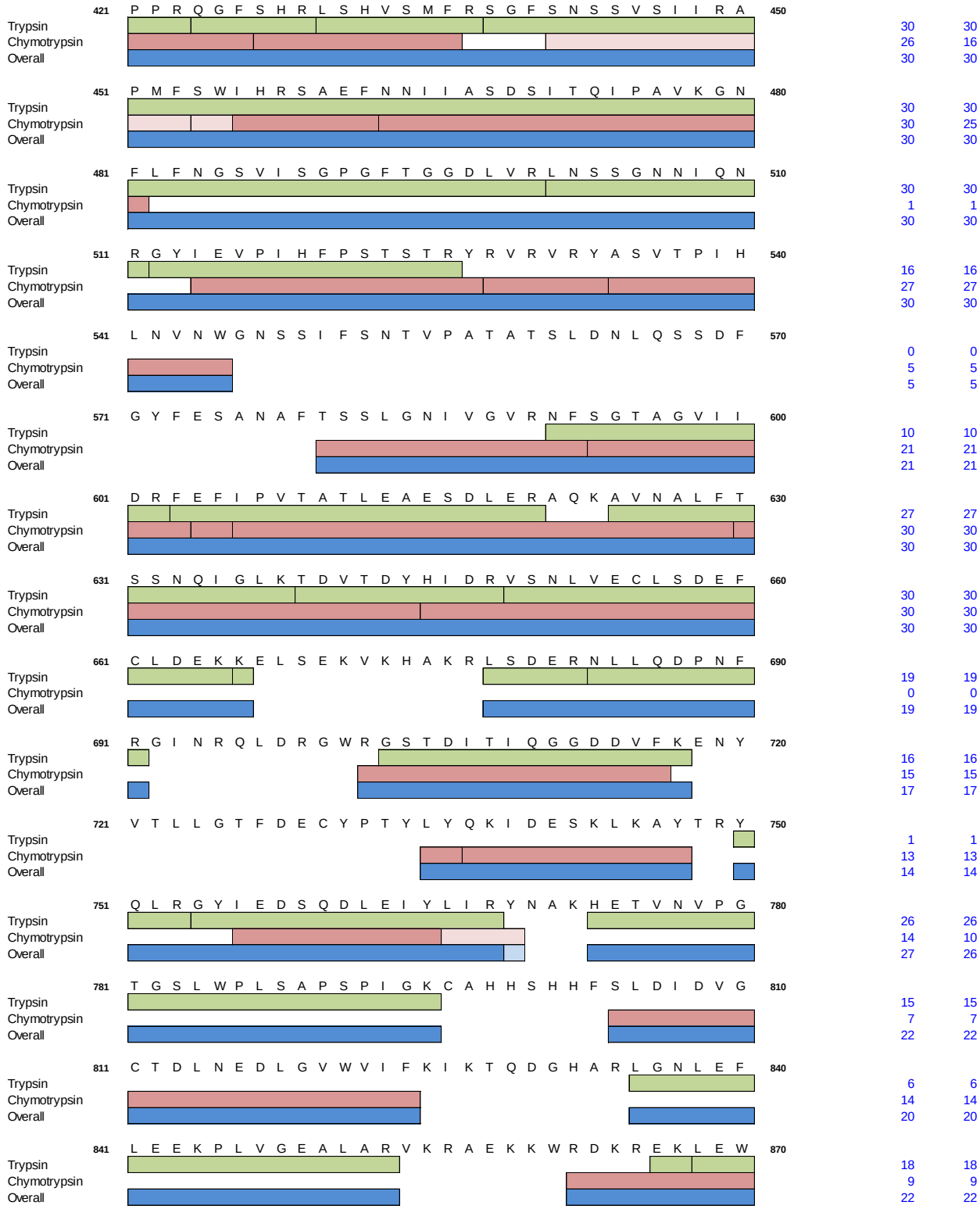

Figure 41 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

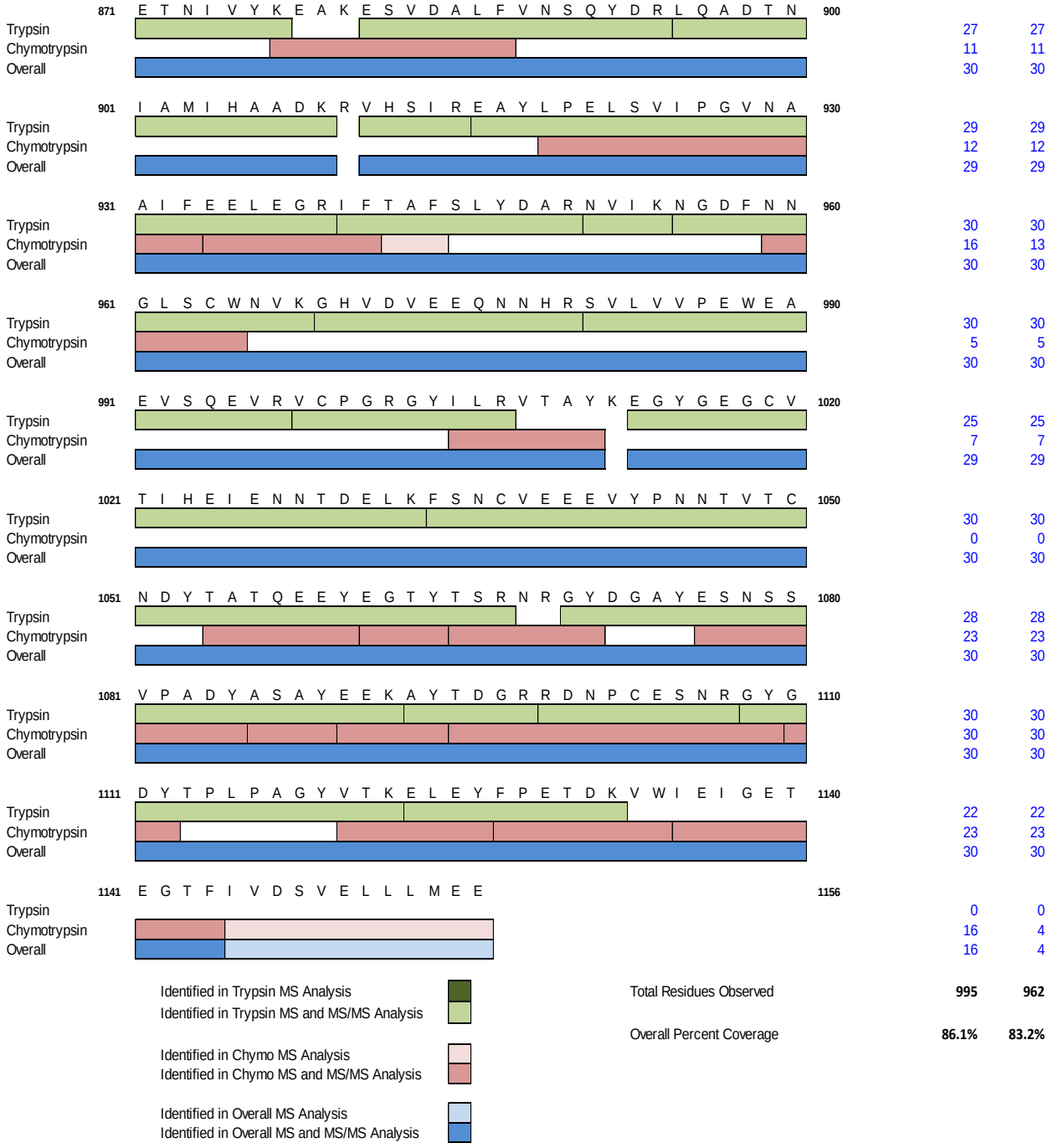

Figure 41 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1Ac (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

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.

(iv) 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 (Embrey 2012b). 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 IN.

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 42). This result was also confirmed by the western blot analysis using polyclonal antibodies specific to the Cry1F protein (Figure 43).

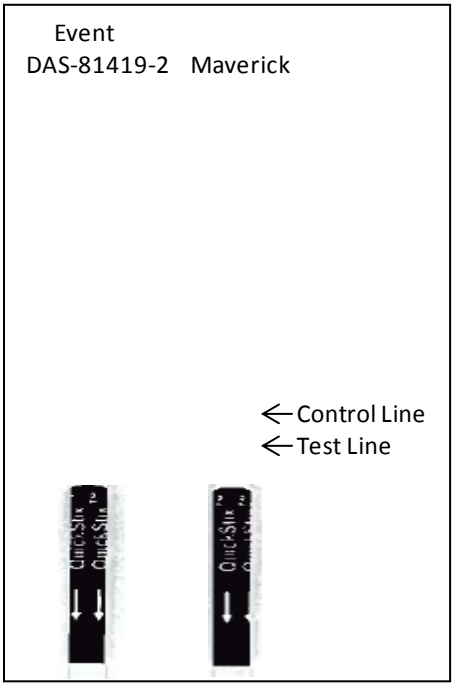

Figure 42. Lateral flow strip assay for the Cry1F protein expression in Event DAS-81419-2 and Maverick grain extracts.

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 43). 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 43). 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.

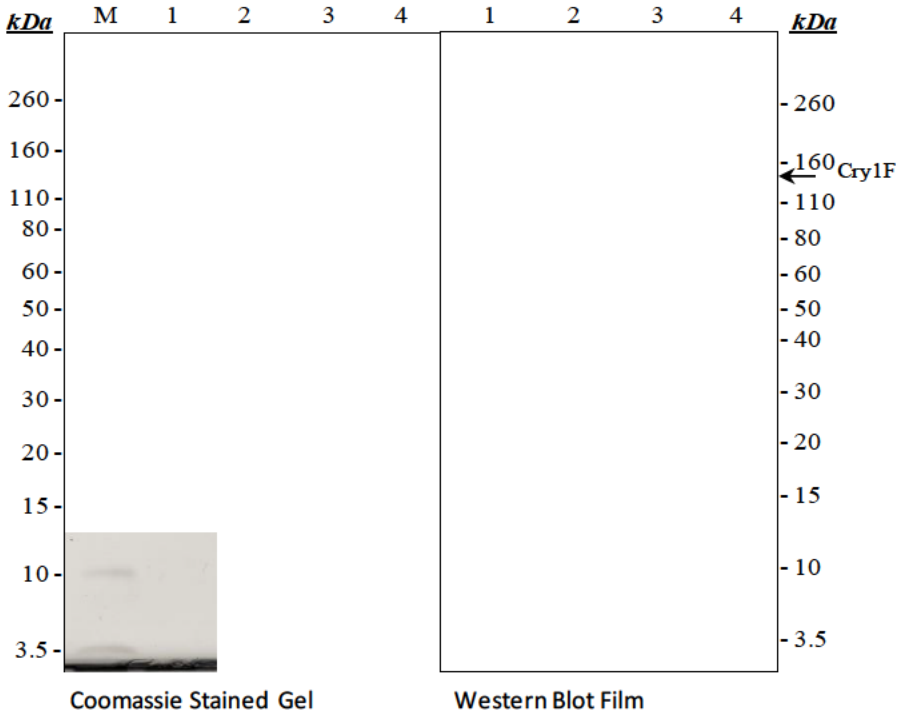

Figure 43. Western blot analysis of microbe- and soybean-derived Cry1F proteins.

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 44). 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 44). 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 & Barnes 1983, Williams et al 2006) as well as antibody leaching from the column (Goldberg et al 1991).

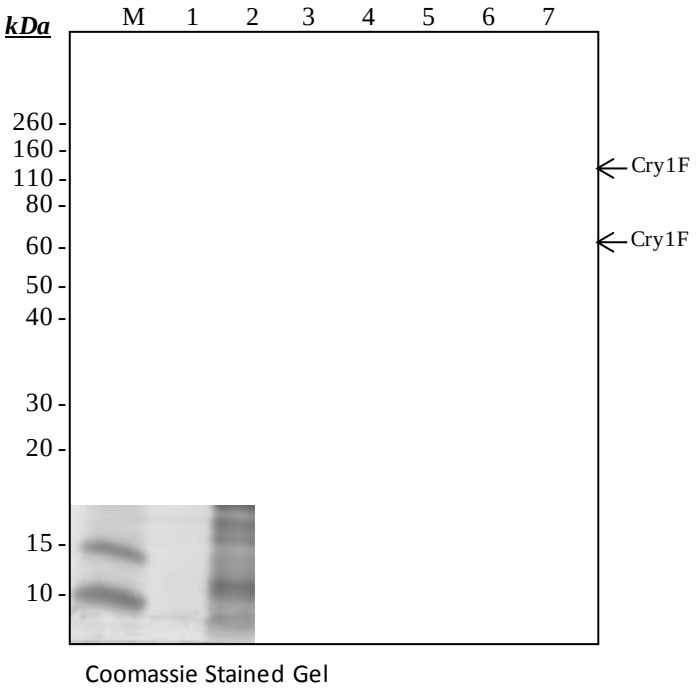

Figure 44. SDS-PAGE analysis of immunoaffinity-purified soybean-derived Cry1F.

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 45).

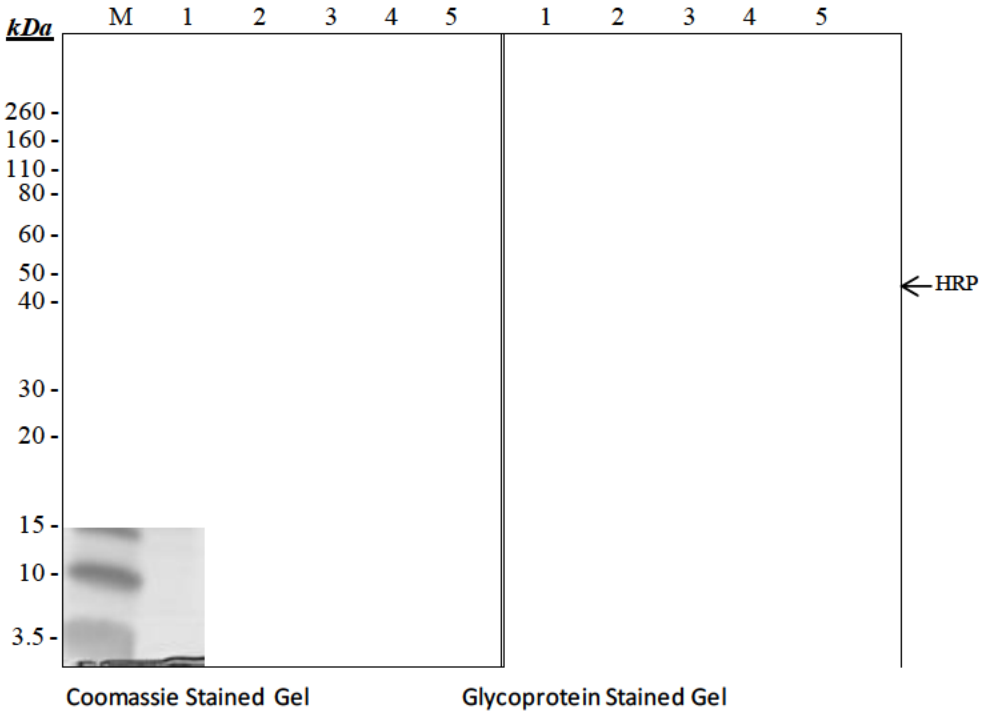

Figure 45. SDS-PAGE analysis of microbe- and soybean-derived Cry1F protein stained with GelCode Blue total protein and glycoprotein stains.

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 44) 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 (Schafer et al 2012b). 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 46 and Figure 47 illustrate the theoretical cleavage of the Cry1F protein which was generated *in silico* using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The theoretical and observed amino acid digest (and molecular weights) of the soybean-derived Cry1F protein is also described in (Schafer et al 2012b). The Cry1F protein, once denatured, is readily digested by endoproteases to yield numerous peptides that are able to be detected using mass spectrometry.

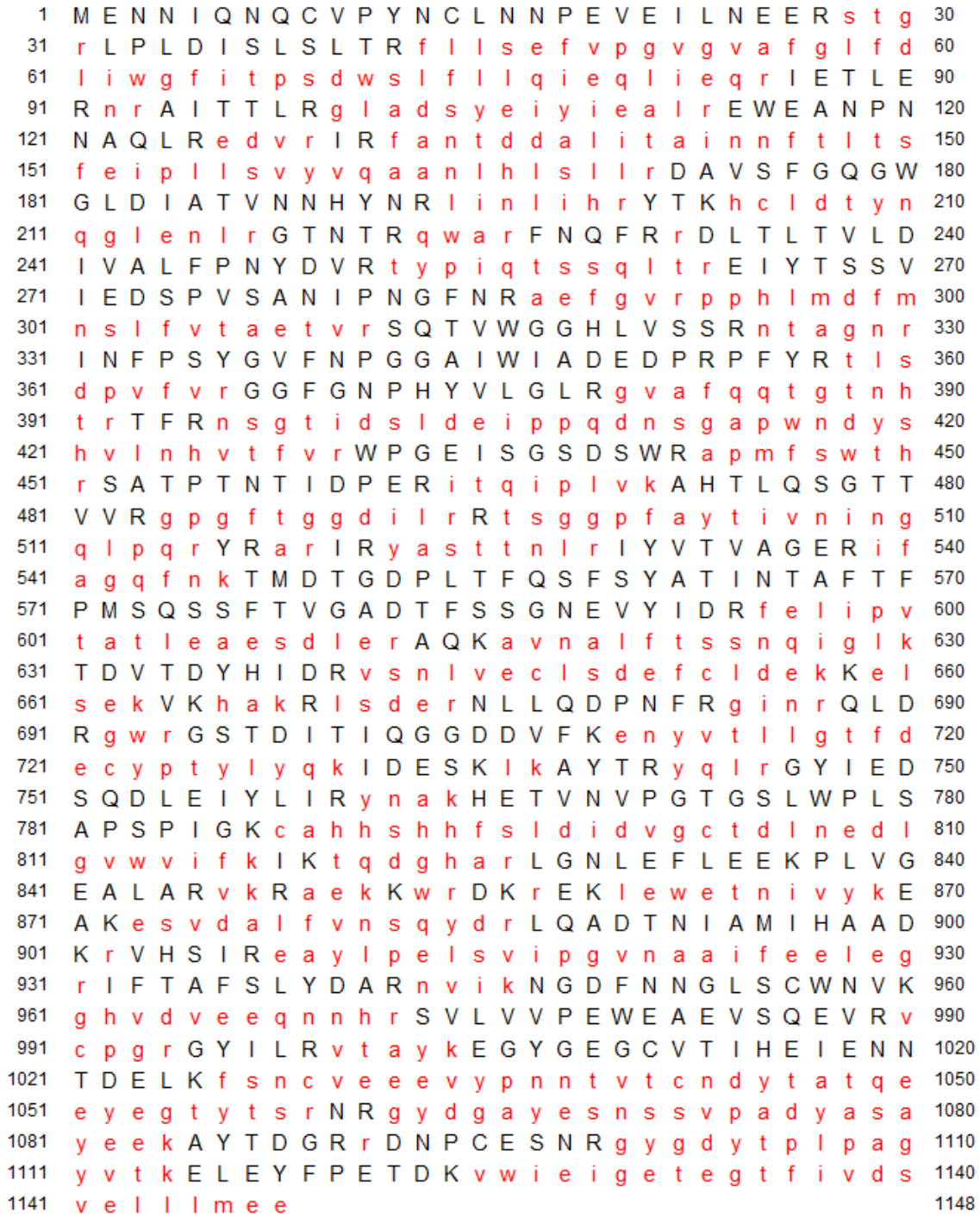

Figure 46. Theoretical cleavage of the Cry1F protein with trypsin generated *in silico* using Protein Analysis Worksheet (PAWS) from Proteometrics LLC.

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 47. 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 48, Figure 49 and Figure 50). 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 (Embrey 2012b, Gao et al 2006).

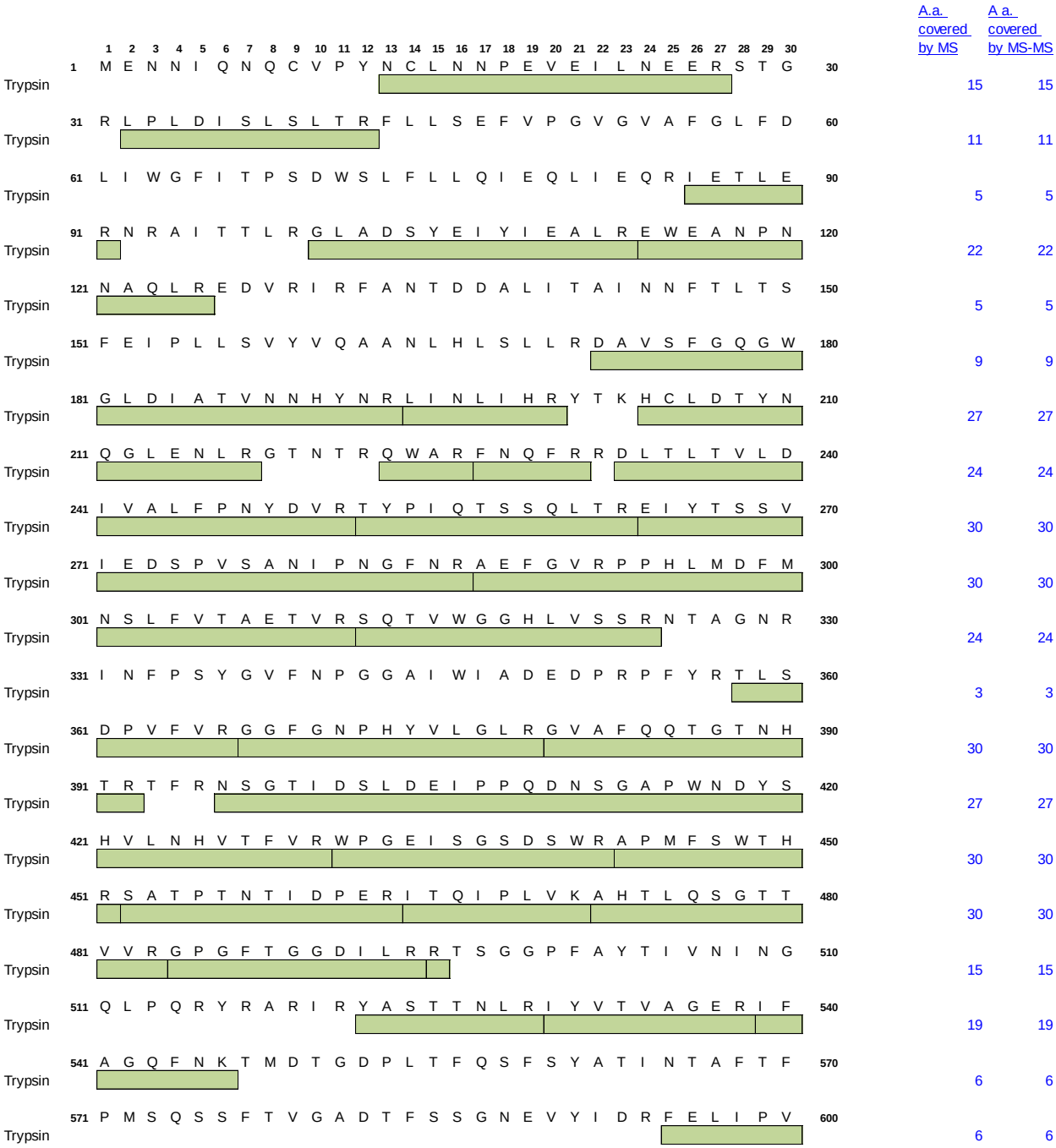

Figure 48. Tryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI-TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 65.1% with PMF data and 65.1% by tandem MS.

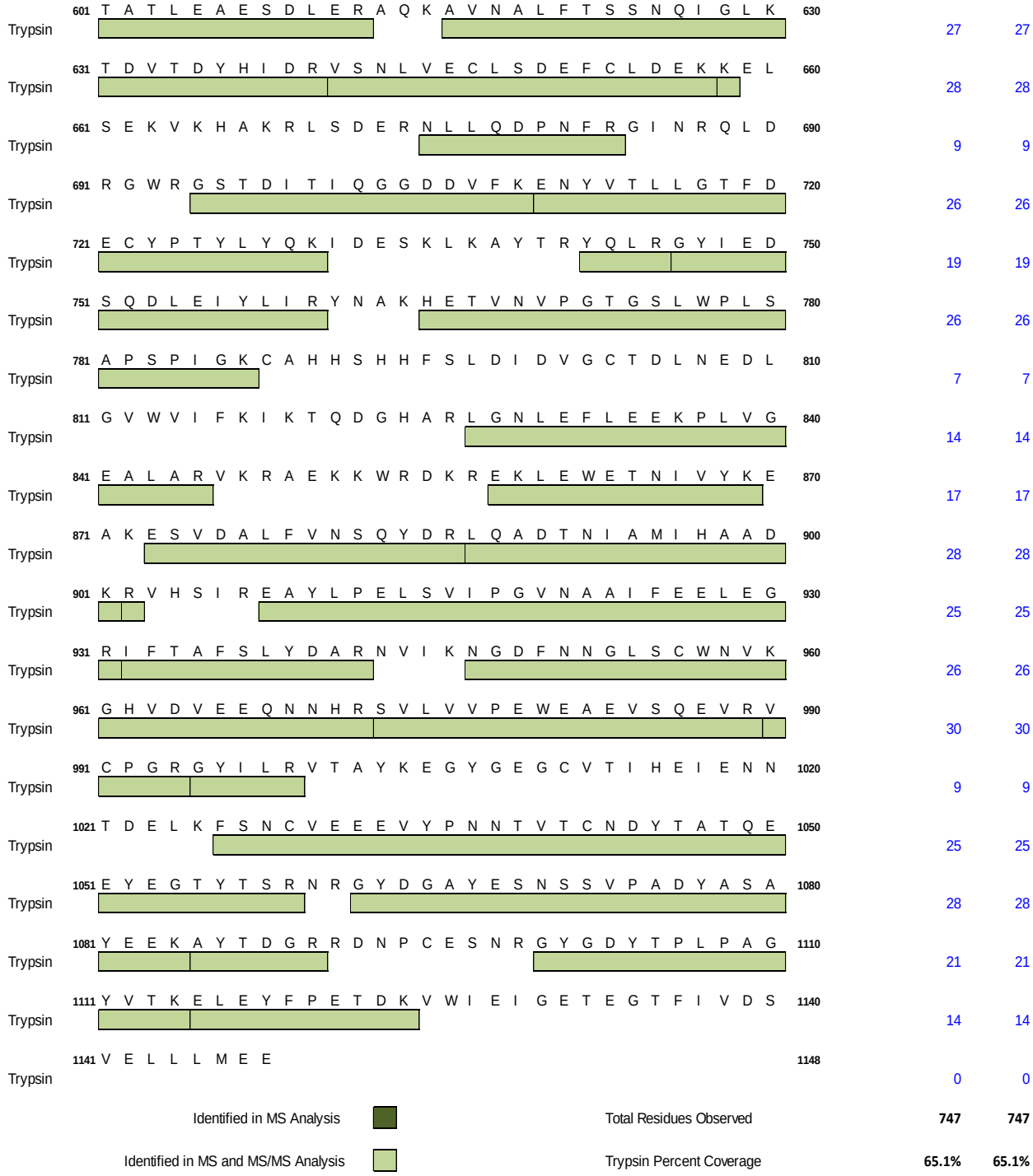

Figure 48 (Cont). Tryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI-TOF/TOF.

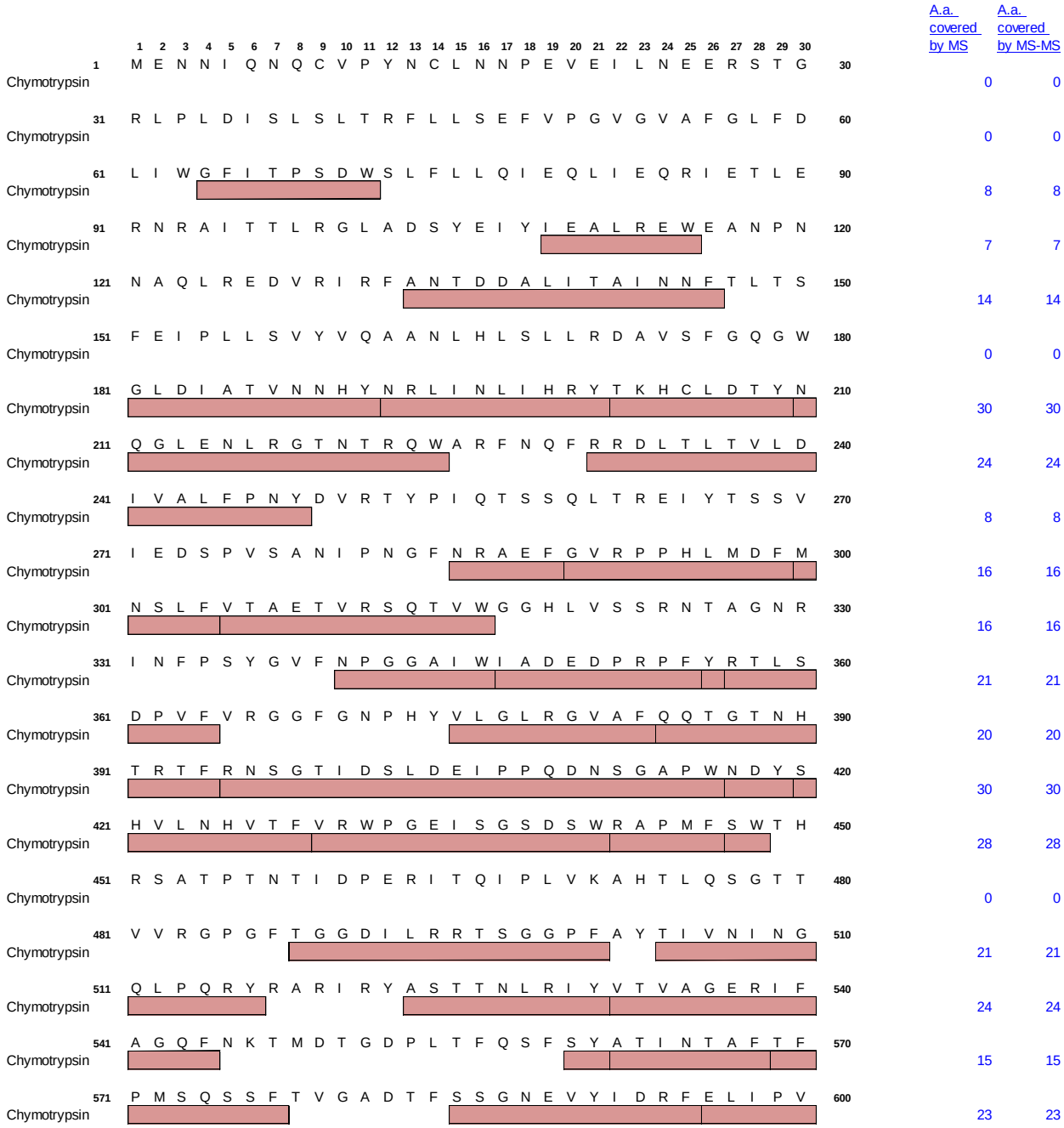

Figure 49. Chymotryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Cys residues were alkylated with iodoacetamide. Sequence coverage was 53.8% with PMF data and 52.4% by tandem MS.

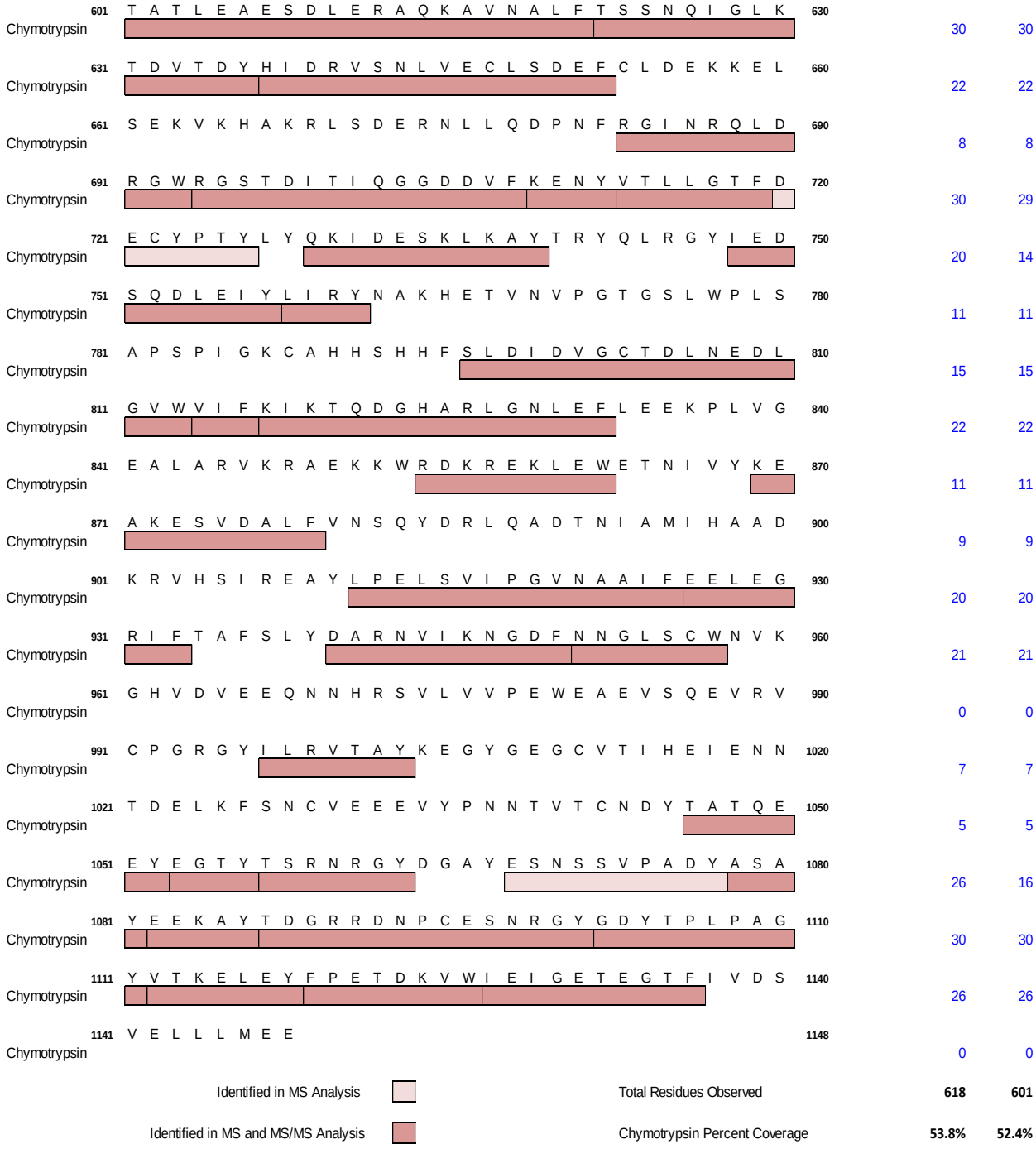

Figure 49 (Cont). Chymotryptic digest sequence coverage map for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

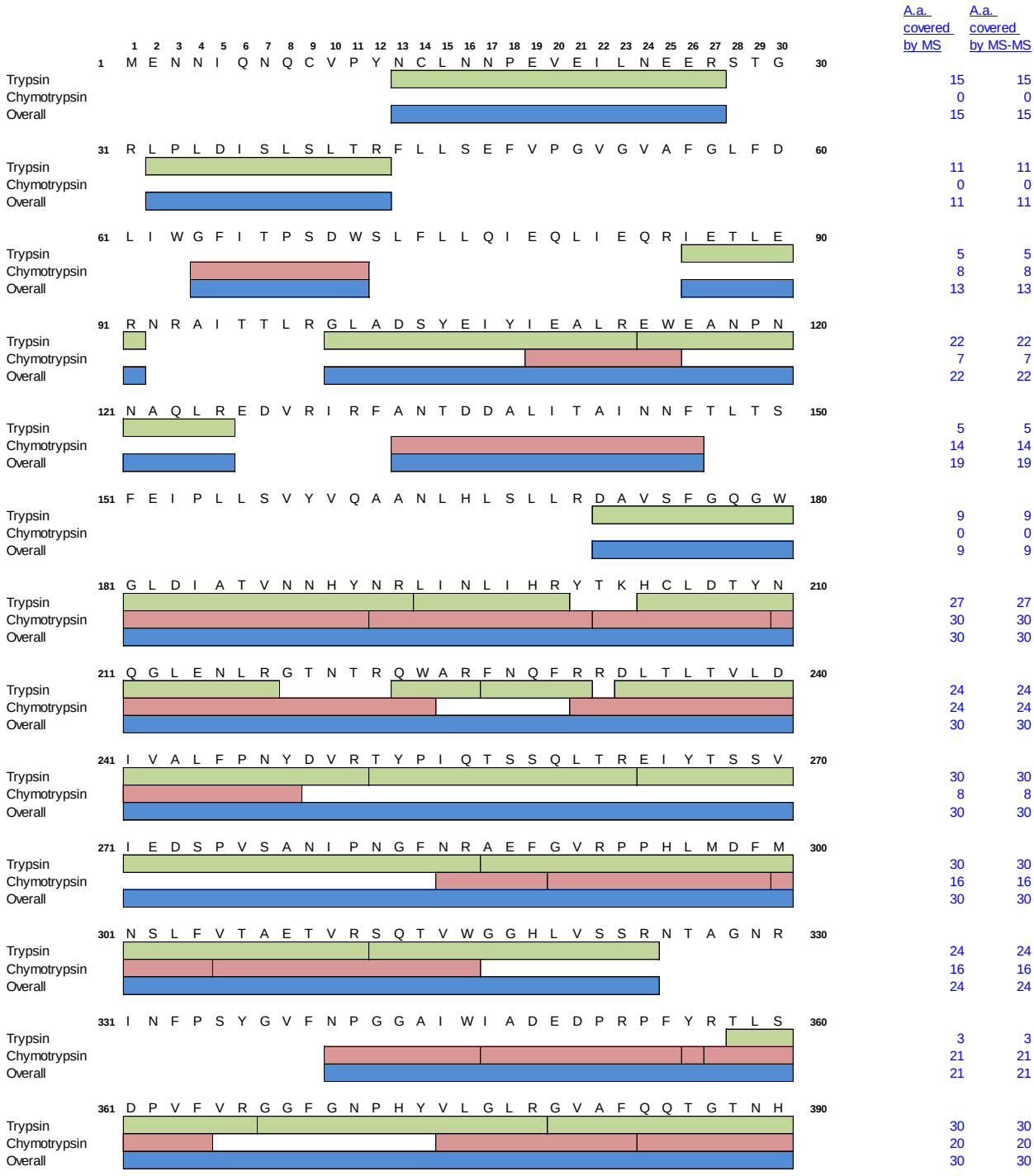

Figure 50. Overall sequence coverage of trypsin and chymotrypsin digests for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF. Overall sequence coverage was 81.7% with PMF data and 81.7% by tandem MS.

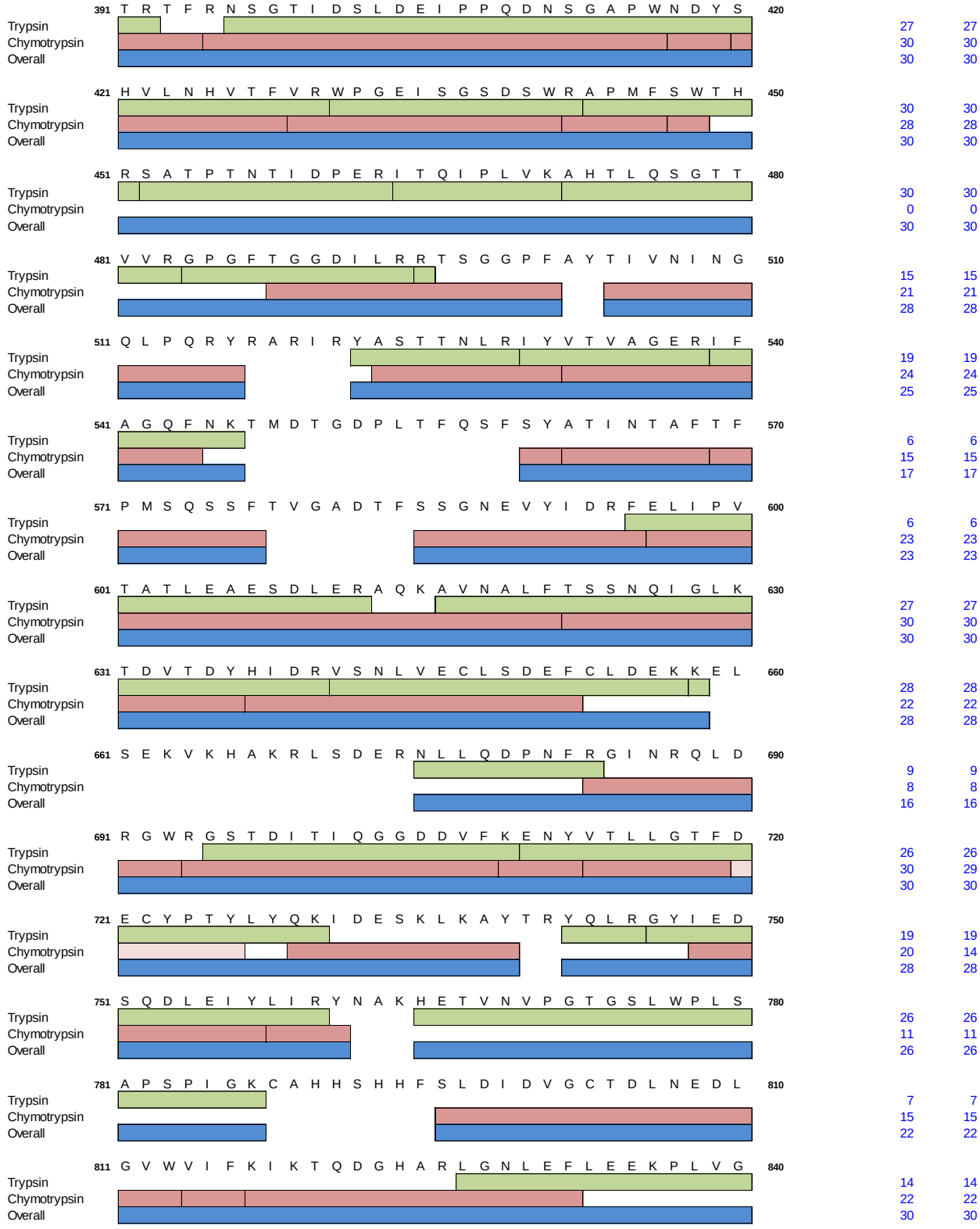

Figure 50 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

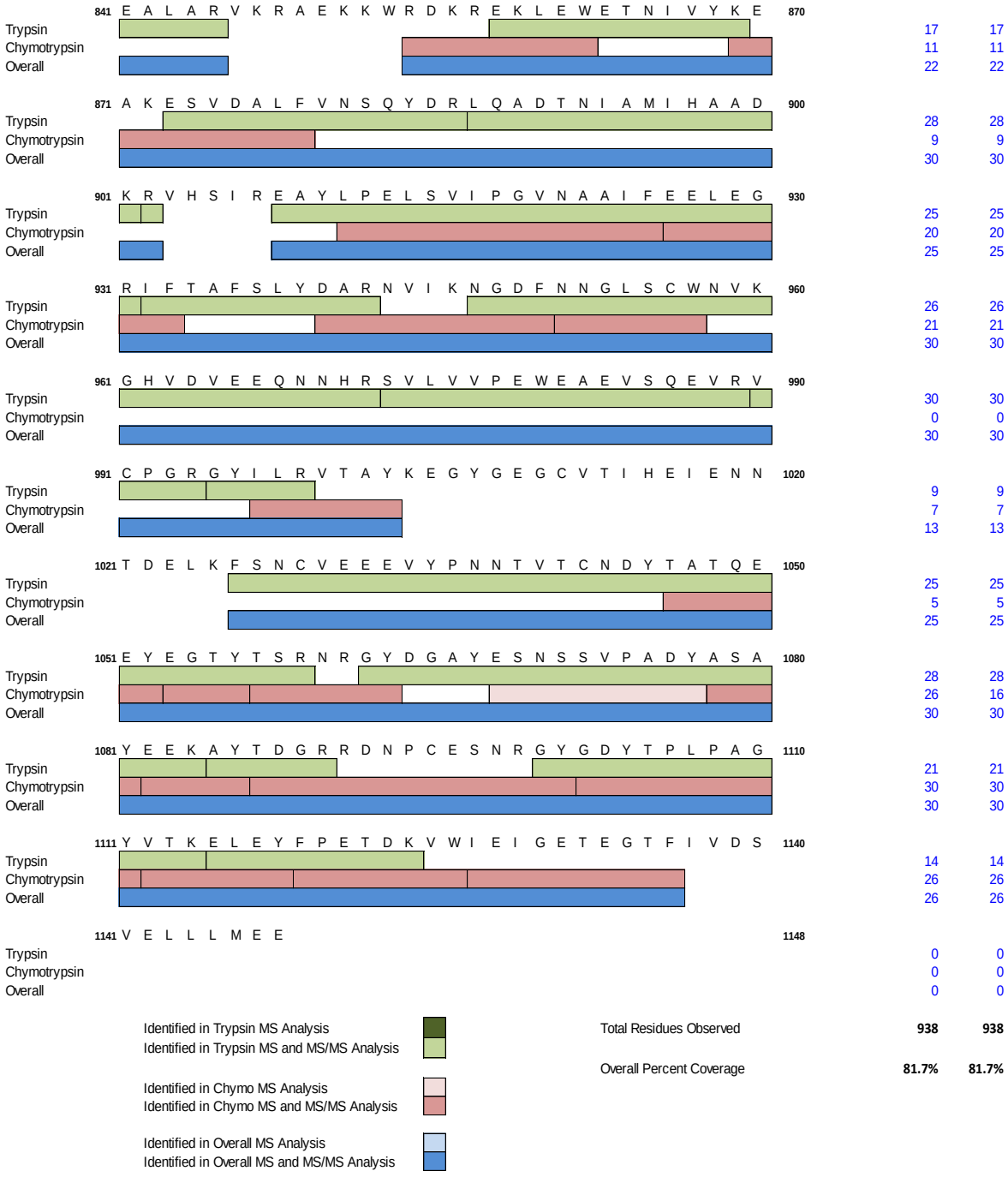

Figure 50 (Cont). Overall sequence coverage of trypsin and chymotrypsin digests for Cry1F (DAS-81419-2) by MALDI-TOF MS and MALDI TOF/TOF.

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.

(v) 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 (Embrey & Schafer 2009). 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 & Snodderley 2007), the *E*. *coli*-derived PAT protein sequence is identical to the proteins expressed in approved transgenic corn, cotton, soybean, canola, event DAS-81419-2, and the native organism *S. viridochromogenes* (AgrEvo 1994, AgrEvo 1996, AgrEvo 1997, Dow AgroSciences 2003a, Dow AgroSciences 2003b, Dow AgroSciences 2003c, Northrup King 1995, Wohlleben et al 1988). 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 7). 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 7. 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 51).

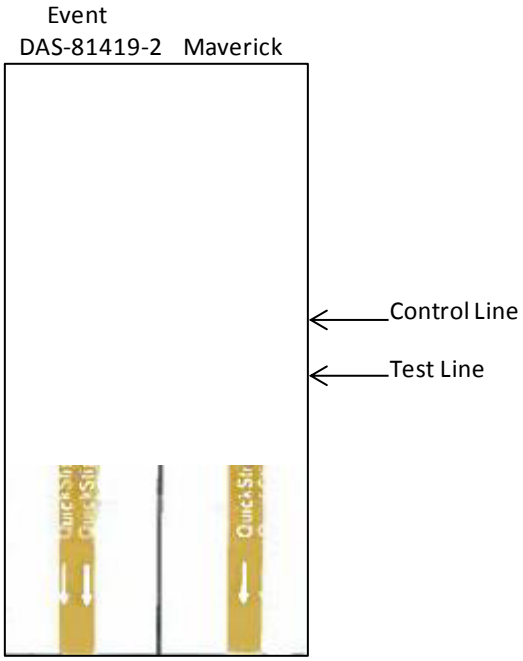

Figure 51. Lateral flow strip assay for PAT protein expression in Event DAS-81419-2 and Maverick leaf extracts.

* 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 52). 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.

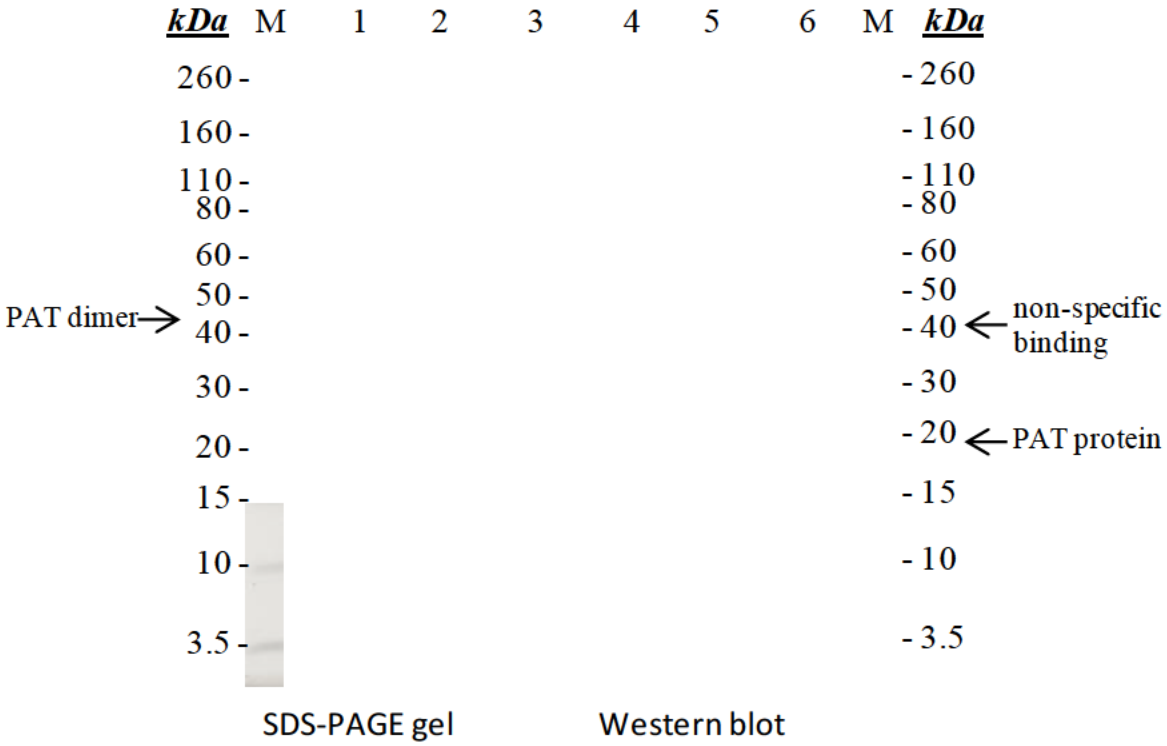

Figure 52. SDS-PAGE and Western blot analysis of microbe-derived PAT, transgenic soybean Event DAS-81419-2, and non-transgenic Maverick extracts.

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.

3.2 b Other Potential Novel Substances

To determine if any putative reading frames were created or any endogenous genes or regulatory elements were disrupted by the integration of the T-DNA insert in DAS-81419-2 soybean, DNA sequences of the insert and its flanking border regions were cloned and analysed (Guttikonda 2012a). In total, 15172 bp of DAS-81419-2 soybean genomic DNA sequence were confirmed, comprising 1297 bp of the 5' flanking border sequence, 1379 bp of the 3' flanking border sequence, and 12496 bp of the T-DNA insert. In addition, 2733 bp of DNA sequences from the parental locus, including the 5' and 3' borders, were confirmed. Sequence comparison of the flanking border with the parental locus indicated that a 57-bp fragment was deleted at the integration site in DAS-81419-2 soybean. According to the currently available sequence information (Schmutz et al 2010), no genes and regulatory elements have been identified in the deleted regions, and there is no evidence to indicate disruption of an endogenous genes or regulatory elements due to the integration of the T-DNA insert in DAS-81419-2 soybean.

Sequence spanning the junctions between the T-DNA insert and its flanking borders was screened for putative reading frames, which were conservatively defined as sequences that begin and end with stop codons and are greater than or equal to eight amino acids in length. A total of nine putative reading frames spanning the junctions between the insert and its flanking borders were identified and were subjected to BLASTp search for sequence similarity with known toxin proteins. The search against the GenBank non-redundant (nr) protein dataset did not detect any significant protein sequence similarity with toxic proteins harmful to humans or animals. For evaluation of potential allergenicity, the putative reading frames were analysed using two search criteria. The first criterion is a search over 80-amino acid stretches (sliding window search) to detect greater than 35% identity between a query protein and known allergens (Codex Alimentarius Commission 2009, Herman et al 2009, Ladics 2008). The minimum size reading frame for this analysis is greater than or equal to 29 amino acids, thus five of the nine putative reading frames spanning the junctions between the insert and its flanking borders were subjected to this analysis.

The second criterion involves evaluating short amino acid stretches for identity between the query protein and known allergens. Each putative reading frame was analysed for any matches of eight contiguous amino acids to the allergens (Silvanovich et al 2006, Stadler & Stadler 2003). Since all nine putative reading frames identified were greater than or equal to 8 amino acids in length, all were subjected to this analysis. Using both search criteria, searches of the putative reading frames against a peer reviewed allergen database (FARRP Allergen Database Version 12, Released in February, 2012) did not generate any significant amino acid sequence similarities with known allergens.

3.2 c Site of Expression of Novel Substances

(i) Expression of the Cry1Ac Protein in Plant Tissues

A field expression study was conducted at ten locations in U.S. during 2011 locations (Maldonado 2012). 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 section 3.2 a(iii).

A summary of the Cry1Ac protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 8. 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 8. 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

(ii) Expression of the Cry1F Protein in Plant Tissues

A field expression study was conducted at ten locations in U.S. during 2011 locations (Maldonado 2012). 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 section 3.2 a(iv).

A summary of the Cry1F protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 9 . 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 9. Expression of Cry1F in DAS-81419-2 soybean.

		Cry1F ng/mg Tissue Dry Weight				
Matrix	Description	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

(iii) Expression of the PAT Protein in Plant Tissues

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 section 3.2 a(v).

A summary of the PAT protein concentrations (averaged across sites) in the various soybean matrices is shown in Table 10. 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 10. Expression of PAT in DAS-81419-2 soybean.

Matrix	Description	PAT ng/mg Tissue Dry Weight				
		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
Root	0.06	0.12
Forage	0.06	0.12
Grain	0.06	0.12

(iv) Materials and Methods for Cry1Ac, Cry1F and PAT Protein Expression Analysis

Experimental Design

This study used the same plots that were used for 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.

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.

3.2 d Post-Translational Modification in the New Host

The methods and results of the biochemical characterization of DAS-81419-2 soybean- and microbe-derived Cry1Ac and Cry1F proteins are described in detail above. Briefly, both the plant and *P. fluorescens*-derived Cry1Ac and Cry1F proteins showed the expected molecular weight of ~130 kDa by SDS-PAGE and were immunoreactive to 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 and Cry1F proteins. 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 (Dow AgroSciences 2003a, Gao et al 2002b).

3.2 e Novel Protein Silencing

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

3.2 f Novel Protein History of Consumption

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 (EPA 2011, Mendelsohn et al 2003, Sanahuja et al 2011). Both proteins are expressed in events comprising Dow AgroSciences' WideStrike® cotton authorized for cultivation in the U.S., Australia and Brazil and for food and feed use in Canada, Australia, Brazil, European Union, Japan, Korea, Mexico, New Zealand and the 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 Canada, 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 USEPA 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.
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 and 600 mg/kg for Cry1Ac and Cry1F, respectively (Brooks & Andrus 1999, Brooks & Yano 2001).

3.3 Potential Toxicity of the Novel Protein

Brooks KJ, Andrus AK. 1999. Cry1F Microbial Protein (Fl): Acute Oral Toxicity Study in CD-1 Mice. Study ID 991178, Dow AgroSciences LLC, Indianapolis, IN

Brooks KJ, Yano BL. 2001. Cry1Ac-(SYNPRO) Microbial Protein: Acute Oral Toxicity Study in CD-1 Mice. Study ID 011126, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012c. Sequence Similarity Assessment of Cry1Ac Protein to Known Allergens by Bioinformatics Analysis (Update, May, 2012). Study ID 120763, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012d. Sequence Similarity Assessment of Cry1Ac Protein to Known Toxins by Bioinformatics Analysis (Update, May, 2012). Study ID 120761, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012e. Sequence Similarity Assessment of PAT Protein to Known Allergens by Bioinformatics Analysis (Update, February, 2012). Study ID 120143, Dow AgroSciences LLC, Indianapolis IN

Guttikonda S. 2012f. Sequence Similarity of PAT Protein to Known Toxins by Bioinformatics Analysis (Update, February, 2012). Study ID 120480, Dow AgroSciences LLC, Indianapolis IN

Song P. 2012. Sequence Similarity Assessment of Cry1F Protein to Known Toxins by Bioinformatics Analysis. Study ID 120762, Dow AgroSciences LLC, Indianapolis, IN

3.3 a Assessment of Toxicity Potential of Cry1Ac and Cry1F

(i) Amino Acid Sequence Comparison to Known Toxins

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 (Guttikonda 2012d, Song 2012).

(ii) Acute toxicity of Cry1Ac and Cry1F

An acute oral toxicity study with the Cry1Ac protein was conducted in mice at a level of 5000 mg Cry1Ac protein/kg-bw (Brooks & Yano 2001) . All animals (5 male and 5 female CD-1 mice) survived and no clinical signs were observed during the study. All animals gained weight by study termination on days 15. There were no treatment related gross pathological observations. The report concludes that under the conditions of this study, the acute oral LD_{50} of Cry1Ac in male and female mice was greater than 5000 mg/kg-bw. No mortality was observed, and there were no observable adverse effects with the Cry1Ac treated animals, therefore the NOEL (No Observed Effect Level) is also considered to be >700mg/ kg-bw after considering Cry1Ac purity level. Cry1Ac protein has a very low acute toxicity potential.
An acute oral toxicity study with Cry1F protein was conducted in mice at a level of 2000 mg Cry1F protein/kg-bw (Brooks & Andrus 1999). All animals (5 male and 5 female CD-1 mice) survived and no clinical signs were observed during the study. All animals gained weight by study termination on day 15. There were no treatment-related gross pathological observations. The report concludes that under the conditions of this study, the acute oral LD_{50} of Cry1F in male and female mice was greater than 2000 mg/kg-bw. No mortality was observed, and there were no observable adverse effects with the Cry1F-

treated animals, therefore, the NOEL (No Observed Effect Level) is also considered to be >600 mg/ kg-bw after considering Cry1Fpurity level. The Cry1F protein has a low acute toxicity potential.

3.3 b Assessment of Toxicity Potential of PAT

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

There is no evidence available indicating that the PAT protein is toxic to either humans or animals. In acute toxicity studies mice gavaged with high levels of PAT protein showed no treatment-related significant toxic effects (OECD 1999, US EPA 1997).

3.4 Potential Allergenicity of the Novel Protein

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

Korjagin VA. 2001b. In vitro simulated gastric fluid digestibility study of microbially derived Cry1F (synpro). Study ID 010081, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012c. Sequence Similarity Assessment of Cry1Ac Protein to Known Allergens by Bioinformatics Analysis (Update, May, 2012). Study ID 120763, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012d. Sequence Similarity Assessment of Cry1Ac Protein to Known Toxins by Bioinformatics Analysis (Update, May, 2012). Study ID 120761, Dow AgroSciences LLC, Indianapolis, IN

Guttikonda S. 2012e. Sequence Similarity Assessment of PAT Protein to Known Allergens by Bioinformatics Analysis (Update, February, 2012). Study ID 120143, Dow AgroSciences LLC, Indianapolis IN

Guttikonda S. 2012f. Sequence Similarity of PAT Protein to Known Toxins by Bioinformatics Analysis (Update, February, 2012). Study ID 120480, Dow AgroSciences LLC, Indianapolis IN

Mo J. 2012b. Sequence Similarity Assessment of Cry1F to Known Allergens by Bioinformatics

Schafer BW, Oman TJ, Clement JM, Juba AN, Embrey SK. 2012a. Characterization of the Full Length Cry1Ac Protein Derived from Transgenic Soybean Event DAS-81419-2. Study ID 110840, Dow AgroSciences LLC, Indianapolis, IN

Schafer BW, Oman TJ, Clement JM, Juba AN, Embrey SK. 2012b. Characterization of the Full Length Cry1F Protein Derived from Transgenic Soybean Event DAS-81419-2. Study ID 110841, Dow AgroSciences LLC, Indianapolis, IN

Xu L, Papineni S. 2012. IgE Reactivity of DAS-81419-2 Soybean. Study ID 120890, Dow AgroSciences LLC, Indianapolis, IN

3.4 a Allergenicity Considerations

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 (Korjagin 2001a, Korjagin 2001b). Glycosylation analysis revealed no detectable covalently linked carbohydrates in the Cry1Ac and Cry1F proteins expressed in DAS-81419-2 soybean (Schafer et al 2012a, Schafer et al 2012b). 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 (Guttikonda 2012c, Mo 2012b).

The food and feed safety of PAT was assessed in these products and in published findings (Herouet et al 2005, OECD 1999, Papineni & Cleveland 2012c) 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,

bioinformatics analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence (Guttikonda 2012e, Guttikonda 2012f).

3.4 b Endogenous Allergen Analysis of DAS-81419-2 Soybean

Soybean is listed as one of the eight most common allergenic foods in the United States and one of the 12 most common allergic foods in Europe (EFSA 2007, FDA 2004d). Therefore, a study was conducted to determine if the genetic modification used to generate DAS-81419-2 soybean altered the endogenous allergen content compared to the non-transgenic counterpart.
IgE binding to extracts of DAS-81419-2 soybean and its non-transgenic control were evaluated with one dimensional (1D) IgE immunoblot (qualitative analysis) and ELISA inhibition (quantitative analysis) using sera from 10 clinically-reactive soy allergic patients (Xu & Papineni 2012). The data demonstrated that the genetic modification used to generate DAS-81419-2 soybean did not alter the endogenous allergenicity compared with its non-transgenic control.

(i) SDS-PAGE with Coomassie blue staining and immunoblot analysis

Extracts were prepared from the ground seed of DAS-81419-2 and its non-transgenic control soybeans. To extract the soluble protein from both control and DAS-81419-2 soybean seeds, approximately 10.0 g of soybean seed powders were combined with approximately 100 mL (1:10 w/v) of extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 8.5) and incubated overnight at 4 ºC. The samples were then homogenized at room temperature for 3 minutes using a Cuisinart Smart Stick followed by centrifugation at 3,000 x g for 30 minutes at 4^{o}C and the resulting supernatants were vacuum filtered through P8 grade filter paper. A 35-mL sub-aliquot of the extracts was further clarified by centrifugation at 3,000 x g for 30 minutes at 4 ºC. The supernatants were then filtered through a 0.22 µm sterile filter, aliquoted into 1-mL sub-aliquots, and stored in a -80 ºC freezer.
All samples were mixed with Laemmli buffer and heated at 95 ºC for 5 min and then subjected to SDS-PAGE with Coomassie blue staining to evaluate the protein content of the seed lots. Gel transfer of the proteins to a nitrocellulose membrane was performed to prepare blots replicating the SDS-PAGE. Transfer was confirmed with the use of pre-stained molecular weight markers. Blots were blocked in 5% milk fat in PBST for at least 1 hour at room temperature followed by overnight incubation at 4 ºC in serum from a pool of 10 soy-allergic patients. Blots were washed with PBST to remove unbound IgE and then incubated in biotinylated goat IgG-anti-human IgE for 1 hr at room temperature with continuous agitation. Additional washing with PBST was carried out and then blots were incubated with NeutrAvidin-HRP conjugate for 1 hr at room temperature. Pierce ECL 2 western blotting substrate was used for development and visualization of the immunoreactive protein bands. The membranes were covered with ECL Plus reagent for 5 minutes, excess solution was removed and membranes were exposed to Thermo Scientific CLX-Posure film in a darkroom and developed.
The protein profiles between DAS-81419-2 and the non-transgenic soybean line examined using SDS-PAGE analysis with Coomassie blue staining did not reveal any differences in protein banding patterns between the two soybean extracts (Figure 53). The IgE binding profiles of DAS-81419-2 and non-transgenic control were compared in the 1D immunoblot using soy-allergic sera and also showed no

difference (Figure 54). These results confirmed that the genetic modification used to generate DAS-81419-2 soybean did not alter the IgE immunoreactivity of soybean.

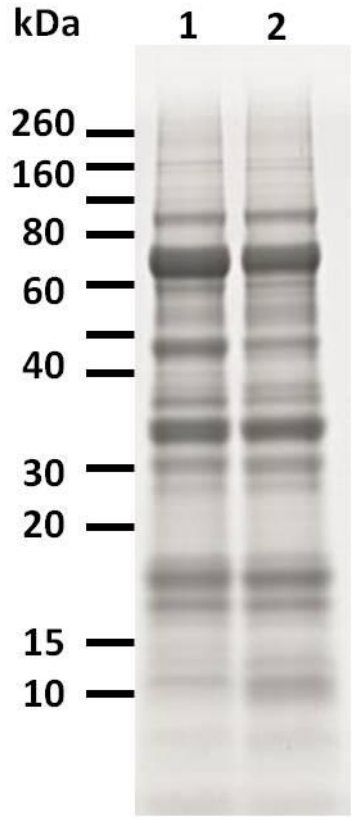

Figure 53. SDS-PAGE analysis of DAS-81419-2 and non-transgenic control soybean seed extract.

Lane	Contents
1	15 µl of control soybean seed extract (15µg)
2	15 µl of DAS-81419-2 soybean seed extract (15 µg)

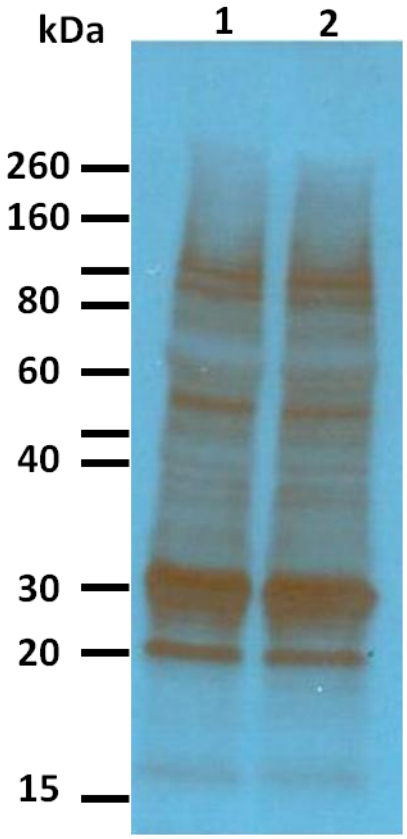

Figure 54. Immunoblot of DAS-81419-2 and non-transgenic control soybean extracts with soybean-allergic patient sera.

Lane	Contents
1	15 µl of control soybean seed extract (15µg)
2	15 µl of DAS-81419-2soybean seed extract (15 µg)

(ii) ELISA Inhibition

ELISA inhibition of IgE binding from a pooled soybean-allergic serum sample was conducted for DAS-81419-2 and non-transgenic control soybean extracts. Extracts from DAS-81419-2 and control at various concentrations (0.002 to 2000 µg/ml of total soluble protein) were pre-incubated with the pooled serum and then transferred to a 96-well plate that was previously coated with the non-transgenic control extracts at 25µg/mL (100µL/well). After a washing step with PBST, biotinylated goat IgG-anti-human IgE and NeutraAvidin-horseradish peroxidase (HRP) conjugate were sequentially incubated on the plate with a PBST washing after each incubation. Finally, tetramethylbenzidine (TMB) is added as a substrate for the peroxidase to initiate the enzymatic reaction, which is stopped by adding 1N HCl. Lastly, the plate was incubated with peroxidase substrate and the reaction was stopped with 1N HCl. A microplate reader was used to read the absorbance in the wells at 450 nm with 650nm background subtraction. The results of the ELISA inhibition experiments were plotted and analyzed using GraphPad Prism 4 (GraphPad Software Inc, La Jolla, CA). Data were analyzed using a non-linear regression curve fit for a

sigmoidal dose-response with a variable slope. This approach uses the following equation, which is identical to the four parameter logistic equation:

$$Y = Bottom + (\frac{Top - Bottom}{1 + 10^{(\log EC_{50} - X) \times HillSlope}})$$

X is the logarithm of the protein concentration, and Y is the percent inhibition. Constraints were applied to set the Bottom ≥ 0% and the Top ≤ 100%. The EC50 value from this analysis represents the protein concentration at which the Y value of the curve (% Inhibition) is halfway between the Top and Bottom plateaus of the curve. The EC50 values and their associated 95% confidence intervals were plotted for the control soybean and DAS-81419-2 extracts.

The ELISA inhibition data with the pooled soy-allergic serum showed the same IgE binding response for the non-transgenic control soybean and the DAS-81419-2 soybean extracts (Figure 55). Furthermore, the associated EC50 values and 95% confidence intervals for control and DAS-81419-2 were similar (Figure 56).

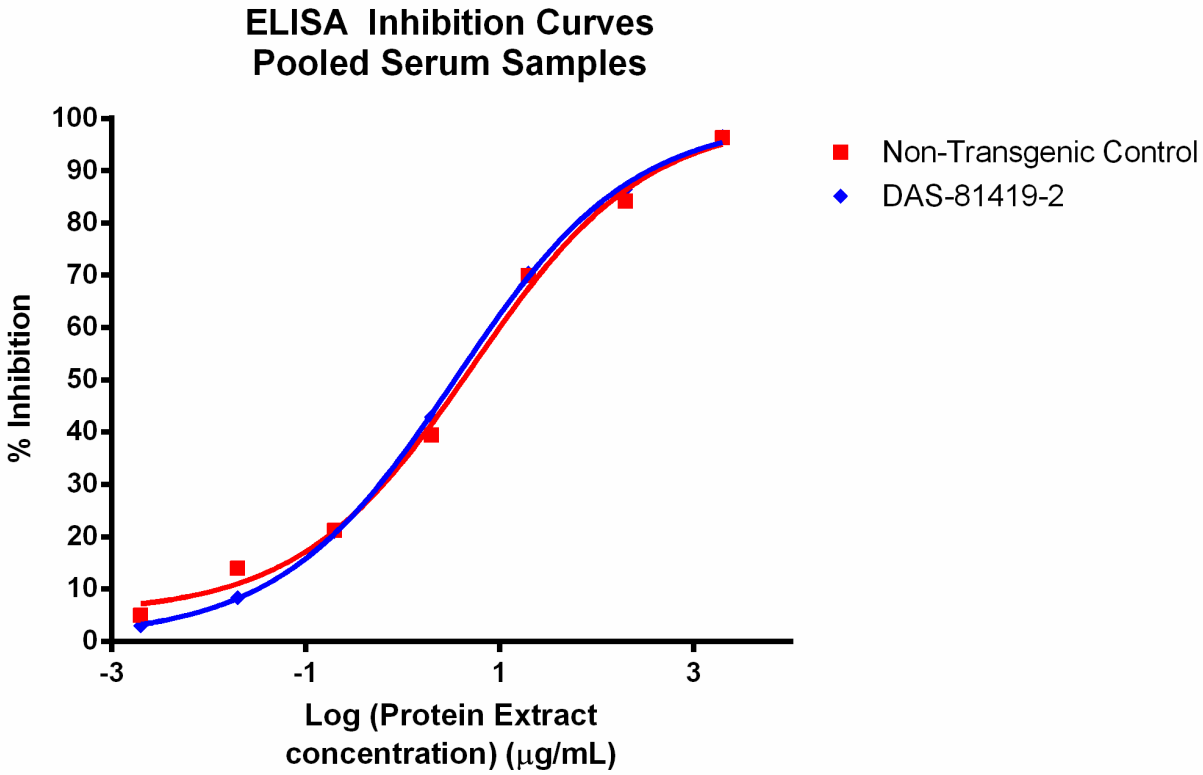

Figure 55. ELISA inhibition with DAS-81419-2 and non-transgenic control soybean extracts using soybean-allergic patient sera.

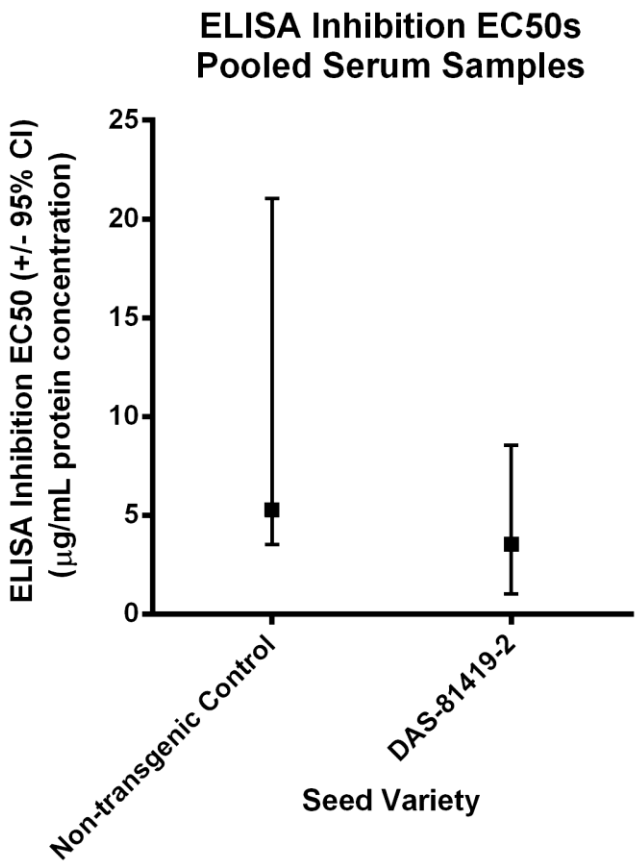

Figure 56. EC50 values from the elisa inhibition data for DAS-81419-22 and non-transgenic control soybean extracts and their 95% confidence intervals[1]

The immunoblot and ELISA inhibition data demonstrate that the genetic modification used to generate DAS-81419-2 soybean did not alter the endogenous allergenicity compared with its non-transgenic control.

[1] Note: Confidence limits are asymmetrical after transformation to the natural scale.

3.5 Compositional Analysis

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

3.5 a 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. (Fast & Johnson 2012). This study used the same plots that were used for protein expression 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.

(i) Field Study Design and 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.

(ii) Composition Analysis

Samples of soybean forage and seed were analyzed for nutrient and antinutrient content (Appendix 6). The analytes examined are presented in Table 12.

Table 11. Composition analytes.

Matrix	Category	Analyte
Forage	Proximate	Protein
Forage	Proximate	Fat
Forage	Proximate	Ash
Forage	Proximate	Moisture
Forage	Proximate	Carbohydrates
Forage	Fibre	ADF
Forage	Fibre	NDF
Forage	Mineral	Calcium
Forage	Mineral	Phosphorus
Grain	Proximate	Protein
Grain	Proximate	Fat
Grain	Proximate	Ash
Grain	Proximate	Moisture
Grain	Proximate	Carbohydrates
Grain	Fibre	ADF
Grain	Fibre	NDF
Grain	Fibre	Total Dietary Fibre
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 (Berman et al 2011, Berman et al 2009, Berman et al 2010, Bilyeu et al 2008, Harrigan et al 2007, Hartwig & Kilen 1991, ILSI 2010, Iskander 1987, Kakade et al 1972, Lundry et al

2008, McCann et al 2005, OECD 2001, Padgette et al 1996, Taylor et al 1999, Zhou et al 2011). A summary of the compositional data from the literature can be found in Table 19.

(iii) 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 ($1\text{-}0.95^{\text{number of comparisons}}$). 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% ($1\text{-}0.95^{71}$).

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 & Hochberg 1995); FDR methods are commonly applied in studies examining transgenic crops (Coll et al 2008, Herman et al 2010, Herman et al 2007, Huls et al 2008, Jacobs et al 2008, Stein et al 2009). 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.

(iv) 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 12 to Table 18, and Figure 57 to Figure 63. 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).

Proximate, Fibre 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, fibre, and minerals (nine analytes). A summary of the results across all locations is presented Table 12 and Figure 57. No statistical differences were observed in the combined-site analysis between the control and DAS-81419-2 entries for all proximate, fibre, 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 12. Summary of the proximate, fibre, 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 (% DW)	0.786	20.4 ± 0.8 13.9 - 29.8	20.6 ± 0.8 15.8 - 25.8 (0.786, 0.828)	14.0 - 35.5	11.2 - 24.71
Fat (% DW)	0.274	2.49 ± 0.19 0.898 - 3.89	2.70 ± 0.20 0.857 - 4.32 (0.274, 0.589)	0.685 - 5.32	1.01 - 9.87
Ash (% DW)	0.926	8.96 ± 0.31 7.01 - 16.9	8.99 ± 0.32 7.51 - 10.9 (0.926, 0.931)	6.80 - 15.2	4.68 - 10.782
Moisture (% FW)	0.578	79.7 ± 0.8 75.8 - 84.3	79.4 ± 0.8 75.5 - 83.6 (0.578, 0.719)	75.3 - 86.6	32.05 - 84.60
Carbohydrates[e] (% DW)	0.510	68.2 ± 1.1 57.2 - 76.4	67.7 ± 1.1 60.3 - 75.7 (0.510, 0.696)	50.1 - 75.6	59.8 - 80.18
Fibre					
Acid Detergent Fibre (ADF) (% DW)	0.310	34.7 ± 2.1 22.4 - 56.7	33.3 ± 2.1 22.2 - 45.4 (0.310, 0.595)	19.4 - 64.1	22.72 - 59.03
Neutral Detergent Fibre (NDF) (% DW)	0.300	41.6 ± 2.6 27.8 - 70.9	39.9 ± 2.6 27.2 - 59.3 (0.300, 0.592)	25.2 - 82.0	19.61 - 73.05
Mineral					
Calcium (mg/100g dry wt.)	0.333	1378 ± 64 940 - 1840	1401 ± 64 908 - 1740 (0.333, 0.615)	874 - 2000	NR
Phosphorus (mg/100g dry wt.)	0.793	266 ± 7 206 - 327	268 ± 7 201 - 342 (0.793, 0.828)	187 - 381	NR

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 Table 19

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

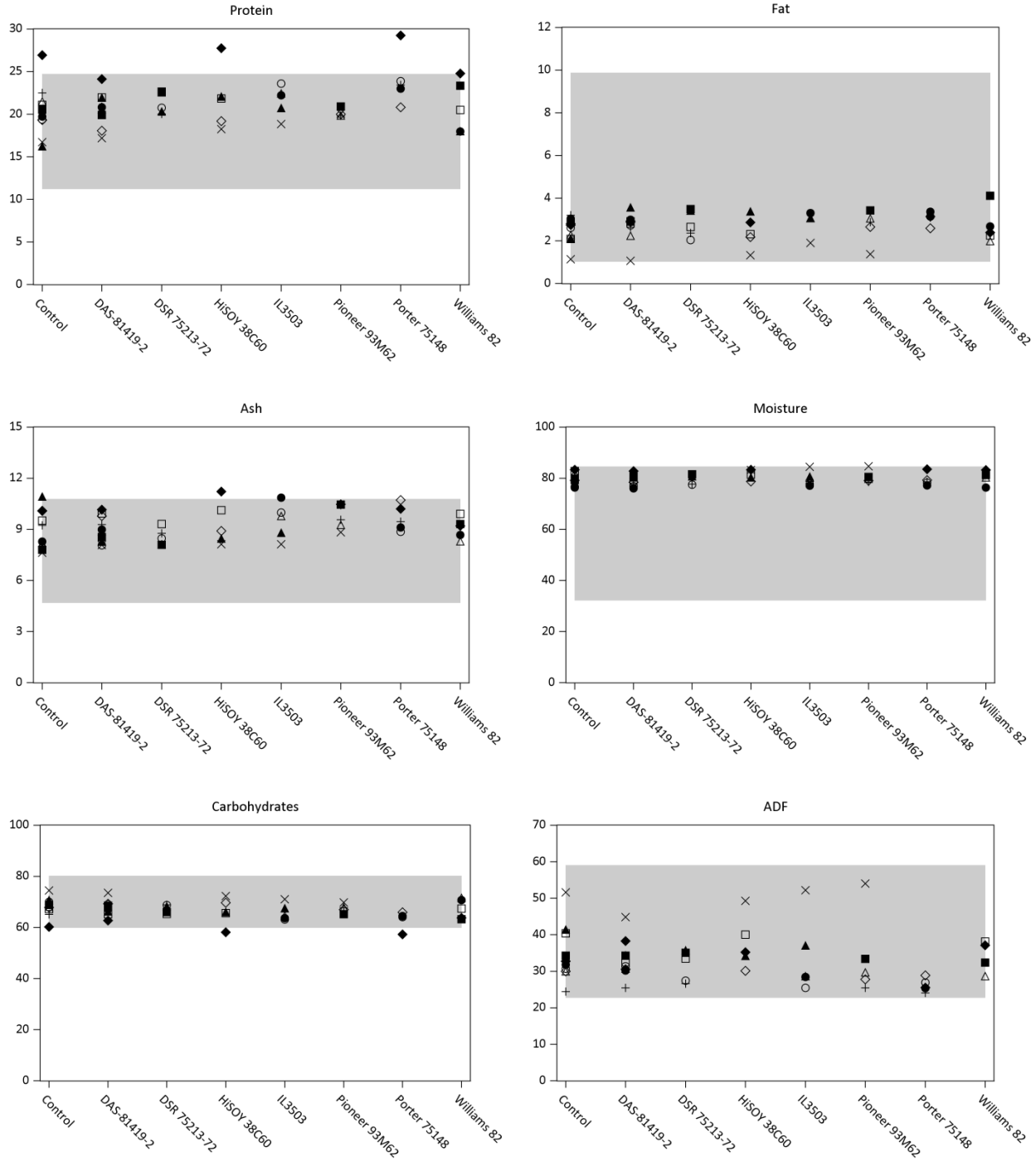

Figure 57. Proximates, fibre and minerals in control, DAS-81419-2 and reference variety soybean forage.

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.

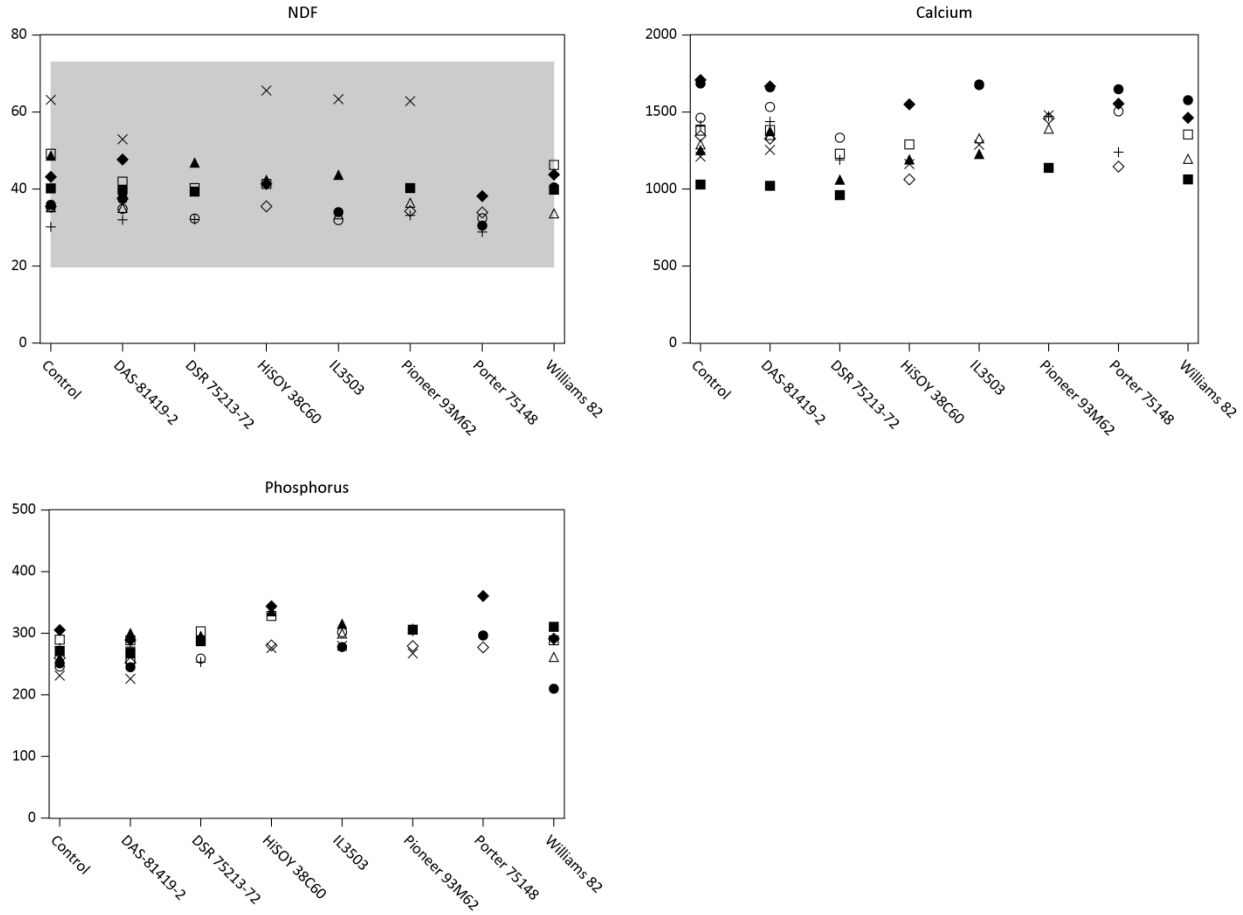

Figure 57 (Cont). Proximates, fibre and minerals in control, DAS-81419-2 and reference variety soybean forage.

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.

3.5 b Composition Analysis of Seed

(i) Proximate and Fibre Analysis of Seed

Soybean seed samples from the control (Maverick), DAS-81419-2, and reference variety entries were analyzed for proximates and fibre (eight analytes). A summary of the results across all locations is presented in Table 13 and Figure 58. 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.

(ii) 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 14 and Figure 59. 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.

(iii) 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 15 and Figure 60. 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 13. Summary of the proximate and fibre 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 (% DW)	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
Fat (% DW)	**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
Ash (% DW)	**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
Moisture (% FW)	**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
Carbohydrates[e] (% DW)	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
Fibre					
Acid Detergent Fibre (ADF) (% DW)	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
Neutral Detergent Fibre (NDF) (% DW)	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
Total Dietary Fibre (% DW)	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

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 Table 19

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

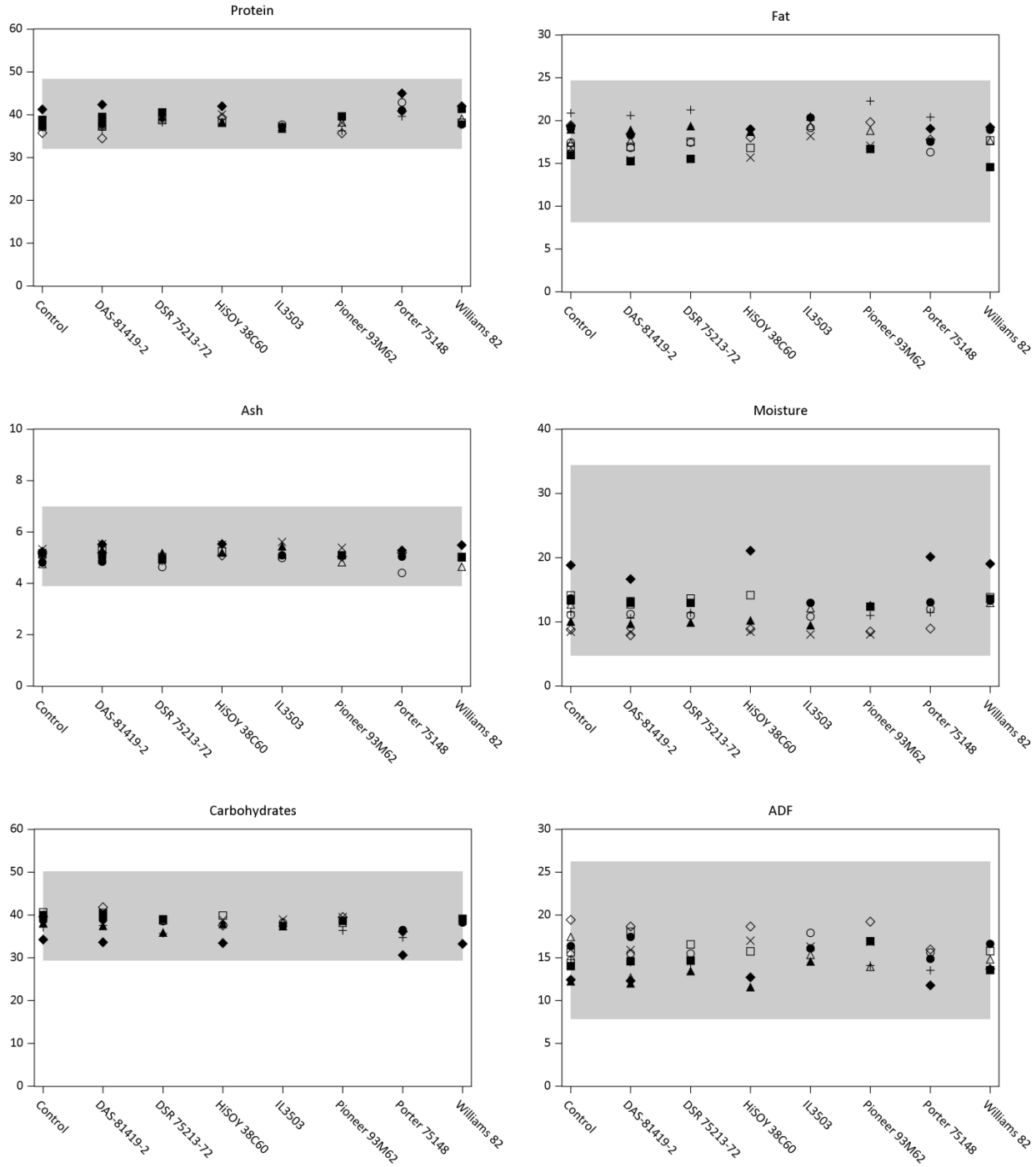

Figure 58. Proximates and fibre in control, DAS-81419-2, and reference variety soybean seed.

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.

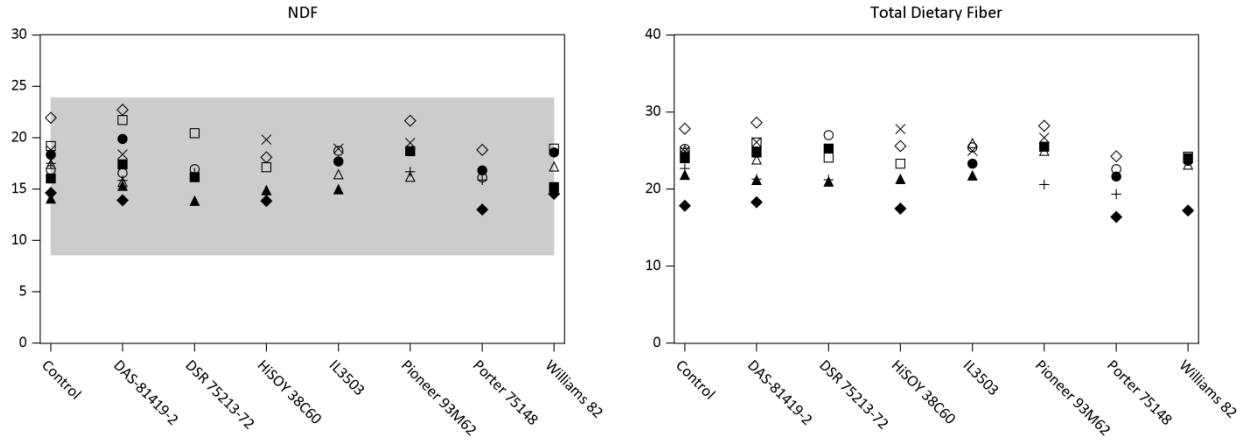

Figure 58 (Cont). Proximates and fibre in control, DAS-81419-2, and reference variety soybean seed.

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.

Table 14. 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 (mg/100 g DW)	0.137	270 ± 8 233 - 335	267 ± 8 205 - 328 (0.137, 0.456)	181 - 339	116.55 - 510
Copper (mg/100 g DW)	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
Iron (mg/100 g DW)	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
Magnesium (mg/100 g DW)	0.663	233 ± 4 204 - 256	232 ± 4 197 - 257 (0.663, 0.747)	205 - 278	219.40 - 312.84
Manganese (mg/100 g DW)	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
Phosphorus (mg/100 g DW)	0.118	607 ± 14 536 - 704	619 ± 14 494 - 708 (0.118, 0.456)	471 - 759	506.74 - 935.24
Potassium (mg/100 g DW)	0.168	1799 ± 21 1660 - 1940	1819 ± 21 1490 - 1980 (0.168, 0.459)	1650 - 2050	1868.01 - 2510
Selenium (ppb DW)	0.401	468 ± 188 <LOQ - 2370	507 ± 187 <LOQ - 2560 (0.401, 0 647)	<LOQ - 3060	NR
Sodium (mg/100 g DW)	NA	NA <LOQ	NA <LOQ	<LOQ - 22.2	4.05 - 30
Zinc (mg/100 g DW)	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

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 Table 19.

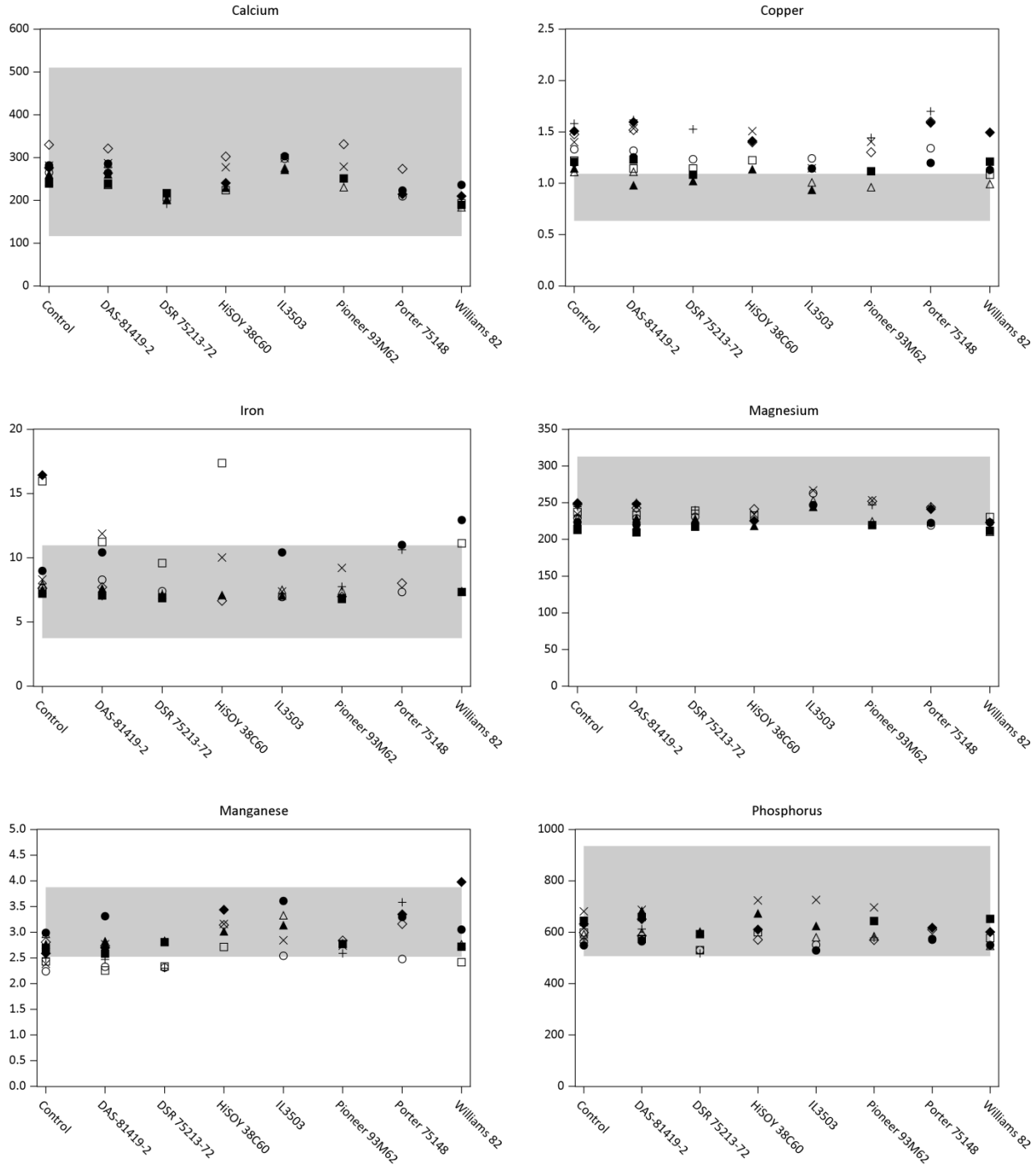

Figure 59. Minerals in control, DAS-81419-2, and reference variety soybean seed.

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.

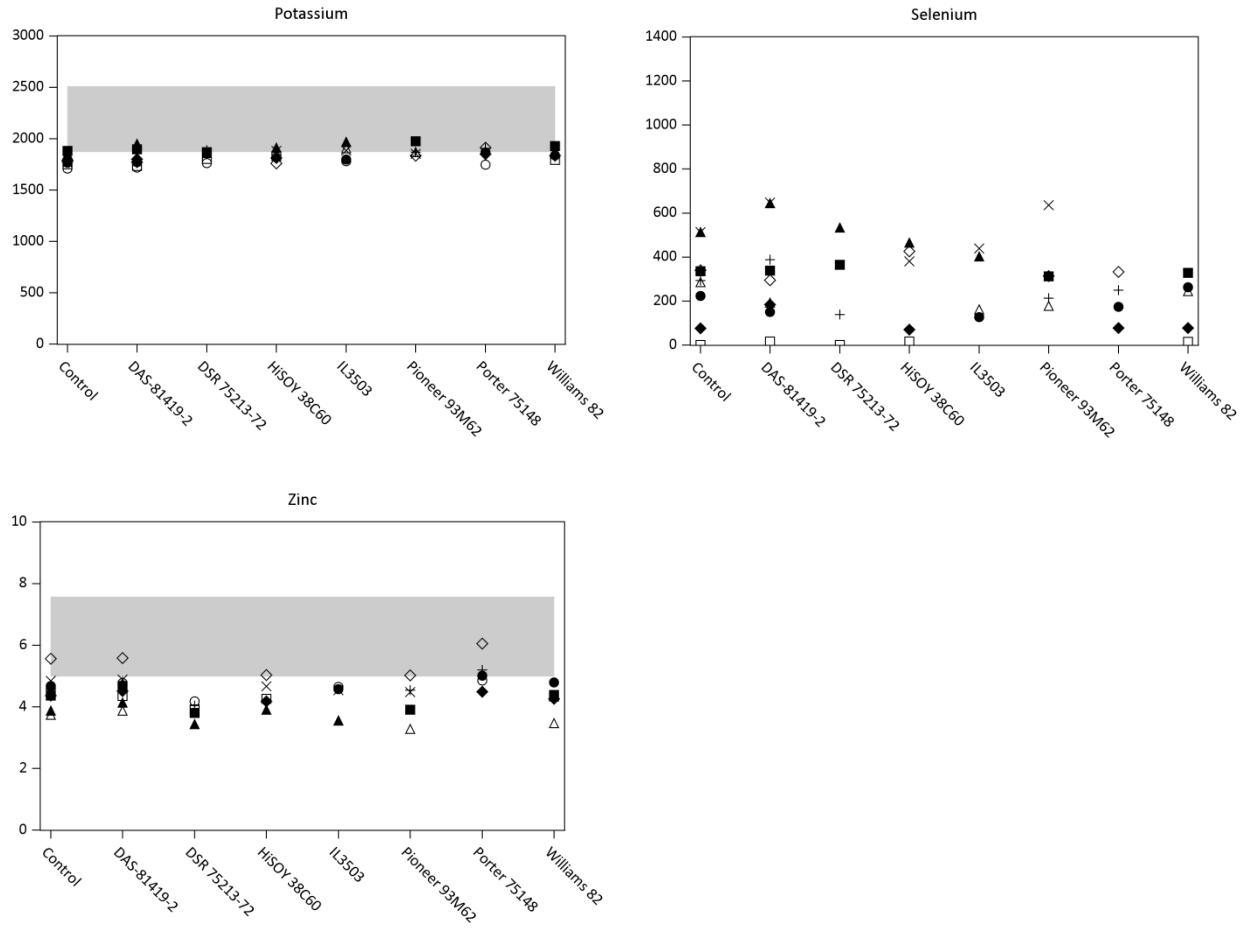

Figure 59 (Cont). Minerals in control, DAS-81419-2, and reference variety soybean seed.

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.

Table 15. 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	4.59 ± 0.03	4.28 - 4.75	4.16 - 4.74
		4.31 - 4.76	4.30 - 4.82		
(% total amino acid)			(0.212, 0.520)		
Arginine	0.111	7.49 ± 0.05	7.44 ± 0.05	7.22 - 8.20	6.41 - 8.41
		7.04 - 7.79	7.05 - 7.74		
(% total amino acid)			(0.111, 0.456)		
Aspartic Acid	0.099	11.51 ± 0.01	11.48 ± 0.01	9.99 - 11.74	11.37 - 12.68
		11.29 - 11.64	11.30 - 11.76		
(% total amino acid)			(0.099, 0.440)		
Cystine	0.283	1.610 ± 0.028	1.629 ± 0.028	1.240 - 1.792	1.02 - 1.87
		1.432 - 1.946	1.448 - 1.861		
(% total amino acid)			(0.283, 0.591)		
Glutamic Acid	0.161	17.32 ± 0.11	17.23 ± 0.11	17.04 - 18.56	17.71 - 20.48
		16.29 - 18.01	16.28 - 17.98		
(% total amino acid)			(0.161, 0.456)		
Glycine	0.745	4.50 ± 0.02	4.50 ± 0.02	4.14 - 4.54	4.19 - 4.62
		4.34 - 4.67	4.30 - 4.66		
(% total amino acid)			(0.745, 0.802)		
Histidine	0.567	2.714 ± 0.016	2.703 ± 0.016	2.434 - 2.776	2.49 - 2.89
		2.583 - 2.818	2.165 - 2.836		
(% total amino acid)			(0.567, 0.719)		
Isoleucine	0.931	4.80 ± 0.02	4.80 ± 0.02	4.61 - 4.99	4.13 - 5.11
		4.65 - 4.96	4.52 - 4.99		
(% total amino acid)			(0.931, 0.931)		
Leucine	0.605	7.65 ± 0.01	7.64 ± 0.01	7.45 - 7.98	7.46 - 8.29
		7.51 - 7.83	7.49 - 7.75		
(% 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 Table 19.

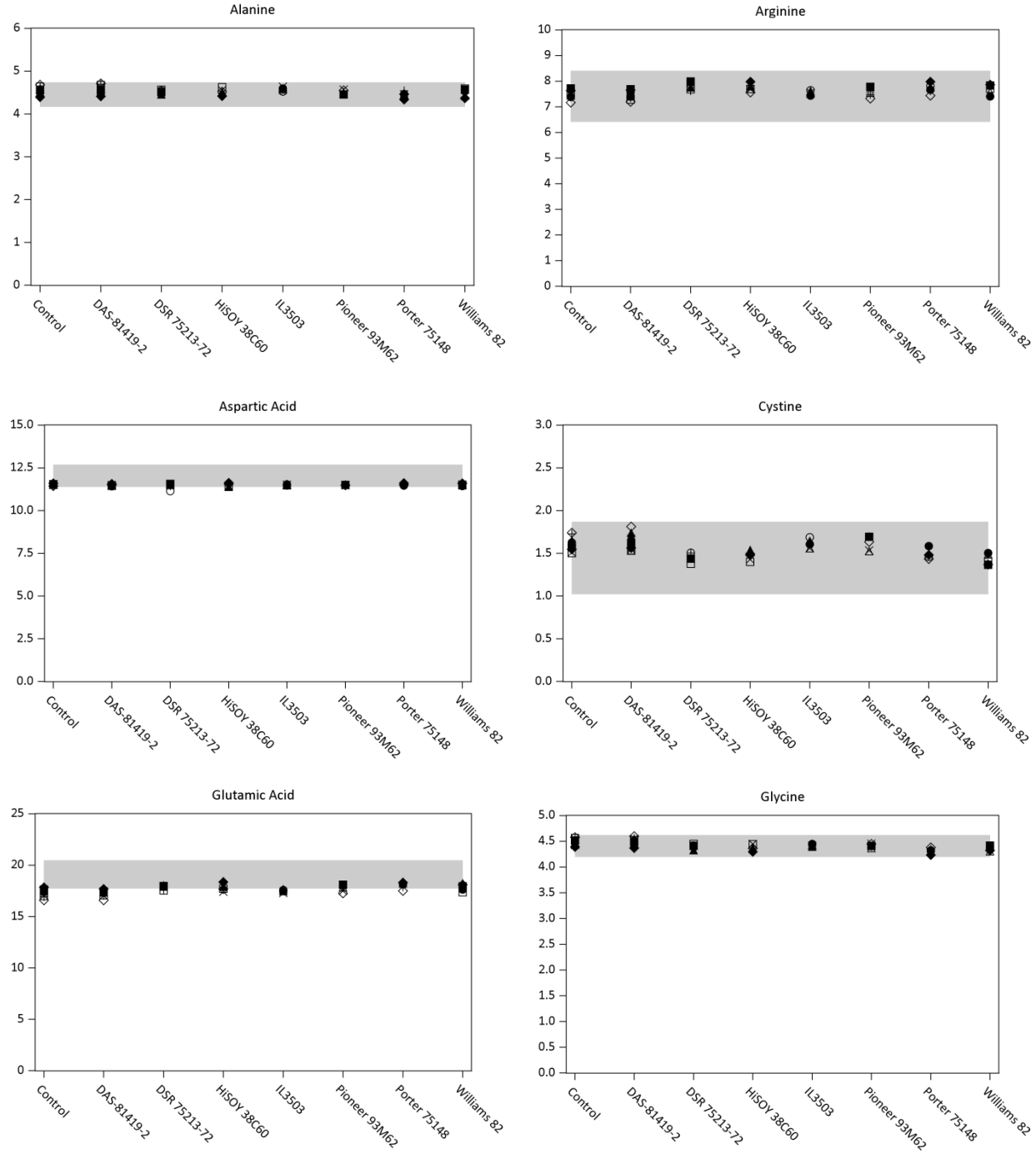

Figure 60. Amino acids in control, DAS-81419-2, and reference variety soybean seed (% total amino acid).

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.

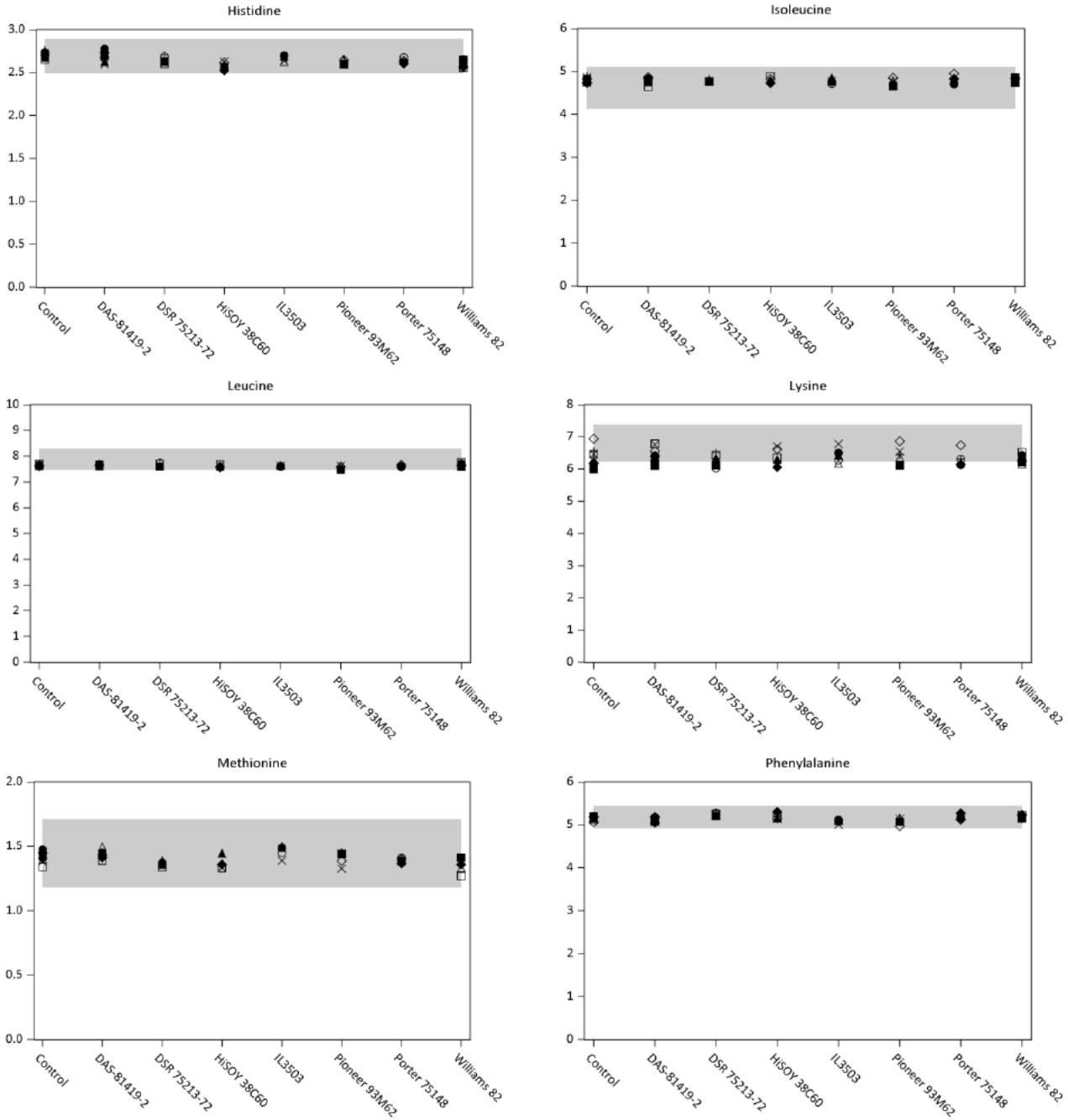

Figure 60 (Cont). Amino acids in control, DAS-81419-2, and reference variety soybean seed (% total amino acid).

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.

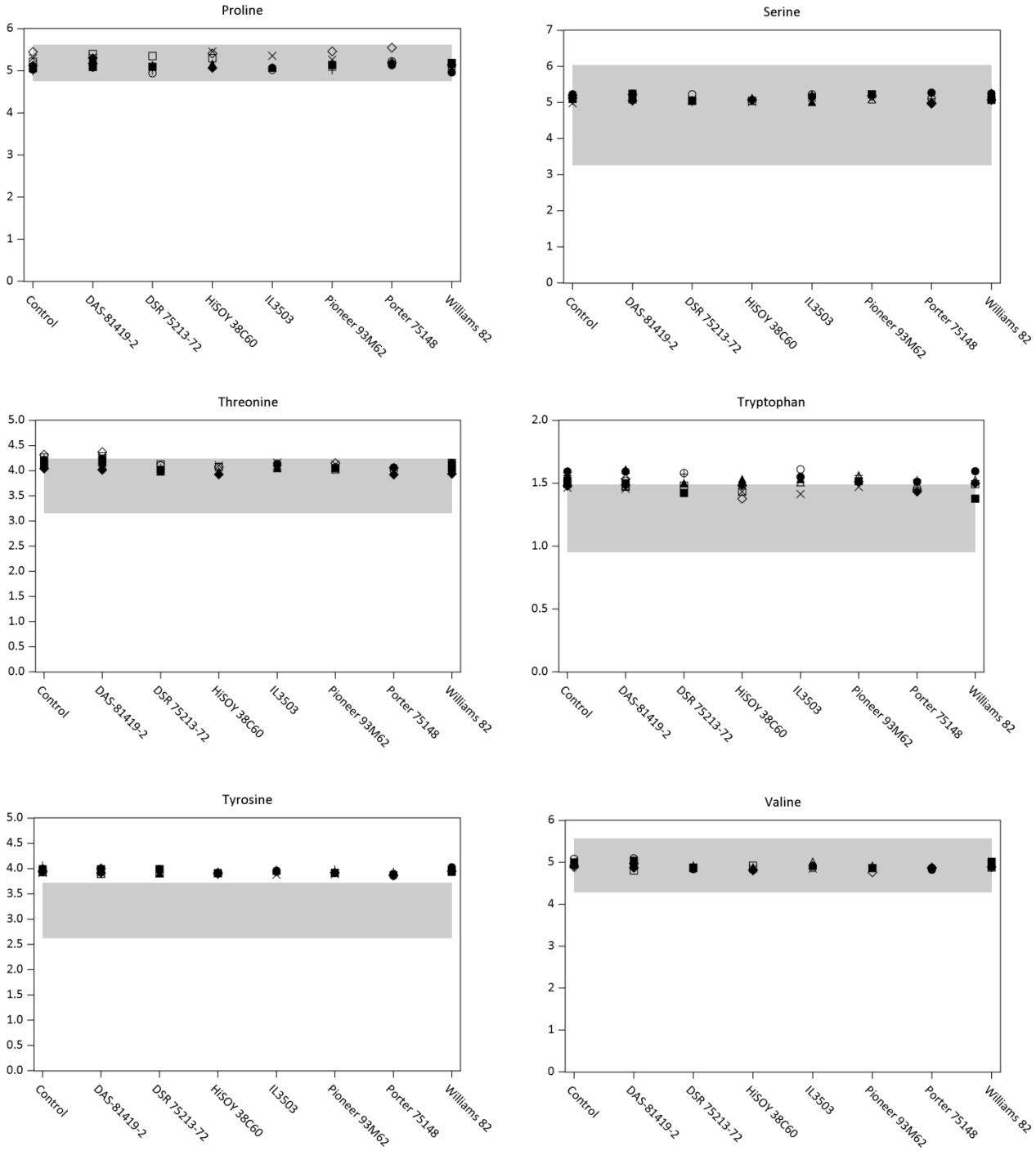

Figure 60 (CONT). Amino acids in control, DAS-81419-2, and reference variety soybean seed (% total amino acid).

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.

(iv) 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 16 and Figure 61. 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 16. 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 (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.148
10:0 Capric (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.27
12:0 Lauric (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.132
14:0 Myristic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.238
14:1 Myristoleic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.125
15:0 Pentadecanoic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
15:1 Pentadecenoic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - <LOQ
16:0 Palmitic (% total fatty acid)	**<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

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 Table 19

Table 16 (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 (% total fatty acid)	NA	NA <LOQ	NA <LOQ - 0.236	<LOQ	<LOQ - 0.194
17:0 Heptadecanoic (% total fatty acid)	NA	NA <LOQ - 0.128	NA <LOQ - 0.132	<LOQ - 0.133	<LOQ - 0.146
17:1 Heptadecenoic (% total fatty acid)	NA	NA <LOQ	NA <LOQ	<LOQ	<LOQ - 0.087
18:0 Stearic (% total fatty acid)	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
18:1 Oleic (% total fatty acid)	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
18:2 Linoleic (% total fatty acid)	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
18:3 Linolenic (% total fatty acid)	**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

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 Table 19

Table 16 (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 Table 19.

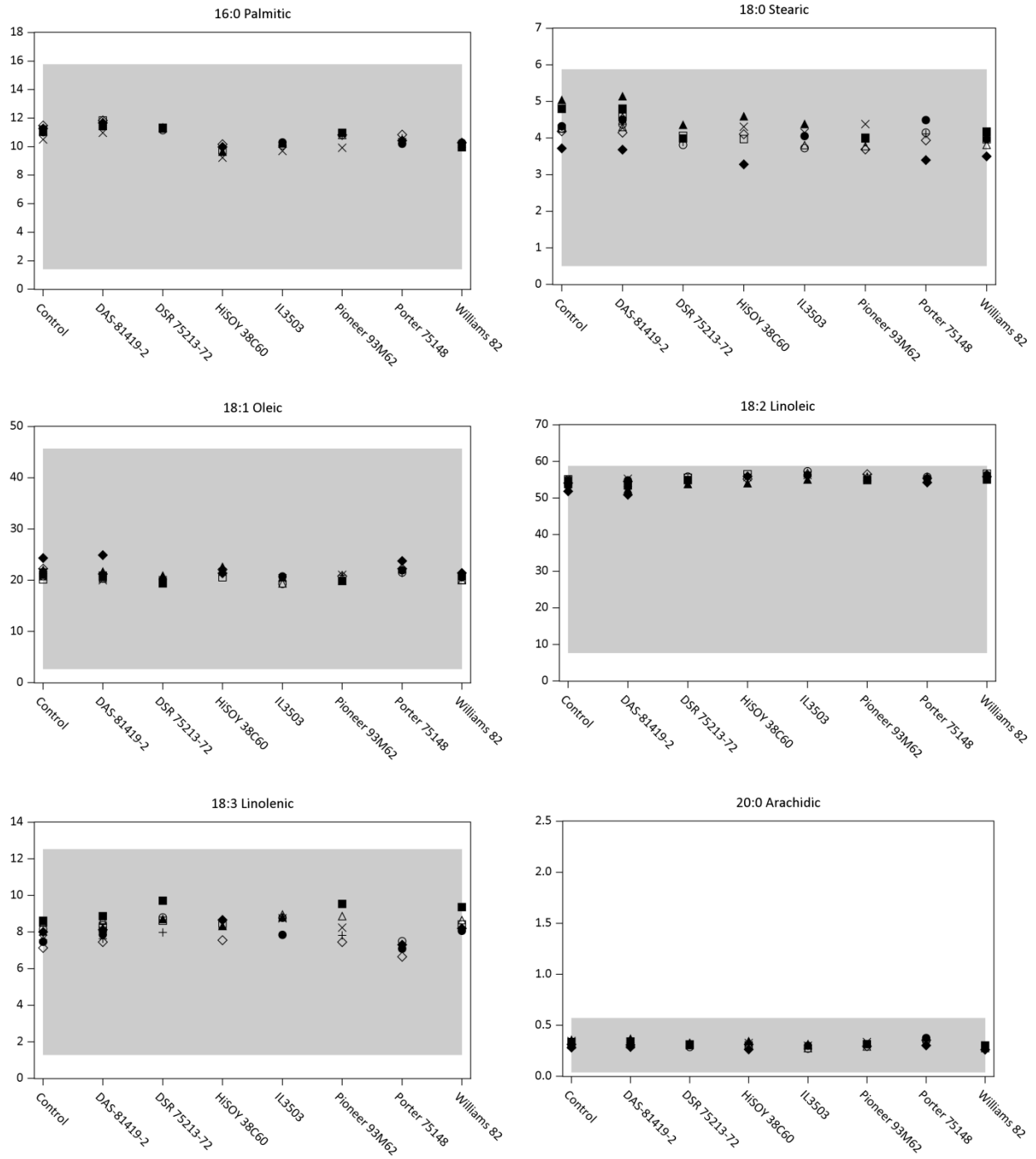

Figure 61. Fatty acids in control, DAS-81419-2, and reference variety soybean seed (% total fatty acid).

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.

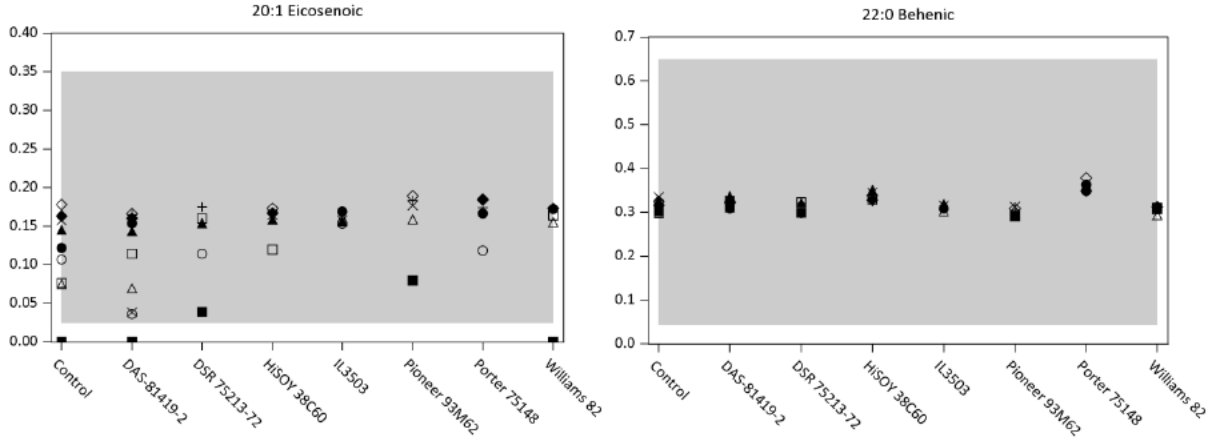

Figure 61 (Cont). Fatty acids in control, DAS-81419-2, and reference variety soybean seed (% total fatty acid).

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.

(v) 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 17 and Figure 62. 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 17. 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 Table 19.

Table 17 (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 Table 19.

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

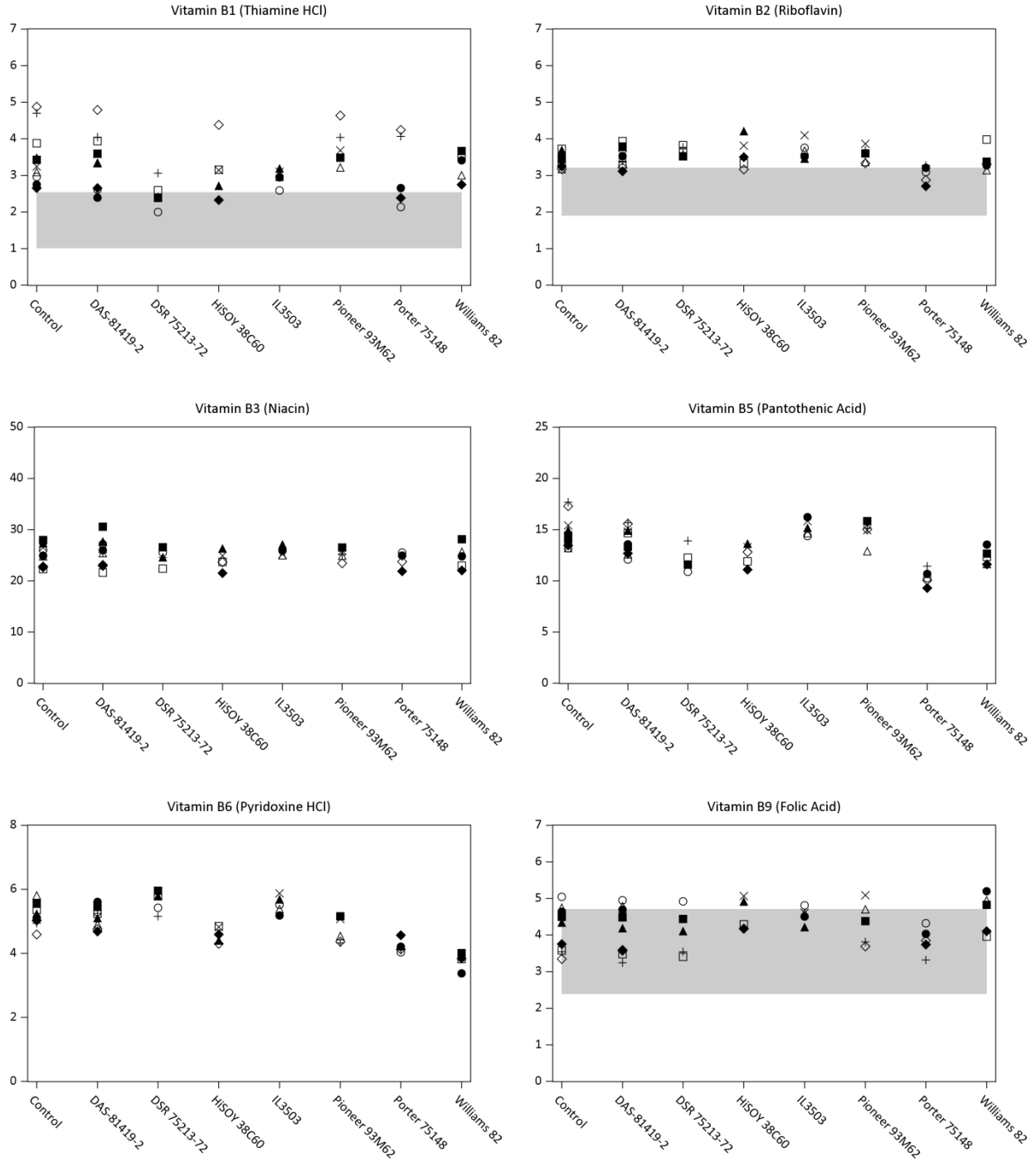

Figure 62. Vitamins in control, DAS-81419-2, and reference variety soybean seed (mg/kg dry weight).

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.

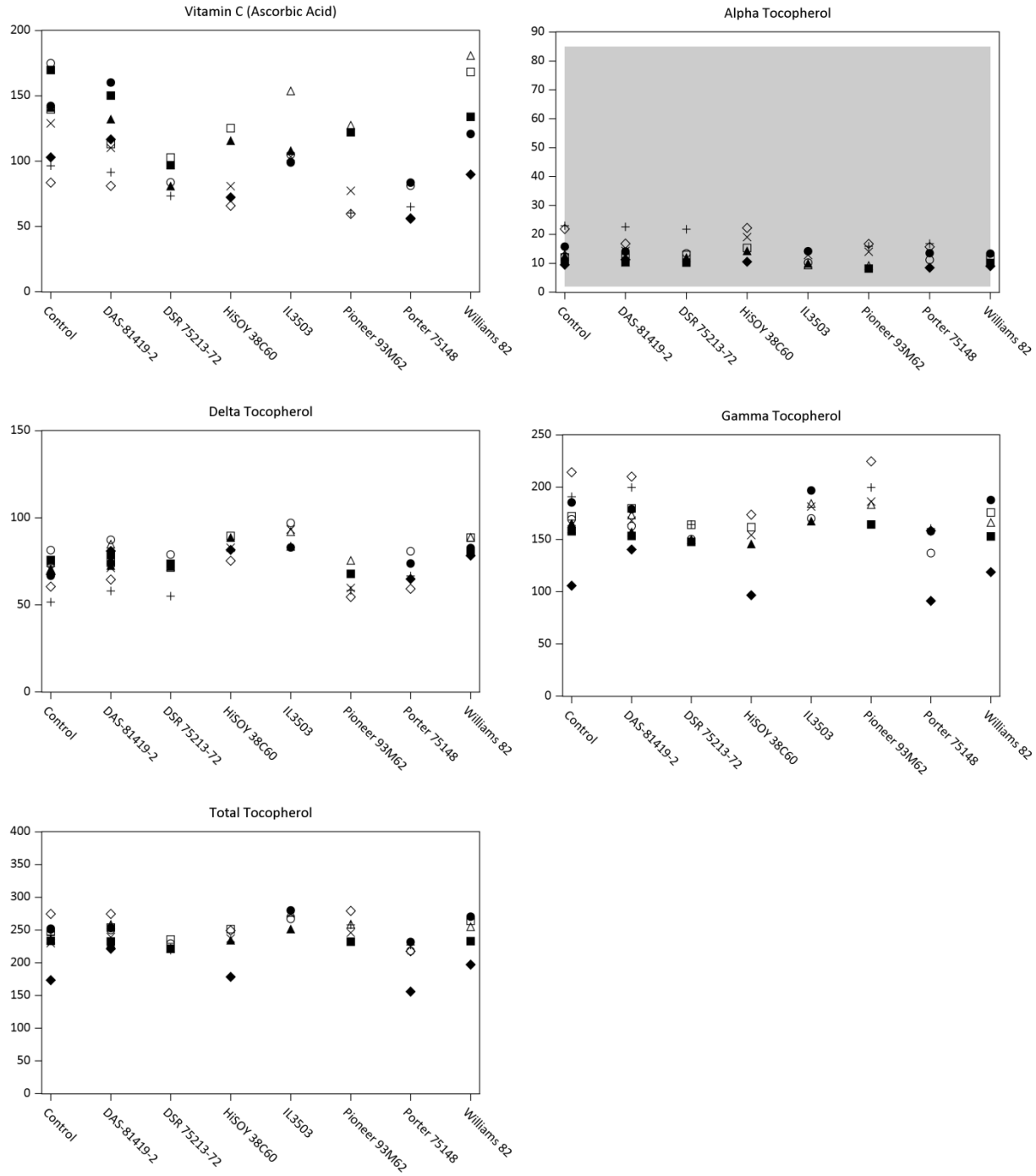

Figure 62 (Cont). Vitamins in control, DAS-81419-2, and reference variety soybean seed (mg/kg dry weight).

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.

(vi) 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 18 and Figure 63. 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 18. 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 Table 19.

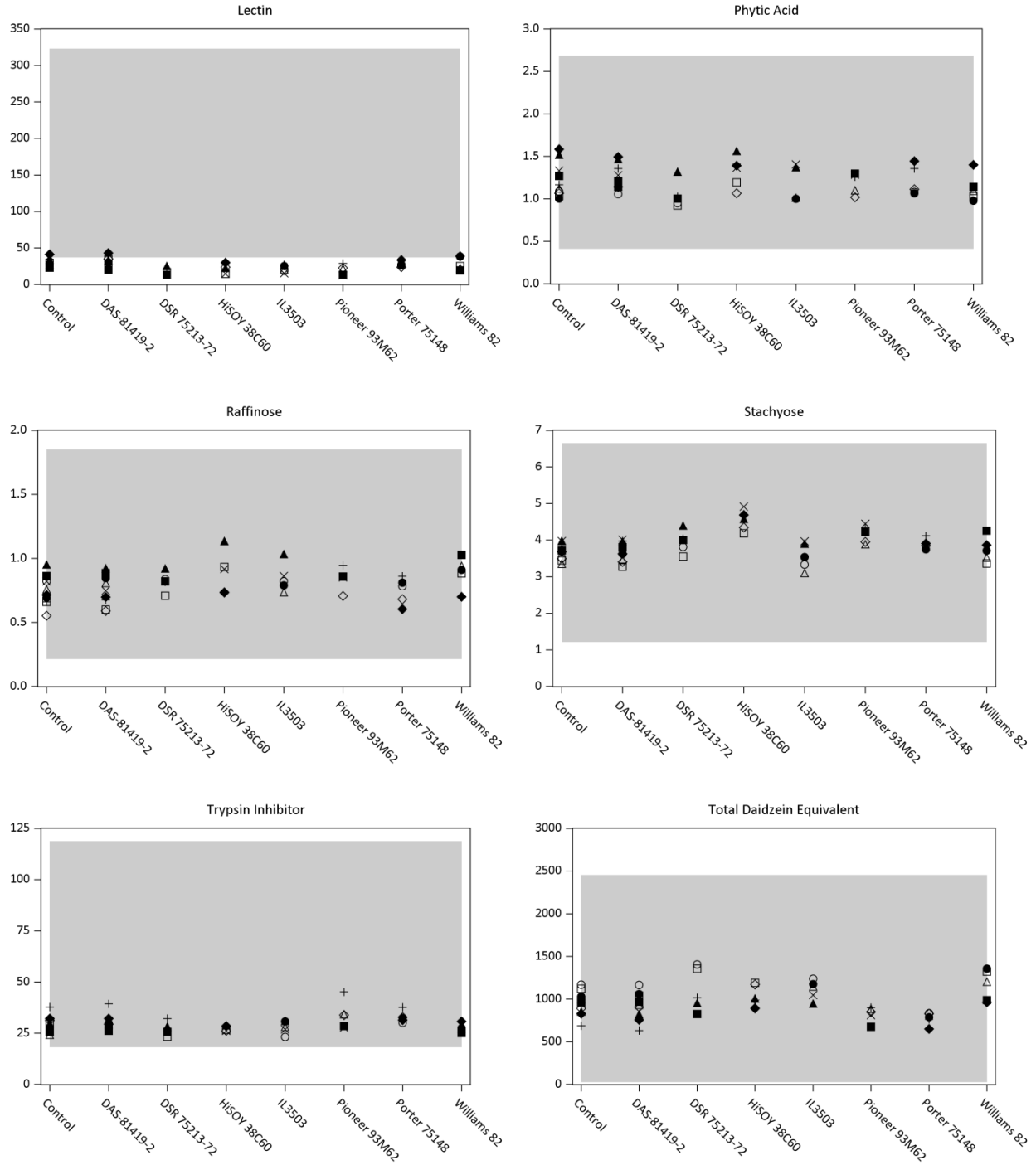

Figure 63. Bioactives in control, DAS-81419-2, and reference variety soybean seed.

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.

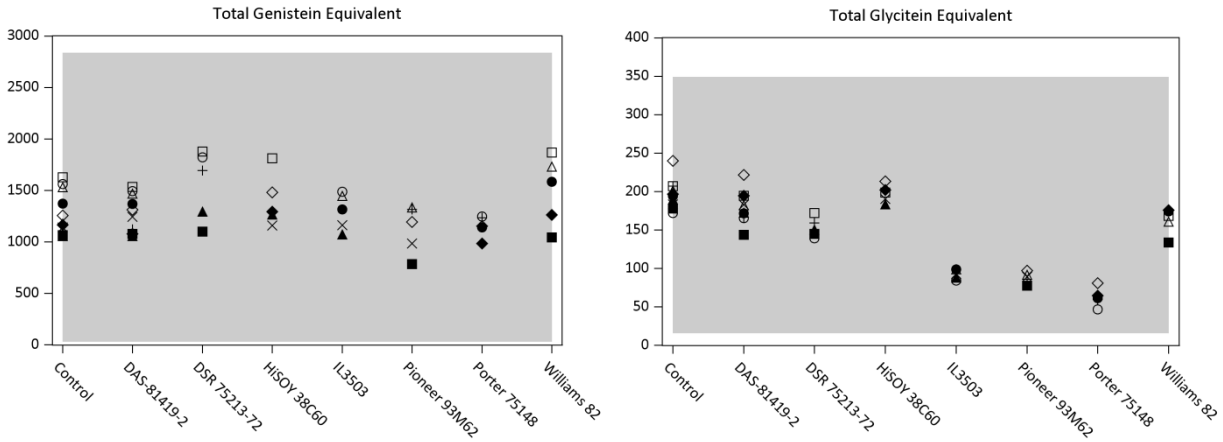

Figure 63 (Cont). Bioactives in control, DAS-81419-2, and reference variety soybean seed.

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

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.

(vii) Methods for Compositional Analysis

Acid Detergent Fibre (ADFA)

The ANKOM2000 Fibre 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
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 Fibre (NDFA)

The ANKOM2000 Fibre 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 (HNO3) 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 Se78 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 Fibre (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 fibre. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fibre in the samples 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 B2 (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

Table 19. 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	Fibre	ADF	% DW	32	38	NR	NR	22.72	59.03	Harrigan et al. 2007	Berman et al. 2010
Forage	Fibre	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	Fibre	ADF	% DW	9.0	11.1	7.81	18.61	9.22	26.26	Lundry et al. 2008	Lundry et al. 2008
Grain	Fibre	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)

4. NUTRITIONAL IMPACT

Maldonado PM. 2012. Protein Expression of a Transformed Soybean Cultivar Containing Cry1Ac, Cry1F, and Phosphinothricin Acetyltransferase (PAT) - Event DAS-81419-2. Study ID 110000.02, Dow AgroSciences LLC, Indianapolis, IN

Papineni S, Cleveland CB. 2012a. Global Dietary and Livestock Assessment of Cry1Ac Protein for DAS Soybean Cultivar Based on Event DAS-81419-2. Study ID 120864, Dow AgroSciences LLC, Indianapolis, IN

Papineni S, Cleveland CB. 2012b. Global Dietary and Livestock Assessment of Cry1F Protein for DAS Soybean Cultivar Based on Event DAS-81419-2. Study ID 120865, Dow AgroSciences LLC, Indianapolis, IN

Papineni S, Cleveland CB. 2012c. Global Dietary and Livestock Assessment of PAT Protein for DAS Soybean Cultivar Based on Event DAS-81419-2. Study ID 120865, Dow AgroSciences LLC, Indianapolis, IN

Smith-Drake JK, Dunville CM, Phillips AM, Herman RA. 2009. Field Expression, Nutrient Composition Analysis and Agronomic Characteristics of a Transformed Soybean Cultivar (DAS-68416-4) Containing Aryloxyalkanoate Dioxygenase (AAD-12) and Phosphinotricin Acetyltransferease (PAT), Dow AgroSciences LLC, Indianapolis IN

4.1 Human Dietary Risk Assessment

4.1 a Cry1Ac Human Dietary Risk Assessment

Expression levels of Cry1Ac protein in plant tissues of DAS-81419-2 soybean were used with conservative (i.e. protective) human dietary consumption data for soybean to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the Cry1Ac protein in DAS-81419-2 soybean, indicating negligible risk for adverse effects from acute dietary exposure (Papineni & Cleveland 2012a).

The field expression of Cry1Ac protein in DAS-81419-2 soybean has been measured using a Cry1Ac specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (Maldonado 2012). Field expression data is available for trials conducted at multiple test sites located within the major soybean-producing regions of the U.S. representing diverse agronomic practices and environmental conditions. Protein expression was analysed in leaf at V5 and V10-12 growth stages, root, forage, and grain tissues collected throughout the growing season from DAS-81419-2 soybean plants.

For human diet, only the grain is relevant and so the protein expression in the soybean grain is the one applicable for human dietary consideration. In soybean grain, the average value of Cry1Ac protein was 1.04 ng Cry1Ac protein/mg tissue on a dry weight basis. The full range of applicable values was 0.793 to 1.40 ng/mg tissue, but the use of an average expression value is most appropriate. This is because grain is a blended commodity, making consumption of single-servings of grain at the maximum expression-level highly unlikely. Use of these values are protective estimates for exposure to the Cry1Ac protein from soybean; actual dietary exposure and impact of the protein will be lower because: 1) there may be protein degradation during transport and storage, 2) grain containing Cry1Ac will be mixed with non-Cry1Ac grain, 3) human consumption of soy products is primarily in food forms which are cooked and

heat is known to denature this protein, and 4) a portion of the consumer dietary exposure to soybeans is in forms that contain very little protein (e.g. soybean oil).

A conservative short term intake (STI) consumption of soybean was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint, the No Observed Effect Level (NOEL) of 700 mg/kg. Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki et al 2005). Nevertheless, to place the Cry1Ac protein exposure estimate in context, a comparison of the exposure information to the lower limit NOEL has been made to provide the MOE.

MOE = NOEL/Exposure

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOEL threshold.

For this assessment, the MOE values were calculated using the DEEM dietary exposure model program (DEEM-FCID version 3.15, Exponent, 2007). In this exercise, the residue value of 1.04 ppm (parts per million) was conservatively applied to all commodities listed in DEEM that are associated with soybean. This is a conservative approach for processed flour and soymilk. No residues were assigned to oil. Using this residue file as input, MOE values were calculated against the acute NOEL of >700 mg/kg bw for several subpopulations (Table 20). In this DEEM estimation, the highest exposed subpopulation is “All Infants” (<1 yr old) with an estimated exposure of 0.0026 mg/kg bw and an MOE value of >268817 at the 97.5^{th} percentile. MOE values >100 are typically considered acceptable. All relevant MOE values are extremely large, indicating negligible risk for adverse effects from dietary exposure to Cry1Ac protein.

Table 20. Summary of human dietary margins of exposure for Cry1Ac protein in soybean based on US DEEM consumption model for short term exposure.

Time frame		Food Intake[a] g/kg-bw	Exposure (mg Cry1Ac /kg-bw/day)	NOEL (mg/kg-bw)	MOE
Short Term					
	US DEEM 97.5th				
	U.S. Population	*0.6308*	0.00066	>700	>1067073
	Children 1-6 yrs	*0.8933*	0.00093	>700	>753498
	Female 13-50	*0.4202*	0.00044	>700	>1601831
	All infants	*2.5038*	0.0026	>700	>268817
	Children 1-2 yrs	*0.8500*	0.00088	>700	>791855
	Children 3-5 yrs	*0.6519*	0.00068	>700	>1032448
	Children 6-12 yrs	*0.2212*	0.00023	>700	>3043478

[a]Consumption for DEEM has been back-calculated based on the exposure estimate results and noted in italics.

4.1 b Cry1F Human Dietary Risk Assessment

Expression levels of Cry1F protein in plant tissues of DAS-81419-2 soybean were used with conservative (i.e. protective) human dietary consumption data for soybean to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the Cry1F protein in DAS-81419-2 soybean, indicating negligible risk for adverse effects from acute dietary exposure (Papineni & Cleveland 2012b).

The field expression of Cry1F protein in DAS-81419-2 soybean has been measured using a Cry1F specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (Maldonado 2012). Field expression data is available for trials conducted at multiple test sites located within the major soybean-producing regions of the U.S representing diverse agronomic practices and environmental conditions. Protein expression was analysed in leaf at V5 and V10-12 growth stages, root, forage, and grain tissues collected throughout the growing season from DAS-81419-2 soybean plants.

For human diet, only the grain is relevant and so the protein expression in the soybean grain is the one applicable for human dietary consideration. In soybean grain, the average value of Cry1F protein was 13.80 ng Cry1F protein/mg tissue on a dry weight basis. The full range of applicable values was 10.41 to 16.95 ng/mg tissue, but the use of an average expression value is most appropriate. This is because grain is a blended commodity, making consumption of single-servings of grain at the maximum expression-level highly unlikely. Use of these values are protective estimates for exposure to the Cry1F protein from soybean; actual dietary exposure and impact of the protein will be lower because: 1) there may be protein degradation during transport and storage, 2) grain containing Cry1F will be mixed with

non-Cry1F grain, 3) human consumption of soy products is primarily in food forms which are cooked and heat is known to denature this protein, and 4) a portion of the consumer dietary exposure to soybeans is in forms that contain very little protein (e.g. soybean oil).

A conservative short term intake (STI) consumption of soybean was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint, the acute No Observed Effect Level (NOEL) of 600 mg/kg. Acute risk assessments are typically not required for substances with acute NOEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki et al 2005). Nevertheless, to place the Cry1F protein exposure estimate in context, a comparison of the exposure information to the lower limit NOEL has been made to provide the MOE.

MOE = NOEL/Exposure

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOEL threshold.

For this assessment, the MOE values were calculated using the DEEM dietary exposure model program (DEEM-FCID version 3.15, Exponent, 2007). In this exercise, the residue value of 13.80 ppm was conservatively applied to all commodities listed in DEEM that are associated with soybean. This is a conservative approach for processed flour and soymilk. No residues were assigned to oil. Using this residue file as input, MOE values were calculated against the acute NOEL of >600 mg/kg for several subpopulations (Table 21). In this DEEM estimation, the highest exposed subpopulation is “All Infants” (<1 yr old) with an estimated exposure of 0.034551 mg/kg bw and an MOE value of >17366 at the 97.5th percentile. MOE values >100 are typically considered acceptable. All relevant MOE values are extremely large, indicating negligible risk for adverse effects from dietary exposure to Cry1F protein.

Table 21. Summary of human dietary margins of exposure for Cry1F protein in soybean based on us deem consumption model for short term exposure.

Time frame		Food Intake[a] g/kg-bw	Exposure (mg Cry1F /kg-bw/day)	NOEL (mg/kg-bw)	MOE
Short Term					
	US DEEM 97.5th				
	U.S. Population	*0.6309*	0.008706	>600	>68918
	Children 1-6 yrs	*0.8930*	0.012324	>600	>48685
	All infants	*2.5037*	0.034551	>600	>17366
	Female 13-50	*0.42*	0.005796	>600	>103520
	Children 1-2 yrs	*0.8503*	0.011734	>600	>51133
	Children 3-5 yrs	*0.6520*	0.008998	>600	>66681
	Children 6-12 yrs	*0.2211*	0.003051	>600	>196657

[a] Consumption for DEEM has been back-calculated based on the exposure estimate results and noted in italics.

4.1 c PAT Human Dietary Risk Assessment

Expression levels of PAT protein in plant tissues of DAS-81419-2 soybean were used with conservative (i.e. protective) human dietary consumption data for soybean to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the PAT protein in DAS-81419-2 soybean, indicating negligible risk for adverse effects from acute dietary exposure (Papineni & Cleveland 2012c).

The field expression of PAT protein in DAS-81419-2 soybean has been measured using a PAT specific enzyme linked immunosorbent assay (ELISA) in several plant tissues at various growth stages of soybean (Maldonado 2012). Field expression data is available for trials conducted at multiple test sites located within the major soybean-producing regions of the U.S representing diverse agronomic practices and environmental conditions. Protein expression was analyzed in leaf at V5 and V10-12 growth stages, root, forage, and grain tissues collected throughout the growing season from DAS-81419-2 soybean plants.

For human diet, only the grain is relevant and so the protein expression in the soybean grain is the one applicable for human dietary consideration. In soybean grain, the average value of PAT protein was 0.86 ng PAT protein/mg tissue on a dry weight basis. The full range of applicable values was 0.63 to 1.12 ng/mg tissue, but the use of an average expression value is most appropriate. This is because grain is a blended commodity, making consumption of single-servings of grain at the maximum expression-level highly unlikely. Use of these values are protective estimates for exposure to the PAT protein from soybean; actual dietary exposure and impact of the protein will be lower because: 1) there may be protein degradation during transport and storage, 2) grain containing PAT will be mixed with non-PAT grain, 3) human consumption of soy products is primarily in food forms which are cooked and heat is

known to denature this protein, and 4) a portion of the consumer dietary exposure to soybeans is in forms that contain very little protein (e.g. soybean oil).

A conservative short term intake (STI) consumption of soybean was determined based on available consumption patterns in the US diet and compared to the acute oral toxicity endpoint, the No Observed Adverse Effect Level (NOAEL) of 5000 mg/kg (OECD 1999). Acute risk assessments are typically not required for substances with acute NOAEL values above 500 mg/kg bw/day or for compounds with no associated mortalities below 1000 mg/kg bw in single dose studies (Solecki et al 2005). Nevertheless, to place the PAT protein exposure estimate in context, a comparison of the exposure information to the lower limit NOAEL has been made to provide the MOE.

MOE = NOAEL/Exposure

The larger the MOE value, the less likelihood there is for adverse effects, because the exposure is well below the established NOAEL threshold.

For a US assessment, the MOE values were calculated using the DEEM dietary exposure model program (DEEM-FCID version 3.15, Exponent, 2007). In this exercise, the residue value of 0.86 ppm was conservatively applied to all commodities listed in DEEM that are associated with soybean. This is a conservative approach for processed flour and soymilk. No residues were assigned to oil. Using this residue file as input, MOE values were calculated against the acute NOAEL of >5000 mg/kg for several subpopulations (Table 22). In this DEEM estimation, the highest exposed subpopulation is “All Infants” (<1yr old) with an estimated exposure of 0.002153 mg/kg bw and an MOE value of >2322341 at the 97.5th percentile. MOE values >100 are typically considered acceptable. All relevant MOE values are extremely large, indicating negligible risk for adverse effects from dietary exposure to PAT protein.

Table 22. Summary of human dietary margins of exposure for PAT protein in soybean based on US deem consumption model for short term exposure.

Time frame		Food Intake[a] g/kg-bw	Exposure (mg PAT /kg-bw/day)	NOAEL (mg/kg-bw)	MOE
Short Term					
	US DEEM 97.5th				
	U.S. Population	*0.6314*	0.000543	>5000	>9208103
	Children 1-6 yrs	*0.8930*	0.000768	>5000	>6510417
	All infants	*2.5035*	0.002153	>5000	>2322341
	Children 1-2 yrs	*0.8500*	0.000731	>5000	>6839945
	Children 3-5 yrs	*0.6523*	0.000561	>5000	>8912656
	Children 6-12 yrs	*0.2209*	0.00019	>5000	>26315789
	U.S. Population	*0.6314*	0.000543	>5000	>9208103
	Children 1-6 yrs	*0.8930*	0.000768	>5000	>6510417

[a] Consumption for DEEM has been back-calculated based on the exposure estimate results and noted in italics.

4.2 Livestock Dietary Risk Assessment

4.2 a Cry1Ac Livestock Dietary Risk Assessment

Expression levels of the Cry1Ac protein in DAS-81419-2 soybean were used with conservative (i.e. protective) livestock dietary consumption data for soybean to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the Cry1Ac protein in DAS-81419-2 soybean, indicating no concern for adverse effects from acute dietary exposure (Papineni & Cleveland 2012a).

The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls and aspirated grain fractions, and optional forage. Numerically, there is little concentration of protein from raw soybean into soybean meal, because the crude protein content of whole soybeans is ~43% and commercial soybean meal is typically sold as either 44 or 48% protein concentrate with a full range from 38 to 48% crude protein depending on the process used (Iowa Soybean Association 2010, LaBudde Group Inc.). Hence a concentration value of 1.1X is used below for soybean meal. Soybean hulls contain ~ 12% crude protein (Boyle 1999), therefore the ratio of 12/43 is used to estimate the 0.28X reduction of protein in hulls relative to the whole seed. A 20X concentration of the seed residue has been assumed for potential aspirated grain exposure used as animal feed in the US (based on EPA OPP default procedures). Hulls are not considered a blended commodity and therefore, use of maximum level, the highest average field trial (HAFT) value for livestock assessments is most appropriate.

An assessment for livestock exposure was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (EPA 2008). All the components relevant for live stock assessments

could be found in Table 1 of Ref (Papineni & Cleveland 2012a). The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average value of 1.14 ng/mg (or ppm) for Cry1Ac protein in DAS-81419-2 soybean meal Ref Table 2 of (Papineni & Cleveland 2012a). This value for soybean meal has also been used to estimate exposure to soybean feeds for which there was no direct expression measurement. A 20X concentration of the seed residue has been assumed for potential aspirated grain exposure. Because meal and seed are both protein concentrates, they are not simultaneously used in a US feed diet and for the assessment in this report meal is considered. The soybean seed HAFT was used with a processing factor of 0.28X to derive an HAFT of 0.34 ng/mg (or ppm) for soybean hulls. When forage was included, a value of 10.28 ng/mg was used as the Highest Residue as the animal feed; forage is only an input for dairy cattle in the US model.

These livestock diets have been built based on the traditional use of the unmodified counterpart per US EPA procedures; and estimates of dietary exposure are conservative (and protective) in that they have assumed 100% replacement of the unmodified counterpart.

US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (EPA 2008):

Beef: Finishing or feedlot beef (body weight at slaughter, 1200 lb or **544 kg,** daily feed intake of 20 lb or **9 kg** dry matter feed). Feedlot rations in the finishing stage consist of high amounts of grain or grain supplements (80% CC), forages (15% R), and protein sources
(5% PC) in last 120 to 180 days (4 to 6 months) before slaughter at **16 to18 months of age.**

Dairy: Mature lactating cow (body weight, 1350 lb or **612 kg**, daily feed intake of 53 lb or **24 kg dry matter feed,** and producing average of 90 lb of milk a day). Feed rations include forages (45% R), grain or grain supplements (45% CC), and protein source (10% PC). Dairy cows generally calve at **24 to 28 months of age**. The usual length of lactation is 250 to
450 days, with a 305 day lactation being the standard. Dairy cows are usually slaughtered after 2 or 3 calves. The average productive life span of the mature lactating dairy cow is 3 to 4 years.

Poultry: Chicken: Laying hen (body weight, 4.2 lb or **1.9 kg**, average daily intake of
52 grams or **0.052 kg of feed**). Laying hens are usually slaughtered **after 18 months**. A daily ration includes grain or grain supplement (75% CC) and protein source (25% PC). Alternate poultry would be frying and rotisserie chickens weighing 3 to 4 lb, with an average life span of 38 to 42 days. The broiler diet contains 85% CC and 15% PC.

Swine: Finishing or Market hog (body weight, up to 250 lb or **113 kg**, average daily intake of 6.8 lb or **3.1 kg of feed**). Hogs are slaughtered in **5 to 8 months**. In general, daily ration consists of high grain or grain supplement (85% CC) and oilseed meal (15% PC).

The above assumptions apply for finishing animals in US feedlots. For cattle, a younger animal would receive a higher percentage of forage than grain, but analysis of younger animals would not result in substantially different overall conclusion given the low toxicity of the Cry1Ac protein. In addition, the

higher values of 10.28 ppm of Cry1Ac protein for forage are assumed at 100% DAS transgenic soybean. In reality, exposure via forage will be lower, given the average values in forage are slightly lower and more importantly market adoption of DAS-81419-2 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled (Table 23).

Table 23. Intake animal dietary burdens for livestock from Cry1Ac.

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	Cry1Ac (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls	R	90	15	20	Nu	Nu	0.34	0.06	0.08	-	-
Aspirated grain**	CC	85	5	Nu	Nu	Nu	20.8	1.22	-	-	-
Soybean seed	PC	89	*Meal used*	*Meal used*	*Meal used*	*Meal used*	1.04	-	-	-	-
Soybean meal*	PC	NA	*5*	*10*	25	15	1.14	0.06	0.12	0.29	0.17
Soybean Forage	R	35	Nu	20	Nu	Nu	10.28	-	5.87	-	-
							Total	**1.34**	**6.07**	**0.29**	**0.17**

* estimate based on measured value for seed

**based on theoretical estimate of 20X the value in soybean seed

Nu – not used

Because only soybean feeds are considered, the nutritional balance of the diets is assumed to be comprised of unmodified feeds. Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal is reported (Table 24).

Table 24. Livestock daily dose estimates of Cry1Ac protein from soybean seeds.

	Cattle		Swine	Poultry
	Beef	**Dairy**	**Finishing**	**Broiler**
Assessment without forage				
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum Cry1Ac intake (mg/kg feed)	1.34	0.20	0.17	0.29
Maximum intake (mg/kg bw)	0.02	0.01	0.005	0.01
MOE vs Mammalian NOEL	31575	89250	150095	88196
Assessment with forage				
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum intake (mg/kg bw)	0.02	0.24	0.00	0.01
Maximum Cry1Ac intake (mg/kg feed)	1.34	6.07	0.17	0.29
Maximum intake (mg/kg bw)	0.02	0.24	0.00	0.01
MOE vs Mammalian NOAEL	31575	2941	150095	88196

The highest exposed US animal is the beef cow with 0.02 mg Cry1Ac /kg bw estimate. Lower estimates for dairy cattle, swine and poultry were $\leq$0.01 mg/kg bw (Table 24). For the worst-case diet with forage, the highest exposed US animal is the dairy cow with a 0.24 mg Cry1Ac /kg bw estimate. When any of these values are compared to the acute mammalian NOEL of >700 mg/kg bw, there is an adequate margin of safety for livestock because the MOEs are very large >2000. Variations in livestock feed diets elsewhere in the world could result in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for Cry1Ac protein in soybean.

4.2 b Cry1F Livestock Dietary Risk Assessment

Expression levels of the Cry1F protein in DAS-81419-2 soybean were used with conservative (i.e. protective) livestock dietary consumption data for soybean to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the Cry1F protein in DAS-81419-2 soybean, indicating no concern for adverse effects from acute dietary exposure (Papineni & Cleveland 2012b).

The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls and aspirated grain fractions, and optional forage. Numerically, there is little concentration of protein from raw soybean into soybean meal, because the crude protein content of whole soybeans is ~43% and commercial soybean meal is typically sold as either 44 or 48% protein concentrate with a full range from 38 to 48% crude protein depending on the process used (Iowa Soybean Association 2010, LaBudde Group Inc.). Hence a concentration value of 1.1X is used below for soybean meal. Soybean hulls contain ~ 12% crude protein (Boyle 1999), therefore the ratio of 12/43 is used to estimate the 0.28X reduction of protein in hulls relative to the whole seed. A 20X concentration of the seed residue has been assumed for potential aspirated grain exposure used as animal feed in the US (based on EPA OPP default procedures). Hulls are not considered a blended commodity and therefore, use of maximum level, the highest average field trial (HAFT) value for livestock assessments is most appropriate.

An assessment for livestock exposure was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (EPA 2008). All the components relevant for live stock assessments could be found in Table 2 of Ref (Papineni & Cleveland 2012b).The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls and aspirated grain fractions, and optional forage. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average value of 15.18 ng/mg (or ppm) for Cry1F protein in DAS-81419-2 soybean meal. This value for soybean meal has also been used to estimate exposure to soybean feeds for which there was no direct expression measurement. A 20X concentration of the seed residue has been assumed for potential aspirated grain exposure. Because meal and seed are both protein concentrates, they are not simultaneously used in a US feed diet and for the assessment in this report meal is considered. The soybean seed HAFT was used with a processing factor of 0.28X to derive an HAFT of 4.54 ng/mg (or ppm) for soybean hulls. When forage was included, a value of 40.23 ng/mg was used as the Highest Residue as the animal feed; forage is only an input for dairy cattle in the US model.

These livestock diets have been built based on the traditional use of the unmodified counterpart per US EPA procedures; and estimates of dietary exposure are conservative (and protective) in that they have assumed 100% replacement of the unmodified counterpart.

US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (EPA 2008):

Beef: Finishing or feedlot beef (body weight at slaughter, 1200 lb or **544 kg,** daily feed intake of 20 lb or **9 kg** dry matter feed). Feedlot rations in the finishing stage consist of high amounts of grain or grain supplements (80% CC), forages (15% R), and protein sources (5% PC) in last 120 to 180 days (4 to 6 months) before slaughter at **16 to18 months of age.**

Dairy: Mature lactating cow (body weight, 1350 lb or **612 kg**, daily feed intake of 53 lb or **24 kg dry matter feed,** and producing average of 90 lb of milk a day). Feed rations include forages (45% R), grain or grain supplements (45% CC), and protein source (10% PC). Dairy cows generally calve at **24 to 28 months of age**. The usual length of lactation is 250 to 450 days, with a 305 day lactation being the standard. Dairy cows are usually slaughtered after 2 or 3 calves. The average productive life span of the mature lactating dairy cow is 3 to 4 years.

Poultry: Chicken: Laying hen (body weight, 4.2 lb or **1.9 kg**, average daily intake of 52 grams or **0.052 kg of feed**). Laying hens are usually slaughtered **after 18 months**. A daily ration includes grain or grain supplement (75% CC) and protein source (25% PC). Alternate poultry would be frying and rotisserie chickens weighing 3 to 4 lb, with an average life span of 38 to 42 days. The broiler diet contains 85% CC and 15% PC.

Swine: Finishing or Market hog (body weight, up to 250 lb or **113 kg**, average daily intake of 6.8 lb or **3.1 kg of feed**). Hogs are slaughtered in **5 to 8 months**. In general, daily ration consists of high grain or grain supplement (85% CC) and oilseed meal (15% PC).

The above assumptions apply for finishing animals in US feedlots. For cattle, a younger animal would receive a higher percentage of forage than grain, but analysis of younger animals would not result in substantially different overall conclusion given the low toxicity of the Cry1F protein. In addition, the higher values of 40.23 ppm of Cry1F protein for forage are assumed at 100% DAS transgenic soybean. In reality, exposure via forage will be lower, given the average values in forage are slightly lower and more importantly market adoption of DAS-81419-2 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled (Table 25).

Table 25. Intake animal dietary burdens for livestock from Cry1F.

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	Cry1F (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls	R	90	15	20	Nu	Nu	**4.54**	0.76	1.01	-	-
Aspirated grain**	CC	85	5	Nu	Nu	Nu	**276**	16.24	-	-	-
Soybean seed	PC	89	*Meal used*	*Meal used*	*Meal used*	*Meal used*	**13.8**	-	-	-	-
Soybean meal*	PC	NA	*5*	*10*	25	15	**15.18**	0.83	1.65	3.80	2.28
Soybean Forage	R	35	Nu	20	Nu	Nu	**40.23**	-	22.99	-	-
							Total	**17.82**	**25.65**	**3.80**	**2.28**

* estimate based on measured value for seed

**based on theoretical estimate of 20X the value in soybean seed

Nu – not used

Because only soybean feeds are considered, the nutritional balance of the diets is assumed to be comprised of unmodified feeds. Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal (Table 26).

Table 26. Livestock daily dose estimates of Cry1F protein from soybean seeds.

	Cattle		Swine	Poultry
	Beef	**Dairy**	**Finishing**	**Broiler**
Assessment without forage				
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum Cry1F intake (mg/kg feed)	17.83	2.66	2.28	3.80
Maximum intake (mg/kg bw)	0.29	0.10	0.063	0.10
MOE vs Mammalian NOEL	2034	5752	9593	5769
Assessment with forage				
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum Cry1F intake (mg/kg feed)	17.83	25.65	2.28	3.80
Maximum intake (mg/kg bw)	0.29	1.01	0.06	0.10
MOE vs Mammalian NOEL	2034	596	9593	5769

The highest exposed US animal is the beef cow with 0.29 mg Cry1F/kg bw estimate. Lower estimates for dairy cattle, swine and poultry were $\leq$0.10 mg/kg bw (Table 26). For the worst-case diet with forage, the highest exposed US animal if the dairy cow with a 1.01 mg Cry1F/kg bw estimate. When any of these values are compared to the acute mammalian NOEL of >600 mg/kg bw, there is an adequate margin of safety for livestock because the MOEs are large >600. Variations in livestock feed diets elsewhere in the world could result in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for Cry1F protein in soybean.

4.2 c PAT Livestock Dietary Risk Assessment

Expression levels of the PAT protein in DAS-81419-2 soybean were used with conservative (i.e. protective) livestock dietary consumption data for soybean to estimate dietary exposure. In addition, the relevance of the exposure estimate is placed into context based on the known mammalian toxicity information. A dietary exposure assessment reveals large margins of exposure (MOE) values for the PAT protein in DAS-81419-2 soybean, indicating no concern for adverse effects from acute dietary exposure (Papineni & Cleveland 2012c).

The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls and aspirated grain fractions, and optional forage. US typically uses highest average field trial (HAFT) value for animal feed inputs but uses average residue values for blended commodities like grain and meal. Numerically, there is little concentration of protein from raw soybean into soybean meal, because the crude protein content of whole soybeans is ~43% and commercial soybean meal is typically sold as either 44 or 48% protein concentrate with a full range from 38 to 48% crude protein depending on the process used (Iowa Soybean Association 2010, LaBudde Group Inc.). Hence a concentration value of 1.1X is used below for soybean meal. Soybean hulls contain ~ 12% crude protein (Boyle 1999), therefore the ratio of 12/43 is used to estimate the 0.28X reduction of protein in hulls relative to the whole seed. A 20X concentration of the seed residue has been assumed for potential aspirated grain exposure used as animal feed in the US (based on EPA OPP default procedures). Hulls are not considered a blended commodity and therefore, use of maximum level, the highest average field trial (HAFT) value for livestock assessments is most appropriate.

An assessment for livestock exposure was conducted based on the Maximum Reasonably Balanced Diet (MRBD) animal burden procedures (EPA 2008). . All the components relevant for live stock assessments could be found in Table 3 of Ref (Papineni & Cleveland 2012c). The soybean commodity forms that are considered potential animal feeds are seed, meal, hulls and aspirated grain fractions, and optional forage. The MRBD guidance has been used to construct a maximum soybean feed contribution for swine, poultry and cattle based on the average value of 0.95 ng/mg (or ppm) for PAT protein in DAS-81419-2 soybean meal. This value for soybean meal has also been used to estimate exposure to soybean feeds for which there was no direct expression measurement. A 20X concentration of the seed residue has been assumed for potential aspirated grain exposure. Because meal and seed are both protein concentrates, they are not simultaneously used in a US feed diet and for the assessment in this report meal is considered. The soybean seed HAFT was used with a processing factor of 0.28X to derive an HAFT of 0.3 ng/mg (or ppm) for soybean hulls. When forage was included, a value of 5.69 ng/mg was used as the Highest Residue as the animal feed; forage is only an input for dairy cattle in the US model.

These livestock diets have been built based on the traditional use of the unmodified counterpart per US EPA procedures; and estimates of dietary exposure are conservative (and protective) in that they have assumed 100% replacement of the unmodified counterpart. The presence of PAT protein in general soybeans is not anticipated to have impact for feed ration formulation, because nutrient composition

analyses have shown that an alternate DAS-68416-4 soybean (which also contains the PAT protein) is substantially equivalent to conventional soybean (Smith-Drake et al 2009), per the general OECD and ILSI guidance (ILSI 2006, ILSI 2010, OECD 2001).

US EPA currently assumes the following for reference animals for dietary assessments based on animals in finishing or feedlots (EPA 2008):
Beef: Finishing or feedlot beef (body weight at slaughter, 1200 lb or **544 kg,** daily feed intake of 20 lb or **9 kg** dry matter feed). Feedlot rations in the finishing stage consist of high amounts of grain or grain supplements (80% CC), forages (15% R), and protein sources
(5% PC) in last 120 to 180 days (4 to 6 months) before slaughter at **16 to18 months of age.**

Dairy: Mature lactating cow (body weight, 1350 lb or **612 kg**, daily feed intake of 53 lb or **24 kg dry matter feed,** and producing average of 90 lb of milk a day). Feed rations include forages (45% R), grain or grain supplements (45% CC), and protein source (10% PC). Dairy cows generally calve at **24 to 28 months of age**. The usual length of lactation is 250 to
450 days, with a 305 day lactation being the standard. Dairy cows are usually slaughtered after 2 or 3 calves. The average productive life span of the mature lactating dairy cow is 3 to 4 years.

Poultry: Chicken: Laying hen (body weight, 4.2 lb or **1.9 kg**, average daily intake of
52 grams or **0.052 kg of feed**). Laying hens are usually slaughtered **after 18 months**. A daily ration includes grain or grain supplement (75% CC) and protein source (25% PC). Alternate poultry would be frying and rotisserie chickens weighing 3 to 4 lb, with an average life span of 38 to 42 days. The broiler diet contains 85% CC and 15% PC.

Swine: Finishing or Market hog (body weight, up to 250 lb or **113 kg**, average daily intake of 6.8 lb or **3.1 kg of feed**). Hogs are slaughtered in **5 to 8 months**. In general, daily ration consists of high grain or grain supplement (85% CC) and oilseed meal (15% PC).

The above assumptions apply for finishing animals in US feedlots. For cattle, a younger animal would receive a higher percentage of forage than grain, but analysis of younger animals would not result in substantially different overall conclusion given the low toxicity of the PAT protein. In addition, the higher values of 5.69 ppm of PAT protein for forage are assumed at 100% DAS transgenic soybean. In reality, exposure via forage will be lower, given the average values in forage are slightly lower and more importantly market adoption of DAS-81419-2 soybean will not be 100 percent. The resulting intake dietary burden for animal feeds is totalled (Table 27).

Table 27. Intake animal dietary burdens for livestock from PAT.

			Dietary Contribution (%)					Animal Dietary Burden (ppm)			
Feedstuff	Type	Dry Matter (%)	Beef	Dairy	Poultry	Pig	PAT (ppm)	Beef	Dairy	Poultry	Pig
Soybean Hulls	R	90	15	20	Nu	Nu	**0.30**	0.05	0.07	-	-
Aspirated grain**	CC	85	5	Nu	Nu	Nu	**17.2**	1.01	-	-	-
Soybean seed	PC	89	*Meal used*	*Meal used*	*Meal used*	*Meal used*	**0.86**	-	-	-	-
Soybean meal*	PC	NA	*5*	*10*	25	15	**0.95**	0.05	0.10	0.24	0.14
Soybean Forage	R	35	Nu	20	Nu	Nu	**5.69**	-	3.25	-	-
							Total	**1.11**	**3.42**	**0.24**	**0.14**

* estimate based on measured value for seed

**based on theoretical estimate of 20X the value in soybean seed

Nu – not used

Because only soybean feeds are considered, the nutritional balance of the diets is assumed to be comprised of unmodified feeds. Use of the reference animal weight and feed consumption allows for a translation to daily dose by animal is reported (Table 28).

Table 28. Livestock daily dose estimates of PAT protein from soybean seeds.

	Cattle		Swine	Poultry
	Beef	**Dairy**	**Finishing**	**Broiler**
Assessment without forage				
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum PAT intake (mg/kg feed)	1.11	0.17	0.14	0.24
Maximum intake (mg/kg bw)	0.02	0.01	0.004	0.01
MOE vs Mammalian NOAEL	272272	750000	1301843	761218
Assessment with forage				
Body weight (kg)	544	612	113	1.9
Daily Maximum Feed [kg Dry Matter (DM)]	9	24	3.1	0.052
Maximum PAT intake (mg/kg feed)	1.11	3.42	0.14	0.24
Maximum intake (mg/kg bw)	0.02	0.13	0.004	0.01
MOE vs Mammalian NOAEL	272272	37281	1301843	761218

The highest exposed US animal is the beef cow with 0.02 mg PAT/kg bw estimate. Lower estimates for dairy cattle, swine and poultry were $\leq$0.01 mg/kg bw (Table 28). For the worst-case diet with forage, the highest exposed US animal is the dairy cow with a 0.13 mg PAT/kg bw estimate. When any of these values are compared to the acute mammalian NOAEL of >5000 mg/kg bw, there is an adequate margin of safety for livestock because the MOEs are very large >37000. Variations in livestock feed diets elsewhere in the world could result in slight changes in the calculated values, but these global variations in diet are not expected to alter the conclusion regarding the large margin of safety afforded livestock animals for PAT protein in soybean.

4.3 DAS-81419-2 Soybean is "As Safe As" Conventional Soybean

The data and information presented in this submission support the conclusion that food and feed derived from DAS-81419-2 soybean will be as safe and nutritious as those derived from conventional soybean. This conclusion was based on 1) detailed molecular characterization of DAS-81419-2 soybean, 2) safety assessment of the introduced proteins including Cry1Ac, Cry1F, and PAT, 3) nutrient composition analysis of DAS-81419-2 soybean forage and grain, and 4) evaluation of endogenous allergen levels in DAS-81419-2 soybean.

DAS-81419-2 soybean was genetically modified to express two insecticidal proteins, Cry1Ac and Cry1F, and phosphinothricin *N*-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; MXB-7 (also described as 3006-210-23 or DAS-21023-5 expressing Cry1Ac) and MXB-9 (also described as 281-24-236 or DAS-24236-5 expressing Cry1F) (FSANZ 2005). 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 A2704-12 (also described as ACS-GMØØ5-3) (FSANZ 2004), Herculex® I corn DAS-01507-1 (also described as TC1507) (FSANZ 2003). MXB-7 (also described as 3006-210-23 or DAS-21023-5 expressing Cry1Ac) and MXB-9 (also described as 281-24-236 or DAS-24236-5 expressing Cry1F) comprising WideStrike® cotton (FSANZ 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.

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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 (EPA 2011, Mendelsohn et al 2003, 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 mouse acute oral toxicity 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.

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 digestibility in simulated gastric fluid. The proteins have a very low acute toxicity potential and are rapidly degraded in simulated gastric fluid. Bioinformatics 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 overall low expression levels of Cry1Ac and Cry1F proteins in DAS-81419-2 soybean present a low exposure risk to humans and animals, and the results of the overall safety assessment of the proteins indicate that they are unlikely to cause allergenic or toxic effects in humans or animals.

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. Bioinformatics analyses revealed no meaningful homologies to known or putative allergens or toxins for the PAT amino acid sequence. The PAT protein hydrolysed rapidly in simulated gastric fluid. There was no evidence of acute toxicity of the PAT protein in mice. 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 soybean 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 demonstrate that DAS-81419-2 soybean is substantially equivalent to conventional soybean. Endogenous allergen analyses indicate that the genetic modification used to generate DAS-81419-2 soybean did not alter the endogenous allergen content compared to the non-transgenic soybean.
Because DAS-81419-2 soybean is compositionally similar to conventional soybean, and Cry1Ac, Cry1F and PAT proteins have a history of safe use, no significant impact is expected on human or animal health via commodity food and feed soybean products.

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