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Request for an Extension of Determination of Nonregulated Status for Lepidopteran-Protected Soybean MON 87751

The undersigned submits this request under 7 CFR 340.6 to request that the Administrator make a determination that the article should not be regulated under 7 CFR 340

November 25, 2013

OECD Unique Identifier: MON-87751-7

Monsanto Submission Number: SY258-13U1

Submitted by:
William R. Reeves, Ph.D.
Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167
Phone: (314) 694-2662
Fax: (314) 694-3080
E-mail: william.r.reeves@monsanto.com

Prepared by:
L. Barberis, B.S., R. Hernan, Ph.D., D. Mahadeo, B.S., W. Oliveira, Ph.D., W. Reeves, Ph.D., D. Stojsin, Ph.D., E. Webb, M.S., J. Whitsel, M.S.

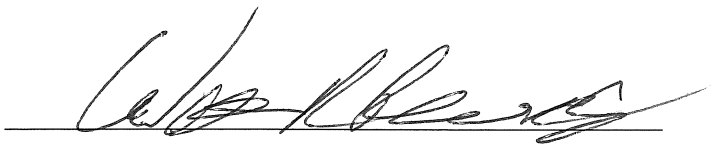
Contributors and/or Principal Investigators:
S. Arackal, M.S., P. Bachman, Ph.D., J. Blankenship, M.S., D. Carson, Ph.D., B. Chen Ph.D., S. Donelson, Ph.D., S. Dubelman, Ph.D., C. Garnaat, M.S., X. Gu, Ph.D., K. Huizinga, Ph.D., C. Jiang, Ph.D., H. Kang, M.S., D. Kovalic, Ph.D., K. Lawry, M.S., S. Levine, Ph.D., E. Leyva-Guerro, Ph.D., K. Liu, Ph.D., S. Martinelli, Ph.D., C. Meng, Ph.D., H. Moon, Ph.D., S. Mozaffar, M.S., I. Nahirna, M.S., A. Nemali, M.S., E. Park, Ph.D., K. Robinson, Ph.D., K. Skottke, Ph.D., R. Wang, M.S., Y. Yan, Ph.D.

RELEASE OF INFORMATION

Monsanto is submitting the information in this request for review by the USDA as part of the regulatory process. Monsanto understands that the USDA complies with the provisions of the Freedom of Information Act (FOIA). In the event the USDA receives a FOIA request, pursuant to 5 U.S.C., § 552, and 7 CFR 1, covering all or some of the information in this request, Monsanto expects that, in advance of the release of the document(s), USDA will provide Monsanto with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, *e.g*., responsiveness, confidentiality, and/or competitive concerns. Monsanto understands that a CBI-deleted copy of this information may be made available to the public in a reading room and upon individual request as part of a public comment period. Monsanto also understands that when deemed complete, a copy of the request may be posted to the USDA-APHIS BRS website or other U.S. government websites (*e.g.*, www.regulations.gov). Except in accordance with the foregoing, Monsanto does not authorize the release, publication or other distribution of this information without Monsanto's prior notice and consent.

CERTIFICATION

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



William R. Reeves, Ph.D.
Regulatory Affairs Manager

Address:
Monsanto Company
800 North Lindbergh Blvd., C3SD
St. Louis, MO 63167

Tel: (314)-694-2662
Fax: (314)-694-3080

EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has responsibility under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S.

APHIS may extend a determination of nonregulated status to additional regulated articles, upon finding that the additional regulated article does not pose a potential for plant pest risk, and should therefore not be regulated (7 CFR 340.6(e)). Such a finding is based on an evaluation of the similarity of the regulated article to the antecedent organism, i.e., an organism that has already been the subject of a determination of nonregulated status by APHIS under § 340.6, and that is used as a reference for comparison to the regulated article under consideration under the regulations.

USDA-APHIS granted Monsanto's petition for the antecedent organism, MON 87701, in 2011 upon finding that MON 87701 did not pose a plant pest risk different from that of conventional soybean. The data and information in this request for an extension demonstrate that MON 87751, likewise does not pose a plant pest risk and the conclusions reached for MON 87701 also apply to MON 87751.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status for the biotechnology-derived soybean product, MON 87751, any progeny derived from crosses between MON 87751 and conventional soybean, and any progeny derived from crosses of MON 87751 with biotechnology-derived soybean that have previously been granted nonregulated status under 7 CFR 340.

Product Description

Monsanto Company has developed insect-protected soybean MON 87751 that produces the CrylA.105 and Cry2Ab2 insecticidal (Cry) proteins. Cry1A.105 is a modified Cry1A protein derived from *Bacillus thuringiensis*. Cry2Ab2 is derived from *B. thuringiensis* subsp. *kurstaki*. The CrylA.105 and Cry2Ab2 proteins provide protection from feeding damage caused by targeted lepidopteran insect pests. Studies conducted with MON 87751 demonstrated efficacy against key soybean pests including *Crocidosema aporema* (bean shoot moth), *Rachiplusia nu* (sunflower looper) and *Spodoptera frugiperda* (fall armyworm). Cry1A.105 and Cry2Ab2 are also known to be active against lepidopteran soybean pests such as *Anticarsia gemmatalis* (velvetbean caterpillar), *Chrysodeixis includens* (soybean looper) and *Helicoverpa zea* (corn earworm). The season-long expression pattern of Cry1A.105 and Cry2Ab2 in MON 87751 is expected to control target insects that are heterozygous for resistance genes specific to one of the proteins and provide an effective tool in managing potential insect resistance, thus prolonging the durability of this product. MON 87751 is expected to provide benefits to growers similar to those obtained by use of other lepidopteran-protected crop varieties, including reduced use of broad spectrum insecticides, increased yield protection and increased worker safety.

MON 87751 will be combined, through traditional breeding methods, with other deregulated biotechnology-derived traits to provide additional protection against lepidopteran soybean pests as well as tolerance to multiple herbicides. These next generation combined-trait soybean products will offer the ability to maximize grower choice, improve production efficiency and increase pest control and weed control durability.

The southeastern states within the U.S. are consistently affected by lepidopteran pests but represent a small portion of total U.S. soybean production. Lepidopteran pressure is greater in South America and accordingly, the initial commercial cultivation of MON 87751 is currently targeted for South America. If MON 87751 were to be grown commercially in the U.S., it would be subject to all U.S. Environmental Protection Agency (U.S. EPA) commercial planting registration requirements.

Data in petition 09-082-01p demonstrated that the antecedent organism, MON 87701, does not pose a plant pest risk. Likewise, data in this request demonstrate that MON 87751 does not pose a plant pest risk as that term is defined by the Plant Protection Act and thus, Monsanto requests full deregulation of MON 87751 at this time.

Data and Information Presented Confirm the Lack of Plant Pest Potential and the Food and Feed Safety of MON 87751 Compared to Conventional Soybean

The data and information presented in this request demonstrate that MON 87751 is agronomically, phenotypically, and compositionally equivalent to commercially cultivated soybean. Moreover, the data and information presented herein demonstrate that MON 87751 is not expected to pose an increased plant pest risk, including weediness, compared to commercially cultivated soybean. Multiple, well-established lines of evidence confirm the food and feed safety and the lack of plant pest potential of MON 87751.

- Soybean is a familiar crop that does not possess any of the attributes commonly associated with weeds and has a history of safe consumption. The conventional variety used for the transformation process was included in studies to serve as an appropriate basis of comparison for MON 87751.
- A detailed molecular characterization of the inserted DNA demonstrated a single, intact copy of the expected T-DNA insert at a single locus within the soybean genome. The genetic elements are present in the expected order and are inherited following Mendelian principles.
- The Cry1A.105 and Cry2Ab2 proteins in MON 87751 exhibit no relevant sequence similarities to known allergenic or toxic proteins and are rapidly degraded by simulated gastric fluid. Additionally, the mode-of-action of Cry proteins has also been extensively studied and is well-documented in numerous publications.
- A comprehensive compositional assessment demonstrated that MON 87751 grain and forage are compositionally equivalent to grain and forage from conventional soybean.

- An extensive evaluation of MON 87751 phenotypic and agronomic characteristics and environmental interactions demonstrated MON 87751 has no increased plant pest potential compared to conventional soybean.
- Based on activity spectrum data and exposure assessments, MON 87751 is not expected to affect NTOs, including organisms beneficial to agriculture, differently from conventional soybean under normal agricultural practices.
- An evaluation of current soybean cultivation and management practices supports a conclusion that, aside from the reduced need for insecticide application and the need for appropriate IRM practices, the introduction of MON 87751 is not likely to have an impact on current agronomic, cultivation and management practices for soybean.

Soybean is a Familiar Crop Lacking Weedy Characteristics

Soybean is the most widely grown oilseed in the world, with approximately 251.5 million metric tons of harvested seed produced in 2011, the most recent year for which global data are available. Soybean is grown as a commercial crop in over 35 countries. In the U.S. soybean was planted on approximately 77.2 million acres in 2012, producing 3.06 billion bushels of soybean with a value of $35.7 billion. The major producers of soybean are the U.S., Brazil, Argentina, China, India, Paraguay and Canada, which accounted for approximately 95% of the global soybean production in 2011.

The commercial soybean species in the U.S. (*Glycine max* L. Merr.) does not exhibit weedy characteristics and does not invade established ecosystems. Soybean is not listed as a weed in major weed references, nor is it present on the lists of noxious weed species maintained by the federal government. Soybean does not possess attributes commonly associated with weeds, such as the ability to disperse, invade, or become a dominant species in new or diverse landscapes, nor does it possess the ability to compete well with native vegetation. Soybean seed has a pronounced lack of dormancy and can germinate quickly under adequate temperature and moisture conditions, potentially leading to its presence as a volunteer plant. Volunteer soybean plants, however, are generally killed by frost during the autumn or winter of the year they germinate. Furthermore, if volunteer plants survive, they do not compete well with the succeeding crop, and are controlled readily via mechanical or chemical means. Finally, wild populations of *Glycine* species are not known to exist in the U.S., therefore there is no opportunity for soybean, including MON 87751, to outcross to wild or weedy relatives.

MON 87751 was derived from a single plant transformant of soybean variety A3555. A3555 was used as the conventional soybean comparator to support the safety assessment of MON 87751. MON 87751 and A3555 have similar genetic backgrounds with the exception of the *cry1A.105* and *cry2Ab2* expression cassettes, allowing the potential effects of the genetic insert and the expressed Cry1A.105 and Cry2Ab2 proteins to be assessed in an unbiased manner using a comparative safety assessment.

Molecular Characterization Verified the Integrity and Stability of the Inserted DNA in MON 87751

Similar to the antecedent organism, MON 87701, MON 87751 was developed through Agrobacterium tumefaciens-mediated transformation of conventional soybean. PV-GMIR13196 was the plasmid vector for MON 87751. PV-GMIR13196 contains two separate T-DNAs that are each delineated by left and right border regions. The first T-DNA, designated as T-DNA I, contains the *crylA.105* and *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains marker genes that allow for simplified selection of transformed tissue. During transformation, both T-DNAs were inserted into the soybean genome. Subsequently, traditional breeding, segregation, selection and screening were used to isolate those plants that contain the *crylA.105* and *cry2Ab2* expression cassettes (T-DNA I) and do not contain the marker gene expression cassettes (T-DNA II), resulting in the production of marker-free, MON 87751.

Characterization of the DNA insert in MON 87751 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 87751 contains one copy of T-DNA I containing the *cry1A.105* and *cry2Ab2* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. The results of this characterization also confirm that T-DNA II is not present. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 87751 by Next Generation Sequencing and Junction Sequence Analysis (NGS/JSA) demonstrated that DNA from PV-GMIR13196 DNA was integrated at a single locus.
- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) determined the complete sequence of the single T-DNA I insert from PV-GMIR13196, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This confirmed that the sequence and organization of the T-DNA I insert is identical to the corresponding region of T-DNA I in PV-GMIR13196. Sequencing also confirmed that no vector backbone, T-DNA II, or other unintended plasmid sequences are present in MON 87751. Furthermore, comparison of the genomic organization at the insertion site in MON 87751 to the sequence of the insertion site in conventional soybean demonstrated that no major DNA rearrangement occurred at the insertion site in MON 87751 upon T-DNA integration.
- Generational stability analysis by NGS/JSA demonstrated that the single PV-GMIR13196 T-DNA I insert in MON 87751 was maintained through five breeding generations, thereby confirming the stability of the intended T-DNA I in MON 87751.
- Segregation data confirm that the inserted T-DNA I segregated following Mendelian inheritance patterns which, corroborates the insert stability demonstrated by NGS/JSA and independently establishes the nature of the T-DNA as a single chromosomal locus.

Taken together, the characterization of the genetic modification in MON 87751 demonstrates that a single copy of the intended T-DNA I was stably integrated at a single locus of the soybean genome and that no plasmid backbone or T-DNA II sequences are present in MON 87751.

Data Confirm Cry1A.105 and Cry2Ab2 Protein Safety

A multistep approach was used to characterize and assess the safety of the Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751. The expression level of the Cry1A.105 and Cry2Ab2 proteins in selected tissues of MON 87751 was determined and exposure to humans and animals through diet was evaluated. In addition, the donor organism for the Cry1A.105 and Cry2Ab2 protein coding sequences, *B. thuringiensis*, is ubiquitous in the environment and is not commonly known for human or animal pathogenicity or allergenicity. Bioinformatics analysis determined that the Cry1A.105 and Cry2Ab2 proteins lack structural similarity to known allergens or protein toxins. Cry1A.105 and Cry2Ab2 proteins are rapidly digested in simulated gastric fluid and demonstrate no acute oral toxicity in mice at the highest levels tested, consistent with findings for other Cry proteins. Both Cry1A.105 and Cry2Ab2 are present in lepidopteran-protected MON 89034 maize and both proteins have tolerance exemptions from U.S. EPA in maize. The Cry1A.105 and Cry2Ab2 proteins from MON 87751 share greater than 99% and 97% amino acid identity, respectively, with the Cry1A.105 and Cry2Ab2 proteins expressed in MON 89034. Hence, the consumption of the Cry1A.105 and Cry2Ab2 proteins from MON 87751 or its progeny poses no meaningful risk to human and animal health or an increased plant pest risk. Similar data submitted to USDA-APHIS in petition 09-082-01p for the antecedent organism, MON 87701, supported the same safety conclusions for Cry1Ac.

MON 87751 is Compositionally Equivalent to Conventional Soybean

Compositional analyses were conducted on forage and harvested seed collected from MON 87751, the genetically similar conventional control and a number of conventional soybean varieties grown in the U.S. during 2012 at eight field sites. The compositional analysis, based on the Organisation for Economic Co-operation and Development (OECD) consensus document for soybean, included measurement of nutrients, anti-nutrients and other metabolites in all varieties, including the conventional commercial reference varieties to provide data on the natural variability of each compositional component analyzed. A total of 66 different analytical components were measured (seven in forage and 59 in seed). Of these, 14 had more than 50% of the observations below the assay limit of quantitation and were excluded from statistical analysis. Moisture values for seed and forage were measured for conversion of components to dry weight, but these two components were not statistically analyzed. Of the 50 remaining components statistically assessed, only eight components (protein, glycine, proline, vitamin E, raffinose, and phosphorus in seed, and total fat and neutral detergent fiber (NDF) in forage) showed a significant difference between MON 87751 and the control. For these eight components, the mean difference in component values between MON 87751 and the control was less than the range of the control values and the reference variety values. Mean component values for MON 87751 were also within the

99% tolerance interval calculated with data from the conventional soybean varieties, the range of values reported in the literature and/or the International Life Sciences Institute Crop Composition Database (ILSI-CCDB). These data indicated that the statistically significant differences in these eight components were not compositionally meaningful from a food and feed safety or nutritional perspective.

These results support the overall conclusion that MON 87751 was not a major contributor to variation in component levels in soybean seed and forage and confirmed the compositional equivalence of seed and forage from MON 87751 to conventional soybean. These results support the overall food and feed safety and lack of plant pest risk of MON 87751. Similar data submitted to USDA-APHIS in petition 09-082-01p supported the same conclusions of compositional equivalence for the antecedent organism, MON 87701.

MON 87751 Does Not Change Soybean Plant Pest Potential or Environmental Interactions

Plant pest potential of a biotechnology-derived crop is assessed from the basis of familiarity that the USDA recognizes as an important underlying concept in risk assessment. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant hybrid or variety whose biological properties and plant pest potential are well known. Familiarity considers the biology of the plant, the introduced trait, the receiving environment and the interactions among these factors. This provides a basis for comparative risk assessment between a biotechnology-derived plant and the conventional control. Thus, the phenotypic, agronomic, and environmental interaction assessment of MON 87751 included a genetically similar conventional control as a comparator. This evaluation used a weight-of-evidence approach and considered statistical differences between MON 87751 and the conventional control with respect to reproducibility, magnitude, and directionality. Comparison to a range of concurrently grown commercial reference varieties established the range of natural variability for soybean, and provided a context from which to further evaluate any observed statistical differences. Characteristics assessed included: seed dormancy and germination, pollen morphology, symbiont interactions, plant phenotypic and agronomic observations, and environmental interactions. In field evaluations of plant phenotypic, agronomic, and environmental interaction assessment demonstrated that MON 87751 is comparable to the conventional control. Thus, like the antecedent organism, MON 87701, MON 87751 is not expected to pose an increased plant pest risk compared to conventional soybean.

In laboratory and greenhouse assessments of seed dormancy and germination, pollen viability and morphology and interactions with symbiotic bacteria, MON 87751 was not different from the conventional control. The lack of differences between MON 87751 and the control for well recognized characteristics associated with weediness such as the percentage of hard seed supports a conclusion of no increased weediness of MON 87751 compared to the conventional control. For pollen characteristic assessments, there were no statistically significant differences detected between MON 87751 and the conventional control for pollen viability and diameter, and no visual differences in

general pollen morphology were observed. In an assessment of the symbiotic relationship between *Bradyrhizobium japonicum* and MON 87751, no significant differences were detected between MON 87751 and the conventional control for the measured parameters, including nodule number, shoot total nitrogen, and weight of nodules, shoot material, and root material.

Field evaluations of phenotypic, agronomic, and environmental interaction observations to characterize the plant support the conclusion that MON 87751 did not exhibit indications of increased weediness or plant pest potential compared to conventional soybean. Evaluations were conducted at 17 replicated field sites across U.S. soybean growing areas. These assessments included 12 plant growth and development characteristics, as well as observations for plant responses to abiotic stressors, plant-disease interactions and plant-arthropod interactions. The observed phenotypic characteristics were not different between MON 87751 and the conventional control. Across all 17 sites, there were no statistically significant differences between MON 87751 and the conventional control for any of the assessed characteristics, including early stand count, 50% flowering date, plant lodging, pod shattering, plant height, final stand count, grain moisture, 100 seed weight, and yield. Thus, the phenotypic characteristics of MON 87751 were not altered and there was no indication of increased weediness or plant pest potential compared to the conventional control.

In an assessment of abiotic stress response and disease damage, no differences were observed between MON 87751 and the control for any of the 193 comparisons for the assessed abiotic stressors. No differences were observed between MON 87751 and the control for any of the 191 comparisons for the assessed disease damage. In an assessment of arthropod-related damage, no differences were observed between MON 87751 and the control for any of the 154 comparisons for the assessed arthropods. In an assessment of stink bug damage at five sites, no statistically significant differences were detected between MON 87751 and the control. The lack of differences in plant response to abiotic stress, disease damage, and non-target arthropod-related damage support the conclusion that MON 87751 is not expected to pose an increased plant pest potential compared to the conventional control.

In an assessment of non-target arthropod abundance, no statistically significant differences were detected between MON 87751 and the control for 157 out of 170 comparisons among the collections at the five sites where these evaluations were made. The mean abundance values for MON 87751 were within the reference ranges for all differences detected in arthropod abundance with the exception of differences in the abundance of big-eyed bug, predatory mite, spiders, kudzu bug, plant bug, and thrips. These differences were not consistently detected across collections or sites. Thus, the differences in abundance were not indicative of a consistent response associated with the trait and are not considered biologically meaningful in terms of increased pest potential of MON 87751 compared to the conventional control.

In summary, the phenotypic, agronomic, and environmental interaction data were evaluated to characterize MON 87751, and to assess whether the introduction of the trait in MON 87751 alters the plant pest potential compared to conventional soybean. The

evaluation, using a weight-of-evidence approach, considered the reproducibility, magnitude, and direction of detected differences between MON 87751 and the conventional control, and comparison to the range of the commercial reference varieties. Results from the phenotypic, agronomic, and environmental interaction assessment indicates that MON 87751 does not possess enhanced weediness characteristics, increased susceptibility or tolerance to specific abiotic stressors, diseases, or arthropods, or characteristics that would confer a plant pest risk compared to conventional soybean. Similar data submitted to USDA-APHIS in petition 09-082-01p supported the same conclusions regarding phenotype and environmental interactions for the antecedent organism, MON 87701.

MON 87751 Will Not Negatively Affect Non-target Organisms (NTOs) Including Those Beneficial to Agriculture

An evaluation of the impacts of MON 87751 on NTOs is a component of the plant pest risk assessment. The NTO assessment considered a number of characteristics to evaluate potential hazards to NTOs that provide important ecological functions (pollinators, detritivores, predators, parasites), including threatened and endangered species and organisms beneficial to agriculture. Characteristics evaluated included mode-of-action (MOA), spectrum of insecticidal activity, and exposure levels to the Cry1A.105 and Cry2Ab2 proteins.

The activity spectrum evaluation for the Cry1A.105 and Cry2Ab2 proteins included a diverse set of NTOs. USDA and U.S. EPA reviewed the data for these two proteins when they were originally submitted in support of MON 89034. USDA deregulated MON 89034 and U.S. EPA registered MON 89034 in 2008. The Cry1A.105 and Cry2Ab2 proteins from MON 87751 share greater than 99% and 97% amino acid identity, respectively, with the Cry1A.105 and Cry2Ab2 proteins expressed in MON 89034. Furthermore, the protease-resistant core domains of the modified Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751 share 100% deduced amino acid identity to MON 89034 expressed core domains of the Cry1A.105 and Cry2Ab2 proteins. These domains are responsible for insecticidal activity and specificity. Finally, the Cry1A.105 and Cry2Ab2 proteins in MON 87751 are functionally equivalent to the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034. Therefore, the previously provided activity spectrum evaluation for the Cry1A.105 and Cry2Ab2 proteins in MON 89034 is applicable to MON 87751. After both extensive testing and wide scale cultivation of MON 89034, no adverse impacts to NTOs have been associated with exposure to Cry1A.105 or Cry2Ab2.

Regarding the NTO testing specifically, no adverse effects were observed from the species tested which included one mammal (mouse), two avian species (bobwhite quail, broiler chickens), soil decomposers (earthworm and Collembola), an aquatic invertebrate (*Daphnia magna*) and four beneficial insect species (honeybee, minute pirate bugs, ladybird beetle, and parasitic wasp). Based on results from these studies there is no evidence that MON 87751will affect NTOs or endangered species under normal agricultural practices. NTO data in petition 09-082-01p supported the same conclusion for the antecedent organism, MON 87701.

Deregulation of MON 87751 is Not Expected to Have Effects on Soybean Agronomic Practices

An assessment of current soybean agronomic practices was conducted to determine whether the cultivation of MON 87751 has the potential to impact current soybean agronomic practices. MON 87751 was developed to provide two effective MOAs against targeted lepidopteran soybean pests. Aside from the potential for reduced insecticide sprays and the need to implement appropriate IRM practices when required, the introduction of MON 87751 is not expected to have an impact on current agronomic or management practices in soybean if it were introduced in the U.S. MON 87751 did not differ from conventional soybean in its agronomic, phenotypic, environmental interactions and compositional characteristics and had no difference in response to diseases and non-lepidopteran pests compared to conventional soybean. Based on this assessment, no changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management and volunteer management from the introduction of MON 87751. Consequently, the introduction of MON 87751 is not expected to result in significant impacts on current soybean agronomic practices. Data and information in petition 09-082-01p supported similar conclusions for the antecedent organism, MON 87701.

Conclusion

Based on the data and information presented in this extension request, it is concluded that, like the antecedent organism, MON 87701, MON 87751 is not expected to be a plant pest. Results also support a conclusion of no increased weediness potential of MON 87751 compared to conventional soybean. Therefore, Monsanto Company requests an extension of determination of nonregulated status from APHIS that MON 87751 and any progeny derived from crosses between MON 87751 and other commercial soybean should no longer be subject to regulation under 7 CFR part 340.

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ABBREVIATIONS AND DEFINITIONS[1]

~	approximately
ADF	acid detergent fiber
ANOVA	analysis of variance
AOSA	Association of Official Seed Analysts
APHIS	Animal and Plant Health Inspection Service
bp	base pairs
Bt	*Bacillus thuringiensis*
CFR	Code of Federal Regulations
CTAB	Hexadecyltrimethylammonium bromide
DAP	days after planting
dATP	deoxyadenosine triphosphate
DDI	daily dietary intake
DEEM-FCID	Dietary Exposure Evaluation Model-Food Commodity Intake Database
DHB	2,5-dihydroxybenzoic acid
DTT	dithiothreitol
dw	dry weight
DWCF	dry weight conversion factor
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EUP	experimental use permit
ETS	Excellence Through Stewardship
FA	fatty acid
FDA	U.S. Food and Drug Administration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMOC	fluorenylmethyl chloroformate
fw	fresh weight
Gb	gigabases
ha	hectares
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
ILSI-CCDB	International Life Sciences Institute-Crop Composition Database
IPM	integrated pest management
IRM	insect resistance management
JSC	junction sequence class
kDa	kilodalton
LOD	limit of detection
LOQ	limit of quantitation
MEEC	maximum expected environmental concentration
Mg/ha	megagrams/hectare
MMT	million metric tons

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

MOA	mode-of-action
MOE	margin of exposure
n	number of samples
NDF	neutral detergent fiber
NFDM	nonfat dry milk
NGS/JSA	Next Generation Sequencing/Junction Sequence Analysis
NHANES	National Health and Nutrition Examination Survey
NOAELs	no observable adverse effect levels
NOEC	no observable effect concentration
nt	nucleotide
NTO	non-target organism
OECD	Organisation for Economic Co-operation and Development
OM	organic matter
OPA	o-phthalaldehyde
OSL	over season leaf
PBST	phosphate buffered saline containing 0.05% (v/v) Tween
PCR	polymerase chain reaction
PIP	plant incorporated protectant
PPA	plant protection act
PTH-AA	phenylthiohydantoin-amino acid
SAP	scientific advisory panel
SBV	soybean variant
SD	standard deviation
SDS	sodium dodecyl sulfate
S.E.	standard error
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
sp.	species
TDF	total dietary fiber
T-DNA	transfer DNA
TFA	trifluoroacetic acid
TSSP	tissue-specific site pool
USDA	United States Department of Agriculture
UTR	untranslated region
UV	ultraviolet
v/v	volume to volume

I. RATIONALE FOR THE DEVELOPMENT OF MON 87751

I.A. Basis for the Request for an Extension of Determination of Nonregulated Status under 7 CFR 340.6

The Animal and Plant Health Inspection Service (APHIS) of the United States (U.S.) Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (Title IV Pub. L. 106-224, 114 Stat. 438, 7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS may extend a determination of nonregulated status to additional regulated articles, upon finding that the additional regulated article does not pose a potential for plant pest risk, and should therefore not be regulated (7 CFR 340.6(e)).

USDA-APHIS granted Monsanto's petition for the antecedent organism, MON 87701, in 2011 upon finding that MON 87701 did not pose a plant pest risk different from that of conventional soybean. The data and information in this request for an extension demonstrate that MON 87751, likewise does not pose a plant pest risk and the conclusions reached for MON 87701 also apply to MON 87751.

I.B. Rationale for the Development of Insect-Protected Soybean MON 87751

Soybean is one of the largest U.S. crops in terms of the acreage planted and quantity harvested. The U.S. soybean acreage in the past ten years has varied from approximately 64.7 to 77.5 million acres, with the lower acreage recorded in 2007 and the higher in 2009. Average soybean yields have varied from 33.9 to 43.5 bushels per acre. Soybean production ranged from 2.45 to 3.36 billion bushels over the past ten years, with 2009 being the largest production year on record. According to data from USDA-NASS (2013a), soybean was planted on approximately 77.2 million acres in the U.S. in 2012.

Over the past 60 years, soybean yield per unit area has almost tripled (Soyatech 2008). This increase is credited to the introduction of improved soybean germplasm, development of new varieties, the availability of better field equipment, and the use of herbicide and other pesticides that have greatly reduced crop losses caused by weeds and pests (Soyatech 2008).

On a regional basis, soybean production in certain areas of the U.S. and other soybean production regions such as South America can suffer considerable economic damage as a result of infestation by various soybean insect pests (Higley 1994; Moscardi 1993). In the U.S., the occurrence of soybean insect pests follows a north-south gradient in terms of severity (Higley 1994). Generally, soybean insect pest problems are less severe in the Midwest states than in other soybean producing areas (Higley 1994). The most damaging lepidopteran insects in southern U.S. states are (in order of economic damage) *Helicoverpa zea* (corn earworm/soybean podworm/cotton bollworm), *Chrysodeixis includens* (soybean looper), armyworms (*Spodoptera spp.*) and *Anticarsia gemmatalis* (velvetbean caterpillar) (Musser, et al. 2013).

I.B.1. Benefits of Insect-Protection Traits

According to USDA-NASS statistics, about 18% of the approximately 75 million U.S. soybean acres received insecticide applications in 2011 (USDA-NASS 2013a). Chemical insecticide applications to control lepidopteran infestations in soybean, however, are not always effective. *C. includens* has developed resistance to every synthetic class of insecticide used against it (Thomas and Boethel 1994), and resistance to pyrethroids is widespread across the southern U.S. (Felland, et al. 1990); (Leonard, et al. 1990). Chemical insecticides remain effective against *A. gemmatalis*; however, infestations can quickly reach damaging levels and cause economic loss if insecticides are not applied promptly.

Biological insecticide formulations containing the crystal (Cry) proteins (δ-endotoxin) produced from *Bacillus thuringiensis* for foliar application have been used widely on many crops, including soybean, since the 1960s. However, field efficacy has often been less than desired, because these materials are subject to weathering and deterioration by the elements and must be regularly reapplied or augmented by the use of other chemicals to achieve desired levels of pest control (Bohorova, et al. 1997). One approach to utilize the efficacy of Cry proteins, while avoiding issues related to field stability, has been to introduce genes that encode Cry proteins into plants using biotechnology. In contrast to a foliar application, these biotechnology-derived plants produce the insect control protein within plant cells. This ensures that target insect pests are exposed to it whenever they feed on plants. As a result, control may be more effective, and applications of other insecticides to control the target lepidopteran species may be reduced or eliminated.

I.B.2. Introduction of Insect-Protection Traits

Since 1995, USDA has deregulated numerous crop plants that express Cry proteins to control lepidopteran pests. MON 87701 expresses Cry1Ac and was the first soybean product developed to control lepidopteran pests. For maize, examples of commercial insect-protected products include MON 810 (Cry1Ab), MON 89034 (Cry1A.105 and Cry2Ab2) and TC1507 (Cry1F). For cotton, examples include MON 531 (Cry1Ac), MON 15985 (Cry1Ac and Cry2Ab2), 3006-210-23 (Cry1Ac), 281-24-236 (Cry1F), T304-40 (Cry1Ab) and T303-3 (Cry2Ae).

I.B.3. Development of Insect-Protected Soybean MON 87751

Monsanto Company has developed insect-protected soybean MON 87751 that produces the CrylA.105 and Cry2Ab2 insecticidal (Cry) proteins derived from *B. thuringiensis*. Cry1A.105 is a modified Cry1A protein and Cry2Ab2 is derived from *B. thuringiensis* subsp. *kurstaki*. The CrylA.105 and Cry2Ab2 proteins provide protection from feeding damage caused by targeted lepidopteran insect pests. Studies conducted with MON 87751 demonstrated that this transformation event was not damaged by infestations of *Crocidosema aporema* (bean shoot moth), *Rachiplusia nu* (sunflower looper) and *Spodoptera frugiperda* (fall armyworm). At the same time, neither Cry1A.105 nor Cry2Ab2 exhibits biological activity against coleopteran, hymenopteran or hemipteran insects at exposure concentrations well above field exposure levels (U.S.

EPA 2010c). The season-long expression pattern for Cry1A.105 and Cry2Ab2 in MON 87751 (Section V.C) is expected to control target insects that are heterozygous for resistance genes specific to one of the proteins thereby providing an effective tool in managing potential insect resistance and prolonging product durability. MON 87751 would be most efficacious in soybean production areas where insecticides are typically applied to control lepidopteran insects. Accordingly, MON 87751 is expected to provide benefits similar to those provided by existing lepidopteran-protected crops, including improved control of lepidopteran pests, reduced use of broad spectrum insecticides (Brookes and Barfoot 2012), increased yield protection and increased worker safety.

Several insect-protected crops derived from biotechnology, including maize MON 89034, expressing both Cry1A.105 and Cry2Ab2 as well as lepidopteran protected cotton MON 15985, expressing Cry2Ab2, have been deregulated and registered for commercial release in the U.S. since 2002. The Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751 have greater than 99% and 97% amino acid identity, respectively, to the Cry1A.105 and Cry2Ab2 expressed proteins in MON 89034. Furthermore, the protease-resistant core domains of the modified Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751 share 100% deduced amino acid identity to MON 89034 expressed core domains of the Cry1A.105 and Cry2Ab2 proteins. These domains are responsible for insecticidal activity and specificity (Gill, et al. 1992; Widner and Whiteley 1989). Therefore, MON 87751, though a new event, contains the proteins and associated modes of action which have already received a determination of non-regulated status (USDA-APHIS 2008a).

MON 87751 will be combined, through traditional breeding methods, with other deregulated biotechnology-derived traits to provide additional protection against lepidopteran soybean pests as well as tolerance to herbicides. These next generation combined-trait soybean products will offer the ability to maximize grower choice, improve production efficiency and increase pest and weed control durability.

The southeastern states within the U.S. are affected by lepidopteran pests but represent a relatively small portion of total U.S. soybean production. Lepidopteran pest pressure in soybean is greater in South America and accordingly, the initial commercial cultivation of MON 87751 is currently targeted for South America. If MON 87751 were to be grown on a commercial scale in the U.S., it would be subject to all U.S. Environmental Protection Agency (U.S. EPA) commercial planting registration requirements including the submission of an insect resistance management (IRM) plan. The plant pest profile of MON 87751 supports a determination of nonregulated status. As this request demonstrates, MON 87751 does not pose a plant pest risk as that term is defined by the Plant Protection Act and APHIS. For these reasons, Monsanto Company seeks deregulation of MON 87751 at this time.

I.C. Mode-of-Action

The specificity of Cry proteins is dependent upon the activated form of the toxin binding to specific receptors present in the insect mid-gut (OECD 2007; Pigott and Ellar 2007). Protease enzymes in the mid gut of susceptible insects degrade full-length Cry proteins

into the activated protein. The activated proteins then bind to midgut membrane receptors in susceptible insects, insert into the apical membrane and form pores. Formation of the pores causes loss of osmotic regulation, and eventually leads to cell lysis, which is thought to be responsible for insect death (Gill et al. 1992; Schnepf, et al. 1998; Zhuang and Gill 2003). Non-target organisms, including mammals, do not have receptors that bind activated Cry proteins and are therefore not adversely affected (OECD 2007; Schnepf et al. 1998).

I.D. Efficacy Against Target Organisms

The specificity of Cry proteins is dependent upon binding to specific receptors present in the insect mid-gut (OECD 2007; Pigott and Ellar 2007). These specific receptors are not present in taxa outside of insects, therefore Cry proteins are not expected to adversely affect wild mammals and no adverse effects have been reported in these organisms as well as in non-target birds (OECD 2007; (Schnepf et al. 1998). It has been widely reported in the literature that the insecticidal activity of Cry1 class proteins is specific for lepidopteran insects (Crickmore, et al. 1998; de Maagd, et al. 2001; Romeis, et al. 2006). Within the Cry2 class of proteins, the activity spectrum is slightly broader than within the Cry1 class (Crickmore et al. 1998). For example, Cry2Aa (formerly CryB1) is active against both lepidopteran and dipteran (mosquito) insects (de Maagd et al., 2001). The Cry2Ab2 (formerly CryB2) protein, however, is only active against lepidopteran insects at field exposure concentrations (Widner and Whiteley 1989).

Activity spectrum studies developed for the Cry1A.105 and Cry2Ab2 proteins have previously been evaluated by USDA-APHIS (2011) and U.S. EPA (2010c) in regulatory submissions for MON 89034. The activity of the Cry1A.105 and Cry2Ab2 proteins was shown to be restricted to the order Lepidoptera at field exposure levels. The Cry1A.105 and Cry2Ab2 proteins in MON 87751 are functionally equivalent to the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034. Therefore, the previously provided activity spectrum evaluation for the Cry1A.105 and Cry2Ab2 proteins in MON 89034 is applicable to MON 87751. Subsequent studies with Cry1A.105 and Cry2Ab2 confirmed that both proteins are active against lepidopteran soybean pests such as *Anticarsia gemmatalis* (velvetbean caterpillar), *Chrysodeixis includens* (soybean looper) and *Helicoverpa zea* (corn earworm).

As part of efforts to evaluate the efficacy of MON 87751, Monsanto conducted screenhouse trials in Fontezuela, Argentina during the 2011/2012 growing season with larvae from three lepidopteran soybean pests that are important in Argentina, *C. aporema*, *R. nu*, and *S. frugiperda*. The trials compared MON 87751 to the conventional control, A3555, and were planted as a randomized complete block design with three replicates per entry.

Individual trials were infested with one of the three pest species. Infestations of *C. aporema* were made with 1,500 pupae 21 days after planting (DAP) and 1,500 pupae 39 DAP. Infestations of *R. nu* were made with 500 pupae 29 DAP and 300 pupae 67 DAP. Infestations of *S. frugiperda* were made with 1,500 pupae 19 DAP. Damage by *C. aporema* was rated 26–36 and 28–38 days post-infestation (1st and 2nd infestations,

respectively) by randomly selecting ten plants from each plot and recording the presence of damage and number of damaged points/pods. Damage by *R. nu* was rated 21–40 and 6–24 days post-infestation (1st and 2nd infestations, respectively) and damage by *S. frugiperda* was rated 28–36 and 26–29 days post-infestation (1st and 2nd infestations, respectively) by estimating percent defoliation in each plot.

For *C. aporema*, maximum damage averaged 5.5 ± 2.2 damaged points per plant[2] in the conventional control and 0 ± 0% in MON 87751. For *R. nu*, maximum damage averaged 33.3 ± 3.3% defoliation in the conventional control and 0 ± 0% in MON 87751. For *S. frugiperda*, maximum damage averaged 15.0 ± 2.9% defoliation in the conventional control and 0 ± 0% in MON 87751. This demonstrates that MON 87751 exhibited less damage by lepidopteran pests than the conventional control and supports a conclusion that Cry1A.105 and Cry2Ab2 provide meaningful control of lepidopteran soybean pests.

I.E. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology (USDA-APHIS 1986), the responsibility for regulatory oversight of biotechnology-derived crops falls primarily on three U.S. agencies: U.S. Food and Drug Administration (FDA), USDA, and in the case of plant incorporated protectants, U.S. EPA. Deregulation of MON 87751 by USDA constitutes only one component of the overall regulatory oversight and review of this product. As a practical matter, MON 87751 cannot be released and marketed until U.S. EPA, FDA and USDA have completed their reviews and assessments under their respective jurisdictions.

I.E.1. Submission to FDA

MON 87751 falls within the scope of the 1992 FDA policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (U.S. FDA 1992). In compliance with this policy, Monsanto submitted a food/feed safety and nutritional assessment summary document to FDA.

I.E.2. Submission to U.S. EPA

Substances that are pesticides, as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) [7 U.S.C. §136(u)], are subject to regulation by U.S. EPA. Pesticides produced *in planta*, referred to as plant-incorporated protectants (PIPs), are also subject to regulation by U.S. EPA under FIFRA.

Pursuant to §408(d) of the Federal Food Drug and Cosmetic Act [21 U.S.C. 346 a(d)] Monsanto Company will petition U.S. EPA for an exemption from the requirement of a tolerance for Cry1A.105 and Cry2Ab2 in or on soybean. On July 2, 2008, U.S. EPA

[2]A damaged "point" refers to any point on the plant showing damage. Generally this occurs at the axillary buds and is evidenced by aborted growth, but it can also occur at the terminus of the main stem or on the pods, as evidenced by tying of unfolding leaves and partially consumed pods.

established an exemption from the requirement of a tolerance for residues of the plant-incorporated protectant Cry2Ab2 in maize and cotton (40 CFR 174.519). On July 16, 2008, U.S. EPA established an exemption from the requirement of a tolerance for residues of the plant-incorporated protectant Cry1A.105 in maize (40 CFR 174.502).

Monsanto plans to file an experimental use permit (EUP) application for MON 87751 and the genetic material necessary for its production with U.S. EPA to facilitate further MON 87751 field testing. Monsanto will apply for a Section 3 seed increase registration for Cry1A.105 and Cry2Ab2, and the genetic material (vector PV-GMIR13196) necessary for its production in soybean to allow seed production activities in the U.S. to support markets in South America.

I.E.3. Submissions to Foreign Government Agencies

To support commercial introduction of MON 87751 in South America, regulatory submissions will be made to the appropriate authorities in those countries. All countries that will plant MON 87751 commercially have their own independent and functioning regulatory system to assess the food and feed safety and conduct environmental risk assessments specific to the planting, use and consumption of MON 87751.

Consistent with our commitments to the Biotechnology Industry Organization and the Excellence Through Stewardship Program, Monsanto intends to obtain import approvals from all key soybean import markets with functioning regulatory systems prior to commercialization of MON 87751. As appropriate, notifications will be made to countries that import significant quantities of soybean and soybean products and do not have formal regulatory review processes for biotechnology-derived crops.

II. THE BIOLOGY OF SOYBEAN

The Organisation for Economic Co-operation and Development (OECD) consensus document on the biology of soybean (OECD 2000) provides key information on:

- general description of soybean biology, including taxonomy and morphology and use of soybean as a crop plant
- agronomic practices in soybean cultivation
- geographic centers of origin
- reproductive biology
- cultivated soybean as a volunteer weed
- inter-species/genus introgression into relatives and interactions with other organisms
- a summary of the ecology of soybean

The taxonomic information for soybean is available in the USDA's PLANTS Profile (USDA-NRCS 2013).

To support the evaluation of the plant pest potential of MON 87751 relative to conventional soybean, additional information regarding several aspects of soybean biology can be found elsewhere in this request. This includes: agronomic practices for soybean in Section VIII; volunteer management of soybean in Section VIII.H; and inter-species/genus introgression potential in Section IX.J.

II.A. Soybean as a Crop

The major producers of soybean are the U.S., Brazil, Argentina, China, India, Paraguay and Canada, which accounted for approximately 95% of the global soybean production in 2011 (Table II-1) (ASA 2012). Approximately 33% of the 2011 world soybean production was produced in the U.S. (ASA 2012). The U.S. exported 34.7 MMT of soybeans in 2011, which accounted for 37% of the world's soybean trade (ASA 2012). Approximately 44 MMT of soybeans were crushed in the U.S. in 2011 and used to supply the feed industry for livestock use or the food industry for edible vegetable oil and soybean protein isolates (ASA 2012).

Soybean is used in various food products, including tofu, soy sauce, soymilk, energy bars, and meat products. A major food use for soybean is purified oil, for use in margarines, shortenings, cooking, and salad oils. Soybean oil accounted for approximately 28% of all the vegetable oils consumed globally, and was the second largest source of vegetable oil worldwide, slightly behind palm oil at approximately 33% share (ASA 2012). Soybean meal is used as a supplement in feed rations for livestock. Soybean meal is the most valuable component obtained from processing the soybean, accounting for roughly 50-75% of its overall value. Industrial edible and industrial uses of soybean range from a carbon/nitrogen source in the production of yeasts via fermentation to the manufacture of soaps, inks, paints, disinfectants, and biodiesel.

Industrial uses of soybean have been summarized by the American Soybean Association (ASA 2012).

Cultivated soybean plants are annuals, and they reproduce solely by means of seeds. Volunteer soybean in rotational crops is typically not a concern in most environments where soybean is cultivated (CFIA 1996; OECD 2000). Although some soybean varieties can exhibit dormancy characteristics, seed remaining in the field after harvest will often imbibe water (Lersten and Carlson 2004), germinate and be killed by frost or field cultivation. If the soybean seed did become established, volunteer plants would not compete well with the succeeding crop, and could be controlled readily by either mechanical or chemical means (OECD 2000).

Table II-1. World Soybean Production in 2011

Country	Production (million metric tons)
U.S.	83.2
Brazil	72.0
Argentina	48.0
China	13.5
India	11.0
Paraguay	6.4
Canada	4.2
Other	13.1

Source: Soy Stats, World Soybean Production (ASA 2012).

II.B. Characteristics of the Recipient Plant

The conventional soybean variety A3555, used as the recipient for the *cry1A.105* and *cry2Ab2* expression cassette insertion that produced MON 87751, was developed by Asgrow Seed Company. A3555 is a mid−maturity group III soybean variety.

II.C. Soybean Varieties Discussed in this Request

Soybean variety A3555 is the parental line of MON 87751 and was used as the conventional soybean comparator (hereafter referred to as the conventional control) in the safety assessment of MON 87751. MON 87751 and A3555 have similar genetic backgrounds with the exception of the *cry1A.105*and *cry2Ab2* expression cassettes, thus, the effect of the *cry1A.*105 and *cry2Ab2* expression cassettes could be assessed in an unbiased manner in the comparative safety assessment. In addition, reference varieties, where appropriate, were used to establish ranges of natural variability or responses representative of commercial soybean varieties. The commercial reference varieties used at each location were selected based on their availability and agronomic fit for the respective geographic region.

III. DESCRIPTION OF THE GENETIC MODIFICATION

Similar to the antecedent organism, MON 87701, MON 87751 was developed through Agrobacterium tumefaciens-mediated transformation of conventional soybean. For MON 87751 the source of conventional soybean meristem tissue for the transformation was A3555 and PV-GMIR13196 was the plasmid vector. This section describes the plasmid vector, the donor genes, and the regulatory elements used in the development of MON 87751 and the deduced amino acid sequences of the CrylA.105 and Cry2Ab2 proteins produced in MON 87751. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

III.A. The Plasmid Vector PV-GMIR13196

PV-GMIR13196 was used for the transformation of conventional soybean to produce MON 87751 and its plasmid map is shown in Figure III-1. The elements included in this plasmid vector are described in Table III-1. PV-GMIR13196 is approximately 24.5 kb and contains two separate T-DNAs, each delineated by Left and Right Border regions. The first T-DNA, designated as T-DNA I, contains the *crylA.105* and *cry2Ab2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *splA* and *aadA* expression cassettes. During transformation, both T-DNAs were inserted into the soybean genome (Section III.B). Subsequently, traditional breeding, segregation, selection and screening were used to isolate those plants that contain the *crylA.105* and *cry2Ab2* expression cassettes (T-DNA I) and do not contain the *splA* and *aadA* expression cassettes (T-DNA II).

splA and *aadA* act as selectable markers to allow selection of transformed plants. *aadA* encodes an aminoglycoside-modifying enzyme that confers spectinomycin and streptomycin resistance (Fling, et al. 1985) and allows selection of transformed tissue. *splA* encodes the sucrose phosphorylase enzyme (Piper, et al. 1999). When *splA* is expressed during embryo development, it interferes with sucrose metabolism, leading to a recognizable seed phenotype to provide a visual demonstration that T-DNA II is absent.

The backbone region of PV-GMIR13196, located outside both of the T-DNAs, contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori-pRi*, *ori-pBR322*), a bacterial selectable marker gene (*nptII*), and a coding sequence for repressor of primer (ROP) protein for the maintenance of the plasmid vector copy number in *Escherichia coli* (*E. coli*). A description of the genetic elements and their prefixes (e.g., B-, P-, TS-, CS-, T-, and OR-) in PV-GMIR13196 is provided in Table III-1.

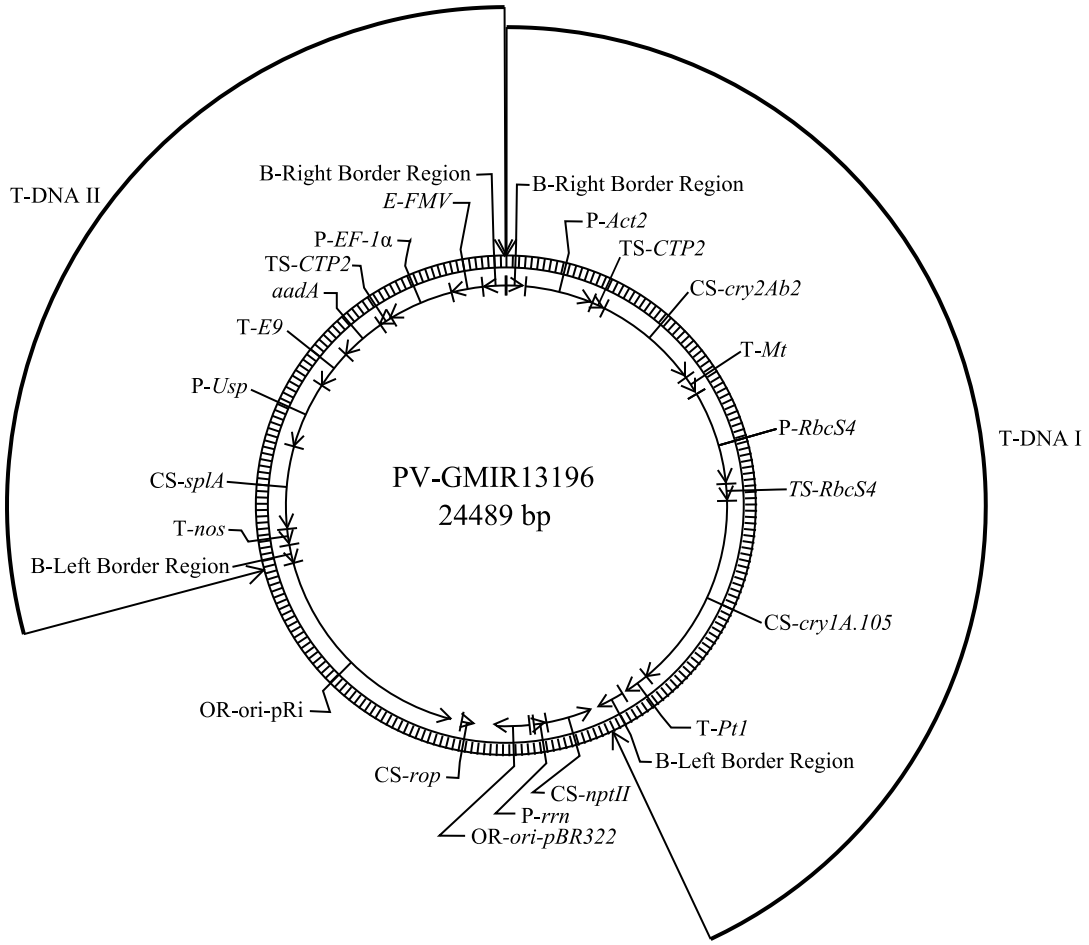

Figure III-1. Circular Map of Plasmid Vector PV-GMIR13196
A circular map of the plasmid vector PV-GMIR13196 used to develop MON 87751 is shown. PV-GMIR13196 contains two T-DNAs, designated as T-DNA I and T-DNA II. Genetic elements are shown on the exterior of the map.

Table III-1. Summary of Genetic Elements in PV-GMIR13196

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA I		
B[1]-Right Border Region	1-285	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker, et al. 1982; Zambryski, et al. 1982)
Intervening Sequence	286-337	Sequence used in DNA cloning
P[2]-*Act2*	338-1545	Promoter, leader and intron sequences from the *act2* gene of *A. thaliana* (An, et al. 1996) that directs transcription in plant cells
Intervening Sequence	1546-1555	Sequence used in DNA cloning
TS[3]-*CTP2*	1556-1783	Targeting sequence of the *ShkG* gene from *A. thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann 1995; Klee, et al. 1987)
Intervening Sequence	1784-1792	Sequence used in DNA cloning
CS[4]-*cry2Ab2*	1793-3697	Coding sequence for the Cry2Ab2 protein of *B. thuringiensis* that provides insect resistance (Donovan 1991)
Intervening Sequence	3698-3700	Sequence used in DNA cloning
T[5]-*Mt*	3701-4000	3' UTR sequence from *O. sativa* (rice) *Mt* gene encoding metallothionein-like protein that directs polyadenylation of mRNA (Hunt 1994)
Intervening Sequence	4001-4045	Sequence used in DNA cloning
P-*RbcS4*	4046-5768	Promoter and leader sequences from *A. thaliana rbcS* gene family encoding small subunit *ats1A* (De Almeida, et al. 1989; Krebbers, et al. 1988) that directs transcription in plant cells
TS-*RbcS4*	5769-6032	Targeting sequence from *A. thaliana rbcS* gene family encoding small subunit *ats1A* (Wong, et al. 1992) that directs transport of the protein to the chloroplast

Table III-1. Summary of Genetic Elements in PV-GMIR13196 (continued)

Genetic Element	Location in Plasmid	Function (Reference)
CS-*cry1A.105*	6033-9566	Coding sequences for the Cry1Ab, Cry1F, and Cry1Ac proteins of *B. thuringiensis* to produce a chimeric protein that provides insect resistance (Monsanto unpublished data)
Intervening Sequence	9567-9569	Sequence used in DNA cloning
T-*Pt1*	9570-9969	3' UTR sequence from *M. truncatula* PT1 gene encoding phosphate transporter that directs polyadenylation of mRNA (Liu, et al. 1998)
Intervening Sequence	9970-10088	Sequence used in DNA cloning
B-Left Border Region	10089-10530	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker, et al. 1983)
Backbone		
Intervening Sequence	10531-10739	Sequence used in DNA cloning
CS-*nptII*	10740-11534	Coding sequence of the *neo* gene from transposon Tn5 of *E. coli* encoding neomycin phosphotransferase II (Beck, et al. 1982) that confers neomycin and kanamycin resistance (Fraley, et al. 1983)
P-*rrn*	11535-11759	Promoter of the ribosomal RNA operon from *A. tumefaciens* (Bautista-Zapanta, et al. 2002)
Intervening Sequence	11760-11835	Sequence used in DNA cloning
OR[6]-*ori-pBR322*	11836-12424	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe 1979)
Intervening Sequence	12425-12851	Sequence used in DNA cloning
CS-*rop*	12852-13043	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang 1989)
Intervening Sequence	13044-13231	Sequence used in DNA cloning
OR-*ori-pRi*	13232-17345	Origin of replication from plasmid pRi for maintenance of plasmid in *Agrobacterium* (Ye, et al. 2011)
Intervening Sequence	17346-17352	Sequence used in DNA cloning

Table III-1. Summary of Genetic Elements in PV-GMIR13196 (continued)

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA II		
B-Left Border Region	17353-17671	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al. 1983)
Intervening Sequence	17672-17703	Sequence used in DNA cloning
T-*nos*	17704-17956	3' UTR sequence of the nopaline synthase (*nos*) gene from *A. tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan, et al. 1983; Fraley et al. 1983)
Intervening Sequence	17957-17972	Sequence used in DNA cloning
CS-*splA*	17973-19430	Coding sequence of the *splA* gene from *A. tumefaciens* strain C58 encoding the sucrose phosphorylase protein that catalyzes the conversion of sucrose to fructose and glucose-1-phosphate (Piper et al. 1999)
Intervening Sequence	19431-19442	Sequence used in DNA cloning
P-*Usp*	19443-20621	5' UTR leader, promoter, and enhancer sequence from *V. faba* (broad bean) *Usp* gene encoding a seed protein that directs transcription in plant cells (Bäumlein, et al. 1991)
Intervening Sequence	20622-20672	Sequence used in DNA cloning
T-*E9*	20673-21315	3' UTR sequence from *P. sativum* (pea) *rbcS* gene family encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi, et al. 1984) that directs polyadenylation of the mRNA
Intervening Sequence	21316-21330	Sequence used in DNA cloning
CS-*aadA*	21331-22122	Bacterial coding sequence for an *aminoglycoside-modifying enzyme, 3''(9)-O-nucleotidyltransferase* from the transposon Tn7 (Fling et al. 1985) that confers spectinomycin and streptomycin resistance

Table III-1. Summary of Genetic Elements in PV-GMIR13196 (continued)

Genetic Element	Location in Plasmid	Function (Reference)
TS-*CTP2*	22123-22350	Targeting sequence of the *ShkG* gene from *A. thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann 1995; Klee et al. 1987)
Intervening Sequence	22351-22359	Sequence used in DNA cloning
P-*EF-1α*	22360-23507	Promoter, leader, and intron sequences of the *EF-1α* gene from *A. thaliana* encoding elongation factor EF-1α that directs transcription in plant cells (Axelos, et al. 1989)
Intervening Sequence	23508-23530	Sequence used in DNA cloning
E[7]-*FMV*	23531-24067	Enhancer from the 35S RNA of figwort mosaic virus (Richins, et al. 1987) that enhances transcription in most plant cells (Rogers 2000)
Intervening Sequence	24068-24117	Sequence used in DNA cloning
B-Right Border Region	24118-24474	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al. 1982; Zambryski et al. 1982)
Backbone		
Intervening Sequence	24475-24489	Sequence used in DNA cloning

[1]B, Border
[2]P, Promoter
[3]TS, Targeting Sequence
[4]CS, Coding Sequence
[5]T, Transcription Termination Sequence
[6]OR, Origin of Replication
[7]E, Enhancer

III.B. Description of the Transformation System

MON 87751 was developed through *Agrobacterium*-mediated transformation of soybean, based on the method described by Martinell et al. (2011), which allows for the generation of transformed plants without the utilization of callus. Briefly, meristem tissues were excised from the embryos of germinated conventional seed. After co-culturing with *Agrobacterium* carrying the transformation construct, the meristems were placed on selection medium containing spectinomycin, carbenicillin disodium salt, cefotaxime sodium salt, and ticarcillin disodium salt/potassium clavulanate mixture to inhibit the growth of untransformed plant cells and excess *Agrobacterium*. The meristems were then placed in media conducive to shoot development followed by a liquid overlay of selection medium and a transfer to a Jiffy Carefree propagation plug for root development. Rooted plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment. As demonstrated in this request, the use of disarmed *A. tumefaciens* strain AB30, a designated plant pest, as the transformation vector has not imparted plant pest characteristics to MON 87751.

The R_0 plants generated through this transformation process were self-pollinated to produce R_1 seed, and the unlinked insertions of T-DNA I and T-DNA II were segregated. The *splA* scorable phenotype and *aadA* coding sequence polymerase chain reaction (PCR) analyses were used to eliminate any seeds or plants from further development that contain T-DNA II. Subsequently, R_1 plants homozygous for T-DNA I were selected for further development and their progenies were subjected to further molecular analysis, insect efficacy and phenotypic assessments. As is typical of a commercial event production and selection process, hundreds of different transformation events (regenerants) were generated in the laboratory using PV-GMIR13196 and related vectors. After careful selection and evaluation of these events in the laboratory, greenhouse and field, MON 87751 was selected as the lead event based on superior agronomic, phenotypic, and molecular characteristics. Studies on MON 87751 were initiated to further characterize the genetic insertion and the expressed products, and to establish the food and feed safety and low environmental risk compared to commercial soybean. The major development steps of MON 87751 are depicted in Figure III-2.

III.C. The *cry1A.105* Coding Sequence and the Cry1A.105 Protein

The *cry1A.105* expression cassette, encodes a 142 kDa CrylA.105 protein consisting of a single polypeptide of 1265 amino acids (Figure III-3). The *cry1A.105* coding sequence is the coding sequence from *B. thuringiensis* that encodes the CrylA.105 protein. The presence of CrylA.105 protein in soybean provides insect resistance.

III.D. The *cry2Ab2* Coding Sequence and the Cry2Ab2 Protein

The *cry2Ab2* expression cassette, encodes a 79 kDa Cry2Ab2 protein consisting of a single polypeptide of 713 amino acids (Figure III-4). The *cry2Ab2* coding sequence is the coding sequence from *B. thuringiensis* that encodes the Cry2Ab2 protein (Donovan 1991). The presence of Cry2Ab2 protein in soybean provides insect resistance.

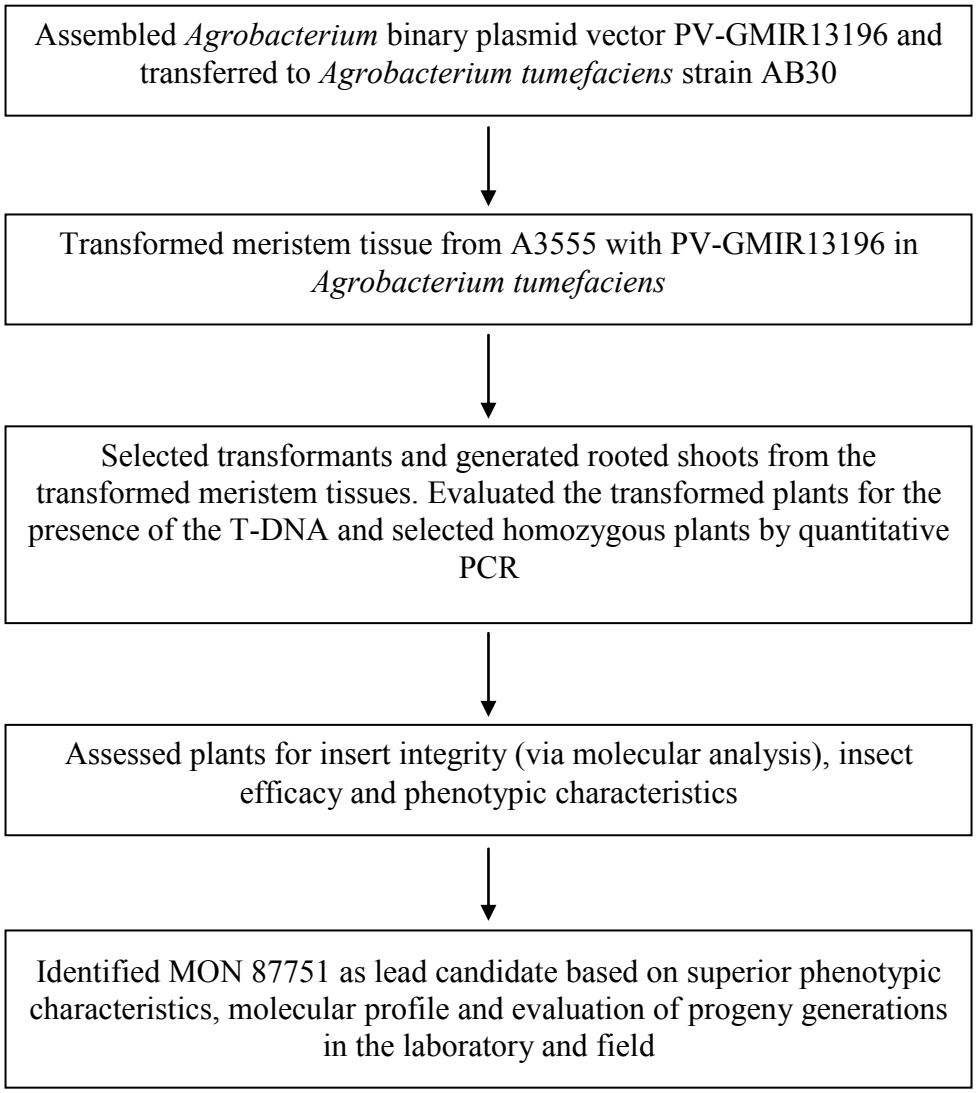

Figure III-2. Schematic of the Development of MON 87751

```
   1 MASSMLSSAT MVASPAQATM VAPFNGLKSS AAFPATRKAN NDITSITSNG GRVNCMQVWP
  61 PIGKKKFETL SYLPDLTDSG GRVNCMQAMD NNPNINECIP YNCLSNPEVE VLGGERIETG
 121 YTPIDISLSL TQFLLSEFVP GAGFVLGLVD IIWGIFGPSQ WDAFLVQIEQ LINQRIEEFA
 181 RNQAISRLEG LSNLYQIYAE SFREWEADPT NPALREEMRI QFNDMNSALT TAIPLFAVQN
 241 YQVPLLSVYV QAANLHLSVL RDVSVFGQRW GFDAATINSR YNDLTRLIGN YTDHAVRWYN
 301 TGLERVWGPD SRDWIRYNQF RRELTLTVLD IVSLFPNYDS RTYPIRTVSQ LTREIYTNPV
 361 LENFDGSFRG SAQGIEGSIR SPHLMDILNS ITIYTDAHRG EYYWSGHQIM ASPVGFSGPE
 421 FTFPLYGTMG NAAPQQRIVA QLGQGVYRTL SSTLYRRPFN IGINNQQLSV LDGTEFAYGT
 481 SSNLPSAVYR KSGTVDSLDE IPPQNNNVPP RQGFSHRLSH VSMFRSGFSN SSVSIIRAPM
 541 FSWIHRSAEF NNIIASDSIT QIPLVKAHTL QSGTTVVRGP GFTGGDILRR TSGGPFAYTI
 601 VNINGQLPQR YRARIRYAST TNLRIYVTVA GERIFAGQFN KTMDTGDPLT FQSFSYATIN
 661 TAFTFPMSQS SFTVGADTFS SGNEVYIDRF ELIPVTATLE AEYNLERAQK AVNALFTSTN
 721 QLGLKTNVTD YHIDQVSNLV TYLSDEFCLD EKRELSEKVK HAKRLSDERN LLQDSNFKDI
 781 NRQPERGWGG STGITIQGGD DVFKENYVTL SGTFDECYPT YLYQKIDESK LKAFTRYQLR
 841 GYIEDSQDLE IYSIRYNAKH ETVNVPGTGS LWPLSAQSPI GKCGEPNRCA PHLEWNPDLD
 901 CSCRDGEKCA HHSHHFSLDI DVGCTDLNED LGVWVIFKIK TQDGHARLGN LEFLEEKPLV
 961 GEALARVKRA EKKWRDKREK LEWETNIVYK EAKESVDALF VNSQYDQLQA DTNIAMIHAA
1021 DKRVHSIREA YLPELSVIPG VNAAIFEELE GRIFTAFSLY DARNVIKNGD FNNGLSCWNV
1081 KGHVDVEEQN NQRSVLVVPE WEAEVSQEVR VCPGRGYILR VTAYKEGYGE GCVTIHEIEN
1141 NTDELKFSNC VEEEIYPNNT VTCNDYTVNQ EEYGGAYTSR NRGYNEAPSV PADYASVYEE
1201 KSYTDGRREN PCEFNRGYRD YTPLPVGYVT KELEYFPETD KVWIEIGETE GTFIVDSVEL
1261 LLMEE
```

Figure III-3. Deduced Amino Acid Sequence of the RbcS4 Targeting Sequence and Cry1A.105 Protein

The chloroplast transit peptide RbcS4 is underlined. Accumulation of the Cry1A.105 protein is targeted to the chloroplasts using cleavable RbcS4, the transit peptide of the *A. thaliana* small subunit *ats1A* protein. The amino acid sequence of the Cry1A.105 protein and RbcS4 targeting sequence was deduced from the full-length coding nucleotide sequence present in PV-GMIR13196.

```
   1 MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG LKKSGMTLIG
  61 SELRPLKVMS SVSTACMLAM DNSVLNSGRT TICDAYNVAA HDPFSFQHKS LDTVQKEWTE
 121 WKKNNHSLYL DPIVGTVASF LLKKVGSLVG KRILSELRNL IFPSGSTNLM QDILRETEKF
 181 LNQRLNTDTL ARVNAELTGL QANVEEFNRQ VDNFLNPNRN AVPLSITSSV NTMQQLFLNR
 241 LPQFQMQGYQ LLLLPLFAQA ANLHLSFIRD VILNADEWGI SAATLRTYRD YLKNYTRDYS
 301 NYCINTYQSA FKGLNTRLHD MLEFRTYMFL NVFEYVSIWS LFKYQSLLVS SGANLYASGS
 361 GPQQTQSFTS QDWPFLYSLF QVNSNYVLNG FSGARLSNTF PNIVGLPGST TTHALLAARV
 421 NYSGGISSGD IGASPFNQNF NCSTFLPPLL TPFVRSWLDS GSDREGVATV TNWQTESFET
 481 TLGLRSGAFT ARGNSNYFPD YFIRNISGVP LVVRNEDLRR PLHYNEIRNI ASPSGTPGGA
 541 RAYMVSVHNR KNNIHAVHEN GSMIHLAPND YTGFTISPIH ATQVNNQTRT FISEKFGNQG
 601 DSLRFEQNNT TARYTLRGNG NSYNLYLRVS SIGNSTIRVT INGRVYTATN VNTTTNNDGV
 661 NDNGARFSDI NIGNVVASSN SDVPLDINVT LNSGTQFDLM NIMLVPTNIS PLY
```

Figure III-4. Deduced Amino Acid Sequence of the CTP2 Targeting Sequence and Cry2Ab2 Protein

The transit peptide CTP2 is underlined. Accumulation of the Cry2Ab2 protein is targeted to the chloroplasts using cleavable CTP2, the transit peptide of the *A. thaliana* EPSPS protein. The amino acid sequence of the Cry2Ab2 protein and CTP2 targeting sequence was deduced from the full-length coding nucleotide sequence present in PV-GMIR13196.

III.E. Regulatory Sequences

The *crylA.105* coding sequence in T-DNA I is under the regulation of the *RbcS4* promoter, *RbcS4* chloroplast targeting sequence and the *Pt1* 3' untranslated region. The *RbcS4* promoter is the promoter for the *rbcS* gene family of *A. thaliana* (Almeida, et al. 1989; Krebbers et al. 1988), which functions to direct transcription in plant cells. The targeting sequence from *A. thaliana rbcS* gene family encodes the small subunit *ats1A* (Wong et al. 1992) that directs transport of the protein to the chloroplast The *Pt1* 3' untranslated region is the 3' untranslated region of the *Pt1* gene from *M. truncatula* encoding phosphate transporter that directs polyadenylation of mRNA (Liu et al. 1998).

The *cry2Ab2* coding sequence in T-DNA I is under the regulation of the *Act2* promoter, *CTP2* targeting sequence and the *Mt* 3' untranslated region. The *Act2* promoter is the promoter for the *act2* gene of *A. thaliana* (An et al. 1996), which functions to direct transcription in plant cells. The *CTP2* targeting sequence is the targeting sequence of the *ShkG* gene from *A. thaliana* encoding the EPSPS transit peptide region (Herrmann 1995; Klee et al. 1987), which functions to direct transport of the protein to the chloroplast. The *Mt* 3′ untranslated region is the 3' untranslated region of the *Mt* gene from *O. sativa* encoding metallothionein-like protein that directs polyadenylation of mRNA (Hunt 1994).

T-DNA II contains the *splA* coding sequence under the regulation of the *Usp* promoter, *Usp* leader, *Usp* enhancer, and the *nos* 3' untranslated region. The *Usp* promoter is the promoter consisting leader, promoter, and enhancer sequences from *V. faba* (broad bean) encoding a seed protein (Bäumlein et al. 1991), which functions to direct transcription in plant cells. The *nos* 3' untranslated region is the 3' untranslated region from *A. tumefaciens* pTi nopaline synthase (*nos*) gene encoding NOS that directs polyadenylation (Bevan et al. 1983; Fraley et al. 1983).

T-DNA II also contains the *aadA* coding sequence under the regulation of the *FMV* enhancer, *EF-1α* promoter, the *CTP2* targeting sequence, and the *T-E9* untranslated region. The *EF-1α* promoter is the promoter consisting leader, promoter, and intron sequences from *A. thaliana* encoding elongation factor *EF-1α* (Axelos et al. 1989), which functions to direct transcription in plant cells. The *FMV* enhancer is the enhancer sequence of the 35S RNA of figwort mosaic virus (FMV) (Richins et al. 1987), which enhances transcription in most plant cells (Rogers 2000). The *CTP2* targeting sequence is the targeting sequence of the *ShkG* gene from *A. thaliana* encoding the EPSPS transit peptide region (Herrmann 1995; Klee et al. 1987), which functions to direct transport of the protein to the chloroplast. The *E9* 3' untranslated region is the 3' untranslated region from *P. sativum* (pea) *rbcS* gene family encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al. 1984), which functions to direct polyadenylation of the mRNA.

III.F. T-DNA Borders

PV-GMIR13196 contains Left and Right Border regions (Figure III-1 and Table III-1) that were derived from *A. tumefaciens* plasmids. The border regions each contain a

24-25 bp nick site that is the site of DNA exchange during transformation (Barker et al. 1983; Depicker et al. 1982; Zambryski et al. 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer of T-DNA into the soybean genome. Because PV-GMIR13196 is a 2T-DNA vector, it contains two Left Border regions and two Right Border regions, where one border region set flanks T-DNA I and the other border region set flanks T-DNA II. As demonstrated in this request, the use of genetic elements from *A. tumefaciens*, a designated plant pest, has not imparted plant pest characteristics to MON 87751.

III.G. Genetic Elements Outside of the T-DNA Borders

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV- GMIR13196 in bacteria and are referred to as plasmid backbone. The origin of replication, *ori pRi*, is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid *pRi* (Ye et al. 2011). The origin of replication, *ori-pBR322*, is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe 1979). Coding sequence *rop* encodes the repressor of primer (ROP) protein which is necessary for the maintenance of plasmid vector copy number in *E. coli* (Giza and Huang 1989). The *rrn* promoter is the promoter for the ribosomal RNA operon from *A. tumefacien*s (Bautista-Zapanta et al. 2002). The selectable marker *nptII* is the coding sequence for an enzyme from transposon Tn5 that confers neomycin and kanamycin resistance (Fraley et al. 1983) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the soybean genome. The absence of the backbone and other unintended plasmid sequence in MON 87751 was confirmed by sequencing and bioinformatic analyses (see Section IV.A).

IV. CHARACTERIZATION OF THE GENETIC MODIFICATION

Characterization of the DNA insert in MON 87751 was conducted using a combination of sequencing, PCR, and bioinformatics. The results of this characterization demonstrate that MON 87751 contains one copy of the intended transfer DNA (T-DNA I) containing the *crylA.105* and the *cry2Ab2* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions are based on several lines of evidence:

- Molecular characterization of MON 87751 by Next Generation Sequencing and Junction Sequence Analysis (NGS/JSA), a whole-genome sequence analysis that provided a comprehensive assessment of MON 87751 to determine the presence of sequences derived from PV-GMIR13196 (DuBose, et al. 2013; Kovalic, et al. 2012), demonstrated that DNA from PV-GMIR13196 was integrated at a single locus in MON 87751.
- Directed sequencing (locus-specific PCR, DNA sequencing and analyses) was performed on MON 87751 which determined the complete sequence of the single DNA insert from PV-GMIR13196, the adjacent flanking DNA, and the 5' and 3' insert-to-flank junctions. This confirmed that the sequence and organization of the DNA insert is identical to the corresponding region in the PV-GMIR13196 T-DNA I. This also confirmed that no vector backbone, or T-DNA II, or other unintended plasmid sequences are present in MON 87751. Furthermore, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87751 to the sequence of the insertion site in conventional soybean. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 87751 upon DNA integration.
- Generational stability analysis by NGS/JSA demonstrated that the single PV-GMIR13196 T-DNA I insert in MON 87751 has been maintained through five breeding generations, thereby confirming the stability of the intended T-DNA I in MON 87751.
- Segregation data confirm that the inserted T-DNA I segregated following Mendelian inheritance patterns which, corroborates the insert stability demonstrated by NGS/JSA and independently establishes the nature of the T-DNA as a single chromosomal locus.

Taken together, the characterization of the genetic modification in MON 87751 demonstrates that a single copy of the intended T-DNA I was stably integrated at a single locus of the soybean genome and that no plasmid backbone or T-DNA II sequences are present in MON 87751. The molecular characterization of the antecedent organism, MON 87701, produced similar results showing stable integration and inheritance of the intended T-DNA at a single locus.

A schematic representation of the NGS/JSA methodology and the basis of the characterization using NGS/JSA and PCR sequencing are illustrated in Figure IV-1

below. Appendix B provides an additional overview of these techniques, their use in DNA characterization in crop plants and the materials and methods.

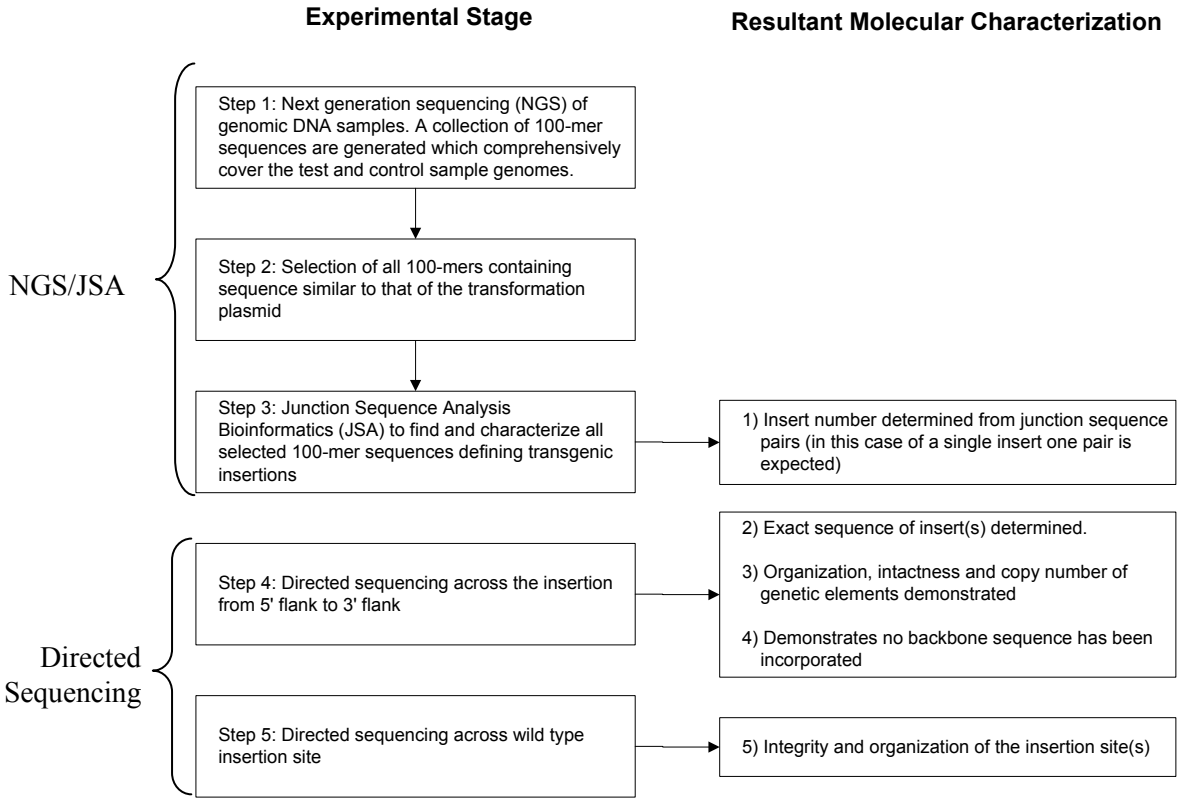

Figure IV-1. Molecular Characterization using Sequencing and Bioinformatics
Genomic DNA from MON 87751 and the conventional control was sequenced using NGS technology that produces a set of short, randomly distributed sequence reads (each approximately 100 bp long) that comprehensively cover the genomes (Step 1). Utilizing these genomic sequences, bioinformatics search tools were used to select all sequence reads (100-mers) that were significantly similar to the transformation plasmid (Step 2) and Junction Sequence Analysis (JSA) bioinformatics was used to determine the insert number (Step 3). Overlapping PCR products are produced which span any insert(s) and their wild type loci (Step 4 and Step 5, respectively). These PCR products are sequenced to provide a detailed characterization of the insertion site(s).

The NGS/JSA method characterized the genomic DNA from MON 87751 and the conventional control using short (~100 bp) randomly distributed sequence fragments (sequencing reads) generated in sufficient number to ensure comprehensive coverage of the sample genomes. It has previously been demonstrated that 75× coverage of the soybean genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic et al. 2012). To confirm sufficient sequence coverage of the genome, the 100-mer sequence reads are analyzed to determine the coverage of a known single-copy endogenous gene, this demonstrates the depth of coverage (the median number of times each base of the genome is independently sequenced). The level of

sensitivity of this method was demonstrated by detection of a positive control spiked at 1 and 1/10th copy-per-genome equivalent, this confirms the method's ability to detect any sequences derived from the transformation plasmid. Bioinformatics analysis was then used to select sequencing reads that contained sequences similar to the transformation plasmid, and these were analysed in depth to determine the number of DNA inserts. NGS/JSA was run on all MON 87751 samples and the conventional controls. Results of NGS/JSA are shown in Sections IV.A and IV.D.

The number of DNA inserts was determined by analyzing sequences for novel junctions. The junctions of the DNA insert and the flanking DNA are unique for each insertion (Kovalic et al. 2012). An example is shown in Figure IV-2. Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions.

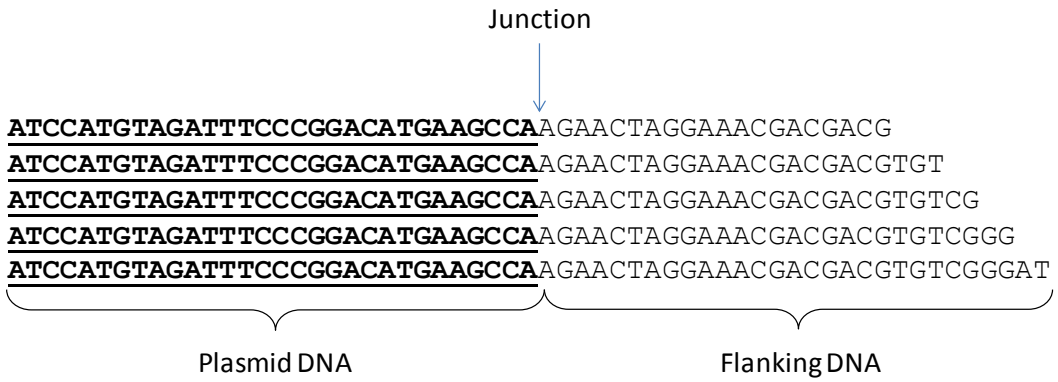

Figure IV-2. Junctions and Junction Sequences
Depicted above are five example junction sequences formatted and labeled to indicate the plasmid/flanking DNA portions of the sequences and with the junction point indicated (plasmid DNA is shown in bold, underlined text and flank DNA is shown in plain text). Junctions are detected by examining the NGS data for sequences having portions of plasmid sequences that span less than the full read. A group of junction sequences which share the same junction point and common flanking sequence is called a Junction Sequence Class (or JSC).

Each insertion will produce two unique junction sequence classes characteristic of the genomic locus, one at the 5' end of the insert (Figure IV-3, named junction sequence class A, or JSC-A, in this case) and one at the 3' end of the insert (junction sequence class B, JSC-B) (Kovalic et al. 2012).

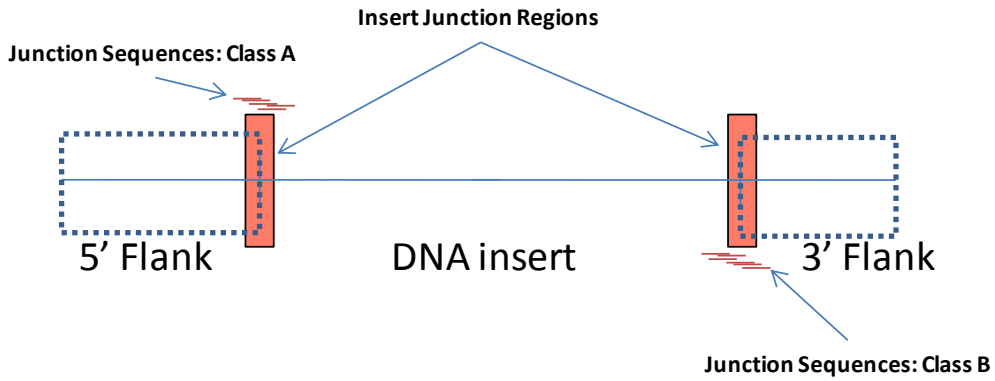

Figure IV-3. Two Unique Junction Sequence Classes are Produced by the Insertion of a Single Plasmid Region
A schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5' and 3' flanks (depicted as areas bounded by dotted lines), and the two distinct regions spanning the junctions between inserted DNA and flanking DNA (shaded boxes). The group of ~100-mer sequences in which each read contains sequences from both the DNA insert and the adjacent flanking DNA at a given junction is called a Junction Sequence Class. In this example, two distinct junction sequence classes (in this case: Class A at the 5' end and Class B at the 3' end) are represented.

By evaluating the number of unique junction classes detected, the number of insertion sites of the plasmid sequence can be determined. For a single insert, two junction sequence classes are expected, each originating from one end of the insert, both containing portions of plasmid DNA insert and flanking sequence.

Directed sequencing (locus-specific PCR and DNA sequencing analyses, Figure IV-1, step 4) complements the NGS/JSA analyses. Sequencing of the insert and flanking genomic DNA determined the complete sequence of the insert and flanks. This analysis evaluates if the sequence of the insert is identical to the corresponding sequence from the T-DNA in PV-GMIR13196, if each genetic element in the insert is intact, if the T-DNA sequence is inserted as a single copy, and establishes no vector backbone or other unintended plasmid sequences were inserted in MON 87751. Results are described in Section IV.B and Section IV.C; methods are presented in Appendix B.

The stability of the T-DNA present in MON 87751 across multiple generations was evaluated by NGS/JSA analyses as described above. This information was used to determine the number and identity of insertion sites. For a single insert, two junction sequence classes are expected; each one originates from either end of the insert, both containing portions of DNA insert and flanking sequence. Results are described in Section IV.D; methods are presented in Appendix B.

Segregation analysis of the T-DNA was conducted to determine the inheritance and stability of the insert in MON 87751. Segregation analysis corroborates the insert stability demonstrated by NGS/JSA and independently establishes the genetic behavior of the T-DNA. Results are described in Section IV.E; methods are presented in Appendix B.

IV.A. Determining the Number of DNA inserts in MON 87751

The number of insertion sites of PV-GMIR13196 DNA in MON 87751 was assessed by performing NGS/JSA on MON 87751 genomic DNA. A plasmid map of PV-GMIR13196 is shown in Figure III-1. Table IV-1 provides descriptions of the genetic elements present in MON 87751. A schematic representation of the insert and flanking sequences in MON 87751 is shown in Figure IV-4. For full details on materials and methods see Appendix B.

IV.A.1. Next Generation Sequencing for MON 87751 and Conventional Control Genomic DNA

Genomic DNA from five generations of MON 87751 (Figure IV-5) and the conventional control was isolated from seed and prepared for sequencing according to the manufacturer's protocol (Illumina, TruSeq library protocol. For material and method details see Appendix B). These genomic DNA libraries were used to generate short (~100 bp) randomly distributed sequence fragments (sequencing reads) of the soybean genome (see Figure IV-1, Step 1).

To demonstrate sufficient sequence coverage the 100-mer sequence reads were analyzed by mapping all reads to a known single-copy endogenous gene (*Glycine max* lectin (*Le1*), GenBank accession version: K00821.1). The analysis showed that the depth of coverage (*i.e.*, the median number of times any base of the genome is expected to be independently sequenced) was 75× or greater for the five generations of MON 87751 (R_3, R_4, R_5, R_6 and R_7) and the conventional control. It has previously been demonstrated that 75× coverage of the soybean genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA (Kovalic et al. 2012).

To demonstrate the method's ability to detect any sequences derived from the PV-GMIR13196 transformation plasmid, a sample of conventional control DNA spiked with PV-GMIR13196 DNA at 1 and 1/10th genome equivalent was analyzed by NGS and bioinformatics. The level of sensitivity of this method was demonstrated to a level of 1 genome equivalent, 100% nucleotide identity was observed over 100% of PV-GMIR13196. This result demonstrates that all nucleotides of the transformation plasmid are observed by the sequencing and bioinformatic assessments performed. Also, observed coverage was adequate (Clarke and Carbon 1976) at a level of at least 1/10th genomic equivalent (100% coverage at 100% identity for the 1/10th genome equivalent spiked control sample) and, hence, a detection level of at most 1/10th genome equivalent was achieved for the plasmid DNA sequence assessment.

IV.A.2. Characterization of insert number in MON 87751 using Bioinformatic Analysis

The number of insertion sites of DNA from PV-GMIR13196 in MON 87751 was assessed by performing NGS/JSA on MON 87751 genomic DNA using the R3 generation (Figure IV-5).

Table IV-1. Summary of Genetic Elements in MON 87751

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
Flanking DNA	1-1334	DNA adjacent to 5' of the DNA insert
B[3]-Right Border Region[r1]	1335-1404	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T DNA (Depicker et al. 1982; Zambryski et al. 1982)
Intervening Sequence	1405-1456	Sequence used in DNA cloning
P[4]-*Act2*	1457-2664	Promoter, leader and intron sequences from the *act2* gene of *A. thaliana* (An et al. 1996) that directs transcription in plant cells
Intervening Sequence	2665-2674	Sequence used in DNA cloning
TS[5]-*CTP2*	2675-2902	Targeting sequence of the *ShkG* gene from *A. thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann 1995; Klee et al. 1987)
Intervening Sequence	2903-2911	Sequence used in DNA cloning
CS[6]-*cry2Ab2*	2912-4816	Coding sequence for the Cry2Ab2 protein of *B. thuringiensis* that provides insect resistance (Donovan 1991)
Intervening Sequence	4817-4819	Sequence used in DNA cloning
T[7]-*Mt*	4820-5119	3' UTR sequence from *O. sativa* (rice) *Mt* gene encoding metallothionein like protein that directs polyadenylation of mRNA (Hunt 1994)
Intervening Sequence	5120-5164	Sequence used in DNA cloning
P-*RbcS4*	5165-6887	Promoter and leader sequences from *A. thaliana rbcS* gene family encoding small subunit *ats1A* (Almeida et al. 1989; Krebbers et al. 1988) that directs transcription in plant cells
TS-*RbcS4*	6888-7151	Targeting sequence from *A. thaliana rbcS* gene family encoding small subunit *ats1A* (Wong et al. 1992) that directs transport of the protein to the chloroplast
CS-*cry1A.105*	7152-10685	Coding sequences for the Cry1Ab, Cry1F, and Cry1Ac proteins of *B. thuringiensis* to produce a chimeric protein that provides insect resistance (Monsanto unpublished data)
Intervening Sequence	10686-10688	Sequence used in DNA cloning

Table IV-1. Summary of Genetic Elements in MON 87751 (continued)

Genetic Element[1]	Location in Sequence[2]	Function (Reference)
T-*Pt1*	10689-11088	3' UTR sequence from *M. truncatula PT1* gene encoding phosphate transporter that directs polyadenylation of mRNA (Liu et al. 1998)
Intervening Sequence	11089-11207	Sequence used in DNA cloning
B-Left Border Region[r1]	11208-11453	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al. 1983)
Flanking DNA	11454-12640	DNA adjacent to 3' of the DNA insert

[1]Although flanking sequences and intervening sequences are not functional genetic elements; they comprise a portion of the sequence.
[2]Numbering refers to the sequence of the insert in MON 87751 and adjacent DNA.
[3]B, Border
[4]P, Promoter
[5] TS, Targeting Sequence
[6]CS, Coding Sequence
[7] T, Transcriptional Terminator
[r1]Superscript in Left and Right Border Regions indicate that the sequence in MON 87751 was truncated compared to the sequences in PV-GMIR13196.

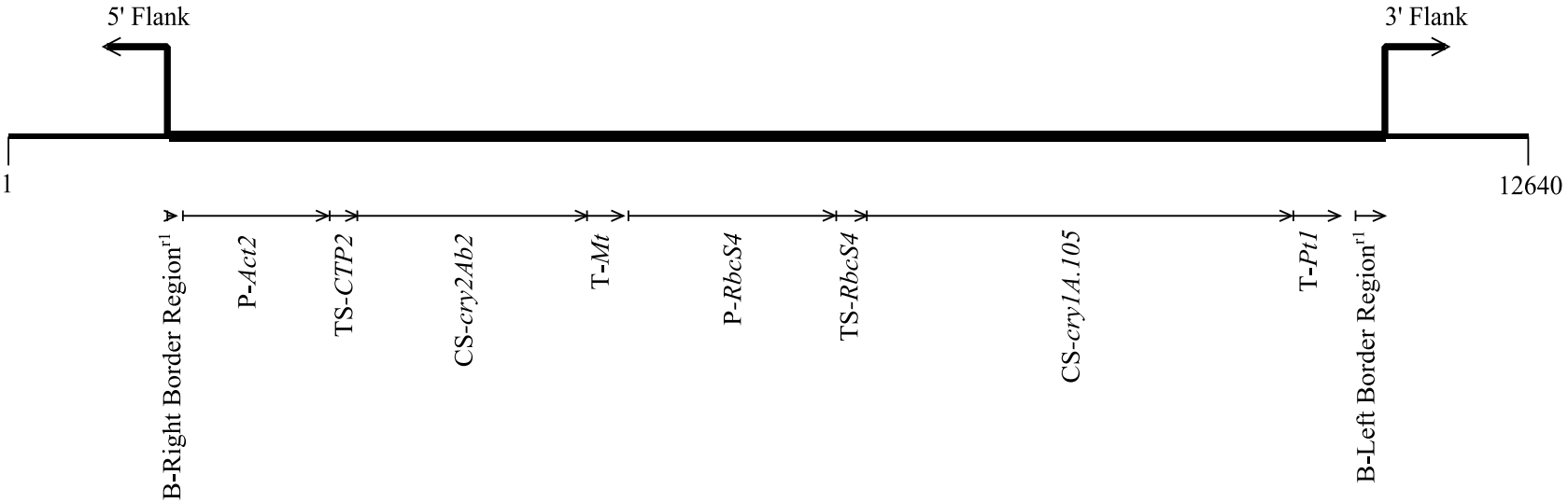

Figure IV-4. Schematic Representation of the Insert and Flanking Sequences in MON 87751

DNA derived from T-DNA I of PV-GMIR13196 integrated in MON 87751. Right-angled arrows indicate the ends of the integrated T-DNA I and the beginning of the flanking sequence. Genetic elements within the insert are identified on the map. This schematic diagram is drawn to scale, the exact coordinates of every element is shown in Table IV-1.

[r1]Superscript in Left and Right Border Regions indicate that the sequence in MON 87751 was truncated compared to the sequences in PV-GMIR13196.

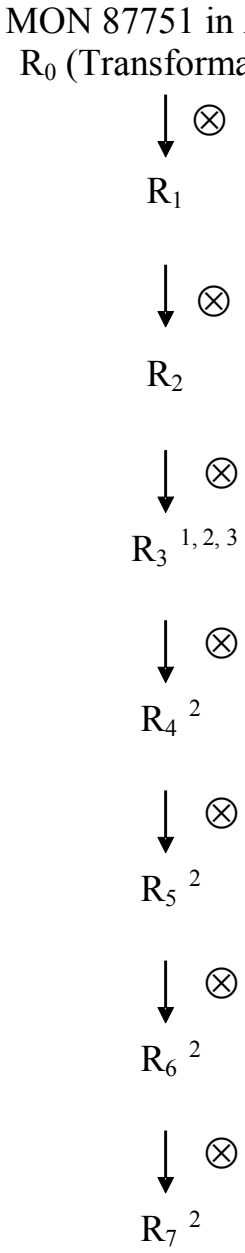

Figure IV-5. Breeding History of MON 87751
R_0 corresponds to the transformed plant, ⊗ designates self-pollination.
[1]Generation used for full molecular characterization
[2] Generations used to confirm insert stability
[3] Generation used for commercial development of MON 87751

IV.A.2.1. Selection of Sequence Reads Containing Sequence of the PV-GMIR13196

PV-GMIR13196 was transformed into the parental variety A3555 to produce MON 87751. Consequently, any DNA inserted into MON 87751 will consist of sequences that are similar to the PV-GMIR13196 DNA sequence. Therefore, to fully characterize the DNA from PV-GMIR13196 inserted in MON 87751, it is sufficient to completely analyze only the sequence reads that have similarity to the transformation plasmid (Figure IV-1, Step 2).

Using established criteria (described in the materials and methods, Appendix B), sequence reads similar to the transformation plasmid were selected from MON 87751 and the conventional control sequence datasets and were then used as input data for bioinformatic junction sequence analysis.

IV.A.2.2. Determination of the Insert Number

The NGS/JSA method described above used the entire plasmid sequence as a query to determine the DNA insertion site number. Any DNA inserts, regardless of their sequence being from backbone or T-DNA, can be detected by junction sequences. Therefore unlike the traditional Southern blot analysis that separately hybridizes T-DNA or backbone probes, in NGA/JSA the determination of the T-DNA insert number and of the absence of backbone or unintended sequences are simply represented by the determination of the overall insert number in the genome followed by determination of the exact identity of any DNA insert using directed sequencing and sequence analysis.

By evaluating the number of unique junction classes, the number of DNA insertion sites can be determined (Figure IV-1, Step 3). If MON 87751 contains a single T-DNA I insert, two junction sequence classes (JSCs) each containing portions of T-DNA I sequence and flanking sequence will be detected.

To determine the insert number in MON 87751, the selected sequence reads described above were analyzed using JSA (Kovalic et al. 2012). JSA uses bioinformatic analysis to find and classify partially matched reads characteristic of the ends of insertions. The number of resultant unique JSCs were determined by this analysis and are shown in Table IV-2.

Table IV-2. Unique Junction Sequence Class Results

Sample	Junction Sequence Classes Detected
MON 87751	2
A3555	0

The location and orientation of the junction sequences relative to the T-DNA insert determined for MON 87751 (as described in Section IV.B) are illustrated in Figure IV-6. As shown in the figure, there are two junction sequence classes identified in MON 87751. Junction Sequence Class A and Class B (JSC-A and JSC-B) both contain the T-DNA

border sequence joined to flanking sequence, indicating that they represent the sequences at the junctions of the intended T-DNA I insert and flanking sequence.

The presence of two, and only two, junction sequence classes (joining T-DNA border and flanking sequences) indicate this single pair of JSCs likely arises from the insertion of the intended PV-GMIR13196 T-DNA I at a single locus in the genome of MON 87751. JSC-A represents the junction of the T-DNA I Left Border sequence to the 3' flank and JSC-B represents the junction of the T-DNA I Right Border sequence to the 5' flank. Complete alignment of the JSCs to the full flank/insert sequence confirm that both of these JSCs originate from the same locus of the MON 87751 genome and are linked by contiguous, known and expected DNA that makes up the single insert.

Based on this comprehensive NGS/JSA study it is concluded that MON 87751 contains one T-DNA inserted into a single locus, as shown in Figure IV-6. The identity of the DNA insert was determined by the sequencing and analysis of overlapping PCR products from this locus as described below in Section IV. B. Additionally, the lack of detectable junction sequences attributable to plasmid backbone sequences leads to the conclusion that no backbone sequences from PV-GMIR13196 are present in MON 87751.

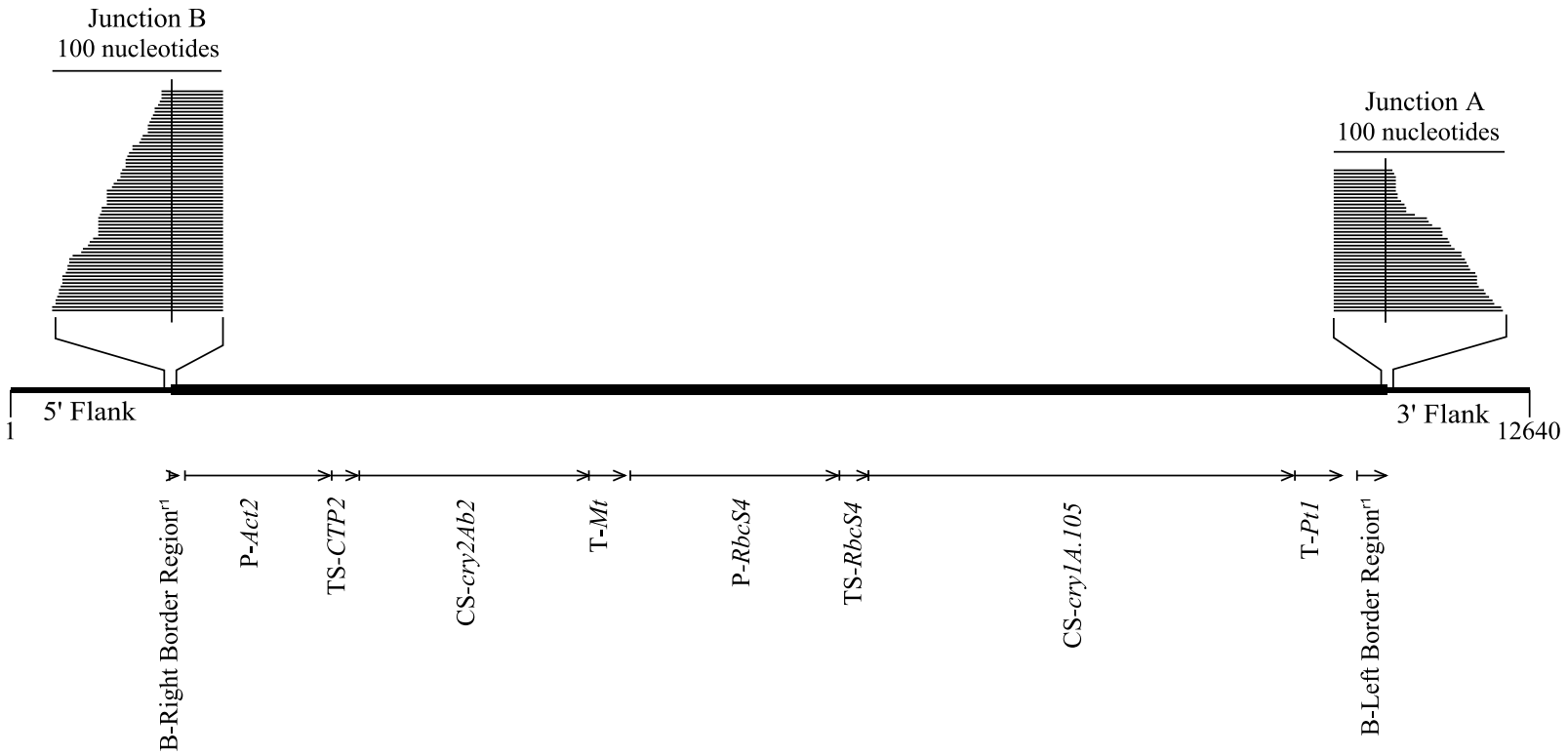

Figure IV-6. Junction Sequences Detected by NGS/JSA

Linear map of MON 87751 illustrating the relationship of the detected junction sequences to the insert locus. The individual junction sequences detected by JSA are illustrated as stacked bars.

[r1]Superscript in Left and Right Border Regions indicate that the sequence in MON 87751 was truncated compared to the sequences in PV-GMIR13196.

IV.B. Organization and Sequence of the Insert and Adjacent DNA in MON 87751

The organization of the elements within the DNA insert and the adjacent genomic DNA was assessed using directed DNA sequence analysis (refer to Figure IV-1, Step 4). PCR primers were designed to amplify four overlapping regions of the MON 87751 genomic DNA that span the entire length of the insert (Figure IV-7). The amplified PCR products were subjected to DNA sequencing analyses. The results of this analysis confirm that the MON 87751 insert is 10,119 bp and that each genetic element in the insert is intact, with the exception of the border regions. The border regions both contain small terminal deletions with the remainder of the inserted border regions being identical to the sequence in PV-GMIR13196. The sequence and organization of the insert was also shown to be identical to the corresponding T-DNA I of PV-GMIR13196, confirming that a single copy of T-DNA I was inserted as intended. This analysis also shows that only T-DNA I elements (described in Table IV-1) are present and no PV-GMIR13196 backbone or T-DNA II elements are present in MON 87751.

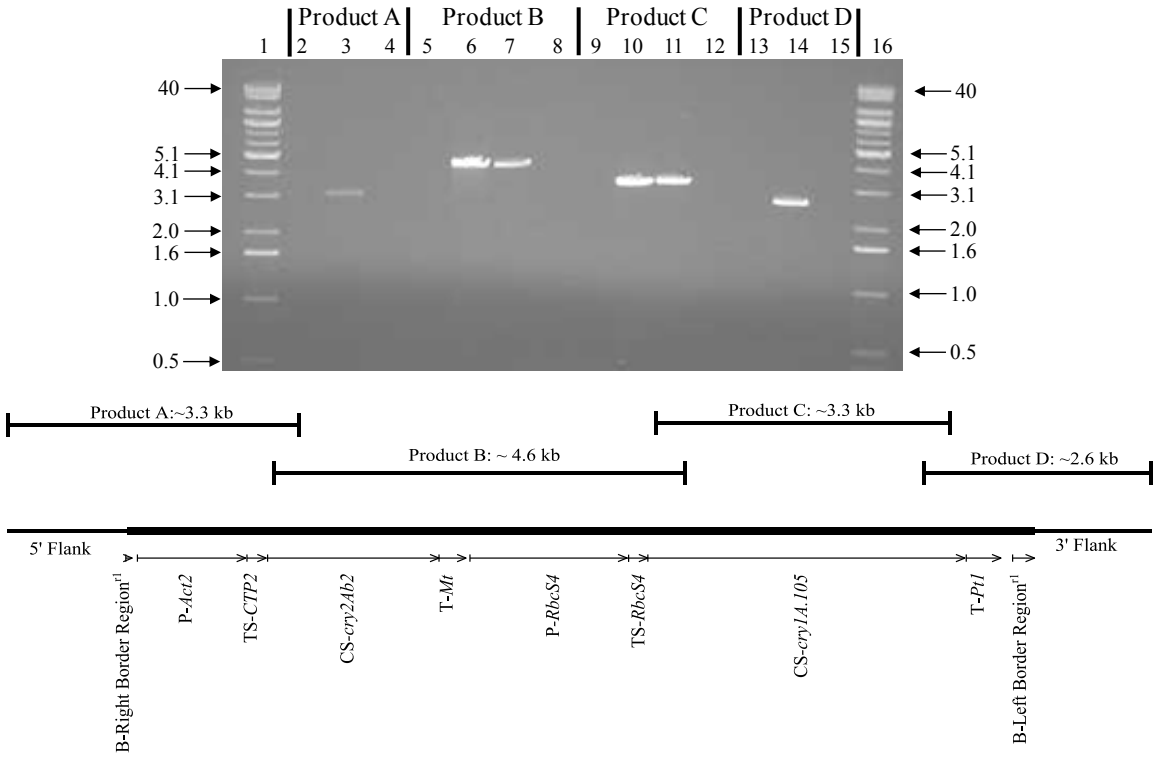

Figure IV-7. Overlapping PCR Analysis across the Insert in MON 87751
PCR was performed on both conventional control genomic DNA and genomic DNA of the R3 generation of MON 87751 using four pairs of primers to generate overlapping PCR fragments from MON 87751 for sequencing analysis. To verify the production of PCR products, 5 µl of each of the PCR reactions was loaded on the gel, except where noted below. The expected product size for each amplicon is provided in the illustration. Lane designations are as follows:

Lane		**Lane**	
1	1 Kb DNA Extension Ladder	9	Conventional Control A3555
2	Conventional Control A3555	10	PV-GMIR13196
3	MON 87751 (10 µl)	11	MON 87751
4	No template control	12	No template control
5	Conventional Control A3555	13	Conventional Control A3555
6	PV-GMIR13196	14	MON 87751
7	MON 87751	15	No template control
8	No template control	16	1 Kb DNA Extension Ladder
Arrows next to the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen, Grand Island, NY) on the ethidium bromide stained gel.			

[r1]Superscript in Left and Right Border Regions indicate that the sequence in MON 87751 was truncated compared to the sequences in PV-GMIR13196.

IV.C. Sequencing of the MON 87751 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from the conventional control to examine the insertion site in conventional soybean (refer to Figure IV-1, Step 5). The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5' end of the MON 87751 insert paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure IV-8). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 87751 indicates both a 1 base pair insertion and a 7 base pair deletion at the insertion site as well as a 16 base pair deletion in the 5′ flanking region that occurred during integration of the T-DNA. The remainder of the flanks in MON 87751 are identical to the conventional control. Such changes are common during plant transformation and these changes presumably resulted from double-stranded break repair mechanisms in the plant during *Agrobacterium* mediated transformation process (Salomon and Puchta 1998).

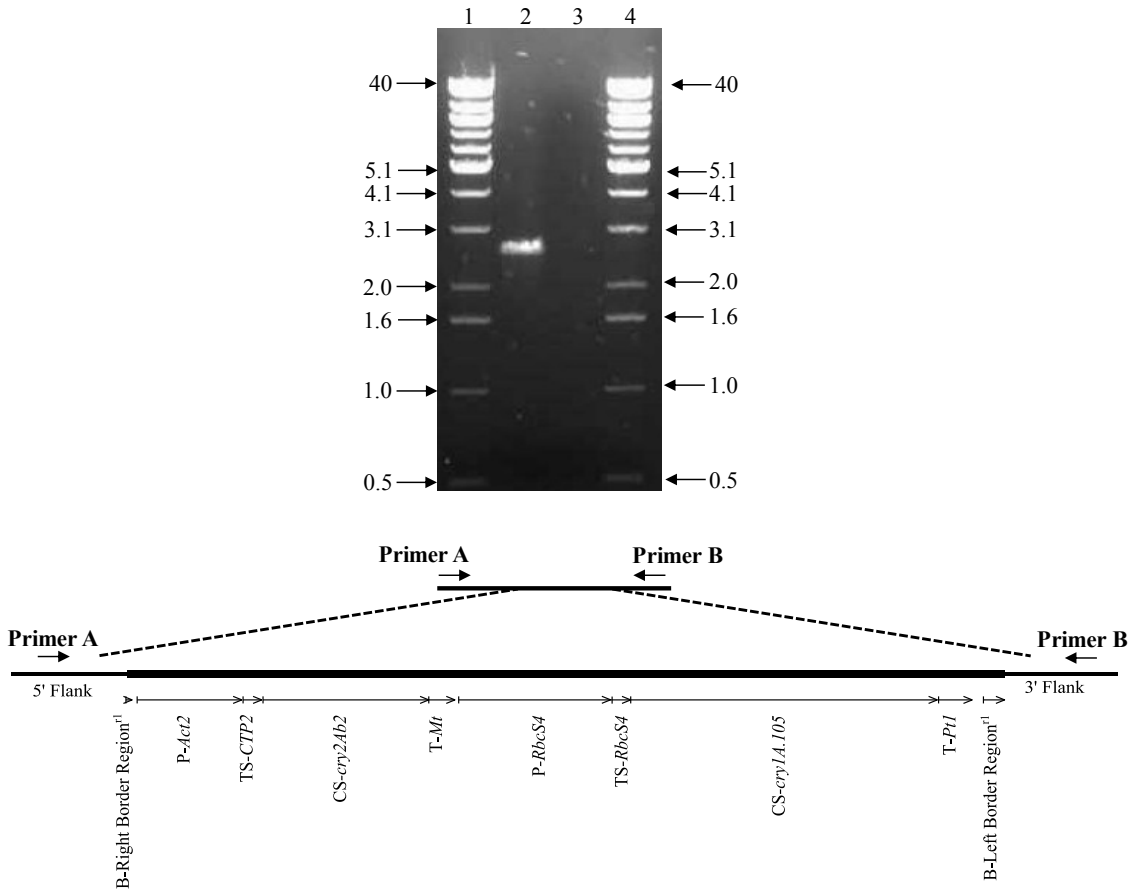

Figure IV-8. PCR Amplification of the MON 87751 Insertion Site

PCR analysis was performed to evaluate the insertion site. PCR was performed on conventional control DNA using Primer A, specific to the 5′ flanking sequence, and Primer B, specific to the 3′ flanking sequence of the insert in MON 87751. The DNA generated from the conventional control PCR was used for sequencing analysis. This illustration depicts the MON 87751 insertion site in the conventional control (upper panel) and the MON 87751 insert (lower panel). Approximately 5 µl of each of the PCR reactions was loaded on the gel. Lane designations are as follows:

Lane	Sample
1	1 Kb DNA Extension Ladder
2	Conventional Control A3555
3	No template DNA control
4	1 Kb DNA Extension Ladder

Arrows next to the gel denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen, Grand Island, NY) on the ethidium bromide stained gel.

[r1]Superscript in Left and Right Border Regions indicate that the sequence in MON 87751 was truncated compared to the sequences in PV-GMIR13196.

IV.D. Determination of Insert Stability over Multiple Generations of MON 87751

In order to demonstrate the stability of the T-DNA present in MON 87751 through multiple generations, NGS/JSA analysis was performed using DNA obtained from five breeding generations of MON 87751. The breeding history of MON 87751 is presented in Figure IV-5, and the specific generations tested are indicated in the figure legend. The MON 87751 (R_3) generation was used for the molecular characterization analyses discussed in Sections IV.A - IV.C and shown in Figure IV-5. To assess stability, four additional generations were evaluated by NGS/JSA analysis as previously described in Section IV.A, and compared to the fully characterized MON 87751 (R_3) generation. The conventional control used for the generational stability analysis was A3555, which has a genetic background similar to the other generations in Table IV-5 and represents the original transformation line. Genomic DNA isolated from each of the selected generations of MON 87751 and conventional control was used for NGS/JSA analysis.

To determine the insert number in the MON 87751 generations, the sequences selected as described in Section IV.A.2.1 were analyzed using JSA (Kovalic et al. 2012). Table IV-3 shows the number of resultant JSCs containing PV-GMIR13196 DNA sequence determined by this analysis.

Table IV-3. Junction Sequence Classes Detected

Sample	Junction Sequence Classes Detected
MON 87751 (R_3)	2
MON 87751 (R_4)	2
MON 87751 (R_5)	2
MON 87751 (R_6)	2
MON 87751 (R_7)	2
A3555	0

Alignment of the JSCs from each of the assessed MON 87751 generations (R_4, R_5, R_6, and R_7) to the full flank/insert sequence and JSCs determined for the MON 87751 R3 generation, confirms that the pair of JSCs originates from the same region of the MON 87751 genome and is linked by contiguous, known and expected DNA sequence. This single identical pair of JSCs is observed as a result of the insertion of PV-GMIR13196 T-DNA I at a single locus in the genome of MON 87751. The consistency of these JSC data across all generations tested demonstrates that this single locus was stably maintained throughout the MON 87751 breeding process.

These results demonstrate that the MON 87751 single integration locus was maintained through several generations of breeding MON 87751; thereby confirming the stability of the insert. Based on this comprehensive sequence data and bioinformatic analysis (NGS/JSA), it is concluded that MON 87751 contains a single and stable T-DNA I insertion.

IV.E. Inheritance of the Genetic Insert in MON 87751

During development of MON 87751, segregation data were generated to assess the heritability and stability of T-DNA I in MON 87751. Chi square (χ^2) analysis was performed over several generations to confirm the segregation and stability of T-DNA I in MON 87751. The Chi square analysis is based on testing the observed segregation ratio to the segregation ratio expected based on Mendelian principles.

The MON 87751 breeding path for generating segregation data is described in Figure IV-9. The transformed R_0 plant was self-pollinated to produce R_1 seed. From the R_1 segregating population, which consisted of 42 total plants containing T-DNA I but not T-DNA II, an individual plant homozygous for the *cry1A.105* and *cry2Ab2* genes was identified via Real-Time TaqMan® PCR and Invader® analyses. Real-Time TaqMan PCR measures the accumulation of the fluorescent signals incorporated into the target sequence throughout the entire PCR assay. The Invader analysis is a signal amplification technology for quantitative analysis or detection of genetic variations. The Invader method uses Cleavase® enzymes, a structure-specific family of endonucleases, and fluorescence detection to detect specific target sequences.

The selected R_1 MON 87751 homozygous plant was self-pollinated to give rise to a population of R_2 plants, which were in turn self-pollinated to obtain the R_3 generation. At each generation, the fixed homozygous plants were tested for the expected segregation pattern of 1:0 (positive: negative) for the MON 87751 T-DNA I using the Real-Time TaqMan® PCR and Invader® analyses.

Homozygous R_3 MON 87751 plants were crossed to a Monsanto proprietary soybean line (MonSoy8329) that did not contain the *cry1A.105* and *cry2Ab2* expression cassettes to produce F_1 hemizygous seed. A hemizygous F_1 plant was selected and then self-pollinated to produce F_2 seed. The resulting F_2 plants were tested for the presence of the T-DNA I by Real-Time TaqMan® PCR assay. This process of self-pollination of hemizygous plants and zygosity determination of the MON 87751 T-DNA I by Real-Time TaqMan PCR analysis was repeated for the F_2, F_3, and F_4 plants. Subsequently, assessment at each of these generations was based on zygosity, and MON 87751 T-DNA I was predicted to segregate at a 1:2:1 (homozygous positive: hemizygous positive: homozygous negative) ratio for progeny derived from a hemizygous parental plant according to Mendelian inheritance principles.

A Chi square (χ^2) analysis was used to compare the observed segregation ratios to the expected ratios according to Mendelian inheritance principles. The χ^2 was calculated as:

$$\chi^2 = \sum [(| o - e |)^2 / e]$$

® TaqMan is a registered trademark of Roche Molecular Systems, Inc.
® Invader and Cleavase are registered trademarks of Third Wave Technologies, Inc.

where o = observed frequency of the phenotype and e = expected frequency of the phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the χ^2 analysis of the segregating progeny of MON 87751 are presented in Table IV-4 The χ^2 value in the F_2, F_3, and F_4 generations indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio. These results support the conclusion that the MON 87751 T-DNA resides at a single locus within the soybean genome and is inherited according to Mendelian inheritance principles. These results are also consistent with the molecular characterization data that indicate MON 87751 contains single, intact copies of the *cry1A.105* and *cry2Ab2* expression cassettes that were inserted into the soybean genome at a single locus.

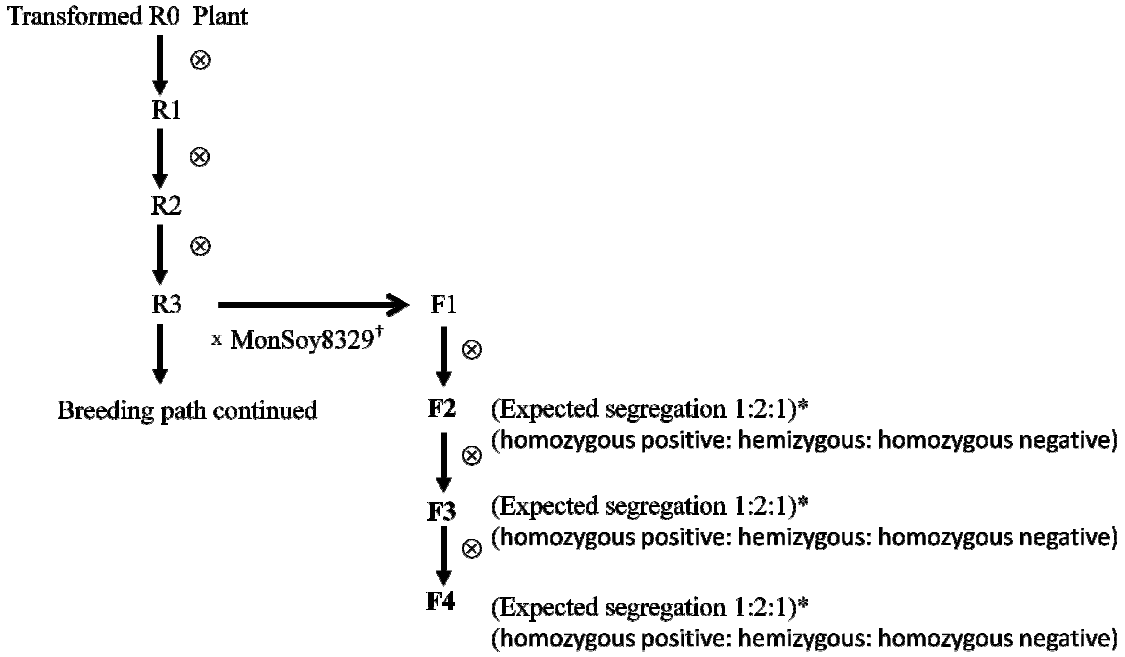

Figure IV-9. Breeding Path for Generating Segregation Data for MON 87751

⊗Self pollinated.

* Chi-square analysis conducted on segregation data from the F_2, F_3, and F_4 generations (bolded text). An individual hemizygous plant from each of the F_1, F_2, and F_3 populations was self-pollinated to product the population for the next generation.

†The soybean line used in the cross that did not contain the *cry1A.105* and *cry2Ab2* genes is MonSoy8329**.**

Table IV-4. Segregation of the Expression Cassette During the Development of MON 87751

					1:2:1 Segregation				
Generation	**Total Plants[2]**	**Observed Homozygous Positive**	**Observed Hemizygous**	**Observed Homozygous Negative**	**Expected Homozygous Positive**	**Expected Hemizygous**	**Expected Homozygous Negative**	**χ^2**	**Probability[3]**
F_2[1]	152	39	72	41	38	76	38	0.47	0.79
F_3[1]	214	49	114	51	53.5	107	53.5	0.95	0.62
F_4[1]	204	58	105	41	51	102	51	3.01	0.22

[1]Segregation was evaluated using results of Real-Time TaqMan analysis.
[2]"Total plants" refers to the total number of plants in which zygosity could be determined using the assay.
[3]Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

IV.F. Characterization of the Genetic Modification Summary and Conclusion

Molecular characterization of MON 87751 by NGS/JSA and directed sequencing demonstrated that a single copy of the intended transfer DNA (T-DNA I) containing the *crylA.105* and the *cry2Ab2* expression cassettes from PV-GMIR13196 was integrated into the soybean genome at a single locus. These analyses also showed no PV-GMIR13196 backbone or T-DNA II elements had been inserted

Directed sequence analyses performed on MON 87751 confirmed the organization and intactness of the full T-DNA I and all expected elements within the insert, with the exception of incomplete Right and Left Border sequences that do not affect the functionality of the *crylA.105* or the *cry2Ab2* expression cassettes. Analysis of the T-DNA I insertion site in soybean showed that the flanks in MON 87751 are identical to the conventional control, except for a 1 base pair insertion and a 7 base pair deletion at the insertion site, and a 16 base pair deletion in the 5′ flanking region of the insertion site.

Generational stability analysis by NGS/JSA demonstrated that the T-DNA in MON 87751 was maintained through five breeding generations, thereby confirming the stability of the insert. Results from segregation analyses show heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA I in MON 87751 at a single chromosomal locus.

V. CHARACTERIZATION AND SAFETY ASSESSMENT OF THE Cry1A.105 AND Cry2Ab2 PROTEINS PRODUCED IN MON 87751

Characterization of the introduced protein(s) in a biotechnology-derived crop is important to establishing food and feed safety and assessing environmental risk. As described in Section IV, MON 87751 contains the *cry1A.105 and cry2Ab2* expression cassettes that, when transcribed and translated, result in the expression of the Cry1A.105 and Cry2Ab2 proteins.

This section summarizes: 1) the identity and function of the Cry1A.105 and Cry2Ab2 proteins produced in MON 87751; 2) assessment of equivalence between the plant-produced and *E. coli*-produced proteins; 3) the level of the Cry1A.105 and Cry2Ab2 proteins in plant tissues from MON 87751; 4) assessment of the potential allergenicity of the Cry1A.105 and Cry2Ab2 proteins produced in MON 87751; and 5) the food and feed safety assessment of the Cry1A.105 and Cry2Ab2 proteins produced in MON 87751. The data support a conclusion that the proteins produced in MON 87751 are safe for human or animal consumption and is based on several lines of evidence summarized below. Similar data submitted to USDA-APHIS in petition 09-082-01p for the antecedent organism, MON 87701, supported the same safety conclusions for Cry1Ac.

V.A. Identity, Function, and Specificity of the Cry1A.105 and Cry2Ab2 Proteins from MON 87751

Cry1A.105 and Cry2Ab2 proteins are derived from *B. thuringiensis*, a ubiquitous gram-positive soil bacterium that accumulates crystal proteins during sporulation. Cry1A.105 and Cry2Ab2 proteins are members of the 3D-Cry family of insecticidal proteins (Crickmore 2012). 3D-Cry proteins are subdivided into different groups based on the high specificity they have for their target category of insects. Because of their narrow spectrum of activity, they lack an impact on broader insect populations or other organisms. For example, both Cry1A.105 and Cry2Ab2 proteins have insecticidal activity specifically against lepidopteran insects (Höfte and Whiteley 1989).

The generalized MOA for Cry proteins was described by English and Slatin (1992). It includes ingestion of the crystals by insects and solubilization of the crystals in the insect midgut, followed by activation through proteolytic processing of the soluble Cry protein by digestive enzymes in their midguts. The activated protein then binds to specific receptors on the surface of the midgut epithelium of target insects and inserts into the membrane, leading to pore formation and generalized disruption of the transmembrane gradients and, therefore, cell integrity. While alternate mechanisms have been proposed, a review of the available data has recently been published and the authors concluded that the original model, pore formation, is the most valid model for Cry protein MOA (Vachon, et al. 2012).

V.A.1. Identity and Function of the Cry1A.105 Protein from MON 87751

Cry1A.105 in MON 87751 is a protein consisting of a single polypeptide of 1181 amino acids containing three domains, with an apparent molecular weight of approximately

133 kDa. Like other Cry proteins, it is synthesized as a prototoxin and is likely cleaved by digestive enzymes in the midgut of target organisms to an approximately 60 kDa activated protein (Bravo, et al. 2007). Cry1A.105 is a chimeric protein that consists of domains I and II from Cry1Ab or Cry1Ac[3], domain III from Cry1F, and the C-terminal domain from Cry1Ac (Figure V-1). Cry1Ac, Cry1Ab and Cry1F are all well known and well characterized insecticidal proteins derived from the soil bacterium *B. thuringiensis*. Cry1A.105 was designed using domain exchange strategy to achieve high levels of activity against target lepidopteran insect pests. Domain exchange is a well known mechanism in nature, resulting in Cry protein diversities that have been described extensively in the literature (de Maagd, et al. 2003; de Maagd et al. 2001). Domain exchange strategy with modern molecular biological tools has been used to switch the functional domains of Cry1 proteins to develop microbial biopesticides with improved specificity to lepidopteran insect pests. (Baum 1998; Baum, et al. 1999; Gao, et al. 2006). Domains I and II of Cry1A.105 are 100% identical to the respective domains of Cry1Ab or Cry1Ac. Domain III of Cry1A.105 is 99% identical to domain III of Cry1F. The C-terminal region of Cry1A.105 is 100% identical to that of Cry1Ac. The overall amino acid sequence identity of Cry1A.105 to Cry1Ac, Cry1Ab, and Cry1F is 93.6%, 90.0%, and 76.7 %, respectively.

The Cry1A.105 protein expressed in MON 87751 is targeted to chloroplasts through the addition of a chloroplast transit peptide (CTP) coding sequence at the 5′ end of the coding sequence (Section III). Following translation and translocation into chloroplasts, the CTP is cleaved. Experimental analysis of the N-terminus of MON 87751-produced Cry1A.105 protein (described below) indicated the presence of four additional amino acids derived from the CTP at the N-terminus compared to the Cry1A.105 protein in MON 89034 (Figure V-2). The additional four amino acids are cysteine (C), methionine (M), glutamine (Q), and alanine (A). While the identities of methionine, glutamine, and alanine were clearly determined by N-terminal sequencing, the identity of the first amino acid, cysteine, was inferred based on the *RbcS*4 targeting sequence in MON 87751. The chemistry employed in N-terminal sequencing is known to degrade cysteine, preventing its clear identification. With the exception of the four additional CTP-derived amino acids, the deduced sequence of the Cry1A.105 protein that accumulates in MON 87751 shares 100% amino acid identity with the deduced sequence of the Cry1A.105 protein present in MON 89034. The presence of these four additional amino acids at the N-terminus of the MON 87751-produced Cry1A.105 protein are unlikely to impact protein specificity because they are not within the trypsin-resistant core that is responsible for target organism specificity and efficacy. Accordingly, this small difference is not expected to result in unanticipated adverse impacts on humans, livestock or NTOs.

[3] Cry1Ab and Cry1Ac share 100% amino acid sequence identity in domains I and II.

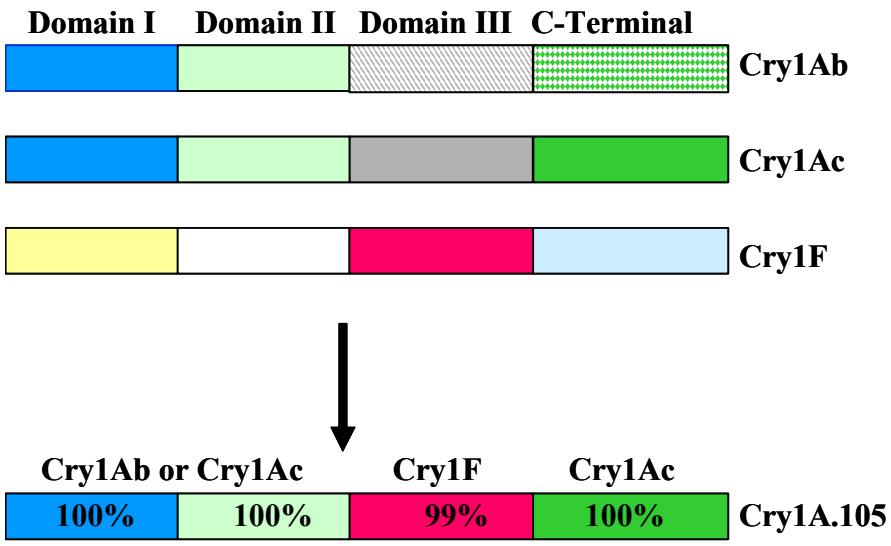

Figure V-1. Schematic representation of the origin of Cry1A.105 protein domains
Different colors and patterns are used to differentiate the origin of domains. For simplicity, the lengths of domains in this illustration are not in proportion to the lengths of amino acid sequence of the respective domains.

V.A.2. Identity and Function of the Cry2Ab2 Protein from MON 87751

Cry2Ab2 protein in MON 87751 is a protein consisting of a single polypeptide of 619 amino acids with an apparent molecular weight of approximately 62 kDa. Like other Cry proteins, it is synthesized as a prototoxin and is likely cleaved by digestive enzymes in the midgut of target organisms to an approximately 60 kDa activated protein (Bravo et al. 2007).

The protein coding region of the *cry2Ab2* coding sequence present in MON 87751 is identical to the *cry2Ab2* coding sequence present in MON 89034 and both are a slight variant of the wild-type *cry2Ab2* coding sequence from *B. thuringiensis*. Accumulation of the Cry2Ab2 protein in MON 87751 is targeted to the chloroplasts due to the addition of a CTP coding sequence at the 5′ end of the coding sequence (refer to Section III). Experimental analysis of the N-terminus of MON 87751-produced Cry2Ab2 protein (described below) indicated that the conjunction of the CTP sequence with the Cry2Ab2 sequence resulted in CTP cleavage at a position 15 amino acids within the Cry2Ab2 protein sequence, likely as a result of the processing of the N-terminal CTP by a general stromal processing peptidase (SPP) (Richter and Lamppa 1998). This deletion results in an amino acid sequence for the MON 87751-derived Cry2Ab2 protein that is 18 amino acids shorter than the MON 89034-derived Cry2Ab2 protein (Figure V-2) because the MON 89034-derived protein is three amino acids longer than wild type Cry2Ab2 (U.S. EPA 2010c). The deletion of these amino acids at the N-terminus of the MON 87751-produced Cry2Ab2 protein are unlikely to impact protein specificity because they are not within the trypsin-resistant core that is responsible for target

organism specificity and efficacy. Accordingly, this small difference is not expected to result in unanticipated adverse impacts on humans, livestock or NTOs.

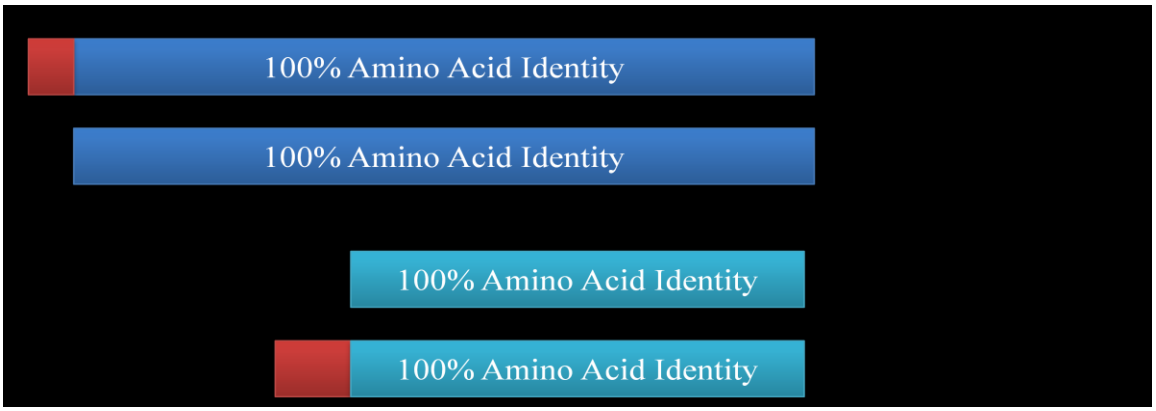

Figure V-2. Schematic representation of the Cry1A.105 and Cry2Ab2 amino acid sequences in MON 87751 and MON 89034

V.B. Characterization and Equivalence of Cry1A.105 and Cry2Ab2 Proteins from MON 87751

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of the protein(s) produced from the inserted DNA, and confirmation of the safety of the protein(s). The expression level of Cry1A.105 and Cry2Ab2 in MON 87751 is low, and insufficient for use in the subsequent safety evaluations. Therefore, recombinant Cry1A.105 and Cry2Ab2 proteins were produced in *Escherichia coli*, using expression vectors with *cry1A.105* or *cry2Ab2* coding sequences that matched those of the *cry1A.105* or *cry2Ab2* coding sequences in MON 87751. The physicochemical and functional characteristics of the MON 87751-produced Cry1A.105 and MON 87751-produced Cry2Ab2 proteins were determined and each was shown to be equivalent to its respective *E. coli*-produced protein. A summary of the analytical results for each protein are shown below and the details of the materials, methods, and results are described in Appendix C.

The MON 87751-produced Cry1A.105 and Cry2Ab2 proteins purified from MON 87751 seed were characterized and assessed for physicochemical and functional equivalence to the *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins using a panel of analytical tests: 1) N-terminal sequence analysis of MON 87751-produced proteins characterized the N-terminus; 2) MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 87751-produced sequences; 3) western blot analysis with antibodies specific for each protein demonstrated that the immunoreactive properties of the MON 87751-produced and *E. coli*-produced proteins were equivalent; 4) SDS-PAGE analysis showed that the electrophoretic mobility and apparent molecular weight of the MON 87751-produced and *E. coli*-produced proteins were equivalent; 5) MON 87751-produced and

E. coli-produced proteins were both determined to be non-glycosylated; and 6) functional activity analysis demonstrated that MON 87751-produced and *E. coli*-produced proteins had equivalent respective insecticidal activities. Additionally, functional activity analysis demonstrated that MON 87751-produced Cry1A.105 and Cry2Ab2 proteins are equivalent to the *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins used for previous toxicity assessments with mice and NTOs in support of MON 89034; thus enabling those previous studies to inform assessments of MON 87751 (Appendix C, Sections C.1.8 and C.2.8).

Taken together, these data provide a detailed characterization of the MON 87751-produced Cry1A.105 and Cry2Ab2 proteins and establish their respective equivalence to *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins. This equivalence justifies the use of protein studies using *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins, summarized in section V.E., to establish the safety of the Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751.

V.C. Expression Levels of Cry1A.105 and Cry2Ab2 Proteins in MON 87751

Cry1A.105 and Cry2Ab2 protein levels in various tissues of MON 87751 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Leaves from four growth stages (Over season leaf (OSL) 1 through OSL4), forage, root, and seed tissue samples of MON 87751 were collected from four replicate plots planted in a randomized complete block field design during the 2012 growing season from the following five field sites in the U.S.: Jackson County, Arkansas (site code ARNE); Jefferson County, Iowa (site code IARL); Pawnee County, Kansas (site code KSLA); Perquimans County, North Carolina (site code NCBD); and Lehigh County, Pennsylvania (site code PAGR). The field sites were representative of soybean producing regions suitable for commercial production. Flowers for the collection of pollen/anther tissue of MON 87751 were also harvested during the 2012 U.S. growing season from a field site in Champaign County, Illinois (site code ILTH). At this site, tissue was collected from one non-randomized plot.

V.C.1. Expression Levels of Cry1A.105 Protein in MON 87751

Cry1A.105 protein levels were determined in all eight tissue types collected. The ELISA results obtained for each sample were averaged across the five sites, except for pollen/anther where only one site was analyzed because of the limited amount of tissue available, and are summarized in Table V-1. The details of the materials and methods are described in Appendix D. Moisture content was not determined for pollen/anther because of a lack of tissue, therefore, pollen/anther results were only reported in fresh weight (fw).

Table V-1. Summary of Cry1A.105 Protein Levels in Tissues from MON 87751 Grown in 2012 United States Field Trials

Tissue Type[1]	Development Stage[2]	Mean(SD) Range (µg/g fw)[3]	Mean(SD) Range (µg/g dw)[4]	LOQ/ LOD (µg/g fw)[5]
OSL1	V3 – V4	130 (50) 61 – 220	580 (250) 260 – 1100	1.500/0.406
OSL2	V5 – V7	120 (54) 13 – 220	590 (270) 68 – 1100	1.500/0.406
OSL3	R2 – R3	79 (45) 8.5 – 160	400 (220) 50 – 780	1.500/0.406
OSL4	R6	230 (82) 120 – 480	790 (280) 430 – 1600	1.500/0.406
Root	R6	<LOD (N/A) N/A – N/A	N/A (N/A) N/A – N/A	0.563/0.322
Forage	R6	62 (21) 31 – 110	230 (91) 110 – 440	1.500/0.524
Seed	R8	2.1 (0.46) 1.5 – 2.9	2.4 (0.50) 1.7 – 3.2	0.900/0.226
Pollen/Anther	R2	11 (N/A) N/A – N/A	N/A (N/A) N/A – N/A	1.500/N.D.[6]

[1] OSL= over season leaf
[2] The crop development stage each tissue was collected.
[3] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20 except for OSL1 where n=19 due to one sample expressing <LOD and pollen/anther where n=1). N/A: Not Applicable
[4] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data. Moisture content was not determined for pollen/anther due to a lack of tissue, therefore, pollen/anther results were only reported in fresh weight.
[5] LOQ=limit of quantitation; LOD=limit of detection.
[6] N.D. = Not determined. Pollen/anther LOD was not determined due to an insufficient amount of tissue.

V.C.2. Expression Levels of Cry2Ab2 Protein in MON 87751

Cry2Ab2 protein levels were determined in all eight tissue types collected. The ELISA results obtained for each sample were averaged across the five sites, except for pollen/anther where only one site was analyzed because of the limited amount of tissue available, and are summarized in Table V-2. The details of the materials and methods are described in Appendix D. Moisture content was not determined for pollen/anther due to a lack of tissue, therefore, pollen/anther results were only reported in fresh weight.

Table V-2. Summary of Cry2Ab2 Protein Levels in Tissues from MON 87751 Grown in 2012 United States Field Trials

Tissue Type[1]	Development Stage[2]	Mean(SD) Range (µg/g fw)[3]	Mean(SD) Range (µg/g dw)[4]	LOQ/LOD (µg/g fw)[5]
OSL1	V3 – V4	5.4 (0.74) 4.4 – 6.8	24 (5.9) 17 – 37	0.625/0.034
OSL2	V5 – V7	5.2 (0.70) 4.0 – 6.6	26 (3.1) 20 – 33	0.625/0.034
OSL3	R2 – R3	6.3 (0.80) 5.2 – 8.0	32 (5.2) 25 – 43	0.625/0.034
OSL4	R6	6.9 (0.79) 5.5 – 8.5	24 (2.7) 18 – 29	0.625/0.034
Root	R6	4.6 (1.0) 3.1 – 7.1	15 (2.7) 11 – 22	1.250/1.241
Forage	R6	3.9 (0.60) 3.0 – 5.1	14 (2.2) 11 – 18	0.313/0.060
Seed	R8	3.6 (0.71) 2.3 – 4.7	4.0 (0.77) 2.6 – 5.1	0.313/0.094
Pollen/Anther	R2	7.7 (N/A) N/A – N/A	N/A (N/A) N/A – N/A	0.313/N.D.[6]

[1] OSL= over season leaf
[2] The crop development stage each tissue was collected.
[3] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20 except for OSL1 where n=19 due to one sample expressing <LOD and pollen/anther where n=1). N/A: Not Applicable
[4] Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data. Moisture content was not determined for pollen/anther due to a lack of tissue, therefore, pollen/anther results were only reported in fresh weight.
[5] LOQ=limit of quantitation; LOD=limit of detection.
[6] N.D. = Not determined. Pollen/anther LOD was not determined due to an insufficient amount of tissue.

V.D. Assessment of Potential Allergenicity of the Cry1A.105 and Cry2Ab2 Proteins

The allergenic potential of an introduced protein is assessed by comparing the physiochemical characteristics of the introduced protein to physiochemical characteristics of known allergens (Codex Alimentarius 2009). Using a weight-of-evidence approach, a protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents a small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; and 4) the protein does not show resistance to pepsin digestion. Cry1A.105 and Cry2Ab2 were both assessed following these guidelines.

V.D.1. Assessment of Potential Allergenicity of the Cry1A.105 Protein

The Cry1A.105 protein has been assessed for its potential allergenicity according to the safety assessment guidelines cited above.

1) The Cry1A.105 protein originates from *B. thuringiensis,* an organism that has not been reported to be a source of known allergens.

2) The Cry1A.105 protein represents no more than 0.0006% of the total protein in the seed of MON 87751.

3) Bioinformatics analyses demonstrated that the Cry1A.105 protein does not share amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* digestive fate experiments conducted with the Cry1A.105 protein demonstrate that the protein is rapidly digested by pepsin in a simulated gastric fluid (SGF) assay.

Taken together, these data support the conclusion that the Cry1A.105 protein in MON 87751 does not pose a significant allergenic risk to humans or animals.

V.D.2. Assessment of Potential Allergenicity of the Cry2Ab2 Protein

The Cry2Ab2 protein has been assessed for its potential allergenicity according to the safety assessment guidelines cited above.

1) The Cry2Ab2 protein originates from *B. thuringiensis,* an organism that has not been reported to be a source of known allergens.

2) The Cry2Ab2 protein represents no more than 0.001% of the total protein in the seed of MON 87751.

3) Bioinformatics analyses demonstrated that the Cry2Ab2 protein in MON 87751 does not share amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes.

4) Finally, *in vitro* digestive fate experiments conducted with the Cry2Ab2 protein demonstrate that the protein is rapidly digested by pepsin in a simulated gastric fluid (SGF) assay.

Taken together, these data support the conclusion that the Cry2Ab2 protein does not pose a significant allergenic risk to humans or animals.

V.E. Safety Assessment Summary of Cry1A.105 and Cry2Ab2 Proteins in MON 87751

A comprehensive set of factors have been considered and evaluated in the safety assessment of the Cry1A.105 and Cry2Ab2 proteins in food and feed or the environment. The results are summarized below along with the conclusions reached from each assessment.

V.E.1. The Donor Organism has a History of Safe Use

The donor organism for *cry1A.105* and *cry2Ab2*, *B. thuringiensis,* has been used commercially in the United States to produce microbial-derived products with insecticidal activity. Applications of sporulated *B. thuringiensis* have a long history of safe use for pest control in agriculture, especially in organic farming (Cannon 1993; IPCS 1999; U.S. EPA 1988). Microbial pesticides containing *B. thuringiensis* Cry proteins have been subjected to extensive toxicity testing showing no adverse effects to human health (Baum et al. 1999; Betz, et al. 2000; McClintock, et al. 1995; Mendelsohn, et al. 2003; U.S. EPA 2001a; 2005). Additionally, there are no confirmed cases of allergic reactions to Cry proteins in microbial-derived *B. thuringiensis* products during more than 50 years of use.

V.E.2. The Cry1A.105 and Cry2Ab2 Proteins have a History of Safe Use

Microbial pesticides that contain Cry1Ac/Cry1F chimeric protein have been used for control of lepidopteran pests since 1997 (Baum 1998; Baum et al. 1999). Cry1A.105 itself is expressed in MON 89034 maize, which was reviewed and approved by regulatory agencies around the world and has been commercially available in the United States since 2009. The MON 87751 Cry1A.105 protein has 99% amino acid identity with the MON 89034-produced Cry1A.105 protein overall and 100% homology with the protease-resistant core which is responsible for target organism specificity and efficacy. U.S. EPA has approved a tolerance exemption for Cry1A.105 in maize (40 CFR 174.502) (U.S. EPA 2010c). In addition, a biotechnology-derived cotton expressing another chimeric protein consisting of domains or sequences from Cry1F, Cry1C, and Cry1Ab was reviewed and approved by regulatory agencies and has been commercialized (Gao et al. 2006). The large scale cultivation of these crops without any indication of harmful impact on the environment, non-target insects, or mammals provides additional evidence for the safety of the Cry1A.105 protein. Taken together, these data demonstrate that the Cry1A.105 protein has a history of safe use and does not pose any adverse effects to human and animal health.

Cry2Ab2 is derived from *B. thuringiensis* subsp. *kurstaki*. *B. thuringiensis* subsp. *kurstaki* has been used as an active ingredient in many commercial microbial pesticide products such as DiPel® and Cutlass®, and Cry2Ab protein is identified as one of the proteins in Cutlass® (Betz et al. 2000). Cry2Ab2 is also expressed in commercially available MON 89034 maize. The MON 87751-produced Cry2Ab2 protein has 97% amino acid identity with the MON 89034-produced Cry2Ab2 protein overall and 100% homology with the protease-resistant core which is responsible for target organism specificity and efficacy. U.S. EPA has approved a tolerance exemption for Cry2Ab2 as expressed in maize and cotton (40 CFR 174.519) (U.S. EPA 2010c). The large scale cultivation of crops expressing Cry2Ab2 without any indication of harmful impact on the environment, non-target insects, or mammals provides additional evidence for the safety of the Cry2Ab2 protein. Taken together, these data demonstrate that the Cry2Ab2 protein has a history of safe use and does not pose any adverse effects to human and animal health.

V.E.3. Cry1A.105 and Cry2Ab2 Proteins Demonstrate Specificity

Cry1A.105 and Cry2Ab2 proteins have insecticidal activity specifically against lepidopteran insects (U.S. EPA 2010c). The history of safe use of Cry1A.105 and Cry2Ab2, without any indication of harmful impact on non-target insects or mammals, provides evidence for the safety of the Cry1A.105 and Cry2Ab2 proteins.

V.E.4. Cry1A.105 and Cry2Ab2 Proteins in MON 87751 are Not Homologous to Known Allergens or Toxins

Bioinformatics analyses were performed to assess the potential for allergenicity, toxicity, or biological activity of Cry1A.105 and Cry2Ab2. The analysis demonstrated that neither protein shares amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins which could have adverse effects on human or animal health.

V.E.5. Cry1A.105 and Cry2Ab2 Proteins in MON 87751 are Labile in *in vitro* Digestion Assays

Both the Cry1A.105 and Cry2Ab2 proteins were readily digestible in simulated gastric fluid (SGF). Digestion in simulated intestinal fluid (SIF) was also assessed. As expected, Cry1A.105 and Cry2Ab2 subjected to SIF were each processed to a trypsin-resistant core that was stable throughout the SIF digestion period. These results are consistent with observations for these and other Cry proteins subjected to SIF digestion. Rapid degradation of the Cry1A.105 and Cry2Ab2 proteins in SGF makes it highly unlikely that either protein would be absorbed in a form other than component nutritional amino acids in the small intestine and have any adverse effects on human or animal health.

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V.E.6. Cry1A.105 and Cry2Ab2 Proteins are Not Acutely Toxic

Acute oral toxicology studies with Cry1A.105 or Cry2Ab2 were conducted previously in support of MON 89034. Results indicate that neither Cry1A.105 nor Cry2Ab2 caused any adverse effects in mice, with No Observable Adverse Effect Levels (NOAELs) for Cry1A.105 at 2072 mg/kg and for Cry2Ab2 at 2198 mg/kg body weight (bw), the highest doses tested (U.S. EPA 2010c). The Cry1A.105 and Cry2Ab2 proteins from MON 87751 are functionally equivalent to the proteins used in these acute toxicity assays, originally conducted in support of MON 89034, and the data are therefore applicable to MON 87751.

V.E.7. Human and Animal Exposure to the Cry1A.105 and Cry2Ab2 Proteins

A common approach used to assess potential health risks for potentially toxic materials is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an appropriate animal toxicity study and an estimate of human exposure. No evidence of mammalian toxicity has been reported for Cry1A.105 or Cry2Ab2. The lack of any demonstrable hazard would normally obviate the need for a subsequent risk assessment step. Nevertheless, dietary risk assessments were conducted for these proteins in order to provide further assurances of safety by calculating a MOE between the NOAELs for the Cry1A.105 and Cry2Ab2 proteins in mouse acute oral toxicity studies (Section V.E.6) and 95th percentile consumption estimates of acute dietary exposure determined using the Dietary Exposure Evaluation Model - Food Commodity Intake Database (U.S. EPA 2013). DEEM-FCID utilizes food consumption data from the National Health and Nutrition Examination Survey (NHANES) conducted in 2003-2008. DEEM-FCID differentiates soybean consumption into five fractions: seed, flour, milk, vegetable, and oil. However, because soybean oil contains negligible amounts of protein (Tattrie and Yaguchi 1973), it would not be a significant source of dietary exposure to the Cry1A.105 and Cry2Ab2 proteins from MON 87751 and was thus excluded from this assessment. Human exposure to Cry1A.105 and Cry2Ab2 from MON 87751 in the U.S. was estimated using a conservative scenario of 95th percentile U.S. soybean consumption on an "eater-only" basis.

Based on levels of Cry1A.105 and Cry2Ab2 on a fresh weight basis (µg/g) discussed above (Sections V.C.1 and V.C.2), the 95th percentile acute intake (eater-only) for Cry1A.105 and Cry2Ab2 protein was estimated to be 0.000561 and 0.000962 mg/kg bw/day, respectively, for the overall U.S. population. The 95th percentile estimate of acute intake (eater-only) for non-nursing infants (< 1 year old), the most highly exposed sub-population, was 0.007207 and 0.012355 mg/kg body weight/day, respectively. The MOEs for acute dietary intake of Cry1A.105 and Cry2Ab2 were estimated to be 3,700,000 and 2,300,000, respectively, for the general population and 290,000 and 180,000, respectively, for non-nursing infants. Actual MOEs will likely be much higher because: 1) the exposure estimates utilized are conservative (95th percentile, assume all dietary sources of soybean were 100% MON 87751) and 2) as described in section V.E.5,

Cry1A.105 and Cry2Ab2 are rapidly digested in simulated gastric fluid, further minimizing exposures. These very large MOEs[4] indicate that there is no meaningful risk to human health from dietary exposure to the Cry1A.105 or Cry2Ab2 proteins produced by MON 87751.

There are a number of steps in the processing of soybean to make food ingredients, including crushing, grinding and heating that can denature a protein (Lusas 2000; Lusas and Riaz 1995) and likely increase the already large MOEs calculated here. Changes in temperature, pH, and physical disruptions associated with food processing and cooking/preparation generally lead to loss of protein structure and functionality (Hammond and Jez 2011). Like other proteins, the Cry1A.105 and Cry2Ab2 proteins in MON 87751 are expected to be similarly susceptible to denaturation when exposed to high temperatures, pH extremes, and digestive environments encountered during processing and cooking of foods containing MON 87751. Thus, there are likely to be significantly lower exposures to the functionally active forms of these proteins through consumption of MON 87751 than the levels estimated above.

The potential Cry1A.105 and Cry2Ab2 protein exposure to animals from consumption of MON 87751 seed in feeds was evaluated by calculating an estimate of daily dietary intake (DDI). Calculations were made for lactating dairy cows, poultry and swine. The highest percentage of Cry1A.105 and Cry2Ab2 proteins (g/kg bw) per total protein consumed was in the lactating dairy cow, 0.03% (g/g) and 0.002% (g/g) of the total dietary protein intake (0.001852 g Cry1A.105/kg bw divided by 6.2 g dietary protein, and 0.000132 g Cry2Ab2/kg bw divided by 6.2 g dietary protein which is the total dietary protein intake for the cow), respectively. The percentages of the Cry1A.105 and Cry2Ab2 proteins consumed as part of the daily protein intake of poultry and swine are much less than for the lactating dairy cow. Under high consumption conditions, per kg bw, poultry, swine and lactating dairy cattle would be consuming 0.06% (g/g) and 0.003% (g/g) or less of their total protein as Cry1A.105 and Cry2Ab2, respectively, from MON 87751. These low consumption numbers support a conclusion that adverse health effects in livestock consuming MON 87751 are unlikely.

In summary, there is no significant risk to human and animal health associated with dietary exposure to the Cry1A.105 and Cry2Ab2 proteins in food and feed products derived from MON 87751.

V.E.8. Non-Target Assessment for Cry1A.105 and Cry2Ab2 Proteins

Previously, USDA and U.S. EPA conducted plant pest and environmental assessments of MON 89034 maize that contains the Cry1A.105 and Cry2Ab2 proteins. In the environmental assessment of MON 89034 (USDA-APHIS 2008b), USDA-APHIS

[4] These MOEs reflect that a human would have to eat thousands of kilograms of soybean in a short time period to achieve exposures to the expressed proteins in MON 87751 that were not toxic to mice, which would be a physical impossibility.

considered the potential impact of the Cry1A.105 and Cry2Ab2 proteins on NTOs, including beneficial organisms and threatened or endangered species concluding:

> *"it [MON 89034] does not pose a risk to non-target organisms, including beneficial organisms and threatened or endangered species, because the insecticidal activity of the Cry1A.105 and Cry2Ab2 proteins are limited to lepidopteran target pest species."*

U.S. EPA also conducted an extensive environmental assessment to support the registration of MON 89034 maize. The assessment was based on studies conducted with the Cry1A.105 and Cry2Ab2 proteins on representative species of bird, fish, and terrestrial non-target insects, including endangered species, (U.S. EPA 2010c) concluding:

> *"At present, the Agency is aware of no identified significant adverse effects of Cry protein on the abundance of non-target organisms in any population in the aquatic or terrestrial field environment, whether they are animals, plants, pest parasites, pest predators, or pollinators."*
>
> *"In addition, no adverse effect on Federally listed endangered and threatened species is expected from the proposed lepidopteran-resistant corn registration."*

The NTO assessment for MON 87751 relied on existing NTO toxicity data for Cry1A.105 and Cry2Ab2 originally generated in support of MON 89034. The Cry1A.105 and Cry2Ab2 proteins in MON 87751 are functionally equivalent to the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034. Therefore, the previously provided activity spectrum evaluation for the Cry1A.105 and Cry2Ab2 proteins in MON 89034 is applicable to MON 87751. The assessment considered NTOs that provide important ecological functions (pollination, detritivores, predation, and parasitism) and organisms beneficial to agriculture. No adverse effects were observed from the NTOs tested, including one mammalian species (mice), two avian species (bobwhite quail, broiler chickens), soil decomposers (earthworm and Collembola), aquatic invertebrate (*Daphnia magna*) and four beneficial insect species (honeybee, minute pirate bugs, ladybird beetle, and parasitic wasp) (U.S. EPA 2010c). The highest dose tested for each species was compared to levels of each protein in MON 87751 to determine whether the existing toxicity data provide sufficient MOEs to conclude that adverse effects on NTOs are unlikely to occur. U.S. EPA guidance states that only MOEs <1 are viewed as an environmental risk (U.S. EPA 2010c). MOEs for Cry1A.105 from MON 87751 ranged from 10.9 to ≥552. MOEs for Cry2Ab2 from MON 87751 ranged from ≥8.8 to ≥38. Based on this assessment, there is no evidence that MON 87751will affect NTOs or endangered species under normal agricultural practices. Section IX.H provides specific results of NTO toxicity testing with Cry1A.105 and Cry2Ab2 as well as details of the MON 87751 NTO risk assessment.

V.F. Cry1A.105 and Cry2Ab2 Proteins Characterization and Safety Conclusion

The data and information provided in this section address questions important to the food and feed safety and environmental risk assessment of the Cry1A.105 and Cry2Ab2 proteins in MON 87751, including their potential allergenicity and toxicity. To summarize, the physicochemical characteristics of the Cry1A.105 and the Cry2Ab2 proteins from MON 87751 were determined and shown to be equivalent to those of their *E. coli*-produced counterparts. The levels of the Cry1A.105 and Cry2Ab2 protein in selected tissues of MON 87751 were determined. An assessment of the allergenic potential of the proteins supports the conclusion that the Cry1A.105 and Cry2Ab2 protein do not pose a significant allergenic risk to humans or animals. The donor organism for the Cry1A.105 and Cry2Ab2 coding sequences, *B. thuringiensis*, is ubiquitous in the environment and not commonly known for human or animal pathogenicity or allergenicity. The Cry1A.105 and Cry2Ab2 proteins lack structural similarity to allergens, toxins or other proteins known to have adverse effects on mammals. The Cry1A.105 and Cry2Ab2 proteins are rapidly digested in simulated gastric fluid and demonstrate no oral toxicity in mice at the highest levels tested. Furthermore, human and animal consumption of Cry1A.105 and Cry2Ab2 are expected to be extremely low even when MON 87751 is assumed to be the source of all consumed soybean. Finally, previous assessments by U.S. EPA and USDA concluded that neither protein is likely to have adverse impacts on NTOs. Based on the above information, the consumption of the Cry1A.105 and Cry2Ab2 proteins from MON 87751 or its progeny is considered safe for humans and animals.

The protein safety data presented herein support the conclusion that food and feed products containing MON 87751 or derived from MON 87751 are as safe as soybean currently on the market for human and animal consumption. Furthermore, there is no evidence that MON 87751 will affect NTOs under normal agricultural practices.

VI. COMPOSITIONAL ASSESSMENT OF MON 87751

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use. Compositional assessments are performed using the principles and analytes outlined in the OECD consensus document for soybean composition (OECD 2012).

A recent review of compositional assessments conducted according to OECD guidelines which encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan, et al. 2010). Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, and anti-nutrients that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan et al. 2010; Reynolds, et al. 2005).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD 2002). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. These quantitative measurements effectively discern any compositional changes that imply potential nutritional or safety (*e.g.,* anti-nutritional) concerns. Levels of the components in seed and forage of the biotechnology-derived crop product are compared to: 1) corresponding levels in a conventional comparator, a genetically similar conventional line, grown concurrently, under similar field conditions, and 2) natural ranges generated from an evaluation of commercial reference varieties grown concurrently and from data published in the scientific literature. The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients, anti-nutrients.

This section provides analyses of concentrations of key nutrients and anti-nutrients, of MON 87751 compared to that of a conventional control grown and harvested under similar conditions, as appropriate. In addition, conventional commercial soybean reference varieties were included in the composition analyses to establish a range of natural variability for each component, defined by the 99% tolerance interval. The production of materials for compositional analyses used a sufficient variety of field trial sites, robust field designs (randomized complete block design with four blocks), and sensitive analytical methods that allow accurate assessments of compositional characteristics over a range of environmental conditions under which MON 87751 is expected to be grown.

The information provided in this section addresses relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius 2009).

VI.A. Compositional Equivalence of MON 87751 Seed and Forage to Conventional Soybean

Seed and forage samples were collected from MON 87751, a conventional control, and a total of 19 different reference varieties grown in the United States during the 2012 field season. The reference varieties were included in the compositional analysis to provide data on the natural variability for each component. The field production was conducted at eight sites. The field sites were planted in a randomized complete block design with four blocks per site. MON 87751, the conventional control and reference soybean varieties were grown under normal agronomic field conditions for their respective geographic regions.

The evaluation of MON 87751 followed considerations relevant to the compositional quality of soybean as defined by the OECD consensus document (OECD 2012). Seed samples were assessed for levels of nutrients including proximates, carbohydrates by calculation, fiber, amino acids, fatty acids, vitamins, and minerals. The anti-nutrients assessed in seed included lectin, trypsin inhibitors, phytic acid, raffinose and stachyose. Other components assessed included isoflavones. Forage samples were assessed for proximates, carbohydrates by calculation and fiber. In all, 66 different components were analyzed.

Of the 66 measured components, 14 fatty acids (caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, pentadecenoic, palmitoleic, heptadecanoic, heptadecenoic, gamma linolenic, eicosadienoic, eicosatrienoic, and arachidonic acids) had more than 50% of the observations below the assay limit of quantitation (LOQ) and as a result, were excluded from the statistical analyses. Moisture values were measured for conversion of components to dry weight in seed and forage, but were not statistically analyzed. Therefore, statistics were provided for 50 components for all samples (six in forage and 44 in seed).

The statistical comparison of MON 87751 and the conventional control was based on compositional data combined across all field sites (the combined-site analysis). Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). The compositional data from the reference varieties were combined across all field sites to calculate a 99% tolerance interval for each component to estimate the natural variability of each component in soybean.

A statistically significant difference between MON 87751 and the conventional control does not necessarily imply biological relevance from a food and feed perspective. Therefore, statistically significant differences observed in the combined-site analysis between MON 87751 and the conventional control were evaluated further to determine whether the detected difference indicated a biologically relevant compositional change or supported a conclusion of compositional equivalence, as follows:

1) Determination of the mean difference between MON 87751 and the conventional control to be used in steps two and three. For protein and amino acids only[5], the relative magnitude of the difference (percent change relative to the control) between MON 87751 and the conventional control was determined to allow an assessment of the difference in amino acids in relation to a difference in protein;

2) Assessment of the relative impact of MON 87751 in the context of variation within the conventional control germplasm grown across multiple sites (*i.e.*, variation due to environmental influence). This assessment evaluates the mean difference between MON 87751 and the conventional control in the context of the range of values for the conventional control (maximum value minus the minimum value) derived from the combined-site analysis. When a mean difference is less than ranges seen due to natural environmental variation within even a single, closely related germplasm, the difference is typically not a food or feed safety concern; and

3) Assessment of the relative impact of MON 87751 compared to natural variation due to multiple sources (*e.g.*, environmental and germplasm influences). This assessment compares the mean difference between MON 87751 and the conventional control to variation in conventional soybean as estimated by in-study reference variety values and assessing whether the mean component value of MON 87751 was within the reference variety 99% tolerance interval, literature values and/or the ILSI Crop Composition Database values (ILSI-CCDB) (ILSI 2011).

These evaluations of natural variation within the conventional control and conventional soybean references are important as crop composition is known to be greatly influenced by environment and variety (Harrigan, et al. 2010). Only if mean differences between MON 87751 and the conventional control are large relative to natural variation inherent to conventional soybean would further assessments be required to establish whether the change in composition would have an impact from a food and feed safety or nutritional perspective. The steps reviewed in this assessment therefore describe whether the differences between MON 87751 and the conventional control are meaningful from a food/feed perspective or whether they support a conclusion of compositional equivalence.

The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and other components in soybean seed and forage of MON 87751 and the conventional control. Of the 50 components statistically assessed for MON 87751 there were no statistically significant differences in 42 components.

[5] Since total amino acids measured in a seed analysis are predominately derived from hydrolysis of protein, changes in protein levels may have a corresponding impact on levels of individual amino acids. However, the mean difference for individual amino acid levels will be less than the mean difference for protein because each amino acid constitutes only a part of the protein. For this reason, the relative magnitudes of difference (percent change relative to the control) for amino acids and protein were determined to allow an assessment of the difference in amino acids in relation to a difference in protein. When the relative magnitudes of difference for amino acids were related to the relative magnitude of difference for protein, then steps 2 and 3 are not discussed for amino acids.

Only eight components (protein, glycine, proline, phosphorus, vitamin E, and raffinose in seed, and total fat and NDF in forage) showed a significant difference ($p<0.05$) between MON 87751 and the conventional control. For these components, the mean difference in component values between MON 87751 and the conventional control was less than the range of the conventional control values. Additionally, the mean difference in component values between MON 87751 and the conventional control was less than the range of reference soybean variety values. Finally, the MON 87751 mean component values were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values. These results support the overall conclusion that MON 87751 was not a major contributor to variation in component levels in soybean seed and forage and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. Similar data submitted to USDA-APHIS in petition 09-082-01p supported the same conclusions of compositional equivalence for the antecedent organism, MON 87701. A detailed description of the assessment of statistically significant differences observed between MON 87751 and the conventional control is provided in the following section. These data indicated that the components with significant differences were not compositionally meaningful from a food and feed safety perspective.

VI.A.1. Nutrient Levels in Soybean Seed

Seed samples were analyzed for levels of nutrients including proximates (four components), carbohydrates by calculation, fiber (two components), amino acids (18 components), fatty acids (22 components), vitamin E (α-tocopherol), vitamin K1 (phylloquinone) and minerals (2 components). Moisture was measured for conversion of components to dry weight, but was not statistically analyzed.

VI.A.1.1 Proteins and Amino Acids

Protein levels in soybean seed generally average ~40% dry weight (dwt), with values reported in the USDA soybean germplasm collection, for example, ranging from 34.1 to 56.8% dwt (Wilson 2004). Protein content is a quantitative trait controlled by many genetic loci (Akond, et al. 2012; Panthee, et al. 2005) and is influenced by both genotype and environment (Rotundo and Westgate 2009).

A statistically significant difference ($p<0.05$) between MON 87751 and the conventional control was observed for protein (Table VI-1). The mean protein value was 40.58% dwt for MON 87751 and 40.12% dwt for the conventional control, a difference of 0.46% dwt. This difference was evaluated in the context of the range of the conventional control values; 5.09% dwt, calculated from the minimum (37.88% dwt) and maximum (42.97% dwt) protein values. The mean difference in protein values between MON 87751 and the conventional control was less than the range of the conventional control values, indicating that MON 87751 does not impact levels of protein more than natural variation within the conventional control grown at multiple locations. The mean difference in protein values between MON 87751 and the conventional control was also less than the variation seen in the reference variety values (ranged 34.68 to 45.22% dwt, a magnitude

of 10.54% dwt), and the MON 87751 mean value was within the 99% tolerance interval, the values observed in the literature, and the ILSI-CCDB (Table VI-8).

Since total amino acids measured in a seed analysis are predominantly derived from hydrolysis of protein, differences in amino acid levels between MON 87751 and the conventional control were assessed relative to the difference in protein levels. The relative magnitude of the difference in mean protein values for MON 87751 and the conventional control was 1.14% (Table VI-1). Correspondingly, relative magnitudes of difference for the 18 amino acids were all ≤ 2.17%. The difference between MON 87751 and the conventional control were significant for two of the amino acids (glycine and proline) (Table VI-1), and reflected small relative magnitudes of differences between MON 87751 and the conventional control, as would be expected based on the small relative magnitude of difference in protein.

The data demonstrated that MON 87751 was not a major contributor to variation in protein and amino acid levels in soybean seed and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values (Table VI-8). These data confirmed that the significant differences in mean values of protein, glycine and proline were not compositionally meaningful from a food and feed safety perspective.

VI.A.1.2 Total Fat and Fatty Acids

Fat levels in soybean seed generally average ~20% dwt, with values reported in the USDA soybean germplasm collection, for example, ranging from 8.3 to 27.9% (Wilson 2004). Total fat content is a quantitative trait controlled by many genetic loci (Akond et al. 2012; Panthee et al. 2005) and is influenced by both genotype and environment (Rotundo and Westgate 2009). There were no significant differences in seed total fat and fatty acid content between MON 87751 and the conventional control (Table VI-2). The data demonstrated that MON 87751 was not a major contributor to variation in total fat and fatty acid levels in soybean seed and confirmed the similarity of MON 87751 to the conventional control in levels of these components.

A total of fourteen fatty acids (caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, pentadecenoic, palmitoleic, heptadecanoic, heptadecenoic, gamma linolenic, eicosadienoic, eicosatrienoic, and arachidonic acids) with more than 50% of observations below the assay LOQ were excluded from statistical analysis. These fatty acids are present in only low amounts in soybean seed, if present at all (Berman, et al. 2009; Lundry, et al. 2008). This study confirmed that this observation extended to MON 87751.

VI.A.1.3 Carbohydrates by Calculation and Fiber

In addition to protein and fat, the major biomass components assessed in soybean seed included carbohydrates by calculation and fiber (ADF and NDF). There were no significant differences in carbohydrate and fiber content between MON 87751 and the

conventional control (Table VI-3). The data demonstrated that MON 87751 was not a major contributor to variation in carbohydrate and fiber levels in soybean seed and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components.

VI.A.1.4 Ash and Minerals

Ash was assessed in soybean seed, in addition to protein, fat, carbohydrates by calculation, and fiber. Mineral components (calcium and phosphorus), a constituent of ash, are discussed in this section.

No statistically significant differences were observed for ash or calcium (Table VI-4). A significant difference ($p<0.05$) between MON 87751 and the conventional control was observed for phosphorus (Table VI-4).

For phosphorus, the mean value was 0.54 g/100g dwt for MON 87751 and 0.53 g/100g dwt for the conventional control, a difference of 0.010 g/100g dwt. This difference was evaluated in the context of the range of the conventional control values, 0.23 g/100g dwt, calculated from the minimum (0.44 g/100g dwt) and maximum (0.67 g/100g dwt) phosphorus values. The mean difference was less than the range of the conventional control values, indicating that MON 87751 does not impact levels of phosphorus more than natural variation within the conventional control grown at multiple locations. The mean difference in phosphorus values between MON 87751 and the conventional control was also less than the variability seen in the respective reference variety values (ranged 0.42 to 0.71 g/100g dwt, a magnitude of 0.29 g/100g dwt), and the MON 87751 mean phosphorus value was within the reference variety 99% tolerance interval, the values observed in the literature and the ILSI-CCDB (Table VI-8).

The data demonstrated that MON 87751 was not a major contributor to variation in ash and mineral levels in soybean seed and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, and the ILSI-CCDB values. These data confirmed that the significant difference in the mean value of phosphorus was not compositionally meaningful from a food and feed safety perspective.

VI.A.1.5 Vitamins

Soybean oil is considered a source of biologically available vitamin K1 with levels ranging from 102.5 to 250µg/100g of oil reported in the published literature (OECD 2012). Vitamin E (α-tocopherol) is an important nutrient and maintains oxidative stability of soybean oil. Vitamin E levels in soybean seed are known to be affected by environment and germplasm (Seguin, et al. 2010); levels in soybean seed harvested from six different locations in Eastern Canada over a single year ranged from 0.87 to 3.32 mg/100g dwt (Seguin, et al. 2009).

No statistically significant difference was observed for vitamin K1 (Table VI-5). A statistically significant difference ($p<0.05$) was observed for vitamin E.

For vitamin E the mean value was 2.59 mg/100g dwt for MON 87751 and 2.78 mg/100g dwt for the conventional control, a difference of -0.19 mg/100g dwt. This difference was evaluated in the context of the range of the conventional control values, 3.04 mg/100g dwt, calculated from the minimum (1.36 mg/100g dwt) and maximum (4.39 mg/100g dwt) vitamin E values. The mean difference was less than the range of the conventional control values, indicating that the presence of MON 87751 does not impact levels of vitamin E more than natural variation within the conventional control grown at multiple locations. The mean difference in vitamin E values between MON 87751 and the conventional control was also less than the variation seen in the respective reference variety values (ranged 1.04 to 4.99 mg/100g dwt, a magnitude of 3.95 mg/100g dwt). The MON 87751 mean vitamin E value was within the 99% tolerance interval, the values observed in the literature and the ILSI-CCDB (Table VI-8).

The data demonstrated that MON 87751 was not a major contributor to variation in vitamin levels in soybean seed and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values. These data confirmed that the significant difference in the mean value of vitamin E was not compositionally meaningful from a food and feed safety perspective.

VI.A.2. Anti-Nutrient Levels in Soybean Seed

Anti-nutrients assessed include lectin, trypsin inhibitors, phytic acid, raffinose and stachyose. Lectins and trypsin inhibitors are proteins; lectins have carbohydrate-binding properties whereas trypsin inhibitors can inhibit protein digestion. Both proteins can negatively impact animal growth and performance and as a result, soybeans are processed to denature these proteins prior to consumption (Qin, et al. 1996). Variation of nearly five-fold in lectin levels was reported based on a screen of over 100 varieties (Pull, et al. 1978). A two-fold variation in trypsin inhibitor activity attributed to genetic and environmental factors has also been observed in soybean (Kumar, et al. 2003; Vollmann, et al. 2003).

Phytic acid is considered an anti-nutrient because of its mineral-chelating properties. Levels in soybean seed are influenced by factors such as genotype, environment, soil type and agronomic practices (Raboy and Dickinson 1993; Raboy, et al. 1984). The oligosaccharides, stachyose and raffinose, are considered anti-nutrients because of their contribution to gas production and resulting flatulence following consumption (OECD 2012). Raffinose and stachyose levels in soybean are quantitative traits and influenced by genotype and environment (Kumar, et al. 2010).

No statistically significant differences were observed for lectin, trypsin inhibitors, phytic acid, and stachyose (Table VI-6). A statistically significant difference ($p<0.05$) was observed for raffinose.

For raffinose, the mean value was 0.88% dwt for MON 87751 and 0.95% dwt for the conventional control, a difference of -0.065% dwt. This difference was evaluated in the

context of the range of the conventional control values (0.88% dwt), calculated from the minimum (0.62% dwt) and maximum (1.50% dwt) raffinose values. The mean difference in raffinose values between MON 87751 and the conventional control was less than the range of the conventional control values, indicating that MON 87751 does not impact levels of raffinose more than natural variation within the conventional control grown at multiple locations. The mean difference in raffinose values between MON 87751 and the conventional control was also less than the variation seen in the respective reference values (ranged 0.54 to 1.45% dwt, a magnitude of 0.91% dwt) and the MON 87751 mean value was within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB (Table VI-8).

The data demonstrated that MON 87751 was not a major contributor to variation in anti-nutrient levels in soybean seed and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. Also the mean values of these components were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values (Table VI-8). These data confirmed that the significant difference in the mean value of raffinose was not compositionally meaningful from a food and feed safety perspective.

VI.A.3. Other Components in Soybean Seed

In addition to the nutrients and anti-nutrients analyzed in soybean seed the levels of isoflavones were also assessed. Isoflavones have been reported to have biological activity as estrogenic and anti-estrogenic compounds and they may impact animal reproduction when consumed in large quantities (OECD 2012). Soybean isoflavones demonstrate considerable variation in levels in mature seed. Eldridge and Kwolek (1983) observed that the concentration of isoflavones varies from variety to variety, and there are also differences when the same variety is grown in different locations; a seven-fold variation was observed for levels of daidzein, while glycitein and genistein presented 2.5 and 76-fold variation respectively. Other studies further demonstrate the extensive variability observed in isoflavone levels (Gutierrez-Gonzalez, et al. 2009; Hoeck, et al. 2000; Morrison, et al. 2008). No statistically significant differences were observed for daidzein, genistein and glycitein (Table VI-6). The data provided here demonstrated that MON 87751 is not a major contributor to variation in isoflavone levels in soybean seed and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components.

VI.A.4. Nutrient Levels in Soybean Forage

Forage samples were assessed for proximates, carbohydrates by calculation, and fiber. No statistically significant differences were observed for ash, carbohydrates by calculation, protein, and ADF (Table VI-7). Statistically significant differences ($p<0.05$) were observed for total fat and NDF.

For total fat, the mean value was 6.03% dwt for MON 87751 and 6.43% dwt for the conventional control, a difference of -0.40% dwt. This difference was evaluated in the context of the range of the conventional control values (5.02% dwt), calculated from the

minimum (4.04% dwt) and maximum (9.06% dwt) values. The mean difference in total fat values between MON 87751 and the conventional control was less than the range of the conventional control values, indicating that MON 87751 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in total fat values between MON 87751 and the conventional control was also less than the variation seen in the reference variety values (ranged 2.74 to 9.74% dwt, a magnitude of 7.00% dwt), and the MON 87751 mean value was within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB (Table VI-8).

For NDF, the mean value was 36.77% dwt for MON 87751 and 34.08% dwt for the conventional control, a difference of 2.69% dwt. This difference was evaluated in the context of the range of the conventional control values (14.36% dwt), calculated from the minimum (27.69% dwt) and maximum (42.05% dwt) NDF values. The mean difference in NDF values between MON 87751 and the conventional control was less than the range of the conventional control values, indicating that MON 87751 does not impact levels more than natural variation within the conventional control grown at multiple locations. The mean difference in NDF values between MON 87751 and the conventional control was also less than the variation seen in the reference variety values (ranged 25.71 to 52.96% dwt, a magnitude of 27.25% dwt), and the MON 87751 mean value was within the 99% tolerance interval and the values observed in the literature (Table VI-8).

The data demonstrated that MON 87751 was not a major contributor to variation in proximates, carbohydrates by calculation, and fiber levels in soybean forage and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. Also, the mean values of these components were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values. These data confirmed that the significant difference in the mean values of total fat and NDF were not compositionally meaningful from a food and feed safety perspective.

Table VI-1. Summary of Soybean Seed Protein and Amino Acids for MON 87751, the Conventional Control, and References

Component (% dwt)[1]	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value	% Relative[6]
Protein	40.58 (0.50) 38.01 - 43.87	40.12 (0.50) 37.88 - 42.97	(34.68 - 45.22) 34.33, 45.17	5.09	0.46 (0.16)	0.023	1.14
Alanine	1.75 (0.018) 1.66 - 1.89	1.75 (0.018) 1.67 - 1.92	(1.58 - 1.95) 1.53, 1.93	0.25	0.0039 (0.0077)	0.611	0.22
Arginine	3.03 (0.047) 2.75 - 3.28	3.00 (0.047) 2.72 - 3.36	(2.48 - 3.62) 2.42, 3.52	0.64	0.028 (0.026)	0.317	0.93
Aspartic Acid	4.51 (0.048) 4.23 - 4.86	4.48 (0.048) 4.17 - 4.89	(3.82 - 5.18) 3.84, 5.07	0.72	0.032 (0.022)	0.185	0.72
Cystine	0.56 (0.017) 0.46 - 0.63	0.57 (0.017) 0.42 - 0.66	(0.42 - 0.67) 0.43, 0.67	0.25	-0.0010 (0.0081)	0.903	-0.18
Glutamic Acid	6.89 (0.085) 6.37 - 7.38	6.85 (0.085) 6.17 - 7.51	(5.64 - 8.18) 5.60, 7.96	1.34	0.044 (0.047)	0.360	0.64

Table VI-1. Summary of Soybean Seed Protein and Amino Acids for MON 87751, the Conventional Control, and References (continued)

Component (% dwt)[1]	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value	% Relative[6]
Glycine	1.73 (0.017) 1.64 - 1.86	1.71 (0.017) 1.63 - 1.83	(1.51 - 1.90) 1.47, 1.89	0.19	0.018 (0.0069)	0.014	1.04
Histidine	1.01 (0.011) 0.89 - 1.09	1.01 (0.011) 0.94 - 1.11	(0.87 - 1.15) 0.90, 1.11	0.17	0.0076 (0.010)	0.469	0.76
Isoleucine	1.92 (0.022) 1.79 - 2.09	1.90 (0.022) 1.78 - 2.06	(1.66 - 2.23) 1.63, 2.13	0.28	0.015 (0.013)	0.296	0.80
Leucine	3.04 (0.032) 2.82 - 3.28	3.02 (0.032) 2.86 - 3.23	(2.70 - 3.43) 2.68, 3.36	0.37	0.023 (0.016)	0.186	0.76
Lysine	2.51 (0.025) 2.35 - 2.77	2.49 (0.025) 2.32 - 2.71	(2.18 - 2.84) 2.16, 2.77	0.40	0.024 (0.014)	0.096	0.97
Methionine	0.56 (0.0092) 0.51 - 0.61	0.55 (0.0092) 0.47 - 0.63	(0.47 - 0.63) 0.45, 0.64	0.16	0.012 (0.0071)	0.135	2.17

Table VI-1. Summary of Soybean Seed Protein and Amino Acids for MON 87751, the Conventional Control, and References (continued)

Component (% dwt)[1]	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value	% Relative[6]
Phenylalanine	2.02 (0.024) 1.87 - 2.17	2.01 (0.024) 1.83 - 2.20	(1.74 - 2.36) 1.75, 2.27	0.37	0.013 (0.011)	0.290	0.65
Proline	2.07 (0.025) 1.91 - 2.24	2.03 (0.025) 1.90 - 2.18	(1.76 - 2.32) 1.72, 2.32	0.28	0.032 (0.011)	0.025	1.59
Serine	1.90 (0.022) 1.74 - 2.10	1.89 (0.022) 1.72 - 2.09	(1.59 - 2.23) 1.60, 2.17	0.37	0.0067 (0.020)	0.734	0.36
Threonine	1.58 (0.011) 1.50 - 1.66	1.57 (0.011) 1.50 - 1.66	(1.42 - 1.74) 1.39, 1.72	0.17	0.012 (0.0076)	0.127	0.75
Tryptophan	0.55 (0.0085) 0.49 - 0.61	0.54 (0.0085) 0.49 - 0.61	(0.46 - 0.61) 0.47, 0.60	0.12	0.0081 (0.0053)	0.131	1.49
Tyrosine	1.55 (0.013) 1.48 - 1.66	1.54 (0.013) 1.47 - 1.62	(1.38 - 1.72) 1.35, 1.71	0.14	0.014 (0.0062)	0.066	0.88

Table VI-1. Summary of Soybean Seed Protein and Amino Acids for MON 87751, the Conventional Control, and References (continued)

Component (% dwt)[1]	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value	% Relative[6]
Valine	1.87 (0.017) 1.73 - 1.99	1.87 (0.017) 1.74 - 1.96	(1.64 - 2.16) 1.66, 2.05	0.22	0.0073 (0.0098)	0.460	0.39

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.
[6]The relative magnitude of the difference in mean values between MON 87751 and the control, expressed as a percent of the control.

Table VI-2. Summary of Soybean Seed Total Fat and Fatty Acids for MON 87751, the Conventional Control, and References

Component	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value
Total Fat (% dwt)[1]	19.21 (0.43) 16.15 - 21.21	19.48 (0.43) 16.84 - 21.08	(18.10 - 22.97) 17.12, 24.20	4.24	-0.27 (0.14)	0.088
16:0 Palmitic[6]	11.69 (0.19) 11.13 - 12.26	11.59 (0.19) 10.03 - 12.36	(9.37 - 12.56) 8.39, 13.35	2.33	0.10 (0.18)	0.603
18:0 Stearic	4.22 (0.12) 3.61 - 4.95	4.29 (0.12) 3.65 - 4.98	(3.27 - 6.11) 2.12, 6.45	1.32	-0.066 (0.036)	0.106
18:1 Oleic	20.81 (0.94) 17.08 - 26.89	21.50 (0.94) 16.98 - 27.41	(17.21 - 34.03) 13.27, 32.25	10.42	-0.69 (0.50)	0.211
18:2 Linoleic	54.57 (0.73) 50.33 - 56.50	53.93 (0.73) 47.96 - 56.99	(45.98 - 58.27) 44.95, 62.64	9.03	0.63 (0.30)	0.074
18:3 Linolenic	7.89 (0.38) 6.09 - 9.97	7.86 (0.38) 6.19 - 10.23	(5.37 - 10.34) 4.29, 10.55	4.04	0.035 (0.10)	0.740

Table VI-2. Summary of Soybean Seed Total Fat and Fatty Acids for MON 87751, the Conventional Control, and References (continued)

Component	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value
20:0 Arachidic	0.32 (0.0087) 0.28 - 0.37	0.33 (0.0087) 0.28 - 0.39	(0.26 - 0.50) 0.17, 0.50	0.11	-0.0066 (0.0039)	0.133
20:1 Eicosenoic	0.19 (0.0084) 0.14 - 0.24	0.19 (0.0084) 0.14 - 0.25	(0.13 - 0.25) 0.12, 0.26	0.11	-0.00023 (0.0037)	0.953
22:0 Behenic	0.31 (0.0090) 0.28 - 0.36	0.32 (0.0090) 0.27 - 0.40	(0.28 - 0.49) 0.20, 0.48	0.13	-0.0075 (0.0075)	0.354

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.
[6]Expressed as % total fatty acid. Prefix numbers refer to number of carbon atoms and number of carbon-carbon double bonds in the fatty acid molecule; 16:0 represents sixteen carbon atoms and zero double bonds. Numbers are not included in text discussion for reasons of clarity. The following fatty acids with more than 50% of observations below the assay LOQ were excluded from statistical analysis: capric acid, lauric acid, myristic acid, myristoleic acid, pentadecanoic acid, pentadecenoic acid, palmitoleic acid, heptadecanoic acid, heptadecenoic acid, gamma linolenic acid, eicosadienoic acid, eicosatrienoic acid, arachidonic acid, and caprylic acid.

Table VI-3. Summary of Soybean Seed Carbohydrates by Calculation and Fiber for MON 87751, the Conventional Control, and References

Component (% dwt)[1]	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value
Carbohydrates by	35.30 (0.58)	35.63 (0.58)	(29.42 - 38.18)	6.63	-0.33 (0.19)	0.131
Calculation	31.27 - 37.69	31.95 - 38.57	29.43, 39.69			
Acid Detergent Fiber	13.99 (0.44)	13.98 (0.44)	(10.02 - 17.59)	4.91	0.010 (0.60)	0.986
	10.75 - 17.44	12.15 - 17.06	10.71, 17.25			
Neutral Detergent Fiber	15.56 (0.33)	15.57 (0.33)	(12.25 - 19.29)	4.71	-0.0088 (0.40)	0.982
	12.56 - 17.65	13.97 - 18.68	12.66, 18.44			

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.

Table VI-4. Summary of Soybean Seed Ash and Minerals for MON 87751, the Conventional Control, and References

Component	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value
Ash (% dwt)[1]	4.91 (0.10)	4.89 (0.10)	(4.16 - 5.69)	1.36	0.013 (0.046)	0.789
	4.46 - 5.56	4.34 - 5.70	4.32, 5.74			
Calcium (g/100g dwt)	0.29 (0.010)	0.29 (0.010)	(0.21 - 0.40)	0.10	-0.0083 (0.0088)	0.376
	0.24 - 0.34	0.25 - 0.34	0.20, 0.41			
Phosphorus (g/100g dwt)	0.54 (0.020)	0.53 (0.020)	(0.42 - 0.71)	0.23	0.010 (0.0041)	0.020
	0.45 - 0.68	0.44 - 0.67	0.40, 0.70			

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.

Table VI-5. Summary of Soybean Seed Vitamins for MON 87751, the Conventional Control, and References

Component	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value
Vitamin E (α-Tocopherol)	2.59 (0.29)	2.78 (0.29)	(1.04 - 4.99)	3.04	-0.19 (0.042)	<0.001
(mg/100g dwt)[1]	1.21 - 4.05	1.36 - 4.39	0, 5.12			
Vitamin K (phylloquinone)	0.64 (0.067)	0.67 (0.067)	(0.28 - 0.98)	0.59	-0.029 (0.016)	0.111
(µg/g dwt)	0.40 - 0.96	0.41 - 1.00	0.036, 1.10			

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.

Table VI-6. Summary of Soybean Seed Anti-nutrients and Isoflavones for MON 87751, the Conventional Control, and References

Component	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	Difference (MON 87751 - Control) p-Value
Anti-nutrient						
Lectin (H.U./mg dwt)[1]	3.42 (0.23) 1.78 - 5.22	3.18 (0.23) 1.71 - 5.61	(1.63 - 7.46) 0.59, 6.30	3.90	0.24 (0.24)	0.353
Phytic Acid (% dwt)	1.22 (0.067) 0.85 - 1.78	1.19 (0.067) 0.72 - 1.68	(0.88 - 1.82) 0.80, 1.68	0.96	0.024 (0.019)	0.234
Raffinose (% dwt)	0.88 (0.087) 0.59 - 1.47	0.95 (0.087) 0.62 - 1.50	(0.54 - 1.45) 0.44, 1.27	0.88	-0.065 (0.018)	0.007
Stachyose (% dwt)	4.09 (0.079) 3.66 - 4.37	4.06 (0.079) 3.60 - 4.47	(3.39 - 4.62) 3.15, 4.80	0.87	0.030 (0.060)	0.628
Trypsin Inhibitor (TIU/mg dwt)	26.21 (1.97) 16.32 - 40.79	26.82 (1.97) 14.90 - 44.39	(14.79 - 42.42) 12.17, 38.77	29.49	-0.61 (1.48)	0.693
Isoflavone (µg/g dwt)						
Daidzein	901.33 (98.11) 223.46 - 1313.65	893.90 (98.11) 288.55 - 1243.53	(164.64 - 1312.77) 0, 1494.46	954.99	7.43 (42.93)	0.867

Table VI-6. Summary of Soybean Seed Anti-nutrients and Isoflavones for MON 87751, the Conventional Control, and References (continued)

Component	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	Difference (MON 87751 - Control) p-Value
Isoflavone (µg/g dwt)						
Genistein	756.78 (77.73) 243.18 - 993.17	755.11 (77.73) 273.13 - 1101.04	(245.14 - 1318.18) 150.41, 1437.69	827.91	1.67 (17.78)	0.925
Glycitein	77.67 (5.37) 37.29 - 117.25	84.03 (5.37) 47.91 - 132.77	(37.32 - 210.17) 8.04, 211.36	84.86	-6.36 (4.13)	0.129

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.

Table VI-7. Summary of Soybean Forage Proximates and Fiber for MON 87751, the Conventional Control, and References

Component (% dwt)[1]	MON 87751 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	Conventional Reference (Range)[3] Tolerance Interval[4]	Control Range Value[5]	Difference (MON 87751 - Control) Mean (S.E.)	p-Value
Ash	6.23 (0.22)	6.11 (0.22)	(4.44 - 7.84)	2.62	0.12 (0.064)	0.061
	4.97 - 7.59	4.88 - 7.50	4.54, 7.78			
Carbohydrates by	68.30 (1.29)	67.62 (1.29)	(58.91 - 76.76)	13.35	0.68 (0.31)	0.065
Calculation	61.33 - 75.86	61.92 - 75.27	59.21, 80.18			
Protein	19.47 (0.96)	19.85 (0.96)	(13.50 - 26.62)	10.23	-0.37 (0.24)	0.164
	14.07 - 25.35	14.50 - 24.73	11.64, 25.13			
Total Fat	6.03 (0.50)	6.43 (0.50)	(2.74 - 9.74)	5.02	-0.40 (0.14)	0.024
	3.35 - 9.65	4.04 - 9.06	0.43, 11.11			
Acid Detergent Fiber	29.52 (0.73)	28.72 (0.73)	(19.97 - 44.27)	15.35	0.79 (0.73)	0.282
	23.66 - 35.95	21.31 - 36.67	18.50, 40.91			
Neutral Detergent Fiber	36.77 (1.18)	34.08 (1.18)	(25.71 - 52.96)	14.36	2.69 (0.88)	0.018
	26.19 - 43.63	27.69 - 42.05	22.42, 50.76			

[1]dwt = dry weight.
[2]Mean (S.E.) = least-square mean (standard error).
[3]Range is the minimum and maximum raw values for the conventional reference soybean varieties.
[4]With 95% confidence, interval contains 99% of the values expressed in the population of conventional reference soybean varieties. Negative limits set to zero.
[5]Maximum value minus minimum value for the control soybean variety.

Table VI-8. Literature and ILSI Database Ranges for Components in Soybean Forage and Seed

Seed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Seed Nutrients		
Proximates (% dw)		
Ash	4.61 – 6.32[a]; 4.32 – 5.88[b]	3.89 – 6.99
Carbohydrates by calculation	32.75 – 40.98[a]; 29.88 – 43.48[b]	29.6 – 50.2
Moisture (% fw)	6.24 – 12.10[a]; 5.44 – 11.70[b]	4.7 – 34.4
Protein	34.78 – 43.35[a]; 32.29 – 42.66[b]	33.19 – 45.48
Total Fat	14.40 – 20.91[a]; 15.10 – 23.56[b]	8.10 – 23.56
Fiber (% dw)		
Acid Detergent Fiber	9.22 – 26.26[a]; 11.81 – 19.45[b]	7.81 – 18.61
Neutral Detergent Fiber	10.79 – 23.90[a]; 13.32 – 23.57[b]	8.53 – 21.25
Amino Acids (% dw)		
Alanine	1.62 – 1.89[a]; 1.43 – 1.93[b]	1.51 – 2.10
Arginine	2.57 – 3.34[a]; 2.15 – 3.05[b]	2.29 – 3.40
Aspartic acid	4.16 – 5.02[a]; 4.01 – 5.72[b]	3.81 – 5.12
Cystine/Cysteine	0.52 – 0.69[a]; 0.41 – 0.71[b]	0.37 – 0.81
Glutamic acid	6.52 – 8.19[a]; 5.49 – 8.72[b]	5.84 – 8.20
Glycine	1.59 – 1.90[a]; 1.41 – 1.99[b]	1.46 – 2.00
Histidine	0.96 – 1.13[a]; 0.86 – 1.24[b]	0.88 – 1.18
Isoleucine	1.59 – 2.00[a]; 1.41 – 2.02[b]	1.54 – 2.08
Leucine	2.79 – 3.42[a]; 2.39 – 3.32[b]	2.59 – 3.62
Lysine	2.36 – 2.77[a]; 2.19 – 3.15[b]	2.29 – 2.84
Methionine	0.45 – 0.63[a]; 0.39 – 0.65[b]	0.43 – 0.68
Phenylalanine	1.82 – 2.29[a]; 1.62 – 2.44[b]	1.63 – 2.35
Proline	1.83 – 2.23[a]; 1.63 – 2.25[b]	1.69 – 2.28
Serine	1.95 – 2.42[a]; 1.51 – 2.30[b]	1.11 – 2.48
Threonine	1.44 – 1.71[a]; 1.23 – 1.74[b]	1.14 – 1.86
Tryptophan	0.30 – 0.48[a]; 0.41 – 0.56[b]	0.36 – 0.50
Tyrosine	1.27 – 1.53[a]; 0.74 – 1.31[b]	1.02 – 1.61
Valine	1.68 – 2.11[a]; 1.50 – 2.13[b]	1.60 – 2.20
Fatty Acids (% total FA)		
8:0 Caprylic	not available	0.148 – 0.148
10:0 Capric	0.15 – 0.27[b]	not available
12:0 Lauric	not available	0.082 – 0.132
14:0 Myristic	0.063 – 0.11[b]	0.071 – 0.238
14:1 Myristoleic	not available	0.121 – 0.125
15:0 Pentadecanoic	not available	not available
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	9.80 – 12.63[b]	9.55 – 15.77
16:1 Palmitoleic	0.055 – 0.14[b]	0.086 – 0.194
17:0 Heptadecanoic	0.076 – 0.13[b]	0.085 – 0.146
17:1 Heptadecenoic	0.019 – 0.064[b]	0.073 – 0.087
18:0 Stearic	3.21 – 5.63[b]	2.70 – 5.88
18:1 Oleic	16.69 – 35.16[b]	14.3 – 32.2
18:2 Linoleic	44.17 – 57.72[b]	42.3 – 58.8

Table VI-8. Literature and ILSI Database Ranges for Components in Soybean Forage and Seed (continued)

Seed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
18:3 Gamma Linolenic	not available	not available
18:3 Linolenic	4.27 – 9.90[b]	3.00 – 12.52
20:0 Arachidic	0.35 – 0.57[b]	0.163 – 0.482
20:1 Eicosenoic	0.13 – 0.30[b]	0.140 – 0.350
20:2 Eicosadienoic	0.016 – 0.071[b]	0.077 – 0.245
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.35 – 0.65[b]	0.277 – 0.595
Vitamins		
Vitamin E (mg/100g dw)	1.29 – 4.80[a]; 1.12 – 8.08[b]	0.19 – 6.17
Vitamin K (µg/g fw)	0.35-0.47[c]	not available
Minerals[4]		
Calcium	0.20-0.22[c], 0.24-0.41[d]	0.12-0.31
Phosphorus	0.48-0.64[c], 0.40-0.61[d]	0.50-0.94
Seed Anti-Nutrients		
Lectin (H.U./mg fw)	0.45 – 10.87[a]; 0.090 – 11.18[b]	0.105-9.038
Trypsin Inhibitor (TIU/mg dw)	20.79 – 59.03[a]; 18.14 – 42.51[b]	19.59 – 118.68
Phytic Acid (% dw)	0.41 – 1.92[a]; 0.81 – 2.66[b]	0.63 – 1.96
Raffinose (% dw)	0.26 – 0.84[a]; 0.43 – 1.85[b]	0.21 – 0.66
Stachyose (% dw)	1.53 – 3.04[a]; 1.97 – 6.65[b]	1.21 – 3.50
Isoflavones	**(µg/g dw)**	**(mg/kg dw)**
Daidzein	224.03 – 1571.91[a]; 198.95 – 1458.24[b]	60.0 – 2453.5
Genistein	338.24 – 1488.89[a]; 148.06 – 1095.57[b]	144.3 – 2837.2
Glycitein	52.72 – 298.57[a]; 32.42 – 255.94[b]	15.3 – 310.0

Forage Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Forage Nutrients		
Proximate (% dw)		
Ash	5.28 – 9.24[a]; 4.77 – 8.54[b]	6.72 – 10.78
Carbohydrates by calculation	62.25 – 72.30[a]; 60.61 – 77.26[b]	59.8 – 74.7
Moisture (% fw)	68.50 – 78.40[a]; 62.76 – 80.20[b]	73.5 – 81.6
Protein	16.48 – 24.29[a]; 12.68 – 23.76[b]	14.38 – 24.71
Total Fat	2.65 – 9.87[a]; 2.96 – 7.88[b]	1.30 – 5.13
Fiber (% dw)		
Acid Detergent Fiber	23.86 – 50.89[a]; 25.49 – 47.33[b]	not available
Neutral Detergent Fiber	19.61 – 43.70[a]; 30.96 – 64.19[b]	not available

[1]fw=fresh weight; dw=dry weight; H.U. = hemagglutinating unit; TIU = trypsin inhibitor unit.
[2]Literature range references; [a]Lundry et al. (2008); [b]Berman et al. (2009), [c]Souci et al. (2008) (in fresh weight) (Accessed July 19, 2013), [d]Bellaloui et al. (2011).
[3]ILSI range is from ILSI Crop Composition Database, 2011 (Accessed August 14, 2013) (ILSI 2011).
[4]Units for minerals are g/100g fw for data obtained from Souci et al. (2008) and in g/100g dw for data obtained from Bellaloui et al. (2011).

VI.B. Compositional Assessment of MON 87751 Conclusion

Compositional analysis was conducted on seed and forage of MON 87751 and a conventional control grown at eight sites in the United States during a 2012 field production. The compositional analysis, based on the OECD consensus document for soybean, also included measurement of nutrients, anti-nutrients and other components in all varieties, including the conventional reference soybean varieties, to provide data on the natural variability of each compositional component analyzed.

Of the 50 components statistically assessed for MON 87751 only eight components (protein, glycine, proline, phosphorus, vitamin E, and raffinose in seed, and total fat and NDF in forage) showed a significant difference between MON 87751 and the conventional control. For these eight components, the mean difference in component values between MON 87751 and the conventional control was less than the range of the conventional control values and the reference variety values. The MON 87751 mean component values were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values.

These results support the overall conclusion that MON 87751 was not a major contributor to variation in component levels in soybean seed and forage and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. These data indicated that the components with statistically significant differences were not compositionally meaningful from a food and feed safety perspective. Similar data submitted to USDA-APHIS in petition 09-082-01p supported the same conclusions of compositional equivalence for the antecedent organism, MON 87701.

VII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides a comparative assessment of the phenotypic, agronomic, and environmental interaction characteristics of MON 87751 compared to the conventional control. The data support a conclusion that MON 87751 is not meaningfully different in plant pest risk from the conventional control. These conclusions are based on the results of evaluations from laboratory, greenhouse, growth chamber and field assessments.

Phenotypic, agronomic, and environmental interaction characteristics of MON 87751 were evaluated in a comparative manner to assess plant pest potential. These assessments included evaluation of seed germination characteristics, plant growth and development characteristics, observations of plant responses to abiotic stress, plant-disease and plant-arthropod interactions, pollen characteristics, and plant-symbiont interaction associations. Results from these assessments demonstrate that compared to the conventional control MON 87751 does not possess a) increased weediness characteristics; b) increased susceptibility or tolerance to specific abiotic stresses, diseases, or arthropods; or c) characteristics that would confer a plant pest risk. Similar data submitted to USDA-APHIS in petition 09-082-01p supported the same conclusions regarding phenotype and environmental interactions for the antecedent organism, MON 87701.

VII.A. Characteristics Measured for Assessment

In the phenotypic, agronomic, and environmental interactions assessment of MON 87751, data were collected to evaluate altered plant pest potential. A detailed description of the regulated article phenotype is requested to support a determination of nonregulated status in 7 CFR 340.6 including differences from the unmodified recipient organism that would "substantiate that the regulated article is unlikely to pose a greater plant pest risk than the unmodified organism from which it was derived". Data were collected to provide a detailed description of the phenotypic, agronomic, and environmental interaction characteristics of MON 87751. A subset of these data were included in an evaluation of specific characteristics related to altered plant pest potential (*e.g.*, seed dormancy, pod shattering, plant lodging, and environmental interactions data).

The plant characterization of MON 87751 encompassed six categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development; 4) seed retention and lodging; 5) plant-environmental interactions; and 6) plant-symbiont interactions. Table VII-1 lists the assessed characteristics. The phenotypic, agronomic, and environmental interactions data were evaluated from a basis of familiarity (OECD 1993) and were comprised of a combination of field, greenhouse, growth chamber and laboratory studies conducted by scientists who are familiar with the production and evaluation of soybean. In each assessment, MON 87751 was compared to an appropriate conventional control that had a genetic background similar to MON 87751 but did not possess insect-protected trait. In addition, commercial soybean varieties were included to provide a range of values for each characteristic that are representative of the variability in commercial soybean varieties. These ranges thereby provide context for interpreting experimental results.

Table VII-1. Phenotypic, Agronomic, and Environmental Interaction Characteristics Evaluated in U.S. Field, Laboratory, Growth Chamber or Greenhouse Studies

Data category	Characteristics measured (associated section where discussed)	Evaluation timing (setting of evaluation)[1]	Evaluation description (measurement endpoints)
Seed germination, dormancy, and emergence	Normal germinated (VII.C.1)	Day 5 and 8 (20/30°C) (laboratory)	Percentage of seed producing seedlings exhibiting normal developmental characteristics
	Abnormal germinated (VII.C.1)	Day 8 (20/30°C) (laboratory)	Percentage of seed producing seedlings that could not be classified as normal germinated
	Germinated (VII.C.1)	Day 5, 8, and 13 (10, 20, 30, 10/20 and 10/30°C) (laboratory)	Percentage of seed producing seedlings exhibiting normal or abnormal developmental characteristics
	Dead (VII.C.1)	Day 5 and 8 (10, 20, 30, 10/20, 10/30, and 20/30°C); Day 13 (10, 20, 30, 10/20 and 10/30°C) (laboratory)	Percentage of seed that had visibly deteriorated and become soft to the touch (also included non-viable hard and non-viable firm-swollen seed)
	Viable hard (VII.C.1)	Day 8 (20/30°C); Day 13 (10, 20, 30, 10/20 and 10/30°C) (laboratory)	Percentage of seed that did not imbibe water and remained hard to the touch (viability determined by a tetrazolium test[2])
	Viable firm-swollen (VII.C.1)	Day 8 (20/30°C); Day 13 (10, 20, 30, 10/20 and 10/30°C) (laboratory)	Percentage of seed that imbibed water and were firm to the touch but did not germinate (viability determined by a tetrazolium test[2])
	Early stand count (VII.C.2.1)	V2 - V4 (Field)	Mean number of emerged plants from three linear meter sub-samples per plot
	Final stand count (VII.C.2.1)	Maturity, R8 (Field)	Mean number of plants from three linear meter sub-samples per plot
Vegetative growth	Plant vigor (VII.C.2.1)	V2 - V4 (Field)	Rated on a 1-9 scale, where 1 = excellent and 9 = poor vigor
	Growth stage assessment (VII.C.2.1)	Four observations taken at approximately 15-20, 40-45, 60-75, and 90-105 days after planting (Field)	Average soybean plant growth stage per plot
	Flower color (VII.C.2.1)	Flowering, R1-R2 (Field)	Color of flowers: purple, white, or mixed
	Plant height (VII.C.2.1)	Maturity, R8 (Field)	Distance (cm) from the soil surface to the uppermost node on the main stem of five representative plants per plot
Reproductive development	Days to 50% flowering (VII.C.2.1)	Flowering, R1 (Field)	The number of days from planting to when approximately 50% of the plants in each plot reach the R1 growth stage
	Pollen viability (VII.C.3)	Flowering, R1-R2 (growth chamber, laboratory)	Percentage of viable pollen based on pollen grain staining characteristics
	Pollen morphology (VII.C.3)	Flowering, R1-R2 (growth chamber, laboratory)	Diameter (µm) of viable pollen grains

Table VII-1. Phenotypic, Agronomic and Environmental Interaction Characteristics Evaluated in U.S. Field, Laboratory, Growth Chamber, or Greenhouse Studies (continued)

Data category	Characteristics measured (associated section where discussed)	Evaluation timing (setting of evaluation)[1]	Evaluation description (measurement endpoints)
Reproductive development	Grain moisture (VII.C.2.1)	Harvest (Field)	Percent moisture content of harvested grain
	100 seed weight (VII.C.2.1)	Harvest (Field)	The weight (g) of 100 seeds harvested from each plot
	Yield (VII.C.2.1)	Harvest (Field)	Grain yield in t/ha and standardized to 13% moisture
Seed retention and lodging	Plant lodging (VII.C.2.1)	Maturity, R8 (Field)	Rated on 1-9 scale, where 1 = completely erect and 9 = completely lodged plants
	Pod shattering (VII.C.2.1)	Maturity, R8 (Field)	Rated on 1-9 scale, where 1 = no shattering and 9 = completely shattered pods
Plant-environment interactions	Plant response to abiotic stress (VII.C.2.2)	Four times per growing season (Field)	Qualitative assessment of each plot, with four ratings: none, slight, moderate and severe plant damage
	Disease damage (VII.C.2.2)	Four times per growing season (Field)	Qualitative assessment of each plot, with four ratings: none, slight, moderate and severe plant damage
	Arthropod-related damage (VII.C.2.2)	Four times per growing season (Field)	Qualitative assessment of each plot, with four ratings: none, slight, moderate and severe plant damage
	Bean leaf beetle damage	Two times per growing season (Field)	Quantitative assessment on 10 plants per plot by examining leaf damage 0-5 rating scale adapted (Koch et al. 2005).
	Stink bug damage	R6-R8 (Field)	Quantitative assessment on 10 plants per plot by determining percent of damaged pods
	Arthropod abundance (VII.C.2.2)	Five times per growing season (Field)	Quantitative assessment of arthropods
Plant-symbiont interactions	Biomass (VII.C.4)	6 weeks after emergence (Greenhouse)	Nodule, root, and shoot dry weight (g/plant)
	Nodule number (VII.C.4)	6 weeks after emergence (Greenhouse)	Nodule number
	Total nitrogen (VII.C.4)	6 weeks after emergence (Greenhouse)	Shoot total nitrogen (% and g/plant)

1 Soybean plant growth stages were determined using descriptions and guidelines outlined in Soybean Growth and Development (Pedersen 2004).
2 Viability of hard and firm-swollen seed were determined by a tetrazolium test (AOSA/SCST 2010).

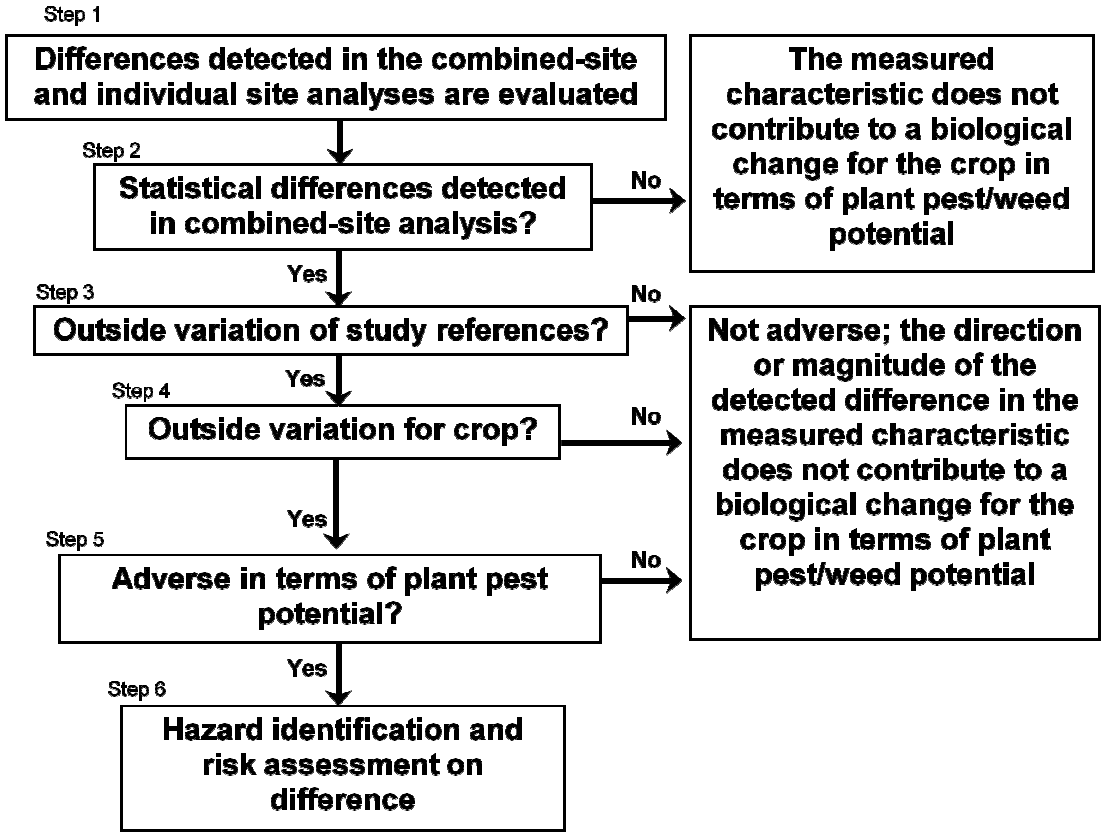
VII.B. Interpretation of Phenotypic and Environmental Interaction Data

Plant pest risk assessments for biotechnology-derived crops are comparative assessments, and are considered from a basis of familiarity. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a well-characterized conventional crop whose biological properties and plant pest potential are well-known. Familiarity considers the biology of the crop, the introduced trait, the receiving environment and the interaction of these factors, and provides a basis for comparative environmental risk assessment between a biotechnology-derived plant and its conventional counterpart.

Expert knowledge and experience with conventionally bred soybean was the basis for selecting appropriate endpoints and estimating the range of responses that would be considered typical for soybean. As such, MON 87751 was compared to the conventional control in the assessment of phenotypic, agronomic, and environmental interaction characteristics. An overview of the characteristics assessed is presented in Table VII-1. A subset of the data relating to well-understood weedy characteristics (*e.g.*, seed dormancy, pod shattering, and plant lodging) was used to assess whether there was an increase in weediness potential of MON 87751 compared to a conventional soybean. Evaluation of environmental interaction characteristics (*e.g.*, plant abiotic stress, plant-disease, and plant-arthropod interactions) was also considered in the plant pest assessment. Prior to analysis, the overall dataset was evaluated for possible evidence of biologically relevant changes and unexpected plant responses. No unexpected observations or issues were identified. Based on all of the data collected, an assessment was made to determine if MON 87751 could be expected to pose an increased plant pest risk compared to conventional soybean.

VII.B.1. Interpretation of Detected Differences Criteria

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest/weed potential as assessed by APHIS. Under the framework of familiarity, characteristics for which no differences are detected support a conclusion of no increased plant pest/weed potential of the biotechnology-derived crop compared to the conventional crop. Characteristics for which differences are detected are considered in a step-wise method (Figure VII-1) or in a similar fashion. All detected differences for a characteristic are considered in the context of whether or not the difference would increase the plant pest/weed potential of the biotechnology-derived crop. Ultimately, a weight-of-evidence approach considering all characteristics and data is used for the overall risk assessment of differences and their significance. In detail, Figure VII-1 illustrates the stepwise assessment process employed:



Note: A "no" answer at any step indicates that the characteristic does not contribute to a biological change for the crop in terms of plant pest/weed potential and subsequent steps are not considered. If the answer is "yes" or "uncertain", the subsequent step is considered.

Figure VII-1. Schematic Diagram of Agronomic and Phenotypic Data Interpretation Methods

Steps 1 and 2 – Evaluate Detected Statistically Significant Differences

Data on each measured characteristic are statistically analyzed, where appropriate, within each individual site and in a combined-site analysis, in which the data are pooled among sites. All statistically significant differences are evaluated and considered in the context of a change in plant pest/weed potential. Differences detected in individual-site analyses that are not detected when data across multiple environments are pooled in the combined-site analysis are considered not biologically meaningful in terms of plant pest/weed potential and, therefore, are not further considered in subsequent steps. Any difference detected in the combined-site analysis is further assessed.

Step 3 – Evaluate differences in the context of commercial reference materials included in the Study

If a difference for a characteristic is detected in the combined-site analysis across multiple environments, then the mean value of the biotechnology-derived crop for the

characteristic is assessed relative to the range of variation of the commercial reference materials included in the study (*e.g.*, reference range).

Step 4 – Evaluate Differences in the Context of the Crop

If the mean value of the characteristics for a biotechnology-derived crop is outside the variation of the commercial reference materials included in the study, the mean value of the biotechnology-derived crop is assessed relative to known values common for the crop (*e.g.*, published values).

Step 5 – Relevance of Difference to Plant Pest/Weed Potential

If the mean value of the characteristics for a biotechnology-derived crop is outside the range of values common for the crop, the difference is then assessed for whether or not it is meaningful in terms of plant pest/weed potential.

Step 6 – Conduct Risk Assessment on Identified Hazard

If an adverse effect (hazard) is identified, risk assessment on the difference is conducted. The risk assessment considers contributions to enhanced plant pest/weed potential of the crop itself, the impact of differences detected in other measured characteristics, and potential for and effects of trait introgression into any populations growing outside of cultivated environments or into a sexually-compatible species.

VII.B.2. Interpretation of Environmental Interactions Data

For the qualitative assessments of abiotic stress response, disease damage, and arthropod damage, the biotechnology-derived crop and conventional control are not considered different in susceptibility or tolerance if the range of injury symptoms of each overlapped across all four replications. Any observed differences are assessed for biological significance in the context of the range of the commercial reference materials, and for consistency in other observation times and sites. Differences that are not consistently observed in multiple environments are considered not biologically meaningful in terms of plant pest potential.

Quantitative assessments of stink bug damage are analyzed within individual sites and pooled across sites in a combined site analysis. Statistically significant differences detected between the biotechnology-derived crop and conventional control materials are evaluated using the method outlined in Figure VII-1.

Quantitative assessments of bean leaf beetle damage and arthropod abundance are only analyzed within each individual site. Statistically significant differences between the biotechnology-derived crop and conventional control are assessed for biological significance in the context of the range of the commercial reference materials, and for consistency in other collection times and collection sites. Differences that are not consistently detected in multiple environments are considered not biologically meaningful in terms of plant pest potential.

VII.C. Comparative Assessments of the Phenotypic, Agronomic, and Environmental Interaction Characteristics of MON 87751

This section provides the results of comparative assessments conducted in replicated laboratory, greenhouse, growth chamber and/or multi-site field experiments to provide a detailed phenotypic, agronomic, and environmental interactions description of MON 87751. The characteristics for MON 87751 evaluated in these assessments included: seed dormancy and germination characteristics (Section VII.C.1), plant phenotypic, agronomic, and environmental interaction observations under field conditions (Section VII.C.2), pollen characteristics (Section VII.C.3), and plant-symbiont interactions (Section VII.C.4). Additional details for each assessment are provided in Appendices F, G, H, and I.

VII.C.1. Seed Dormancy and Germination Characteristics

USDA-APHIS considers the potential for weediness to constitute a plant pest factor (7 CFR 340.6). Seed germination and dormancy mechanisms vary with species and their genetic basis tends to be complex. Seed dormancy (*e.g.*, hard seed) is an important characteristic that is often associated with plants that are considered weeds (Anderson 1996; Lingenfelter and Hartwig 2007). However, it is important to note that it is not uncommon to observe low levels of hard seed in soybean (Mullin and Xu 2001; Potts, et al. 1978). Information on germination and dormancy characteristics is therefore useful when assessing a plant for increased weediness potential. To assess germination characteristics, standardized germination assays are available and routinely used. The Association of Official Seed Analysts (AOSA), an internationally recognized seed testing organization, recommends a temperature range of 20/30°C as optimal for testing the germination and dormancy characteristics of soybean seed (AOSA 2012).

Comparative assessments of seed dormancy and germination characteristics were conducted on MON 87751 and the conventional control A3555. In addition, 12 commercial reference varieties were included to provide a range of comparative values that are representative of existing commercial soybean varieties (Table F-1). The seed used for this assessment was produced in 2012 in replicated field trials at three sites in Nebraska, Iowa and Illinois (representing environments where soybean seed is produced). In addition to the AOSA recommended temperature (20°C /30°C), seed was tested at five additional temperature regimes of 10°C, 20°C, 30°C, 10°C /20°C, and 10°C /30°C to assess dormancy and germination characteristics. The study was conducted as a split-plot design. The details on materials, methods and individual site results associated with this study are presented in Appendix F, while the combined-site analyses are summarized below.

In the combined-site analysis in which data were pooled from the three production sites, no statistically significant differences ($\alpha = 0.05$) were detected between MON 87751 and the conventional control for any characteristic at five temperature regimes (10°C, 20°C, 30°C, 10°C/20°C, and 10°C/30°C) (Table VII-2). MON 87751 had higher percent normal germinated seed than the conventional control (91.2% vs. 88.3%) at 20°C/30°C. The magnitude of the difference for percent normal germinated seed of MON 87751 was

small and the mean value was within the reference range (62.6 - 99.3%). MON 87751 had lower percent abnormal germinated seed compared to the conventional control (8.1% vs. 10.6%) at 20°C/30°C. The magnitude of the difference for percent abnormal germinated seed of MON 87751 was small and the mean value was within the reference range (0.5 - 37.4%). Therefore, these differences are unlikely to be biologically meaningful in terms of altered dormancy and germination characteristics.

The germination and dormancy characteristics evaluated were used to assess MON 87751 in the context of plant pest risk. Based on the dormancy and germination characteristics assessed and the results of this study, particularly the lack of increased hard seed, it can be concluded that there were no changes indicative of increased weediness or plant pest potential of MON 87751 compared to conventional soybean.

Table VII-2. Germination Characteristics of MON 87751 and the Conventional Control

Temperature Regime	Germination Characteristic (%)[1]	Mean (S.E.)[2]		Reference Range[3]
		MON 87751	Control	Min. - Max.
10°C	Germinated	98.6 (0.31)	97.8 (0.52)	77.5 - 99.8
	Viable Hard	0.8 (0.22)	0.8 (0.34)	0.0 - 15.8
	Dead	0.5 (0.23)	0.9 (0.34)	0.0 - 3.5
	Viable Firm-Swollen	0.2 (0.11)	0.4 (0.19)	0.0 - 3.3
20°C	Germinated	99.2 (0.27)	99.1 (0.50)	88.7 - 100.0
	Viable Hard	0.2 (0.11)	0.0 (0.00)	0.0 - 3.3
	Dead	0.7 (0.28)	0.8 (0.42)	0.0 - 8.0
	Viable Firm- Swollen	0.0 (0.00)	0.1 (0.08)	0.0 - 0.3
30°C	Germinated	99.6 (0.19)	99.3 (0.22)	92.0 - 100.0
	Viable Hard	0.0 (0.00)	0.0 (0.00)	0.0 - 4.3
	Dead	0.4 (0.19)	0.7 (0.22)	0.0 - 3.0
	Viable Firm- Swollen	0.0 (0.00)	0.0 (0.00)	0.0 - 0.8
10°C/20°C	Germinated	99.1 (0.42)	98.8 (0.37)	84.0 - 100.0
	Viable Hard	0.5 (0.19)	0.8 (0.28)	0.0 - 13.8
	Dead	0.4 (0.26)	0.3 (0.14)	0.0 - 0.8
	Viable Firm-Swollen	0.0 (0.00)	0.1 (0.08)	0.0 - 1.5
10°C/30°C	Germinated	99.4 (0.23)	99.1 (0.26)	89.5 - 100.0
	Viable Hard	0.1 (0.08)	0.1 (0.08)	0.0 - 4.8
	Dead	0.5 (0.23)	0.8 (0.24)	0.0 - 4.8
	Viable Firm-Swollen	0.0 (0.00)	0.0 (0.00)	0.0 - 1.0
20°C/30°C (AOSA)	Normal Germinated	91.2 (1.72)*	88.3 (2.23)	62.6 - 99.3
	Abnormal	8.1 (1.87)*	10.6 (2.34)	0.5 - 37.4
	Viable Hard	0.3 (0.13)	0.3 (0.14)	0.0 - 6.0
	Dead	0.5 (0.29)	0.7 (0.28)	0.0 - 5.0
	Viable Firm-Swollen	0.0 (0.00)	0.1 (0.08)	0.0 - 1.5

Note: The experimental design was a split-plot with four replications and statistical analysis consisted of an analysis of variance (ANOVA).

* Statistically significant differences detected ($\alpha = 0.05$) between MON 87751 and the conventional soybean control.

[1] In some instances, the total percentage of the MON 87751 and the control did not equal 100% due to numerical rounding of the means.

[2] Test and control values represent means with standard error in parentheses.

[3] Reference range was calculated from the minimum and maximum mean values from among 12 references.

VII.C.2. Field Phenotypic, Agronomic Characteristics, and Environmental Interactions

Phenotypic, agronomic characteristics and environmental interactions were evaluated under field conditions as part of the plant characterization assessment of MON 87751. These data were developed to provide USDA-APHIS with a detailed description of MON 87751 relative to the conventional control and reference varieties. According to 7 CFR 340.6, as part of the petition to seek deregulation, a petitioner must submit "a detailed description of the phenotype of the regulated article." This information is being provided to assess whether there are phenotypic differences between MON 87751 and the conventional control that may impact its plant pest/weed potential. Specific characteristics that are related to weediness (*e.g.*, plant lodging, pod shattering), were used to assess whether there is a potential increase in weediness of MON 87751 compared to conventional soybean. Environmental interactions including plant response to abiotic stress, disease damage, specific arthropod-related damage, pest-arthropod abundance, and beneficial-arthropod abundance were also assessed as an indirect indicator of phenotypic changes to MON 87751 and are also considered in the plant pest assessment.

The results of the assessments of agronomic and phenotypic characteristics demonstrated that the introduction of the insect-protected trait did not meaningfully alter the plant pest/weed potential of MON 87751 compared to conventional soybean. Furthermore, the lack of meaningful differences in plant response to abiotic stress, disease damage, arthropod-related damage, non-target pest-arthropod abundance, and beneficial-arthropod abundance also support the conclusion that the introduction of the insect-protected trait is not expected to result in increased plant pest/weed potential for MON 87751 compared to conventional soybean.

VII.C.2.1. Field Phenotypic and Agronomic Characteristics

Data were collected at 17 field locations in the U.S. during 2012 to evaluate phenotypic, agronomic, and environmental interaction characteristics. These 17 field sites provided a range of environmental and agronomic conditions representative of commercial soybean production areas in the U.S. (Table VII-3). The experimental design at each site was a randomized complete block with four replications. All plots of MON 87751, the conventional control A3555, and the commercial reference varieties within each site were uniformly managed in order to assess whether the introduction of insect-protected trait altered the phenotypic and agronomic characteristics or the environmental interactions of MON 87751 compared to the conventional control soybean. A description of the evaluated phenotypic and environmental interaction characteristics and the designated developmental stages when evaluations occurred are listed in Table VII-1. The methods and detailed results of the individual-site data comparisons are presented and discussed in Appendix G, while the combined-site analyses are summarized below.

In the combined-site analysis of phenotypic data, no statistically significant differences were detected ($\alpha = 0.05$) between MON 87751 and the control A3555 for any of the evaluated characteristics: early stand count, 50% flowering date, plant lodging, pod

shattering, plant height, final stand count, grain moisture, 100 seed weight, and yield (Table VII-4).

Plant growth stage, plant vigor and flower color data were categorical and were not statistically analyzed. MON 87751 and the control were within the same range for growth stage and plant vigor across all the sites (Tables G-4 and G-5). The flower color for MON 87751 and the control was purple as expected across all the sites (Table G-4). Thus, there were no differences in plant development observed between MON 87751 and the conventional control soybean.

The plant phenotypic and agronomic characteristics evaluated were used to provide a plant characterization assessment of MON 87751 compared to the conventional control A3555. A subset of these characteristics was useful to assess the weediness potential of MON 87751. Based on the assessed phenotypic and agronomic characteristics, the results support a conclusion that the introduction of the insect-protected trait in MON 87751 did not meaningfully alter the plant pest/weed potential of MON 87751 compared to commercial soybean.

Table VII-3. Field Phenotypic Evaluation Sites for MON 87751 during 2012

Site Code	County, State	Country
ARNE	Jackson, Arkansas	U.S.
GACH	Tift, Georgia	U.S.
IABG	Greene, Iowa	U.S.
IAHU	Story, Iowa	U.S.
IARL	Jefferson, Iowa	U.S.
ILAG	Vermilion, Illinois	U.S.
ILCY	Clinton, Illinois	U.S.
ILMN	Warren, Illinois	U.S.
ILTH	Champaign, Illinois	U.S.
KSLA	Pawnee, Kansas	U.S.
LACH	Rapides, Louisiana	U.S.
MOFI	Butler, Missouri	U.S.
NCBD	Perquimans, North Carolina	U.S.
NECC	Merrick, Nebraska	U.S.
OHTR	Miami, Ohio	U.S.
PAGR	Lehigh, Pennsylvania	U.S.
SCEK	Barnwell, South Carolina	U.S.

Table VII-4. Combined-Site Comparison of MON 87751 to Conventional Control for Phenotypic and Agronomic Characteristics During 2012

	Mean (S.E.)[2]		Reference Range[3]	
Phenotypic Characteristic (units)[1]	MON 87751	Control	Min	Max
Early stand count (#/linear meter)	23.5 (0.60)	24.3 (0.55)	13.0	35.1
Days to 50% Flowering	44.0 (1.19)	43.6 (1.19)	27.8	63.0
Plant lodging (1-9 scale)	2.4 (0.18)	2.2 (0.16)	1.0	5.8
Pod shattering (1-9 scale)	1.5 (0.10)	1.7 (0.16)	1.0	2.8
Plant height (cm)	85.4 (2.02)	83.9 (2.14)	58.6	125.2
Final stand count (#/linear meter)	21.2 (0.46)	21.6 (0.49)	12.0	25.7
Grain moisture (%)	11.7 (0.33)	12.0 (0.35)	9.1	16.4
100 Seed weight (g)	18.2 (0.19)	17.8 (0.22)	15.5	21.8
Yield (t/ha)	3.5 (0.11)	3.6 (0.12)	1.4	4.0

Note: The experimental design was a randomized complete block with four replicates per site. Means based on n = 68 for MON 87751 and the conventional control A3555 for all characteristics except where noted in Table G-3.

No statistically significant differences were detected between MON 87751 and the control (α =0.05) using ANOVA.

[1] Phenotypic data were collected on a per plot basis except for early and final stand counts which were on a per linear meter basis and plant height data collected from five plants per plot.

[2] Test and control values represent means with standard error in parentheses.

[3] Reference range was calculated from the minimum and maximum mean values from among 34 reference varieties.

VII.C.2.2. Environmental Interaction Characteristics

USDA-APHIS considers the environmental interaction of the biotechnology-derived crop compared to its conventional control to determine the potential for increased plant pest characteristics. Evaluations of environmental interactions were conducted as part of the plant characterization for MON 87751. In the 2012 U.S. field trials conducted to evaluate the phenotypic and agronomic characteristics of MON 87751, data were also collected on plant response to abiotic stress (*e.g.*, drought, wind, nutrient deficiency, etc.), disease damage, arthropod-related damage, and arthropod abundance (Tables G-6, G-7, G-8, G-9 and G-10). These data were used as part of the environmental analysis (Section IX) to assess plant pest potential and provide an indication of potential adverse effects of MON 87751 on non-target organisms (NTOs) compared to the conventional control. The results of the field evaluations showed that the insect-protected trait did not unexpectedly alter the assessed environmental interactions of MON 87751 compared to the conventional control. The lack of significant biological differences in plant responses to abiotic stress, disease damage, arthropod-related damage, non-target pest-arthropod abundance and beneficial-arthropod abundance support the conclusion that the introduction of the insect-protected trait is not expected to result in increased plant pest potential from MON 87751 compared to commercial soybean.

VII.C.2.2.1. Qualitative Environmental Interactions Assessment

Plant responses to abiotic stressors, disease damage, and arthropod damage were assessed at natural levels, *i.e.*, no artificial infestation or imposed abiotic stress; therefore these levels typically varied among observations at a site and among sites. As a result, individual site analysis was most appropriate to evaluate these responses. Plant responses to abiotic stress, disease damage, and arthropod damage data were collected from each plot using a categorical scale (none, slight, moderate, and severe) of increasing severity of observed damage for each stressor. This scale was utilized to allow for the evaluation of the wide variety of potential abiotic stressor, disease damage, and arthropod damage symptoms potentially occurring across the season and across sites. These data were categorical and therefore were summarized and not subjected to ANOVA. For a particular stressor, all comparisons of the range of responses for MON 87751 to the range of responses for the conventional control across all observation times and sites are reported.

Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, additional details concerning the qualitative environmental interactions assessments, and detailed results of the qualitative data comparisons are presented and discussed in Appendix G (Tables G-6, G-7, and G-8).

In the individual-site assessment, no differences were observed between MON 87751 and the control for any of the 193 comparisons for the assessed abiotic stressors, (Table VII-5 and Table G-6).

In the individual-site assessment, no differences were observed between MON 87751 and the control for any of the 191 comparisons for the assessed diseases (Table VII-5 and Table G-7).

In the individual-site assessment, no differences were observed between MON 87751 and the control for any of the 154 comparisons for the assessed arthropods (Table VII-5 and Table G-8).

The lack of differences observed between MON 87751 and the conventional control for plant responses to abiotic stressors, disease damage, and arthropod-related damage in multiple environments across the U.S. supports the conclusion that the introduction of the insect-protected trait is not expected to cause a biologically meaningful change in terms of plant pest potential compared to the conventional control (See Section VII.B.2.).

Table VII-5. Summary of Qualitative Environmental Interactions Assessments during 2012

Stressor	Number of observations across all sites	Number of observations with no differences between MON 87751 and the conventional control across all sites[1]
Abiotic stressors	193	193
Disease damage	191	191
Arthropod-related damage	154	154
Total	538	538

Note: The experimental design was a randomized complete block with four replicates per site.
No differences were observed between MON 87751 and the conventional control during any observation for damage caused by any of the assessed stressors.
[1]MON 87751 and the conventional control were considered different in susceptibility or tolerance if the range of injury symptoms across four replications did not overlap between MON 87751 and the conventional control.

VII.C.2.2.2. Quantitative Environmental Interactions Assessment

Quantitative arthropod assessments on bean leaf beetle damage, stink bug damage, and arthropod abundance were conducted at five sites (GACH, IABG, ILAG, LACH, and SCEK). Bean leaf beetle damage was assessed twice, and stink bug damage was assessed once during the growing season at each site. Arthropod abundance was assessed from collections performed five times during the growing season at each sites using vertical beat sheet samples.

Quantitative assessments of stink bug damage are analyzed within individual sites and pooled across sites in a combined site analysis. Descriptions of the evaluated environmental interactions characteristics and the timing of the evaluations are listed in Table VII-1. The materials, methods, additional details concerning the specific arthropod

damage assessments, and detailed results of the individual-site data comparisons are presented and discussed in Appendix G (Table G-9).

In the combined-site analysis for plant damage caused by stink bug there were no statistically significant differences detected between MON 87751 and the control variety (Table VII-6). These results indicate there was no biological difference in stink bug damage that would contribute to increased plant pest potential of MON 87751 compared to the conventional control (Figure VII-1, Step 2, answer "no").

In an assessment of bean leaf beetle damage, no or minor damage (as indicated by average damage rating per plant) was detected for all entries across observations and sites. Lack of damage prevented statistical comparisons between MON 87751 and control for bean leaf beetle damage in the combined-site analysis.

The lack of differences in plant response to stink bug damage support the conclusion that the introduction of the insect protected trait in MON 87751 is not expected to pose an increased plant pest potential compared to the control soybean.

Arthropod abundance was assessed from collections performed using vertical beat sheet samples at five sites in 2012. Variations in temporal activity and geographical distribution of arthropod taxa occurred among sites, therefore, only individual-site analyses were conducted for arthropod abundance data. Additional details of the arthropod abundance assessments and detailed results of the individual-site data comparisons are provided in Appendix G (Table G-10). The results of these analyses are summarized below and in Table VII 7.

A total of 170 statistical comparisons were made between MON 87751 and the control for arthropod abundance involving the following pest and beneficial arthropods: aphids, bean leaf beetles, corn rootworm beetles, Japanese beetles, kudzu bugs, minute brown scavenger beetles, leaf beetles, leafhoppers, tarnished plant bugs, plant bugs, spider mites, stink bugs, thrips, treehoppers, whiteflies, ant-like flower beetles, spiders, assassin bugs, big-eyed bugs, brown lacewings, green lacewings, damsel bugs, ladybird beetles, micro-Hymenoptera, minute pirate bugs, and predatory mites. Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87751 and the control for 127 additional comparisons; however, descriptive statistics were provided for these comparisons (Table G-10).

No statistically significant differences were detected between MON 87751 and the control for 157 out of 170 comparisons. The 13 differences detected between MON 87751 and the conventional control included spiders, big-eyed bugs, damsel bugs, predatory mites, bean leaf beetle, plant bugs, kudzu bugs, stink bugs and thrips. These differences are summarized in Table VII-7 and Table G-10. In cases where mean abundance values for MON 87751 were outside the reference range, the statistical differences were not consistently detected across collections or sites (Table VII-7 and Table G-10). Thus, the statistical differences in abundance were not indicative of a consistent response associated with the trait and are not considered biologically

meaningful in terms of increased pest potential of MON 87751 compared to the conventional control (See Section VII.B.2.).

Table VII-6. Combined-Site Comparison of Stink Bug Damage to MON 87751 Compared to the Conventional Control during 2012

Pest	Damage assessment	Mean (S.E.)[1]		Reference range[2]	
		MON 87751	Control	Min	Max
Stink bug (Pentatomidae)[3]	Damaged pods per plot (%)	21.2 (5.61)	21.5 (5.63)	3.1	63.9

Note: The experimental design was a randomized complete block with four replication (n = 20 except where noted in Table G-3).
No statistically significant differences were detected between MON 87751 and the control (α=0.05) using ANOVA.
[1] MON 87751 and control values represent means with standard error in parentheses.
[2] Reference range is calculated from the minimum and maximum mean values from among reference materials across five sites (GACH, IABG, ILAG, LACH, and SCEK).
[3] Damage assessments for stink bugs were conducted once at R6-R8 growth stage.

Table VII-7. Summary of Arthropod Abundance Assessments and Detected Differences during 2012

Summary of Statistical Comparisons[1]

Arthropod Abundance Assessment	Number of sites	Number of comparisons across sites	Number of comparisons where no differences were detected
Vertical beat sheet	5	170	157

Summary of Detected Differences[2]

Arthropod	Site	Collection Number	Within reference range?	Consistently detected across collections or sites?
Spiders	IABG	3	Yes	No
	SCEK	5	No	No
Big-eyed bug	GACH	2	Yes	No
	SCEK	5	No	No
Damsel bug	SCEK	4	Yes	No
Predatory mites	ILAG	1	No	No
Bean leaf beetle	ILAG	4	Yes	No
Kudzu bug	SCEK	4	No	No
Plant bug	GACH	5	Yes	No
	LACH	3	No	No
Stink bug	SCEK	4	Yes	No
Thrips	GACH	2	No	No
	SCEK	3	No	No

[1] Quantitative arthropod abundance assessments were statistically analyzed at α=0.05 using ANOVA. Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87751 and the conventional control for additional 127 comparisons; however, descriptive statistics were provided for these comparisons in Appendix G (Table G-10).

[2] Thirteen statistically significant differences were detected. These differences are further assessed following guidance in Section VII.B.2.

VII.C.3. Pollen Characteristics

USDA-APHIS considers the potential for gene flow and introgression of the biotechnology-derived trait(s) into other soybean plants and wild relatives to determine the potential for increased weedy or invasive characteristics of the receiving species. Pollen morphology and viability information are pertinent to this assessment and, therefore, were assessed for MON 87751. In addition, morphological characterization of pollen produced by MON 87751 and the conventional control is relevant to the plant pest risk assessment because it adds to the detailed description of the phenotype of MON 87751 compared to the conventional control.

The viability and morphology of pollen was assessed for MON 87751 and compared to the conventional control (A3555). Pollen was collected from MON 87751, the control, and four commercial reference varieties (Table H-1) grown under similar growth chamber conditions. The study was arranged in a randomized complete block design with four replications and with seven plants per replication per entry. Pollen was collected from each entry and stained for assessment. Pollen viability was evaluated for each sample on a minimum of 75 pollen grains, and pollen grain diameter was evaluated using ten representative viable pollen grains per replication. Two perpendicular diameter measurements were made on each of the ten pollen grains for a total of twenty diameter measurements per replication. General morphology of the pollen was observed for each of the four replications of MON 87751, the control, and the reference soybean varieties. A reference range was calculated from the minimum and maximum mean values of the reference varieties to provide pollen viability and pollen grain diameter values representative of commercial soybean. The details on materials and methods associated with this study are presented in Appendix H.

No statistically significant differences were detected (α=0.05) between MON 87751 and the control for percent viable pollen or pollen grain diameter (Table VII-8). Furthermore, no visual differences in general pollen morphology were observed between MON 87751 and the control (Figure H-1). Based on the assessed characteristics, these results support a conclusion that neither viability nor morphology of pollen of MON 87751 was altered compared to the conventional soybean control.

Table VII-8. Pollen Characteristics of MON 87751 Compared to the Conventional Control during 2012

Pollen Characteristic (unit)	Mean (S.E.)[1]		Reference Range[2]	
	MON 87751	Control	Minimum	Maximum
Viability[3] (%)	99.7 (0.3)	98.8 (0.5)	98.8	99.7
Diameter[4] (μm)	24.0 (0.2)	24.2 (0.2)	23.5	24.6

Note: No significant differences were detected between the MON 87751 and the conventional control (α=0.05) using analysis of variance (ANOVA).

[1] MON 87751 and control values represent means with standard error in parentheses.

[2] Reference range is the minimum and maximum mean value observed among the four reference soybean varieties.

[3] Evaluated for each of the four replications of MON 87751, the conventional control, and reference varieties.

[4] Evaluated for ten representative viable pollen grains per replication.

VII.C.4. Symbiont Interactions

As part of the plant pest risk assessment, USDA-APHIS considers the impact of the biotechnology-derived crop on plant pest potential and the environment compared to its conventional counterpart. Potential changes in the symbiotic relationship with members of the bacterial families, *Rhizobiaceae* and *Bradyrhizobiaceae*, which inhabit the rhizosphere, could directly impact pest potential or the environment. Thus, the purpose of this evaluation was to assess whether the introduction of the insect-protected trait altered the symbiotic interaction of MON 87751 with *B. japonicum* compared to that of the conventional control.

Members of the bacterial families *Rhizobiaceae* and *Bradyrhizobiaceae* form a highly complex and specific symbiotic relationship with leguminous plants, including soybean (Gage 2004). The nitrogen-fixing plant-microbe symbiosis results in the formation of root nodules, which provide an environment in which differentiated bacteria called bacteroids are capable of reducing or "fixing" atmospheric nitrogen. The product of bacterial nitrogen fixation, ammonia, can then be utilized by the plant to support growth and development. As a result of this relationship, external nitrogen inputs are typically not necessary for agricultural production of soybeans.

The relative effectiveness of the symbiotic relationship between a leguminous plant and its rhizobial symbiont can be assessed by various methods. Measurement of nodule number and mass along with plant growth and nitrogen status are commonly used to assess differences in the symbiotic relationship between a legume and its associated rhizobia (Israel, et al. 1986). It should be noted, however, that nodule number relative to nodule dry weight may be variable in soybean experiments because some nodules may be larger in diameter and less numerous, while others are not as developed (smaller) but more abundant (Appunu and Dhar 2006; Israel et al. 1986).

MON 87751, the parental conventional control A3555, and six commercial reference varieties were produced from seeds planted in pots containing nitrogen-deficient potting medium and grown in the greenhouse. Seeds were inoculated with a solution of *B. japonicum*. The pots were arranged in a randomized complete block design with eight replicates. At six weeks after emergence, plants were excised at the surface of the potting medium, and shoot and root plus nodule material were removed from the pots. Nodules were separated from roots prior to enumeration and determination of dry weight. MON 87751 was compared to the conventional control A3555 for key characteristics related to their association with the soybean *B. japonicum* symbiosis. Detailed information on materials and methods used for the symbiont evaluation is presented in Appendix I.

No statistically significant differences were detected (α=0.05) between MON 87751 and the conventional control A3555 for each measured parameter, including nodule number, shoot percent total nitrogen, shoot total nitrogen (g), and dry weight of nodules, shoot material, and root material (Table VII-9).

Based on the assessed characteristics, the results support the conclusion that the introduction of the insect-protected trait does not alter the symbiotic relationship between *B. japonicum* and MON 87751 compared to that of conventional soybean. Thus, these data further support a conclusion of no change in plant pest potential for MON 87751 compared to conventional soybean.

Table VII-9. Symbiont Interaction Assessment of MON 87751 and Conventional Control

Measurements	Mean (S.E.)			Reference Range[1]	
	MON 87751	A3555	p-Value	Minimum	Maximum
Nodule Number (per plant)	283 (18)	282 (21)	0.9896	191	302
Nodule Dry Wt (g)	0.64 (0.04)	0.65 (0.04)	0.8660	0.54	0.65
Root Dry Wt (g)	1.84 (0.16)	1.95 (0.19)	0.6423	1.65	2.31
Shoot Dry Wt (g)	9.28 (0.68)	9.83 (0.70)	0.4941	8.21	10.04
Shoot Percent Total Nitrogen (% dwt)	3.87 (0.07)	3.71 (0.10)	0.1658	3.42	4.00
Shoot Total Nitrogen (g)	0.36 (0.02)	0.36 (0.02)	0.8668	0.31	0.36

Note: Pots were arranged in eight replicated blocks (n = 8) in a greenhouse using a randomized completed block design. S.E. = Standard Error.
No significant differences were detected between MON 87751 and the conventional control A3555 (α=0.05).
[1] Reference range is the minimum and maximum mean value observed among six commercial reference varieties.

VII.D. Conclusions for Phenotypic, Agronomic, and Environmental Interactions Evaluation

Comparative plant characterization data between a biotechnology-derived crop and the conventional control are interpreted in the context of contributions to increased plant pest/weed potential as assessed by USDA-APHIS. Based on the concept of familiarity, characteristics for which no differences are detected support a conclusion of no increased plant pest/weed potential of the biotechnology-derived crop compared to the conventional crop. Ultimately, a weight-of-evidence approach that considers all characteristics and data is used for the overall risk assessment of differences and their significance.

An extensive and robust set of agronomic, phenotypic, and environmental interactions data, including specific weedy characteristics, were used to assess whether the introduction of the insect-protected trait altered the plant pest potential of MON 87751 compared to the conventional control, considered within the context of the variation among the reference varieties. These assessments included six general data categories: 1) seed germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive development; 4) seed retention and lodging; 5) plant-environment interactions; and 6) plant-symbiont interactions. Within these data categories, data relevant to understanding specific characteristics associated with weediness were also assessed to determine whether there was a potential increase in weediness of MON 87751 compared to conventional soybean.

Results from these assessments comparing MON 87751 and the conventional control demonstrate that MON 87751 does not possess: 1) increased weediness characteristics; 2) increased susceptibility or tolerance to specific abiotic stress, diseases, or arthropods; or 3) characteristics that would confer a plant pest risk compared to conventional soybean. Therefore, based on the results of multiple assessments discussed above and presented in the appendices, the weight of evidence indicates that MON 87751 is not meaningfully different from conventional soybean with the exception of the insect-protected trait and is not expected to pose a plant pest/weed risk compared to conventional soybean.

VIII. U.S. AGRONOMIC PRACTICES

VIII.A. Introduction

As part of the plant pest assessment required by 7 CFR 340.6(c)(4), impacts of deregulation on agricultural and cultivation practices must be considered. This section provides a summary of current agronomic practices in the U.S. for producing soybean, and is included in this request as a baseline to assess possible impacts to agricultural practices due to the cultivation of MON 87751. Discussions include soybean production, seed production, plant growth and development, general management practices during the season, management of weeds, insects and diseases, soybean rotational crops, and volunteer management. Information presented in the previous section demonstrated that MON 87751 is no more susceptible to diseases or pests than commercially cultivated soybean. Additionally, data presented in Section VII show that MON 87751 is not expected to pose a plant pest risk compared to conventional soybean. Aside from the potential to reduce broad spectrum insecticide sprays, a common benefit of all insect-protected crops, there are no changes to the inputs needed for MON 87751, and no specific impacts to most of the agronomic practices employed for production of soybean. Where there is potential impact on agronomic practices from the deregulation of MON 87751, discussion delineating the scope and magnitude of those impacts is provided. Overall, given the documented phenotypic characteristics of MON 87751, the impacts on soybean agronomic practices are not expected to be different from those of MON 87701.

VIII.B. Overview of U.S. Soybean Production

In the 2011/12 growing seasons, the U.S. led global soybean production with approximately 35% (USDA-FAS 2013) followed by Brazil with 28%. The U.S. exported 1.36 billion bushels (37.1 million metric tons) of soybean in 2011/12, which accounted for 41% of the world's soybean exports (USDA-FAS 2013). In total, the U.S. exported $21.5 billion of soybeans, soybean meal and soybean oil in the marketing year 2011 (ASA 2012). China is the largest export market for U.S. soybeans, followed by Mexico. Other significant markets include Japan and Taiwan.

The majority of the soybean acres are planted in the Midwest and Midsouth states (Figure VIII-1). Over the past ten years, U.S. soybean acreage has varied from approximately 64.7 to 77.5 million acres, and there is no consistent trend (Table VIII-1). The value of U.S. soybeans reached a record high of $43.19 billion in 2012, primarily as a result of the record high soybean price of $14.30 per bushel (USDA-NASS 2013b).

For purposes of this discussion, soybean production is divided into three major soybean growing regions – Midwest region (IL, IN, IA, KS, KY, MI, MN, MO, NE, ND, OH, SD, and WI), South region (AL, AR, GA, LA, MS, NC, OK, SC, TN, and TX) and the Northeast region (DE, MD, NJ, NY, PA, VA, and WV) (Table VIII-2). Table VII-2 provides acreage and production for each of these regions in 2012.

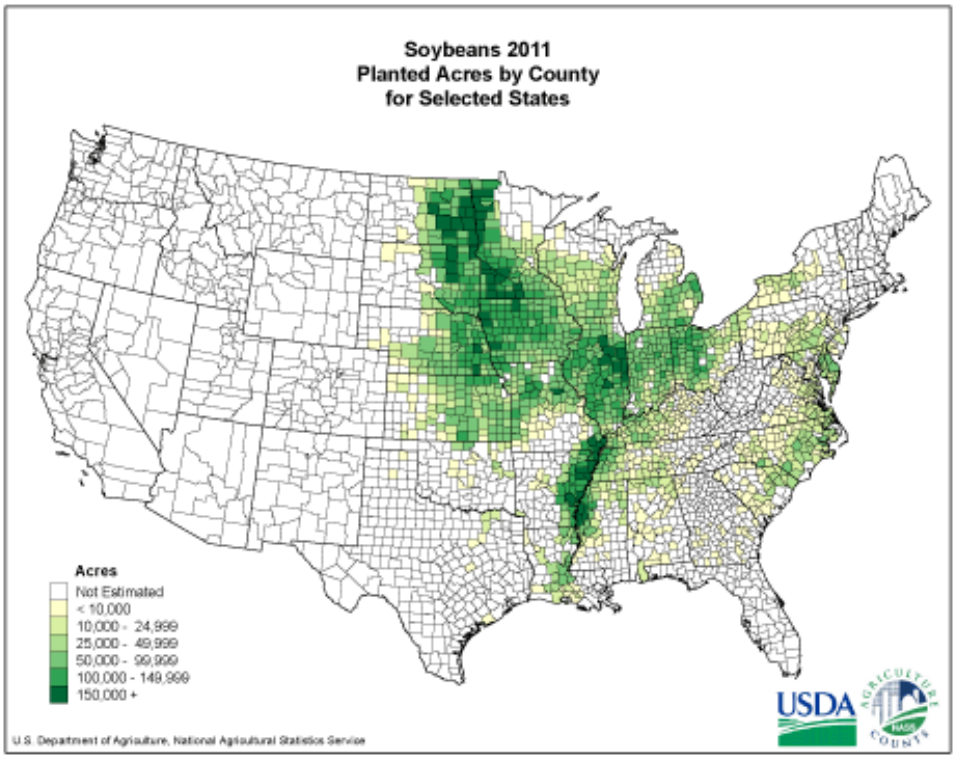


Figure VIII-1. Planted Soybean Acres by County in the U.S. in 2012
Source: USDA-NASS, (2012b).

Table VIII-1. Soybean Production in the U.S., 2003 – 2012

Year	Acres Planted (×1000)	Acres Harvested (×1000)	Average Yield (bushels/acre)	Total Production (×1000 bushels)	Value (billions $)
2012	77,198	76,104	39.6	3,014,998	43.19
2011	75,046	73,776	41.9	3,093,524	38.50
2010	77,404	76,610	43.5	3,329,181	37.55
2009	77,451	76,372	44.0	3,359,011	32.15
2008	75,718	74,681	39.7	2,967,007	29.46
2007	64,741	64,146	41.7	2,677,117	26.97
2006	75,522	74,602	42.9	3,196,726	20.47
2005	72,032	71,251	43.1	3,086,342	17.30
2004	75,208	73,958	42.2	3,123,790	17.90
2003	73,404	72,476	33.9	2,453,845	18.02

Source: USDA-NASS, (2013a; b).

Table VIII-2. U.S. Soybean Production by Region and State in 2012

Region/State	Acres Planted[1] (thousands)	Acres Harvested[1] (thousands)	Average Yield[1] (bushels/acre)	Total Production[1] (×1000 bushels)	Value[2] (billions $)
Midwest Region					
Illinois	9,050	8,920	43.0	383,560	5.60
Indiana	5,150	5,140	43.5	223,590	3.26
Iowa	9,350	9,300	44.5	413,850	5.92
Kansas	4,000	3,810	22.0	83,820	1.20
Kentucky	1,480	1,470	40.0	58,800	0.85
Michigan	2,000	1,990	43.0	85,570	1.20
Minnesota	7,050	6,990	43.0	300,570	4.27
Missouri	5,400	5,260	29.5	155,170	2.25
Nebraska	5,050	4,990	41.5	207,085	2.90
North Dakota	4,750	4,730	30.0	160,820	2.28
Ohio	4,600	4,580	45.0	206,100	2.99
South Dakota	4,750	4,710	42.0	141,300	1.99
Wisconsin	1,710	1,700	41.5	705,550	0.98
Region Totals	**64,340**	**63,590**	**39.2**	**2,490,785**	**35.69**
South Region					
Alabama	340	335	45.0	15,075	0.22
Arkansas	3,200	3,160	43.0	135,880	1.96
Florida	21	20	39.0	780	0.01
Georgia	220	215	37.0	7,955	0.12
Louisiana	1,130	1,115	46.0	51,290	0.75
Mississippi	1,970	1,950	45.0	87,750	1.26
North Carolina	1,590	1,580	39.0	61,620	0.86
Oklahoma	420	260	15.0	3,900	0.06
South Carolina	380	370	34.0	12,580	0.18
Tennessee	1,260	1,230	38.0	46,740	0.68
Texas	125	110	26.0	28,60	0.04
Region Totals	**10,656**	**10,345**	**41.2**	**426,430**	**6.14**

Table VIII-2. U.S. Soybean Production by Region and State in 2012 (continued)

Region/State	Acres Planted[1] (thousands)	Acres Harvested[1] (thousands)	Average Yield[1] (bushels/acre)	Total Production[1] (×1000 bushels)	Value[2] (billions $)
Northeast Region					
Delaware	170	168	42.5	7,140	0.10
Maryland	480	475	47.0	22,325	0.32
New Jersey	96	94	39.0	3,666	0.05
New York	315	312	46.0	14,352	0.20
Pennsylvania	530	520	48.0	24,960	0.35
Virginia	590	580	42.0	24,360	0.33
West Virginia	21	20	49.0	980	0.01
Region Totals	**2,202**	**2,169**	**45.1**	**97,783**	**1.36**
U.S. Totals	**77,198**	**76,104**	**39.6**	**3,014,998**	**43.19**

[1] Source: USDA-NASS, (2013a).
[2] Source: USDA-NASS, (2013b).

VIII.C. Production Management Considerations

Other than the specific insertion of the coding sequences for Cry1A.105 and Cry2Ab2 that provide protection against targeted lepidopteran insect pests, MON 87751 is not different from many other soybean varieties in terms of its production management requirements. USDA has deregulated numerous crop plants that express Cry proteins to protect against insect pests since 1995 and deregulated the first insect-protected soybean in 2011 (USDA-APHIS 2011). Although there are no insect-protected soybean varieties currently being cultivated commercially in the U.S., based on experience with widespread use of other insect-protected crops, Monsanto anticipates no unique production management considerations from introducing MON 87751 above and beyond those in current use.

VIII.D. Management of Insect Pests and Insect Resistance Management

Although insects are rated as less problematic than weeds in U.S. soybean production, management of insect pests during the growth and development of soybean is important for protecting soybean yield (Aref and Pike 1998). Insect injury can impact yield, plant maturity, and seed quality. Injury may produce stress, which is a departure from optimal physiological conditions (Higley 1994). In the U.S., insect injury in soybean seldom reaches levels to cause an economic loss as indicated by the low percentage (18%) of soybean acreage that receives an insecticide treatment (USDA-NASS 2013c).

Most often, soybean insect pests are categorized or defined by the plant parts they injure, namely root-feeding, stem-feeding, leaf-feeding, or pod-feeding insects. The root- and stem-feeding insect groups are often the hardest to scout and typically are not detected until after they have caused soybean damage. The leaf-feeding insects comprise the largest group of insects, but are not necessarily the most damaging (Higley 1994). Soybean can withstand considerable defoliation early in the season without significant yield loss. Defoliation during the flowering and pod filling stages poses a greater threat to yield because the soybean has less time to compensate for injury compared to other growth stages (Higley 1994). Extension entomologists indicate that the soybean plant can sustain a 40 to 50% leaf-area loss during the vegetative stages (to about V7); 15 to 20% during flowering, pod development, and pod fill; and more that 25% from pod fill to harvest before an insecticide treatment is necessary (Steffey and Gray 2013).

The occurrence of soybean insects follows a north-south gradient in terms of severity (Higley 1994). Soybean in the Northeast region is relatively free of insect pests although some soybean fields may require insect control on occasion. In this region, the most prevalent pests are foliage feeding insects including *Aphis glycines* (soybean aphid), *Plathypena scabra* (green cloverworm), *Popillia japonica* (Japanese beetle), *Empoasca fabae* (potato leafhopper), *Epilachna varivestis* (Mexican bean beetle), *Cerotoma trifurcate* (bean leaf beetle), and *Melanoplus* spp. (grasshopper) (Curran, et al. 2013). Table VIII-3 lists the most common soybean insect pests in the Midwest region. *P. scabra* is the only lepidopteran insect that occurs frequently in the Midwest.

Table VIII-4 provides a summary of soybean insect pest infestations, yield losses, and costs in the southern states based on a 2012 survey of crop consultants and extension personnel. Overall, the yield loss from all soybean insect pests in the surveyed states was 5.59% or approximately 23.3 million bushels down slightly from 2011. Yield losses and control costs (seed treatment, foliar insecticides, and scouting) resulted in total costs of $506.8 million or $50.43 per acre. In the midsouth states (AR, LA, MS, and TN) insecticide seed treatments were used on 51 to 90% of the soybean acreage while seed treatments were only used on 5 to 20% of the acreage in AL, NC, and VA (Musser et al. 2013). The average number of foliar insecticide applications also varied among southern states. In 2012, soybean growers in midsouth states averaged 1.07 to 4.21 foliar applications of insecticide, while in AL, NC, and VA the average number of applications per year ranged from 0.44 to 1.25 (Musser et al. 2013). Lepidopteran pests made up five of the top ten most damaging insect pests in the southern states in 2012. *H. zea* was the most costly insect pest in the southern states in terms of lost yield and cost of control in 2012 (Musser et al. 2013) and resulting in losses and control costs of an estimated $201 million or $20.04 per acre. *C. includens*, *Spissistilus festinus* (three-cornered alfalfa hopper) and *Spodoptera* spp. (armyworms) were also among the top five most damaging insect pest in the southern states. As noted in Table VIII-2, soybean acres in southern states represent less than approximately 14% of total U.S. soybean acres.

Management of insect pests in soybean integrates chemical with biological control, cultural control and plant resistance to reduce overall dependence on chemical insecticides (Funderburk, et al. 1999). Preventive pest management practices are most important where the pest problem can be anticipated each year. Changes in cultural practices can adversely affect pest species or aid beneficial species. Variety selection, crop rotation, tillage, planting dates and adjacent crops all play a role in pest outbreaks in a particular field or influence the degree to which natural enemies are effective in suppressing pest populations.

According to USDA-NASS statistics, about 18% of the U.S. planted soybean acreage in 2012 received an insecticide treatment and 4.06 million pounds on insecticide active ingredient were applied to this acreage (Table VIII-5). Three insecticides (bifenthrin, chlorpyrifos, lambda-cyhalothrin) account for most of the soybean treated acreage. Each of these insecticides controls a similar broad spectrum of insect pests including lepidopteran and non-lepidopteran pests. USDA-NASS statistics are not available to determine the targeted insect pests for these insecticide treatments. According to USDA-NASS (2013c) statistics, the percentage of treated acres in 2012 in Midwest region states ranged from 0 to 29% while the percentages of South region states ranged from 12 to 84%. In the southern states, insecticide treatments are used to control a number of important lepidopteran and non-lepidopteran soybean insect pests including *H. zea*, *C. includens*, *S. festinus*, *Melanoplus* spp., *P. scabra*, and *C. trifurcate* (Musser et al. 2013).

Chemical insecticides are not always effective for controlling lepidopteran infestations in soybean. Narrow application windows, the emergence of insecticide resistance, harmful

effects on beneficial insects, and public pressure for reduced pesticide use limit the desirability of this approach to pest management (Boethel, et al. 1992; Funderburk et al. 1999). *C. includens* has developed resistance to every synthetic class of insecticide used against it (Boethel et al. 1992), and resistance to pyrethroids is widespread across the southern U.S. (Felland et al. 1990; Leonard et al. 1990).

Insect control strategies that rely on biological insecticides and natural enemies are available but not widely used because of narrow host range, slow killing speed, technical and economical difficulties for *in vitro* commercial production, timing of application based on host population monitoring, variability of field efficacy to climatic conditions, and farmers' attitude toward pest control (Moscardi 1999). Additionally, predators of soybean insect pests are sometimes uses as alternative control agents (Funderburk et al. 1999).

There has been limited success over the past 30 years in the development of superior soybean cultivars with insect resistance (Boethel 1999; Narvel, et al. 2001). A resistant conventionally-bred variety can have many advantages in insect management including effectiveness, selectivity against pest, relatively long stability, compatibility with other tactics and human and environmental safety (Pedigo 1996). In addition, resistant varieties can be adopted into crop production systems easily and economically. The successful introduction of resistant varieties may be hindered by the quantitative nature of resistance and by linkage drag from resistance trait donor parents (Narvel et al. 2001). The length of time to develop conventionally-bred resistant varieties is also a significant limitation. New techniques in selection and breeding, however, have shortened development times. Several soybean varieties are currently available that contain loci that confer resistance to soybean aphid (McCarville, et al. 2012) while others have resistance to soybean cyst nematode, brown stem rot or phytophthora root rot.

Integrated Pest Management (IPM) programs provide a viable method to minimize economic losses from insect pests. IPM programs integrate preventive pest management with insecticidal control, integrate chemical control and biological control, cultural control, and plant resistance to minimize insecticide resistance and reduce dependence on insecticides (Pedigo 1996). IPM programs involve scouting or monitoring fields during periods of risk for insect damage. Fields are monitored for growth stage, insect development and population density, and occasionally natural enemy development and population density. Management decisions for insect populations in individual fields are based on economic injury level, which is defined as the lowest population density of each insect likely to cause economic damage. The economic injury level usually changes during the growing season. For example, control of velvetbean caterpillar and similar caterpillars with chemical insecticides is normally not warranted until greater than 30% of the foliage is destroyed prior to bloom, or when 20% of the foliage is destroyed during the bloom, pod set or fill stages (NDSU 2002).

Biotechnology-derived insect-protected crop products have become important tools for insect pest management since their introduction in maize and cotton. The adoption of

insect-protected maize has reduced the impact of lepidopteran stalk borers while reducing insecticide use (Armstrong, et al. 1995; Pilcher and Rice 2003). Likewise, the adoption of insect-protected cotton has enabled more effective management of lepidopteran pests and significantly reduced chemical insecticide use (Carrière, et al. 2001; Perlak, et al. 2001). MON 87701 is a biotechnology derived insect-protected soybean, deregulated by USDA, that offers an effective alternative to chemical insecticides for control of certain lepidopteran pests in soybean. MON 87701 expresses Cry1Ac to provide protection against lepidopteran soybean pests including corn earworm, velvetbean caterpillar, and soybean looper (MacRae, et al. 2005). Varieties containing MON 87701 have not been commercialized in the U.S. MON 87751 is also expected to provide an effective means of lepidopteran control in soybean but as it does not control non-lepidopteran pests, strategies to control those pests will still be necessary.

MON 87751 will be offered as part of combined-trait breeding stacks with other selected biotechnology-derived traits. As these selected insect-protected products are identified and developed and if Monsanto decides to pursue commercialization in the U.S., Monsanto will develop appropriate IRM program(s) for these products and submit them to U.S. EPA as part of its registration package(s).

Table VIII-3. Important Soybean Insect and Mite Pests in the Midwest Region of the U.S.

Common name	Scientific name/Order	Primary Feeding Site
	Key Insect Pests[1]	
Bean leaf beetle	*Cerotoma trifurcate* /C	Leaf, pods
Japanese beetle	*Popillia japonica /C*	Leaf
Soybean aphid	*Aphis glycines* /H[2]	Leaf, stems
Two-spotted spider mite	*Tetranychus urticae* /A	Leaf
	Less Frequently Occurring/Damaging Pests	
Blister beetles[3]	*Epicauta spp. /C*	Leaf
Corn earworm	*Helicoverpa zea* /L	Pods, seeds
Cutworms[3]	*Agrotis ipsilon*, *Peridroma saucia* /L	Stems
European corn borer	*Ostrinia nubilalis* /L	Stem
Fall armyworm	*Spodoptera frugiperda* /L	Leaf
Grape colaspis	*Colaspis brunnea* /C	Leaf
Grasshoppers[3]	*Melanoplus spp.* /O	Leaf, pods, seeds
Green cloverworm	*Plathypena scabra* /L	Leaf
Plant bugs[3]	*Lygus lineolaris* /H	Leaf, stems, pods
Potato leafhopper	*Empoasca fabae* /H	Leaf and veins
Seedcorn maggot	*Delia platura* /D	Seed
Soybean thrips	*Sericothrips variablis* /T	Leaf
Soybean stem borer	*Dectes texanus* /C	Stem
Stalk borer	*Papaipema nebris* /L	Stem
Stink bugs[3]	*Acrosternum hilare*, *Euschistus servus /H*	Pods, seeds
Painted lady	*Cynthia cardui* /L	Leaf
Webworms[3]	*Loxostege cerealis* /L	Leaf
Western corn rootworm	*Diabrotica virgifera* /C	Leaf
White flies[3]	*Bemisia tabaci* /H	Leaf
White grubs[3]	*Phylophaga spp.* /C	Seeds
Wireworms[3]	*Melanotus spp., Agriotes mancus, Limonius dubitans* /C	Seeds
Woolly bear caterpillers[3]	*Spilosoma virginica* /L	Leaf
Yellowstriped armyworm	*Spodoptera ornithogalli* /L	Leaf

Source: Steffey and Gray, 2013.
[1]Not in order of importance
[2]Insect Orders: A-Acari; C-Coleoptera; D-Diptera; H-Hemiptera; L-Lepidoptera; O-Orthoptera, T-Thysanoptera
[3]More than one species.

Table VIII-4. Soybean Insect Infestations and Losses in Southern States (MS, TN, AR, AL, LA, NC, VA) in 2011

Insect Pest	Acres Infested	% Acres Infested	% Acres treated	% Yield Loss	Yield Loss + Control Cost Total $	Yield Loss + Control Cost $/Acre
Corn earworm /L[1]	6,009,200	59.8	25.6	2.72	201,384,682	20.04
Soybean looper /L	6,808,500	67.7	30.0	1.02	107,918,944	10.74
Stink bugs /H	7,815,800	77.8	29.1	0.82	87,817,289	8.74
Threecornered alfalfa hopper /H	8,441,800	84.0	14.3	0.18	23,760,880	2.36
Armyworms /L	4,047,000	40.3	6.7	0.19	17,707,000	1.76
Grasshopper / O	5,459,800	54.3	7.8	0.11	14,573,010	1.45
Green cloverworm /L	7,866,300	78.3	6.3	0.13	12,623,554	1.26
Velvetbean caterpillar /L	2,748,600	27.3	7.4	0.10	11,621,255	1.16
Spider mites /A	1,288,300	12.8	0.2	0.10	5,960,566	0.59
Blister beetle /C	1,752,100	17.4	3.2	0.03	5,576,425	0.55
Bean leaf Beetle /C	5,447,600	54.2	3.1	0.04	5,129,528	0.51
Thrips /T	7,454,900	74.2	1.6	0.04	4,020,333	0.40
Lesser cornstalk borer /L	200,100	2.0	0	0.04	2,466,047	0.25
Dectes stem borer /C	4,534,500	45.1	0	0.02	1,484,262	0.15
Garden webworms /L	1,240,000	12.3	0.5	0.01	1,282,150	0.13
Cutworms /L	333,500	3.3	1.0	0	988,553	0.10
Kudzu bug /H	366,600	3.6	0.6	0.01	988,034	0.10
Spotted cucumber beetle /C	5,693,100	56.6	0	0.01	577,755	0.06
Saltmarsh caterpillar /L	2,355,700	23.4	0	0	253,374	0.03
Grape colaspis /C	4,669,100	46.5	0	0	51,800	0.01
Other[2]	7,349,500	NA	NA	0.01	643,053	0.06
All Insects				**5.59**	**506,829,397**	**50.43**

Source: Musser et al., 2013.

[1] Insect Orders: A-Acari; C-Coleoptera; H-Hemiptera; L-Lepidoptera; O-Orthoptera, T- Thysanoptera.

[2]Other insects include: banded cucumber beetle, Japanese beetle, Mexican bean beetle, potato leafhopper, soybean aphid, and tochanter mealybug.

Table VIII-5. Insecticide Use in soybean in the U.S. in 2012

Insecticide	Chemical Family	Mode of Action (MOA)	Area Applied (Percent)	Total Area Applied (Percent/MOA)	Quantity Applied (1000 lbs)	Total Quantity Applied (1000 lbs/MOA)
Acephate	organophosphate	Acetylcholine esterase inhibitors	1	8	989	3,355
Chlorpyrifos	organophosphate		6		2,090	
Dimethoate	organophosphate		1		276	
Beta-cyfluthrin	pyrethroid	Sodium channel modulators	<0.5	12	4	372
Bifenthrin	pyrethroid		3		153	
Cyfluthrin	pyrethroid		1		44	
Cypermethrin	pyrethroid		<0.5		10	
Esfenvalerate	pyrethroid		<0.5		10	
Gamma-cyhalothrin	pyrethroid		1		6	
Lambda-cyhalothrin	pyrethroid		6		141	
Zeta-cypermethrin	pyrethroid		1		4	
Methoxyfenozide	Diacylhydazine	Ecdysone agonists/moulting disruptors	<0.5	<1	130	130
Imidacloprid	Neonicotinoid	Nicotine Acetylcholine receptor antagonists	<0.5	1	13	32
Thiamethoxam	Neonicotinoid		1		19	
Flubendiamide	Flubendiamide	Ryanodine receptor modulators	<0.5	<0.5	21	21
Diflubenzuron	benzoylureas	Inhibitors of chitin Biosynthesis	<0.5	<0.5	6	6
Total				**18**		**4,060**

[1]USDA-NASS (2013c).

VIII.E. Management of Diseases and Other Pests

More than 100 pathogens are known to affect soybean, of which 35 are considered to be of economic importance (Bowers and Russin 1999). The estimated yield losses to soybean diseases in the U.S. were 10.9, 11.9 and 14.0 million metric tons in 1996, 1997 and 1998, respectively (Wrather, et al. 2001). Pathogens can affect all parts of the soybean plant resulting in reduced quality and yield. The extent of losses depends upon the pathogen, the state of plant development and health when infection occurs, the severity of the disease on individual plants, and the number of plants affected (Bowers and Russin 1999).

Selecting resistant varieties is the first line of defense of disease control (Bowers and Russin 1999). Resistant varieties may have morphological or physiological characteristics that provide immunity, resistance, tolerance or avoidance to certain pathogens. Cultural practices play an important role in disease management by reducing initial inoculums or reducing the rate of disease development (Bowers and Russin 1999). Preplant tillage can bury crop residue, which encourages the decomposition of fungal-resting structures. Crop rotation is routinely recommended as a disease management strategy. Rotating crops interrupts the disease cycle and allows time for the decomposition of inocula. *Rhizoctonia*, a soil-inhabiting pathogen, may grow on a wide variety of crops and can survive sufficiently in the soil to make crop rotation an impractical means of control. Row spacing, plant population, and planting date can also be changed to manage soybean diseases.

Foliar fungicide applications can effectively reduce the incidence of many diseases (Bowers and Russin 1999). The economic return from a fungicide application for most diseases, however, may be limited to select production programs. According to USDA-NASS statistics, fungicides were applied on approximately 11% of the soybean acreage in 2012 (USDA-NASS 2013c).

In field studies conducted in the U.S. during 2012, MON 87751 did not differ from commercially available soybean varieties in terms of its interaction with plant diseases (Section VII.C.2.2.1). It is therefore not expected that MON 87751 will impact soybean disease management.

VIII.F. Weed Management

Annual weeds are perceived to be the greatest pest problem in soybean production, followed by perennial weeds (Aref and Pike 1998). Soybean insects and diseases are rated less problematic but may reach economic thresholds requiring treatment. Weed control in soybean is essential to optimizing yields. Weeds compete with soybean for light, nutrients, and soil moisture (Loux, et al. 2013). Weeds can reduce profits by hindering harvest operations, and produce chemicals, which are harmful to crop plants. Weeds can also harbor insects and diseases (Loux et al. 2013). The primary factors affecting soybean yield loss from weed competition are the weed species, weed density, and the duration of the competition (Hoeft, et al. 2000). When weeds are left to compete

with soybeans for the entire growing season, yield losses can exceed 75% (Dalley, et al. 2001).

Cultural and mechanical weed control practices are important components of an effective weed management program. Crop rotation, narrow row spacing, planting date, insect and disease control and adequate fertility are commonly employed to provide the crop a competitive advantage over weeds (Loux et al. 2013). Although the primary purpose of tillage is for seedbed preparation, tillage is still used to supplement weed control with selective herbicides in soybean production. Approximately 98% of soybean acreage received a herbicide application in 2012 indicating the importance of weed control in maximizing soybean yield (USDA-NASS 2013c). Currently, herbicide-tolerant varieties are planted on 93% of the soybean acreage (USDA-NASS 2012a). Glyphosate-tolerant soybean varieties provide tolerance to in-crop postemergence applications of glyphosate and currently account for almost all of the herbicide-tolerant soybean varieties. Subsequently, glyphosate is the most widely used herbicide being applied on 98 percent of the soybean acreage in 2012 (USDA-NASS 2013c). Approximately 31 to 52% of growers apply non-glyphosate herbicides in glyphosate-tolerant soybean depending on the crop rotation (Prince, et al. 2011). Preemergence residual herbicides, such as flumioxazin, metolachlor or acetochlor, are applied in glyphosate-tolerant soybean to provide early season weed control and control glyphosate-resistant weeds.

MON 87751 is not herbicide tolerant and is therefore no different from other non-herbicide tolerant soybean in terms of its weed management considerations. Commercial products containing MON 87751 combined with deregulated herbicide tolerant soybean varieties would have weed management practices consistent with those of the deregulated herbicide tolerant variety.

VIII.G. Crop Rotation Practices in Soybean

The well-established farming practice of crop rotation is an important management tool for farmers. The purposes of growing soybean in rotation are to improve yield and profitability of one or both crops over time, decrease the need for nitrogen fertilizer on the crop following soybean, increase residue cover, mitigate or break disease, insect, and weed cycles, reduce soil erosion, increase soil organic matter, improve soil tilth, and reduce runoff of nutrients, herbicides, and insecticides (Al-Kaisi, et al. 2003; Heatherly and Elmore 2004). Crop rotations also afford farmers the opportunity to diversify farm production in order to minimize market risks. Although the benefits of crop rotations can be substantial, growers make cropping decisions by evaluating both agronomic and economic returns.

Continuous soybean production is not a common practice in the Midwest and is discouraged by most extension soybean specialists to reduce the risk of diseases and nematodes (Al-Kaisi et al. 2003; Hoeft et al. 2000). Maize and soybean occupy more than 90% of the cropland in some of the Midwestern states, and the two-year cropping sequence of soybean-maize is used most extensively in this region. The yields of both maize and soybeans are approximately 10% higher when grown in rotation than when either crop is grown continuously (Hoeft et al. 2000). The insect protection trait in

MON 87751 is not expected to impact crop rotation practices because its agronomic requirements are not different from currently available soybean varieties (Section VII.C).

VIII.H. Soybean Volunteer Management

Soybean seeds can remain in a field as a result of pods splitting before or during harvest. Soybean seeds also can remain in a field when pod placement on the plants is too close to the ground for the combine head to collect all the pods or when the combine is improperly adjusted for efficient harvesting. Volunteer soybean in rotational crops is not a concern in because soybean seed rarely exhibits dormancy and any volunteer plants do not compete well with succeeding crops (OECD 2000).

Volunteer soybean normally is not a concern in rotational crops because control measures are available for volunteer soybean when they arise. Preplant tillage and/or herbicides are the first management tool for control of emerging volunteer soybean in the spring, where this may be an issue, such as in the south. If volunteer soybean should emerge after planting, shallow cultivation and/or use of another herbicide will control volunteers and effectively reduce competition with the crop. Several postemergence herbicides also are available to control volunteer soybean (conventional or herbicide-tolerant soybean) in each of the major soybean rotational crops. Given the low potential for soybean to volunteer in subsequent crops, the availability of multiple herbicidal and cultivation methods for controlling volunteers, as well as the demonstrated lack of difference in germination of MON 87751 compared to conventional soybean (see Section VII.C.1), the introduction of MON 87751 alone or in combination with other deregulated soybean products is not expected to impact the management of soybean volunteer plants.

VIII.I. Stewardship of MON 87751

Monsanto develops effective products and technologies and is committed to assuring that its products and technologies are safe and environmentally responsible. Monsanto demonstrates this commitment by implementing product stewardship processes throughout the lifecycle of a product and by participation in the Excellence Through Stewardship® (ETS) Program. ETS policies and practices include rigorous field compliance and quality management systems and verification through auditing. Monsanto's Stewardship Principles are also articulated in Technology Use Guides (Monsanto Company 2013) and Monsanto Technology Stewardship Agreements that are signed by growers who utilize Monsanto branded traits, to ensure stewardship compliance.

As an integral action of fulfilling this stewardship commitment, Monsanto will seek biotechnology regulatory approvals for MON 87751 in all key soybean importing countries with functioning regulatory systems to assure global compliance and support the flow of international trade. These actions will be consistent with the Biotechnology Industry Organization (BIO) Policy on Product Launch (BIO 2010). Monsanto continues to monitor other countries that are key importers of soybean for the development of formal biotechnology approval processes. If new functioning regulatory processes are developed, Monsanto will make appropriate and timely regulatory submissions.

Monsanto also commits to industry best practices on seed quality assurance and control to ensure the purity and integrity of MON 87751 seed. As with all of Monsanto's products, before commercializing MON 87751 products in any country, the appropriate detection methods will be made available to soybean producers, processors, and buyers.

VIII.J. Impact of the Introduction of MON 87751 on Agricultural Practices

MON 87751 has been developed to provide protection from feeding damage caused by targeted lepidopteran insect pests. Biotechnology-derived traits that protect against lepidopteran insect pests are present in many currently available biotechnology-derived crops and their management requirements are well known. Aside from the reduced need for broad spectrum insecticide application and the need for appropriate IRM practices, the introduction of MON 87751 is not expected to have an impact on current agronomic, cultivation and management practices for soybean. No changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management, and volunteer management from the introduction of MON 87751. USDA-APHIS reached similar conclusions in its assessment of the antecedent organism, MON 87701 (USDA-APHIS, 2008).

IX. PLANT PEST ASSESSMENT

This section provides a brief review and assessment of the plant pest potential of MON 87751 and its impact on agronomic practices and the environment. USDA-APHIS has responsibility, under the Plant Protection Act (PPA) (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Regulation 7 CFR 340.6 provides that an applicant may petition APHIS to evaluate submitted data to determine that a particular regulated article does not present a plant pest risk and should no longer be regulated. If APHIS determines that the regulated article does not present a plant pest risk, the petition is granted, thereby allowing unrestricted introduction of the article. APHIS may extend a determination of nonregulated status to additional regulated articles, upon finding that the additional regulated article does not pose a potential for plant pest risk, and should therefore not be regulated (7 CFR 340.6(e)).

USDA-APHIS granted Monsanto's petition for the antecedent organism, MON 87701, in 2011 upon finding that MON 87701 did not pose a plant pest risk different from that of conventional soybean. The data and information in this request for an extension demonstrate that MON 87751, likewise does not pose a plant pest risk and the conclusions reached for MON 87701 also apply to MON 87751.

According to PPA, the definition of "plant pest" includes the living stage of any of the following, or a similar article that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogens (7 U.S.C. § 7702[14]).

The regulatory endpoint under the PPA for biotechnology-derived crop products is not zero risk, but rather a determination that deregulation of the article in question is not expected to pose a plant pest risk. Information in this request related to plant pest risk characteristics includes: 1) mode-of-action and changes to plant metabolism; 2) composition; 3) expression and characteristics of the gene product; 4) potential for weediness of the regulated article; 5) impacts to NTOs; 6) disease and pest susceptibilities; 7) impacts on agronomic practices; and 8) impacts on the weediness of any other plant with which it can interbreed, as well as the potential for gene flow. Using the assessment above, the data and analysis presented in this request lead to a conclusion that MON 87751 does not pose any unique plant pest concerns not already considered by USDA-APHIS during its review of the antecedent organism, MON 87701. MON 87751 therefore should no longer be subject to regulation under 7 CFR part 340.

IX.A. Characteristics of the Genetic Insert

As described in Section III, MON 87751 was developed by *A. tumefaciens*-mediated transformation of soybean meristem tissue using plasmid vector PV-GMIR13196. Characterization of the DNA insert in MON 87751 was conducted using a combination of sequencing, PCR, and bioinformatics methods. The results of this characterization demonstrate that MON 87751 contains one copy of the intended T-DNA containing the *cry1A.105* and *cry2Ab2* expression cassettes that is stably integrated at a single locus and

is inherited according to Mendelian principles over multiple generations. These methods also confirmed that no vector backbone or other unintended plasmid sequences are present in MON 87751. Additionally, the genomic organization at the insertion site was assessed by comparing the sequences flanking the T-DNA insert in MON 87751 to the sequence of the insertion site in conventional soybean. This analysis determined that no major DNA rearrangement occurred at the insertion site in MON 87751 upon DNA integration.

IX.B. Mode-of-Action of Gene Products

Cry1A.105 and Cry2Ab2 proteins are derived from *B. thuringiensis*, a ubiquitous gram-positive soil bacterium that accumulates crystal proteins during sporulation. The generalized MOA for Cry proteins was described by English and Slatin (1992). It includes ingestion of the crystals by insects and solubilization of the crystals in the insect midgut, followed by activation through proteolytic processing of the soluble Cry protein by digestive enzymes in their midguts. The activated protein then binds to specific receptors on the surface of the midgut epithelium of target insects and inserts into the membrane, leading to pore formation and generalized disruption of the transmembrane gradients and, therefore, cell integrity. While alternate mechanisms have been proposed, a review of the available data has recently been published which concluded that the original model, pore formation, is the most valid model for Cry protein MOA (Vachon et al. 2012).

IX.C. Characterization of Gene Products

Cry1A.105 and Cry2Ab2 have established histories of safe use, having been assessed previously by USDA and U.S. EPA (Section V.E.). Neither protein originates from an organism known to be a source of allergens, a bioinformatic assessment of each protein shows no shared amino acid sequence similarities to known allergens or toxins, and each protein is rapidly digested in a simulated gastric fluid assay (Sections V.D.1 and V.D.2), indicating that neither protein is expected to cause allergenic effects or toxicity in mammals.

IX.D. Expression Levels of Gene Products

Levels of Cry1A.105 and Cry2Ab2 were measured in eight tissue types from trials conducted in the U.S. (Section V.C). Cry1A.105 protein levels in MON 87751 across all samples analyzed ranged from below the limit of detection (<LOD) to 1600 µg/g dw. Cry2Ab2 protein levels in MON 87751 across all samples analyzed ranged from 2.6 µg/g dw to 43 µg/g dw. These proteins are present at a very small percentage of the total protein in soybean seed (no more than 0.0006% and 0.001% for Cry1A.105 and Cry2Ab2, respectively).

IX.E. Compositional Characteristics

The compositional analysis based on OECD guidance (Section VI) provided a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and other components in soybean seed and forage of MON 87751 and the conventional

control. Of the 50 components statistically assessed for MON 87751 there were no statistically significant differences in 42 components. Only eight components (protein, glycine, proline, phosphorus, vitamin E, and raffinose in seed, and total fat and NDF in forage) showed a significant difference ($p<0.05$) between MON 87751 and the conventional control. For these components, the mean difference in component values between MON 87751 and the conventional control was less than the range of the conventional control values and the reference soybean variety values. The MON 87751 mean component values were within the 99% tolerance interval, the values observed in the literature, or the ILSI-CCDB values. These results support the overall conclusion that MON 87751 was not a major contributor to variation in component levels in soybean seed and forage and confirmed the compositional equivalence of MON 87751 to the conventional control in levels of these components. These data indicated that the components with significant differences were not compositionally meaningful from a food and feed safety perspective.

IX.F. Weediness Potential of MON 87751

The commercial *Glycine* species in the U.S. (*Glycine max* L.) does not exhibit weedy characteristics and is not effective in invading established ecosystems. Soybean is not listed as a weed in the major weed references (Crockett 1977; Holm, et al. 1997; Holm, et al. 1979), nor is it present on the lists of noxious weed species distributed by the federal government (7 CFR 360). Soybean does not possess any of the attributes commonly associated with weeds (Baker 1974), such as the ability to disperse, invade, and become a dominant species in new or diverse landscapes or the ability to compete well with native vegetation. Soybean seed typically germinates quickly under adequate temperature and moisture conditions, potentially resulting in volunteer plants. If volunteer soybean plants did become established, they would not compete well with the succeeding crop, and could be controlled readily by either mechanical or chemical means (OECD 2000). In addition, because wild populations of *Glycine* species are not known to exist in the U.S., the potential does not exist for MON 87751 to outcross to wild or weedy relatives and to alter their weediness potential.

Comparative plant characterization data were used to assess whether the introduction of the Cry1A.105 and Cry2Ab2 proteins altered the plant pest potential, including weediness, of MON 87751 compared to the conventional control A3555 (Section VII). Phenotypic, agronomic, and environmental interaction characteristics of MON 87751 were evaluated and compared to those of the conventional control. As described below, these assessments included: seed dormancy and germination characteristics; plant growth and development characteristics; observations for abiotic stress response, disease damage, arthropod-related damage; arthropod abundance and pollen characteristics. Results from the phenotypic, agronomic, and environmental interaction assessments demonstrated that MON 87751 possesses neither weedy characteristics, nor increased susceptibility or tolerance to diseases, insects, or abiotic stressors compared to conventional soybean. Taken together, the results of the analysis support a determination that MON 87751 is no more likely to exhibit weediness than conventional soybean.

IX.F.1. Seed Dormancy and Germination

Seed dormancy and germination characterization demonstrated that MON 87751 seed had germination characteristics that did not differ from those of the conventional control (Section VII). In particular, no difference in percentage of hard seed between the MON 87751 and the conventional control, supports a conclusion of no increased weediness or plant pest potential of MON 87751 compared to conventional soybean.

IX.F.2. Plant Growth and Development

Evaluations of plant growth and development characteristics in the field are useful for assessing potential weediness characteristics such as lodging and pod shattering (Section VII). In the combined-site analysis of phenotypic data, no statistically significant differences were detected between MON 87751 and the conventional control for any of the evaluated characteristics: early stand count, 50% flowering date, plant lodging, pod shattering, plant height, final stand count, grain moisture, 100 seeds weight and yield (Table VII-4). Plant growth stage, plant vigor, and flower color data were categorical and were not statistically analyzed. MON 87751 and the conventional control were within the same range for plant growth stage and plant vigor across all the sites. The flower color of MON 87751 and the conventional control was purple as expected. Thus, there were no differences in plant growth and development observed between MON 87751 and conventional soybean.

IX.F.3. Response to Abiotic Stressors

No biologically meaningful differences were observed during comparative field observations between MON 87751 and the conventional control for responses to abiotic stressors, such as drought, flood, frost, hail injury, heat, mineral toxicity, nutrient deficiency, soil compaction, sun scald and wind (Section VII). The lack of biologically meaningful differences in the MON 87751 responses to abiotic stress supports the conclusion that the introduction of the Cry1A.105 and Cry2Ab2 proteins is unlikely to result in increased weediness or plant pest potential compared to conventional soybean.

IX.F.4. Response to Disease Damage and Arthropod-Related Damage

The lack of differences observed between MON 87751 and the conventional control for plant responses to disease damage and arthropod-related damage in multiple environments across the U.S. supports the conclusion that the introduction of the insect-protected trait is not expected to cause a biologically meaningful change in terms of plant pest potential compared to the conventional control.

IX.F.5. Arthropod Abundance

A total of 170 statistical comparisons were made between MON 87751 and the control for arthropod abundance involving the following pest and beneficial arthropods: aphids, bean leaf beetles, corn rootworm beetles, Japanese beetles, kudzu bugs, minute brown scavenger beetles, leaf beetles, leafhoppers, tarnished plant bugs, plant bugs, spider mites, stink bugs, thrips, treehoppers, whiteflies, ant-like flower beetles, spiders, assassin bugs, big-eyed bugs, brown lacewings, green lacewings, damsel bugs, ladybird beetles, micro-Hymenoptera, minute pirate bugs, and predatory mites. Lack of sufficient

arthropod abundance precluded statistical comparisons between MON 87751 and the control for 127 additional comparisons; however, descriptive statistics were provided for these comparisons.

No statistically significant differences were detected between MON 87751 and the control for 157 out of 170 comparisons. The 13 differences detected between MON 87751 and the conventional control included spiders, big-eyed bugs, damsel bugs, predatory mites, bean leaf beetle, plant bugs, kudzu bugs, stink bugs and thrips. In cases where mean abundance values for MON 87751 were outside the reference range, the statistical differences were not consistently detected across collections or sites. Thus, the statistical differences in abundance were not indicative of a consistent response associated with the trait and are not considered biologically meaningful in terms of increased pest potential of MON 87751 compared to the conventional control.

IX.F.6. Pollen Morphology and Viability

Evaluations of pollen morphology and viability from field-grown plants provide information useful in a plant pest assessment as it relates to the potential for gene flow and introgression of the biotechnology-derived trait into other soybean varieties and wild relatives. Pollen morphology and viability evaluations demonstrated no statistically significant differences ($\alpha = 0.05$) between MON 87751 and the conventional control (Table VII-8). Based on the assessed characteristics, these results support a conclusion that neither viability nor morphology of pollen of MON 87751 was altered compared to the conventional soybean control.

IX.G. Impacts on Non-Target Organisms

Evaluation of the potential risks to NTOs is an important component of APHIS's plant pest risk assessment of a biotechnology-derived crop. Assessment of the potential risks to NTOs associated with the introduction of a biotechnology-derived crop producing an insecticidal trait is based on the characteristics of the crop and the introduced trait. Risk is a function of hazard and exposure and it is therefore critical to determine the potential hazard and exposure scenarios that are most likely and that require evaluation through experimental studies. Selection of the test organisms and test material are important decisions that are based on the characteristics of the trait and the product (Romeis, et al. 2008; Romeis, et al. 2013). In the U.S., regulatory guidelines for NTO testing and risk assessment of insect-protected crops have been developed by U.S. EPA and testing is conducted according to a tier-based system (U.S. EPA 2010b). Additionally, U.S. EPA has convened several Scientific Advisory Panel (SAP) meetings to make recommendations and provide guidance for NTO testing and risk assessment for agricultural products produced by methods of biotechnology (U.S. EPA 2001b; 2002; 2004a; 2010b). Section V.E.8 summarizes previous reviews by U.S. EPA and USDA of the potential impacts of Cry1A.105 and Cry2Ab2 on NTOs that concluded adverse effects are unlikely.

The specificity of Cry proteins is dependent upon binding to specific receptors present in the insect mid-gut (OECD 2007; Pigott and Ellar 2007). These specific receptors are not

present in taxa outside of insects, therefore Cry proteins are not expected to adversely affect wild mammals and no adverse effects have been reported in these organisms as well as in non-target birds (OECD 2007(Schnepf et al. 1998). It has been widely reported in the literature that the insecticidal activity of Cry1 class proteins is specific for lepidopteran insects (Crickmore et al. 1998; de Maagd et al. 2001; Romeis et al. 2006). Within the Cry2 class of proteins, the activity spectrum is slightly broader than within the Cry1 class (Crickmore et al. 1998). For example, Cry2Aa (formerly CryB1) is active against both lepidopteran and dipteran (mosquito) insects (de Maagd et al. 2001). However, the Cry2Ab2 (formerly CryB2) protein is only active against lepidopteran insects at field exposure concentrations (Widner and Whiteley 1989).

The activity spectrum studies developed for the Cry1A.105 and Cry2Ab2 proteins have previously been evaluated by USDA-APHIS (2011) and U.S. EPA (U.S. EPA 2010c) in regulatory submissions for MON 89034. The activity of the Cry1A.105 and Cry2Ab2 proteins was shown to be restricted to the order Lepidoptera at field exposure levels. The Cry1A.105 and Cry2Ab2 proteins in MON 87751 are functionally equivalent to the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034. Therefore, the previously provided activity spectrum evaluation for the Cry1A.105 and Cry2Ab2 proteins in MON 89034 is applicable to MON 87751.

IX.G.1 Assessment of Potential Interaction between the Cry1A.105 and Cry2Ab2 Proteins

The potential for interaction among the Cry1A.105 and Cry2Ab2 proteins was evaluated in insect bioassays with two pest species that were susceptible to the Cry1A.105 and Cry2Ab2 proteins, European corn borer (ECB, *Ostrinia nubilalis*) (Lepidoptera: Crambidae) and corn earworm (CEW, *Helicoverpa zea*) (Lepidoptera: Noctuidae) (U.S. EPA 2010c). The study provided evidence that the proteins do not interact in either an antagonistic or synergistic manner, and that there will not be any unexpected interactions with regard to target and non-target insects (U.S. EPA 2010c). Demonstrating no interaction between these proteins (*i.e.*, the proteins act additively) using sensitive species allows for each of the proteins to be tested independently in safety assessment studies. The principle of independent assessment has been used for many years for microbial risk assessments (U.S. EPA 2004b). Demonstrating the lack of synergism permits the application of the principle of independent assessment which has a long history of use in toxicology. This principle provides that if each substance in a mixture acts independently and the substances are below their no observed adverse effect level, their toxicity can be assessed independently (U.S. EPA 2009).

IX.G.2. Non-Target Organism Risk Assessment for MON 87751

The NTO risk assessment for MON 87751, including an assessment of threatened and endangered species, took into consideration several components, including familiarity with the MOA of Cry proteins; the known specificity of Cry1A.105 and Cry2Ab2 to lepidopterans; levels of the two proteins in MON 87751; the lack of synergistic or antagonistic interaction between the two proteins; feeding tests with each of the two

proteins and proximity and habitat requirements for individual species to representative NTOs.

The Cry1A.105 and Cry2Ab2 proteins produced by MON 87751 share greater than 99% and 97% amino acid sequence, respectively with the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034. Additionally, the protease-resistant core domains of the modified Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751 share 100% amino acid identity to their counterparts expressed in MON 89034. Protease-resistant core domains are responsible for insecticidal activity and specificity (OECD 2007). Furthermore, diet incorporation assays conducted with *H. zea* confirmed that the Cry1A.105 and Cry2Ab2 proteins produced by MON 87751 are functionally equivalent to the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034 (Section V). Therefore, NTO data produced for MON 89034 are applicable to MON 87751.

The potential for adverse effects of the Cry1A.105 and Cry2Ab2 proteins on NTOs was evaluated with a standard battery of test organisms. Two soil decomposers [earthworm (*Eisenia fetida*) and Collembola (*Folsomia candida*)], and four beneficial insect species [honeybee (*Apis mellifera*), parasitic wasp (*Ichneumon promissorius*), ladybird beetle (*Coleomegilla maculata*), and minute pirate bugs (*Orius insidiosus*)] were previously reviewed for the deregulation and registration of MON 89034 (U.S. EPA 2010c). Bioassay procedures used in these studies varied according to insect and insects were exposed to dietary concentrations that greatly exceeded environmentally relevant concentrations. The no observed effect concentrations (NOECs) used in the NTO risk assessment for MON 87751 are summarized in Table IX-1.

The results from the MON 87751 expression studies (Section V.C) were used to determine the Expected Environmental Concentration (EEC) for the Cry1A.105 or Cry2Ab2 proteins produced in MON 87751. EECs reflect expression in tissue types that the NTOs would most likely be exposed to in the environment. For several NTOs (honeybee, ladybird beetle, parasitic wasp, and minute pirate bug), pollen represents the primary or most relevant route of exposure. As a result of difficulties with pollen collection from soybean flowers, only mean expression values could be determined. The most ecologically relevant route of exposure for soil-dwelling organisms, such as earthworms and Collembola, was considered to be from decomposing root tissue in the soil environment. Consequently, for these species, EECs were based on the level of the Cry1A.105 or Cry2Ab2 proteins in MON 87751 roots. The Collembola study was conducted using lyophilized leaf tissue from MON 89034 maize in a 50% mix with yeast. Though roots are the most appropriate tissue for consideration in the risk assessment, the use of the higher expressing leaf tissue provided a worst-case scenario for exposure. Therefore, the NOECs reflect 50% of the maximum leaf expression in MON 89034. Mean expression values from MON 87751 root were used for the EEC rather than maximum because Collembola would likely feed on roots from multiple plants and their exposures would therefore be closer to the mean values reported in Section V.C. For other EECs the maximum expression values were used to reflect a worst-case exposure scenario for the NTO assessment. For Cry1A.105, levels in root tissue were below the limit of detection (LOD) so the LOD was used EECs for MON 87751 roots (Table IX-1).

To assess potential risk to NTOs, the results of the effects testing studies have been evaluated against the EECs. In Tier 1 ecological effects tests, LC_{50} values ≥ 10× EEC are sufficient to conclude negligible risk and a lack of adverse effects (U.S. EPA 2010c). It is recognized, however, that ≥ 10× the EEC presents a highly conservative approach and studies performed with test concentrations that are less than 10× the EEC can be used to evaluate whether adverse effects might be detected at realistic field concentrations [1× EEC] (U.S. EPA 2010c). An EEC that is lower than the NOEC is generally indicative of negligible risk (U.S. EPA 2010c). U.S. EPA guidance states that only adverse effects to NTOs at 1× the field exposure are viewed as an environmental risk (U.S. EPA 2010c).

MOEs were calculated based on the ratio of the NOECs for the specific non-target organism to the relevant EEC values. For all NTOs examined, the EECs for MON 87751 were significantly below the NOECs for species tested in the NTO battery. U.S. EPA's published Level of Concern (LOC) is 50% mortality at 5× EEC (U.S. EPA 2010c). For MON 87751, calculated MOEs were all >5× of the EECs (Table IX-1), demonstrating that the Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751 are not likely to produce adverse effects on terrestrial beneficial invertebrate species at field exposure levels. This conclusion is in agreement with a prior assessment of the Cry1A.105 or Cry2Ab2 proteins expressed in MON 89034 which determined that the Cry1A.105 or Cry2Ab2 proteins were not likely to have detrimental effects on non-lepidopteran insects at relevant field exposure levels (U.S. EPA 2010c).

The 2002 U.S. EPA SAP report (U.S. EPA 2002) recommended that non-target testing should be focused on species exposed to the crop being evaluated (*i.e.*, for MON 87751 beneficial insects found in soybean fields). Though aquatic habitats may be located near agricultural areas, U.S. EPA concluded that exposure of aquatic organisms to biotech crops is limited temporally and spatially and that the potential exposure of aquatic organisms is therefore low to negligible (U.S. EPA 2010b). Aquatic testing, therefore, is not warranted for MON 87751.

Table IX-1. Expected Environmental Concentrations (EECs), No Observed Effect Concentrations (NOECs) from Non-Target Organism Studies and Margins of Exposure (MOEs) for the Cry1A.105 or Cry2Ab2 Proteins

Test Organism	Order	Cry1A.105 EEC[1]	NOEC	MOE[2]	Cry2Ab2 EEC[1]	NOEC	MOE[2]
Folsomia candida	Collembola	<0.322 µg/g fw root[3]	≥80 µg/g[4]	≥248	7.5 µg/g dw root[3]	≥70 µg/g[4]	≥9.3
Eisenia fetida	Haplotaxida	< 0.322 µg/g fw root[4]	≥178 mg/kg dry soil	≥552	22 µg/g dw root	≥330 mg/kg dry soil	≥15
Apis mellifera larvae	Hymenoptera	0.022 µg[5]	≥12 µg/larva[6]	≥500	0.0154 µg[5]	≥0.60 µg /larva[6]	≥39
Apis mellifera adult	Hymenoptera	11 µg/g fw pollen	≥550 µg/ml	≥50	7.7 µg/g fw pollen	≥68 µg/ml	≥8.8
Ichneumon promissorius	Hymenoptera	11 µg/g fw pollen	≥240 µg/ml	≥21	7.7 µg/g fw pollen	≥100 µg/ml	≥12
Orius insidiosus	Hemiptera	11 µg/g fw pollen	120 µg/g	10.9	7.7 µg/g fw pollen	≥100 µg/g	≥12
Coleomegilla maculata	Coleoptera	11 µg/g fw pollen	≥240 µg/g	≥21	7.7 µg/g fw pollen	≥120 µg/g	≥15

[1] Maximum expression levels obtained from MON 87751.

[2] Margins of Exposure (MOE) were calculated based on the ratio of the NOEC to MEEC. The MOE was determined based on the expression level of the Cry1A.105 and Cry2Ab2 proteins in the tissue of MON 87751 deemed most relevant to non target insect exposure.

[3] The EEC was calculated as 50% of the soybean root mean expression for Cry2Ab2. For Cry1A.105, the LOD was used because this protein was not detectable in roots. These studies are considered chronic feeding studies and as such the assumption of 50% of the Collembola diet to be exclusively soybean root is conservative. Soybean roots (Cry1A.105 expression < 0.322 µg/g fw and Cry2Ab2 mean expression of 15 µg/g dw) are the ecologically relevant growth stage for dietary exposures of Collembola.

[4] This study was conducted using lyophilized leaf tissue from MON 89034 maize in a 50% mix with yeast, and no adverse effects were observed. Therefore, the NOEC is calculated as 50% the maximum Cry1A.105 and Cry2Ab2 expression in the MON 89034 leaf used in this study. The maximum protein expression values were 160 µg Cry1A.105/g dw and 140 µg Cry2Ab2/g dw, which results in NOEC of 80 µg Cry1A.105/g and 70 µg Cry2Ab2/g . For Cry1A.105, only fresh weight root expression values were available.

[5] MEEC based upon maximum amount of Cry protein expressed in 2 mg of MON 87751 pollen (fw). The average consumption of pollen by honey bee larvae is 2 mg during development (Babendreier, et al. 2004). The MEEC was calculated for Cry1A.105 as follows: (2 mg pollen × 11 µg Cry1A.105/1000 mg pollen) = 0.022 µg Cry1A.105). The MEEC was calculated for Cry2Ab2 as follows: (2 mg pollen × (7.7 µg Cry2Ab2/1000 mg pollen) = 0.0154 µg Cry2Ab2).

[6] No Observed Effect Concentration represents the concentration of the test solution used for dosing individual larval cells. Ten microliters of 1200 Cry1A.105 µg/ml solution was added to each larval cell for a total mass of 12 µg Cry1A.105/cell. Ten microliters of 100 µg/ml solution was added to each larval cell for a total mass of 0.60 µg Cry2Ab2/cell.

IX.G.3. Potential Impact on Threatened and Endangered Species

A review of the literature indicates that Cry proteins have a limited host range (*i.e.*, high degree of specificity) and will not pose a significant hazard to non-target insects (Federici 2002; Romeis et al. 2006). This conclusion has been confirmed through effects testing with a standard battery of NTOs, including mammals, birds, earthworms and beneficial insects for Cry protein containing crops (Mendelsohn et al. 2003). Based on the low hazard of Cry proteins to non-insect animals, no adverse effects are expected for endangered mammals, birds, non-insect aquatic animals, and non-insect soil organisms. This conclusion has been acknowledged in earlier regulatory decisions for MON 89034, a commercial crop containing the Cry1A.105 and Cry2Ab2 proteins (U.S. EPA 2010c).

As discussed in section IX.H, the Cry1A.105 and Cry2Ab2 proteins expressed in MON 87751 are highly specific in insecticidal activity against lepidopteran insects and there is no evidence that they will affect non-lepidopteran terrestrial beneficial invertebrate species at field exposure levels. These activity spectrum studies indicate that the only potential effects to endangered species resides with endangered butterflies and moths in the order Lepidoptera. Soybean pollen is not expected to drift beyond the planted soybean field and its immediate margins, therefore any exposure to lepidopterans would be expected to occur within those areas (U.S. EPA 2010a).

Soybean is highly self-pollinated and its pollen is essentially contained in the flower (Caviness 1966; U.S. EPA 2010a). Exposure of threatened and endangered lepidoptera to MON 87751 pollen could occur via direct consumption of soybean plants or pollen that deposits on non-soybean plants within the soybean field and its immediate margins. Exposure to significant amounts of Cry1A.105 and Cry2Ab2 proteins via pollen consumption is not likely because little pollen is expected to be released from the self-pollinated flowers. In soybean fields, airborne pollen densities have been measured at a mean level of 0.18 grains/cm^2/day, with a maximum exposure of 0.368 grains/cm^2/day (Yoshimura, et al. 2006). Additionally, U.S. EPA recently assessed the potential risk to several threatened and endangered lepidoptera from MON 87701, a commercial soybean expressing the Cry1Ac protein (U.S. EPA 2010a). The assessment focused on three federally listed lepidoptera species, the Karner blue butterfly (*Lycaeides melissa samuelis*), St. Francis' Satyr Butterfly *(Neonympha mitchellii fransisci*), and Mitchells' Satyr Butterfly (*Neonympha mitchellii mitchellii*) known to be present in U.S. counties in which soybean are grown (U.S. EPA 2010a). Analysis of the feeding ecology, habitat preferences, ovipositing sites and data on dispersal and movement of each species resulted in the conclusion of negligible exposure and therefore no direct effects from cultivation of the Cry1Ac-expressing soybean on these federally listed lepidoptera (U.S. EPA 2010a). Furthermore, U.S. EPA concluded that there are no known federally listed insectivores that are obligate feeders on lepidoptera found in soybean fields, and therefore no indirect effects on federally listed species would occur as a result of lost food resources in a field cultivated with soybean expressing a lepidopteran-active trait (U.S. EPA 2010a).

Based upon soybean flower morphology preventing wide pollen dispersal, and the lack of significant habitat overlap with soybean fields, federally listed threatened and endangered

lepidopteran species will have an exceedingly low likelihood of exposure to the Cry1A.105 and Cry2Ab2 proteins produced in pollen from MON 87751. Therefore, it is reasonable to conclude no effect to threatened and endangered species will occur from the cultivation of MON 87751. This conclusion is further supported by ecological assessments conducted for maize products containing Cry1A.105 and Cry2Ab2 proteins that indicated no effects on endangered species (U.S. EPA 2010c).

IX.H. Environmental Fate of MON 87751 Products Expressing Cry1A.105 and Cry2Ab2

Soil organisms may be exposed to the Cry1A.105 and Cry2Ab2 proteins from MON 87751 by contact with roots, or with above-ground plant biomass deposited on the soil or tilled into the soil. Soil organisms may also be exposed to the Cry1A.105 and Cry2Ab2 proteins from other biotechnology-derived products (*e.g.*, MON 89034). In addition, feeding on living or dead crop biomass or ingesting or absorbing the Cry proteins after their release into the soil may represent a route of exposure to soil-dwelling organisms.

Several soil factors (*e.g.*, pH and clay content) can influence the degradation rate of Cry proteins. Published studies suggest that Cry proteins may bind to the clay components of soil and become more resistant to degradation by soil microorganisms (Fiorito, et al. 2008; Stotzky 2004). Laboratory and field studies, however, show that only a very small fraction of the Cry protein derived from post-harvest residues persists long enough to be stabilized by soil colloids or clay minerals (Hopkins and Gregorich 2005). In addition, soil pH near or above neutrality substantially increases the degradation rate of Cry proteins (Tapp and Stotzky 1998). Under most production conditions, crops expressing the Cry1A.105 and Cry2Ab2 proteins are grown on soils that are near neutral pH, *i.e.*, under conditions favorable to degradation of Cry proteins. A soil pH of 6.3 - 6.5 is recommended for soybean cultivation to maximize nutrient availability and nitrogen fixation while minimizing soybean cyst nematode (SCN) population growth (Staton 2012). Some experts suggest increasing soil pH to the 6.3 - 7.0 range with lime and advise that soil pH below 5.2 can be very detrimental to soybean production (Peters, et al. 2005).

U.S. EPA issued a Biopesticide Registration Action Document (BRAD) for the Cry1A.105 and Cry2Ab2 proteins expressed in MON 89034. A soil degradation study using clay, silt loam, and loamy sand soils was found to be acceptable under OPPTS guideline 885.5200, and results indicated that the Cry1A.105 and Cry2Ab2 proteins do not persist in soil beyond approximately three weeks (U.S. EPA 2010c).

Many laboratory soil degradation studies have been conducted with Cry proteins from a variety of biotechnology-derived crops (*e.g.*, Cry1Ab, Cry1Ac, Cry1A.105, Cry2Ab2, Cry3Bb1, Cry1F, Cry34/35), the weight of evidence indicating that Cry proteins do not persist in soil (Herman, et al. 2002; Icoz and Stotzky 2008a; b; Sims and Holden 1996; Sims and Ream 1997). Furthermore, a number of field monitoring studies have been conducted to assess the dissipation of Cry proteins following several years of sustained maize or cotton production. These studies have shown no persistence or accumulation of

Cry proteins in fields where maize expressing Cry1Ab protein (Dubelman, et al. 2005) or Cry3Bb1 protein (Ahmad, et al. 2005) and cotton expressing Cry1Ac protein (Head, et al. 2002) were grown continuously for several years.

Commercial experience with the cultivation of biotechnology-derived insect protected crops for more than 15 years, as well as the results of the laboratory and field studies cited here strongly suggest that the Cry1A.105 and Cry2Ab2 proteins produced in MON 87751 will not persist or accumulate under soybean production conditions, indicating negligible exposure to NTOs.

IX.I. Potential for Pollen Mediated Gene Flow and Introgression

Gene introgression is a process whereby one or more genes successfully integrate into the genome of a recipient plant population. Introgression is affected by many factors, including the frequency of the initial pollination event, environmental factors, sexual compatibility of pollen donor and recipient plants, pollination biology, flowering phenology, hybrid stability and fertility, selection, and the ability to backcross repeatedly. Because gene introgression is a natural biological process, it does not constitute an environmental risk in and of itself. Gene introgression must be considered in the context of the transgene(s) inserted into the biotechnology-derived plant, and the likelihood that the presence of the transgene(s) and their subsequent transfer to recipient plants will result in increased plant pest potential (Sutherland and Poppy 2005).

The assessment for gene introgression from MON 87751 with other cultivated or wild relatives of soybean, discussed in detail below, indicates that MON 87751 is no more likely to become a weed than conventional soybean and MON 87751 is expected to be similar to conventional soybean regarding its potential for and impacts from gene flow. Soybean lacks sexually-compatible relatives in the U.S.; therefore, the only pollen-mediated gene flow would be within cultivated soybean.

IX.I.1. Hybridization with Cultivated Soybean

Although soybean is largely a self-pollinated species, low levels of natural cross-pollination can occur (Caviness 1966; OECD 2000; Ray, et al. 2003; Yoshimura et al. 2006). In studies with cultivated soybean, where conditions have been optimized to ensure close proximity and flowering synchrony, natural cross-pollination generally has been found to be very low. Most outcrossing occurred with surrounding plants, and cross-pollination frequencies varied depending on growing season and genotype. Insect activity does increase the outcrossing rate, but soybean generally is not a preferred plant for pollinators (Abrams, et al. 1978; Erickson 1975; Jaycox 1970a; c; b).

Numerous studies on soybean cross-pollination have been conducted, and the published results, with and without supplemental pollinators, are summarized in Table IX-2. Under natural conditions, cross-pollination among adjacent plants in a row or among plants in adjacent rows ranged from 0 to 6.3%. In experiments where supplemental pollinators (usually bees) were added to the experimental area, cross-pollination ranged from 0.5 to 7.74% in adjacent plants or adjacent rows. However, cross-pollination does not occur at

these levels over long distances. Cross-pollination rates decrease to less than 1.5% beyond one meter from the pollen source, and rapidly decrease with greater distances from the source. The following cross-pollination rates at extended distances have been reported: 0.05% at 5.4 meters (Ray et al. 2003), 0% at 6.5 meters (Abud, et al. 2003), 0% at 10.5 meters (Yoshimura et al. 2006), and 0.004% at 13.7 meters of separation (Caviness 1966).

The potential for cross-pollination in soybean is limited. This is recognized in certified seed regulations for foundation seed in the U.S., which permit any distance between different soybean cultivars in the field as long as the distance is adequate to prevent mechanical mixing (USDA-APHIS 2006). Similarly the likelihood of introgression of the T-DNA from MON 87751 into other soybean varieties is expected to be negligible.

Table IX-2. Summary of Published Literature on Soybean Cross Pollination

Distance from Pollen Source (meters)	Cross-Pollination (%)	Comments	Reference
0.3	0.04 (estimated per pod)	Interspaced plants within a row. Experiment conducted in a single year. Single male and female parental varieties. Percent outcrossing calculated per pod rather than per seed.	(Woodworth 1922)
0.8	0.07 to 0.18	Adjacent rows. Experiment conducted over two years. Several male and female parental varieties.	(Garber and Odland 1926)
0.1	0.38 to 2.43	Adjacent plants within a row. Experiment conducted in a single year. Several male and female parental varieties.	(Cutler 1934)
0.1	0.2 to 1.2	Adjacent plants within a row. Experiment conducted in single year at two locations. Several male and female parental varieties.	(Weber and Hanson 1961)
0.9 2.7–4.6 6.4–8.2 10–15.5	0.03 to 0.44 0.007 to 0.06 0 to 0.02 0 to 0.01	Frequency by distance was investigated. Experiment conducted over three years. Single male and female parental varieties.	(Caviness 1966)
0.8 m	0.3 to 3.62	Various arrangements within and among adjacent rows. Experiment conducted over three years. Several male and female parental varieties.	(Beard and Knowles 1971)
One row (undefined)	1.15 to 7.74	Bee pollination of single-row, small-plots of pollen receptor surrounded by large fields (several acres) of pollen donor soybean. Soybean is not a preferred flower for alfalfa leaf-cutting bees.	(Abrams et al. 1978)
0.1 – 1.8 m	0.11 to 1.42 depending on planting design	Bee pollination of soybean grown in various spatial arrangements. Experiment conducted over four years. Several soybean cultivars.	(Chiang and Kiang 1987)
1.0	0.09 to 1.63	Adjacent rows. Experiment conducted over two years. Several male and female parental varieties.	(Ahrent and Caviness 1994)
0.5 1.0 6.5	0.44 to 0.45 0.04 to 0.14 none detected	Frequency by distance was investigated. Experiment conducted in a single year. Single male and female parental varieties.	(Abud et al. 2003)
0.9 5.4	0.29 to 0.41 0.03 to 0.05	Frequency by distance was investigated. Experiment conducted in a single year. Single male and female parental varieties.	(Ray et al. 2003)
0.15	0.65 to 6.32 (avg. 1.8)	Interspaced plants within a row. Experiment conducted in a single year. Single male and female parental varieties.	(Ray et al. 2003)

Table IX-2. Summary of Published Literature on Soybean Cross Pollination (continued)

Distance from Pollen Source (meters)	Cross-Pollination (%)	Comments	Reference
0.7 1.4 2.1 2.8 3.5 7.0 10.5	0 to 0.19 0 to 0.04 0 to 0.05 0 to 0.08 0 to 0.04 0 to 0.04 0	Interspaced plants within a row arranged in small plots. Experiment conducted in a four year period. Single male and two female parental varieties.	(Yoshimura et al. 2006)
0.5, 1, 2, 4, and 8 m	Max rate of 0.83%	Two cultivars (glyphosate tolerant and conventional) planted beside each other.	(Pereirá, et al. 2012)
5 29	0.03 0.001	Glyphosate tolerant pollen donor cultivar; mean outcrossing of 0.01% to semi-rampant landrace; mean outcrossing of 0.45% to selected cultivar.	(Liu, et al. 2012)
1	0.035	Transgenic pollen donor surrounded by non-transgenic cultivar; conducted over 2 years.	(Zhang, et al. 2011)

IX.I.2. Hybridization with Wild Annual Species of Subgenus *Soja*

The subgenus *Soja* includes the cultivated soybean *Glycine max* and the wild annual species *Glycine soja*. *G. soja* is the only known true species with which *G. max* can interbreed. *Glycine soja* is found in China, Taiwan, Japan, Korea, and Russia (Hymowitz 2004; Lu 2004). Hybridization between female *G. soja* and male *G. max* was less successful than hybridization in the opposite direction (Dorokhov, et al. 2004), where frequency of spontaneous cross pollination in reciprocal combinations of *G. max* and *G. soja* varied from 0.73 (♀ *G. soja* × ♂ *G. max*) to 12.8% (♀ *G. max* × ♂ *G. soja*). Species relationships in the subgenus soja indicated that F_1 hybrids of *G. max* and *G. soja* carry similar genomes and are fertile (Singh and Hymowitz 1989). Abe et al. (1999) note that "natural hybrids between *G. max* and *G. soja* are rare and hybrid swarms involving both species have never been reported." This is also supported by work from Kuroda et al. (2008) in which molecular markers were used and no gene flow from *G. max* to *G. soja* was detected. Many barriers to natural hybridization exist between soybean and wild relatives, including the highly selfing nature of both plants, required proximity of wild soybean to cultivated soybean, synchrony of flowering, and presence of pollinators. As such, it is highly unlikely that naturally occurring, pollen-mediated gene flow and transgene introgression into wild soybean relatives from incidentally released biotechnology-derived soybean will occur at any meaningful frequency.

The subgenus *Soja* also contains an unofficial species, *G. gracilis* (Hymowitz 2004). *G. gracilis* is known only from Northeast China, and is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*. *G. gracilis* may be a hybrid between *G. soja* and *G. max* (Hymowitz 1970; Lu 2004). Interspecific fertile hybrids formed by intentional crosses between *G. max* and *G. soja* and between *G. max* and *G. gracilis* have been obtained (Dorokhov et al. 2004; Singh and Hymowitz 1989). Although hybridization between *G. max* and members of the subgenus *Soja* can take place, *G. soja* is not found in North America, and it is highly unlikely that gene transfer will occur.

IX.I.3. Hybridization with the Wild Perennial Species of Subgenus *Glycine*

Wild perennial species of the *Glycine* subgenus occur in Australia; West, Central and South Pacific Islands; China; Papua New Guinea; Philippines; and Taiwan (Hymowitz, et al. 1992; Hymowitz and Singh 1992). Therefore, the only opportunities for this interspecific hybridization would occur in areas where those species are endemic. Nonetheless, the likelihood of interspecific hybridization between *G. max* and wild perennial *Glycine* species is extremely low because they are genomically dissimilar (Hymowitz 1970; Lu 2004) and pod abortion is common. From time to time, immature seeds of the crosses have been germinated aseptically *in vitro*, but the resulting F_1 hybrids are slow-growing, morphologically weak, and completely sterile. Their sterility is caused by poor chromosome pairing. Furthermore, species distantly related usually produce nonviable F_1 seeds that either have premature death of the germinating seedlings or suffer from seedling and vegetative lethality (Kollipara, et al. 1993). In North and South America, it is not possible for gene transfer to occur between cultivated soybean and wild

perennial species of *Glycine* subgenera because these wild species do not exist in these regions.

IX.J. Transfer of Genetic Information to Species with which Soybean Cannot Interbreed (Horizontal Gene Flow)

Monsanto is unaware of any reports regarding the unaided transfer of genetic material from soybean species to other sexually-incompatible plant species. The likelihood for horizontal gene flow to occur is exceedingly small. Therefore, potential ecological risk associated with horizontal gene flow from MON 87751 is not expected. The consequence of horizontal gene flow of the *cry1A.105* and *cry2Ab2* genes from MON 87751 into other plants that are sexually-incompatible is negligible because, as data presented in this request confirm, the gene and trait confer no increased plant pest potential to soybean. Thus, in the highly unlikely event that horizontal gene transfer was to occur, the presence of *cry1A.105* and *cry2Ab2* would not be expected to increase pest potential in the recipient species.

IX.K. Potential Impact on Soybean Agronomic Practices

MON 87751 has been developed to provide two MOA against the targeted lepidopteran pests. As insect protection is a widely used technology in many crops, the introduction of MON 87751 is not expected to have major impacts on current agronomic, cultivation and management practices for soybean aside from a potential reduction in the need to apply broad spectrum insecticides. No changes are anticipated in crop rotations, tillage practices, planting practices, fertility management, weed and disease management, and volunteer management from the introduction of MON 87751.

MON 87751 has been shown to be comparable to conventional soybean in its compositional, phenotypic, and agronomic characteristics (Sections VI and VII). Biotechnology-derived lepidopteran-protected crops have been available in the U.S. since 1995 and have reduced broad spectrum insecticide applications (U.S. EPA 2011). MON 87751 provides two MOA and is expected to prolong the durability of existing lepidopteran-protected soybean varieties. MON 87751 is expected to provide benefits to growers similar to those obtained by use of other lepidopteran-protected crop varieties, including reduced use of broad spectrum insecticides, increased yield protection and increased worker safety.

IX.L. Summary of Plant Pest Assessments

Plant pests, as defined in the PPA, are the living stage of any of the following, or a similar article, that can directly or indirectly injure, damage, or cause disease in any plant or plant product: (A) a protozoan; (B) a nonhuman animal; (C) a parasitic plant; (D) a bacterium; (E) a fungus; (F) a virus or viroid; or (G) an infectious agent or other pathogens (7 U.S.C. § 7702[14]). Characterization data presented in Sections III through VII of this request confirm that MON 87751, with the exception of insect-protected trait, is not fundamentally different from conventional soybean, in terms of plant pest potential.

Monsanto is not aware of any study results or observations associated with MON 87751 that would suggest an increased plant pest risk would result from its introduction.

The plant pest assessment was based on multiple lines of evidence developed from a detailed characterization of MON 87751 compared to conventional soybean, followed by a risk assessment on detected differences. The plant pest risk assessment in this request was based on the following lines of evidence: 1) insertion of a single functional copy of the *cry1A.105* and *cry2Ab2* expression cassettes; 2) characterization and safety of the expressed product; 3) compositional equivalence of MON 87751 seed and forage compared to a conventional control; 4) phenotypic, agronomic, and environmental characteristics demonstrating no increased plant pest potential compared to conventional soybean; 5) negligible risk to NTOs including organisms beneficial to agriculture; 6) familiarity with soybean as a cultivated crop and 7) no greater likelihood to impact agronomic practices, including land use, cultivation practices, or the management of weeds, diseases and insects, than conventional soybean.

Based on the data and information presented in this extension request, it is concluded that, like the antecedent organism, MON 87701, MON 87751 is not expected to be a plant pest. Results also support a conclusion of no increased weediness potential of MON 87751 compared to conventional soybean. Therefore, Monsanto Company requests an extension of determination of nonregulated status from APHIS that MON 87751 and any progeny derived from crosses between MON 87751 and other commercial soybean should no longer be subject to regulation under 7 CFR part 340.

X. ADVERSE CONSEQUENCES OF INTRODUCTION

Monsanto knows of no study results or observations associated with MON 87751 indicating that there would be adverse consequences from its introduction. MON 87751 produces the Cry1A.105 and Cry2Ab2 proteins. The Cry1A.105 and Cry2Ab2 proteins produced in MON 87751 are functionally equivalent to the same proteins present in MON 89034 that was previously granted a determination of nonregulated status by USDA-APHIS. As demonstrated by field results and laboratory tests, the only phenotypic difference between MON 87751 and conventional soybean is protection from feeding by larval lepidopteran pests.

The data and information presented in this request demonstrate that MON 87751 is unlikely to pose an increased plant pest risk compared to conventional soybean. This conclusion is reached based on multiple lines of evidence developed from a detailed characterization of the product compared to conventional soybean, followed by risk assessment on detected differences. The characterization evaluations included molecular analyses, which confirmed the insertion of one copy of the intended DNA containing the *cry1A.105* and *cry2Ab2* expression cassettes that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations.

Analysis of key nutrients, anti-nutrients, and secondary metabolites of MON 87751 demonstrate that MON 87751 is compositionally equivalent to conventional soybean. The phenotypic evaluations, including an assessment of seed germination and dormancy characteristics, plant growth and development characteristics, pollen characteristics, ecological interaction characteristics, symbiont interactions and environmental interactions also indicated MON 87751 is unchanged compared to conventional soybean. There is no indication that MON 87751 would have an adverse impact on beneficial or non-target organisms, including threatened or endangered species. Therefore, based on the lack of increased pest potential compared to conventional soybean, the risks for humans, animals, and other NTOs from MON 87751 are negligible.

The introduction of MON 87751 will not adversely impact cultivation practices or the management of weeds, diseases, and insects in soybean production systems. Farmers familiar with lepidopteran-protected products currently available will be advised to continue to employ the same crop rotational practices currently in place for these products.

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APPENDICES

Appendix A: USDA Notifications and Permits

Field trials of MON 87751 have been conducted in the U.S. since 2010. The protocols for these trials include field performance, breeding and observation, agronomics, and generation of field materials and data necessary for this request. In addition to the MON 87751 phenotypic assessment data, observational data on pest and disease stressors were collected from these product development trials. The majority of the final reports have been submitted to the USDA. However, some final reports, mainly from the 2011 - 2013 seasons, are still in preparation. A list of trials conducted under USDA notifications or permits and the status of the final reports for these trials are provided in Table A-1.

Table A-1. USDA Notifications and Permits Approved for MON 87751 and Status of Trials Planted under These Notifications

USDA No.	Effective date	Release State (Site)	Trial Status
2010 Field Trials			
09-349-101n	07-Jan-2010	HI (1), PR (1)	Submitted to USDA
09-351-101rm	10-Mar-2010	IA (10), IL (15), IN (3), KS (6), MO (2), MS (1), NE (1)	Submitted to USDA
10-069-101n	09-Apr-2010	AL (1), IL (2), LA (1), MS (2), PR (1)	Submitted to USDA
10-091-101rm	21-May-2010	PR (2)	Submitted to USDA
10-257-101rm	09-Nov-2010	HI (2), PR (2)	Submitted to USDA
2011 Field Trials			
10-351-105rm	01-Feb-2011	HI (2), PR (3)	Submitted to USDA
10-354-101rm	15-Mar-2011	IA (9), IL (14), IN (3), KS (7), MO (2), NE (1)	Submitted to USDA
11-088-108n	28-Apr-2011	AL (1), GA (1), LA (2), MS (6)	Submitted to USDA
11-045-104rm	11-Jun-2011	HI (2), PR (3)	Submitted to USDA
11-154-104rm	30-Sep-2011	HI (2), PR (3)	Submitted to USDA
2012 Field Trials			
11-336-103n	01-Jan-2012	PR (2)	Submitted to USDA
11-277-102rm	01-Feb-2012	HI (2), PR (3)	In Progress
11-326-102rm	16-Mar-2012	IA (12), IL (15), IN (2), KS (7), MO (2), MS (4)	In Progress
12-053-111n	23-Mar-2012	AR (1), GA (1), LA (1), MO (1), NC (1), SC (1)	In Progress
12-060-103n	29-Mar-2012	IA (1), IL (2), MS (1), NE (1)	In Progress
12-061-107n	31-Mar-2012	IA (1), IL (4), IN (3), KS (2), OH (1)	In Progress
12-061-106n	31-Mar-2012	IA (3), IL (4), NE (4)	In Progress
12-074-117n	12-Apr-2012	AL (1), AR (1), GA (1), IL (2), LA (2), MS (2), PR (1), TN (1)	In Progress
12-087-107n	26-Apr-2012	PR (2)	In Progress

Table A-1. USDA Notifications and Permits Approved for MON 87751 and Status of Trials Conducted under These Notifications (continued)

USDA No.	Effective date	Release State (Site)	Trial Status
2012 Field Trials (continued)			
12-089-108n	28-Apr-2012	AR (2), IA (2), IL (2), NE (1), PA (2)	In Progress
12-032-106rm	01-Jun-2012	HI (2), PR (3)	In Progress
12-241-101n	27-Sep-2012	AR (1), GA (1), IN (1), KS (2), LA (1), NC (1), NE (2), PA (1)	In Progress
12-166-101rm	01-Oct-2012	HI (2), PR (3)	In Progress
12-262-102n	18-Oct-2012	IL (1)	In Progress
12-292-101n	17-Nov-2012	PR (2)	In Progress
12-303-102n	28-Nov-2012	PR (1)	In Progress
2013 Field Trials			
12-275-104rm	01-Feb-2013	HI (2), PR (2)	In Progress
13-064-103n	30-Mar-2013	AR (1), IL (2), LA (2), MS (2), NC (2), SC (1), TN (1)	In Progress
13-065-105n	04-Apr-2013	IA (3)	In Progress
13-065-101n	05-Apr-2013	AR (1), IA (2), IL (8), IN (3), KS (3), MO (3), NC (2), NE (4), OH (1), PA (1), WI (1)	In Progress
12-334-101rm	11-Apr-2013	IA (13), IL (19), KS (8), TN (1)	In Progress
13-074-116n	17-Apr-2013	HI (1), IA (2), PR (3)	In Progress
13-036-105rm	01-Jun-2013	HI (2), PR (2)	In Progress
13-158-103n	06-Jul-2013	PR (2)	In Progress
13-144-104rm	01-Oct-2013	HI (2), PR (2)	In Progress
13-254-110n	17-Oct-2013	HI (1), IA (2), PR (5)	In Progress

Appendix B: Overview, Materials, Methods, and Supplementary Results for Molecular Analyses of MON 87751

B.1. NGS/JSA Overview

Safety assessments of biotechnology-derived crops include a detailed molecular characterization of the inserted DNA sequence and its location within the genome (Codex Alimentarius 2009). Typically, molecular characterization has relied on Southern blot analysis to establish locus and copy number along with targeted sequencing of polymerase chain reaction products spanning any inserted DNA to complete the characterization process. With the advent of next-generation sequencing (Shendure and Ji 2008; Zhang et al. 2011), improvements in sequencing technologies have enabled alternative methods for molecular characterizations which do not require Southern blot analysis. Next-Generation Sequencing and Junction Sequence Analysis bioinformatics (NGS/JSA) utilizes sequencing (both next-generation technologies and traditional methods) and bioinformatics to produce characterizations equivalent to those achieved by current Southern blot based methods.

There are multiple advantages to using next-generation sequencing and bioinformatics, most notably the robustness, simplicity and consistency of the method compared with Southern blot studies, which require customized experimental design for every transformation event. The new sequencing-based method overcomes many technical challenges inherent in Southern blot analyses (*e.g.*, false positive hybridization bands resulting from incomplete digestion or star activity (Wei et al. 2008)) and the need for radioactive ^{32}P-labeled probes. This new method provides higher reproducibility, because it is less dependent on complex lab based procedures. The method described here is essentially identical for all transformation events and it robustly establishes molecular characteristics of genetically engineered crops (Kovalic et al. 2012). Additionally, similar techniques are being used to characterize transgene integration sites and insert molecular anatomy in mammalian systems (DuBose et al. 2013; Zhang et al. 2012).

Method Synopsis

Molecular characterization of the inserted DNA and associated native flanking sequences consists of a multistep approach to determine:

1. the number of insertion sites;
2. the presence/absence plasmid backbone;
3. insert copy number at each insertion site;
4. DNA sequence of each inserted DNA;
5. sequence of the native locus at each insertion site.

Additionally, current methods also establish a description of any genetic rearrangements that may have occurred at the insertion site as a consequence of transformation. Generational stability analysis, which demonstrates the stable heritability of inserted DNA sequences over a number of breeding generations, is also routinely conducted.

The first step of the molecular characterization, determination of number of insert sites, is conducted using a combination of next-generation sequencing technologies (NGS) and Junction Sequence Analysis (JSA) bioinformatics (DuBose et al. 2013; Kovalic et al. 2012). A schematic representation of the basis of the characterization, including the NGS/JSA methodology and the directed sequencing, is presented in Figure B-1 (Kovalic et al. 2012).

Genomic DNA from the transformation event and the conventional control are used to generate short (~100 bp) randomly distributed sequence fragments (sequencing reads) in sufficient numbers to ensure comprehensive coverage of the genomes (Shendure and Ji 2008) (Figure B-1 box 1). Sufficient numbers of sequence fragments are obtained (≥75× effective genome coverage) to comprehensively cover the genomes of the sequenced samples (Ajay et al. 2011; Clarke and Carbon 1976; Wang et al. 2008). Previous studies with a variety of transformation events demonstrate that 75× coverage of the genome is adequate to provide comprehensive coverage and ensure detection of inserted DNA, producing results equivalent to Southern blot analysis (Kovalic et al. 2012). The 75× coverage used in this method is predicted, based on established and accepted methods (Clarke and Carbon 1976; Lander and Waterman 1988), to provide genome coverage that would be expected to not miss a single basepair in complex genomes (Kovalic et al. 2012). Furthermore, even with known biases in next-generation sequencing techniques, including the Illumina sequencing by synthesis method employed here (Minoche et al. 2011), it has previously been established experimentally that given deep next-generation sequencing, it is possible to achieve comprehensive coverage of complex genomes that form the foundation for accurate whole genome studies (Ajay et al. 2011; Wang et al. 2008).

To confirm sufficient sequence coverage in both the transformation event and the control, the 100 bp sequence reads are analyzed to determine the coverage of a known single-copy endogenous gene, this analysis demonstrates coverage at ≥75× median depth in each sample. Furthermore, in order to confirm the method's ability to detect any sequences derived from the transformation plasmid, plasmid DNA is spiked into conventional control DNA at a single copy genome equivalent ratio and 1/10 copy genome equivalent ratio. This analysis demonstrates that any portion of the plasmid may be detected at a single copy per genome level and 1/10 copy genome equivalent level, which is adequate sensitivity to observe any inserted fragment.

Also of note is that although the method presented here provides 75× or greater coverage of the genomes under study, accurate assembly of complete genome sequences for the transformation event and conventional control is not technically possible using currently available sequence assembly tools. This is due to the nature of the sequences generated in this study, short reads of a single short insert length (Miller et al. 2010), in addition to limitation on available sequence assembly algorithms (Zhang et al. 2011). The sequences generated with this method represent datasets sufficient for achieving precise molecular characterization of transformed DNA in transformation events where reference to a template sequence (plasmid DNA) is utilized for comparison (Kovalic et al. 2012).

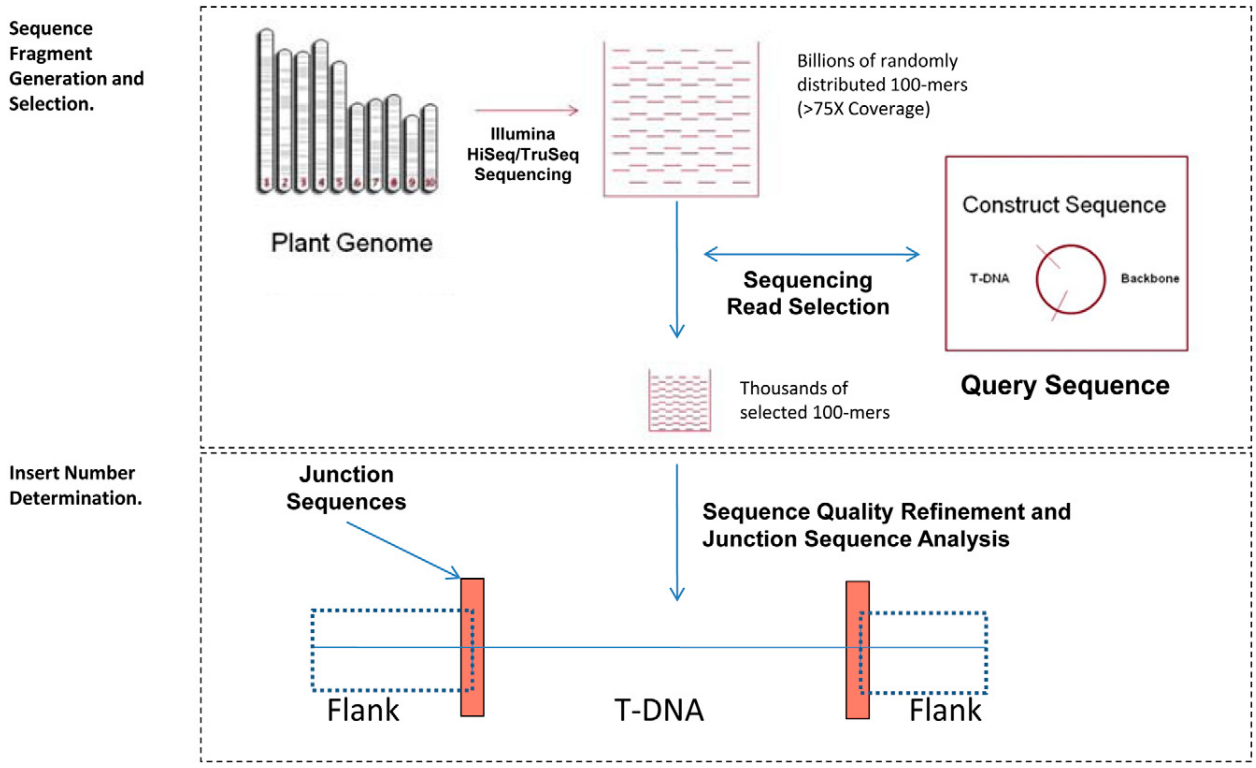

Figure B-1. Sequencing and Sequence Selection
Genomic DNA from the test and control material were sequenced using Illumina HiSeq/TruSeq technology (Illumina, Inc.) that produces large numbers of short sequence reads approximately 100 bp in length. Sufficient numbers of these sequence fragments were obtained to comprehensively cover the genomes of each sample at ≥75× median coverage. Using these genome sequence reads, bioinformatics search tools were used to select all sequence reads that are significantly similar (as defined in the text) to the transformation plasmid. Only the selected sequence reads were used in further bioinformatics analysis to determine the insert number by detecting and characterizing all junction sequences and the presence or absence of the plasmid backbone sequences by lack of detectable sequences, including the use of suitable controls for experimental comprehensiveness and sensitivity.

Using bioinformatics tools, the sequence reads that are derived from the plasmid vector are selected for further analysis out of the comprehensive genomic sequence dataset produced from the transformation event. To determine the insert number, the known sequence of the transformation vector plasmid is used as a query sequence in the bioinformatics analysis to search for and select the sequences that contain any portion of sequence of the plasmid. The DNA sequencing reads with a match to the query sequence having an e-value of 1×10^{-5} or less and having a match length of at least 30 bases with at least 96.7% sequence identity are collected. The results of a parameter optimization study that systematically evaluated many different potential parameter sets established these selection criteria as providing the best possible combination of sensitivity and specificity.

The number of DNA inserts is determined by analyzing the selected sequences for novel junctions. The junctions of the DNA insert and flanking DNA are unique for each

insertion and an example is shown in Figure B-2 below (Kovalic et al. 2012). Therefore, insertion sites can be recognized by analyzing for sequence reads containing such junctions. Each insertion will produce two unique junction sequence classes characteristic of the genomic locus, with one at the 5' end of the insert, in this case named Junction Sequence Class A (JSC-A), and similarly one at the 3' end of the insert, JSC-B (as illustrated in Figure 3 from Kovalic et al., 2012). By evaluating the number and the sequences of all unique junction classes detected, the number of insertion sites of the plasmid sequence can be determined. For a single insert, two junction sequence classes are expected, one each originating from either end of the insert, both containing portions of T-DNA and flanking sequence.

Figure B-2. Junctions and Junction Sequences
Depicted above are five example junction sequences formatted and labeled to indicate the plasmid/flanking DNA portions of the sequences and with the junction point indicated (plasmid DNA is shown in bold, underlined text and flank DNA is shown in plain text). Junctions are detected by examining the NGS data for sequences having portions of plasmid sequences that span less than the full read. Detected junctions are typically characteristic of plasmid insertions in the genome. A group of junction sequences which share the same junction point and common flanking sequence (as shown above) is called a Junction Sequence Class (or JSC).

The next step in the molecular characterization is determination of the insert copy number, integrity of the insert, lack of backbone or other unintended plasmid sequences, and flanking sequence of the native locus at the insertion site. This analysis is conducted using directed sequencing, locus-specific PCR and DNA sequencing analyses, which complements the NGS/JSA analyses, and is common to both the Southern-based and the NGS/JSA characterization methods. Directed sequencing (locus-specific PCR and DNA sequencing analyses) of the transformation event determines the complete sequence of the insert and flanks. This determines if the sequence of the insert is identical to the corresponding sequence in plasmid vector, if each genetic element in the insert is intact, if the plasmid vector sequence is inserted as a single copy, and establishes no vector backbone or other unintended plasmid sequences were inserted in the event. This comparison allows a determination of whether the T-DNA elements are present in the intended order. Furthermore, the genomic organization at the insertion site is assessed by comparing the insert and flanking sequence to the sequence of the insertion site in conventional control genome.

Finally, the stability of the T-DNA across multiple generations is evaluated by NGS/JSA analyses. Genomic DNA from multiple generations of the transformation event is assayed for the number and sequences of all unique junction classes, as described above. This information is used to determine the number and identity of insertion sites. For a single insert, two junction sequence classes are expected, both containing portions of T-DNA and flanking sequence (Figure B-2), with one each originating from either end of the insert (Figure B-3). In the case of an event where a single locus is stably inherited over multiple generations, two identical junction sequence classes are expected in all the generations tested.

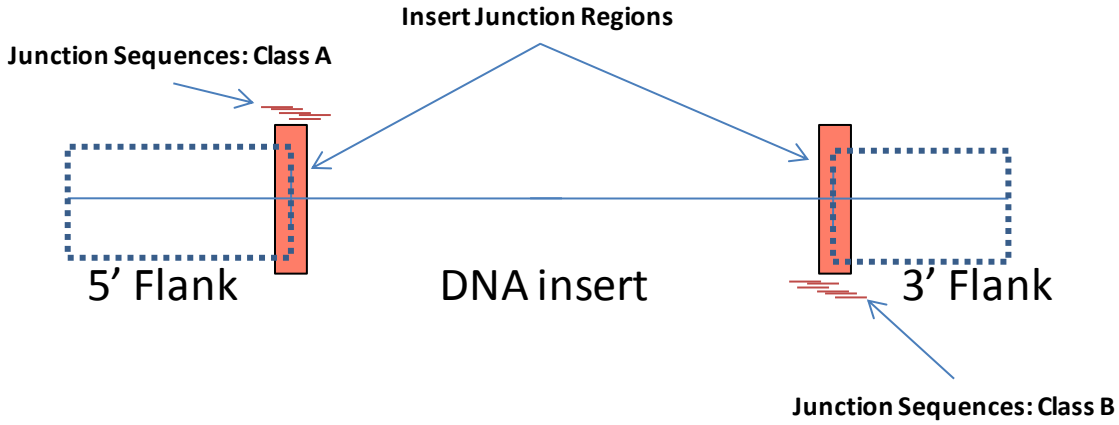

Figure B-3. Two Unique Junction Sequence Classes are Produced by the Insertion of a Single Plasmid Region
A schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5' and 3' flanks (depicted as areas bounded by dotted lines), and the two distinct regions spanning the junctions between inserted DNA and flanking DNA (shaded boxes). The group of ~100-mer sequences in which each read contains sequences from both the DNA insert and the adjacent flanking DNA at a given junction is called a Junction Sequence Class. In this example, two distinct junction sequence classes (in this case: Class A at the 5’ end and Class B at the 3’ end) are represented.

B.2. Materials and Methods

B.2.1 Test Substance

The test substance in this study was MON 87751. Genomic DNA for use in this study was extracted from tissue listed in the table below.

Generation	Seed ORION[6] ID	Leaf Production Plan
R3	11338716	PPN-2012-0287
R4	11338714	n/a
R5	11338715	n/a
R6	11338717	n/a
R7	11332614	n/a

B.2.2 Control Substance

The control substance is the conventional soybean variety A3555, which has similar genetic background as the test substances. Genomic DNA for use in this study was extracted from tissue listed in the table below.

Control Substance	Seed ORION ID	Leaf Production Plan
A3555	11332613	PPN-2012-0287

B.2.3 Reference Substance

The reference substance was plasmid vector PV-GMIR13196, which was used to develop MON 87751. Whole plasmid served as a positive control for sequencing and bioinformatic analyses. The identity of the reference plasmid was confirmed by restriction enzyme digestion prior to the study. Documentation of the confirmation of the plasmid vector identity was archived with the raw data. Appropriate molecular size markers from commercial sources were used for size estimations on agarose gels. The unique identity of the molecular weight markers was documented in the raw data.

[6] ORION is a proprietary database used at Monsanto Company to track Regulatory plant samples

B.2.4 Characterization of Test, Control, and Reference Substances

The seed for the test and control substances used in this study were obtained from Monsanto Trait Development. The synthesis records for these materials are located in the MIDAS[7] system. The identities of the test substance and the conventional control substance were confirmed by the sequencing in the study. No certificates of analysis (COA) or verification of identity (VOI) certificates were generated for these materials. The Study Director reviewed the chain of custody documentation to confirm the identity of the test and control substances prior to the use of these materials in the study.

Test, control and reference DNA substance were considered stable during storage if they yielded interpretable signals in sequencing experiments and/or did not appear visibly degraded on the stained gels.

B.2.5 Genomic DNA Isolation

For sequencing library construction, genomic DNA was isolated from seed tissues of the test and control substances. First the seeds were decontaminated by vigorously agitating them by hand for 30 seconds with 0.05% (v/v) Tween-20 sufficient to cover the seeds. Then the Tween-20 was discarded and the seeds were rinsed with tap water to remove residual Tween-20. Next the tube containing the seeds was filled with 0.5% (w/v) NaOCl sufficient to cover the seeds, vigorously agitated by hand, and then allowed to stand for one minute at room temperature. The solution was poured off and seeds were again rinsed with tap water. The tube containing the seeds was filled with a volume of 1% (v/v) HCl sufficient to cover the seeds, vigorously agitated by hand, and allowed to stand for one minute at room temperature. The solution was poured off and seeds were again rinsed with tap water. The 1% (v/v) HCl rinse was repeated one time, and then the seeds were rinsed with distilled water and spread in a single layer on a clean paper towel. The seeds were placed in a drying oven at 75°C to dry. The dried seeds were ground to a fine powder in a Harbil paint shaker. Genomic DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) extraction protocol. Briefly, CTAB buffer (1.5% (w/v) CTAB, 75 mM Tris HCl (pH 8.0), 100 mM EDTA (pH 8.0), 1.05 M NaCl, 0.75% (w/v) PVP, and 2% (w/v) 2-mercaptoethanol) were added to ground seed tissue. The samples were incubated at ~65°C for 60 minutes with intermittent mixing. The samples were cooled to room temperature and subjected to three rounds of phenol:chloroform:isoamyl alcohol (25:24:1, PCI) extraction followed by one round of extraction with chloroform. Genomic DNA was precipitated with 100% (v/v) ethanol, washed with 70% (v/v) ethanol, and dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) with 40 µg RNAse A added. The DNA was then precipitated by the addition of equal volumes of precipitation buffer (20% (w/v) PEG (MW 5,000), 2.5 M NaCl) washed with 70% (v/v) ethanol, and re-dissolved in TE buffer.

[7] MIDAS is a proprietary database used at Monsanto Company to track plant synthesis records

For insert and insertion site sequencing, genomic DNA was isolated from MON 87751 and the conventional control leaf tissues tissue using a CTAB extraction protocol. Briefly, CTAB buffer and RNase A were added to ground leaf tissue and incubated at ~60-70°C for ~40-50 minutes with intermittent mixing. The samples were cooled to room temperature and subjected to three rounds of chloroform extraction. Genomic DNA was precipitated with 100% (v/v) ethanol, washed with 70% (v/v) ethanol, and dissolved in TE buffer.

All extracted DNA was stored in 4°C refrigerators or -20°C freezers.

B.2.6 DNA Quantification

PV-GMIR13196 DNA and extracted genomic DNA were quantified using a Qubit™ Fluorometer (Invitrogen, Carlsbad, CA). For directed sequencing, genomic DNA was quantified using a Nanodrop™ Spectrophotometer (Thermo Scientific, Wilmington, DE) according to manufacturer's instructions.

B.2.7 Agarose Gel Electrophoresis

After quantification, approximately 0.5-1 µg of the extracted DNA for NGS/JSA sequencing library construction was run on a 1% (w/v) agarose gel to check the quality.

B.2.8 Shearing of DNA

Approximately 1 µg of DNA from the test, control and reference substances were sheared using a Covaris S-220 ultrasonicator. The DNA was diluted to ~ 20 ng/µl in Buffer EB (Qiagen Inc., Valencia, CA) and fragmented using the following settings to create approximately 325 bp fragments with 3' or 5' overhangs: duty cycle of 10; peak incident power of 175; intensity of 5.0, 200 bursts per cycle, in the frequency sweeping mode at ~ 6°C for 80 seconds for test and control DNA or 60 seconds for reference DNA.

B.2.9 Bioanalyzer Analysis

One microliter of sheared genomic DNA was diluted 1:10 in Buffer EB and run on a DNA High Sensitivity chip on an Agilent 2100 Bioanalyzer to check the quality of the shearing. After preparing the chip according to the manufacturer's instructions, 1 µl of each diluted DNA sample or water was added to individual wells and the chip was run on the Bioanalyzer using the dsDNA, High Sensitivity Assay reagents.

B.2.10 Paired End Library Preparation

Paired end genomic DNA libraries were prepared for the test, control, and reference substances using the Illumina TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's instructions for the low-throughput procedure with the following exception: a Sage Science Pippin Prep DNA Size Selection system (Sage Science Inc., Beverly, MA) was used to size select the DNA fragments instead of agarose gel electrophoresis.

First, the 3' and 5' overhangs of the DNA fragments generated by the shearing process were converted into blunt ends by adding 10 µl of Illumina Resuspension Buffer and 40 µl of Illumina End Repair mix to each sample and mixing thoroughly by pipette.

Then the libraries were incubated for 30 minutes at 30°C. The end-repaired samples were purified using AMPure XP beads (Beckman Coulter, Inc., Brea, CA) and resuspended in 17.5 µl of Illumina Resuspension Buffer.

Fifteen microliters of each library was transferred to a new tube for adenylation, which adds a single adenosine nucleotide to the 3' ends of the blunt fragments. Then 2.5 µl of Illumina Resuspension Buffer and 12.5 µl of Illumina A-Tailing Mix were added to each library and mixed thoroughly by pipetting. The libraries were incubated for 30 minutes at 37°C. After incubation, 2.5 µl each of individual adapter index, Illumina Resuspension Buffer, and Illumina DNA Ligase Mix was immediately added to each tube, and mixed thoroughly by pipetting to begin ligation of each library. The libraries were incubated for 10 minutes at 30°C. Then 5 µl of Illumina Stop Ligase Buffer was added to each tube and mixed thoroughly by pipetting to stop the ligation reaction. Next, another AMPure XP bead cleanup was performed on the libraries which were then resuspended in 32.5 µl of Illumina Resuspension Buffer prior to size selection.

The libraries were run on the Sage Science Pippin Prep Size Selection system using 2% gel cassettes according to the manufacturer's instructions. Ten microliters of loading solution were added to 30 µl of each of the purified libraries and mixed thoroughly by pipetting. Forty microliters of Marker B was loaded in the cassette well designated for the reference sample, and 40 µl of each DNA library was loaded in the remaining wells for analysis. After elution of the desired size range (~445 bp) of DNA fragments, the DNA sample in the elution chamber of the cassette was removed from the cassette by pipette and transferred into PCR strip tubes.

After removal from the Pippin Prep, the libraries were again put through the AMPure XP bead cleanup procedure and resuspended in 22.5 µl of Illumina Resuspension Buffer. Twenty microliters of the resuspended library was added to five microliters of Illumina PCR Master Mix and 25 µl of Illumina PCR Primer Cocktail and mixed thoroughly by pipetting. The DNA fragments were enriched through PCR using the following cycling conditions: 1 cycle at 98°C for 30 seconds; 10 cycles at 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 30 seconds; 1 cycle at 72°C for 5 minutes. Following PCR amplification, a final AMPure XP bead cleanup was performed on the libraries which were resuspended in 32.5 µl of Illumina Resuspension Buffer. Finally, 1 µl of each DNA library was diluted 1:10 in Buffer EB for running in a DNA High Sensitivity chip on an Agilent 2100 Bioanalyzer as described above. All purified library DNA was stored in a -20°C freezer.

B.2.11 Next-Generation Sequencing

The library samples described above were sequenced by The Genome Analysis Center (TGAC, Monsanto) using Illumina HiSeq technology that produces short sequence reads (~100 bp long). Sufficient numbers of these sequence fragments were obtained (≥75x

genome coverage) to comprehensively cover the entire genomes of the test event, the conventional control and the spiked-in control (Kovalic et al. 2012). Sequencing runs performed by the TGAC passed standard QC criteria. No sequence data in this study failed to meet these QC acceptance criteria.

B.2.12 Junction Sequence Analysis Bioinformatics

High-throughput sequence reads were enriched by mapping to the PV-GMIR13196 transformation plasmid sequence using the local alignment software BlastAll (V2.2.21) in order to collect all reads that were sourced from the plasmid as well as reads with sequences representing integration point. All collected reads were further refined by removing sequencing artefacts of sequencing adapters, redundant reads and low quality read ends. All quality refined reads were then used to identify junction points with custom developed bioinformatics tools as detailed below. All significant junctions are reported for both the test and the control samples. All software versions were documented in the archived data package and the software versions which were used in this study have been archived.

Sequencing Read Enrichment

The transformation plasmid PV-GMIR13196 sequence was used as reference to find all reads that were either fully matched to the insert plasmid fragments or partially matched as junction sequences. The sequence used was obtained from the MEGA8 system. A junction sequence is characterized by a combination of transformation plasmid sequence and flanking sequence that is likely to be host genome flanking sequence or any other co-inserted sequence. Local alignment with BlastAll (V2.2.21) was performed to collect all sequencing reads with an *E*-score of less than 1e-5 and at least 30 bases match of greater than 96.7% identity to the transformation plasmid (Kovalic et al. 2012). Both reads of the paired-end sequences were collected in all cases.

Read Quality Refinement

In order to identify all duplicate read pairs, a high quality segment (bases 3-42) of all collected pairs was compared to all others with short sequence alignment software (Bowtie v.0.12.3) allowing up to 1 mismatch. If multiple read pairs were matched at both paired reads, such read pairs were deemed redundant and only the best quality pair of reads was kept for further analysis.

Computer software Novoalign (v.2.06.09) was used to remove any adapter sequences at either end of the sequencing reads. Low quality read ends (with phred scores of 12 or lower) were trimmed. Only reads of 30 bases or longer after adapter and quality trimming were collected. A custom developed Perl script

[8] MEGA is a proprietary database used at Monsanto Company to track sequences and annotations.

"farm_gen_sm_bucket.pl" was used to perform read enrichment and read quality refinement as described above.

Junction Detection

Enriched and quality refined reads of both test and control samples were aligned against the whole PV-GMIR13196 transformation plasmid sequence in order to detect junction sequences using custom developed Perl script "farm_blast_map.pl". Reads with partial match to the transformation plasmid of at least 30 bases match and 96.7% identity were collected as potential junction sequences and their match cutoff position on the plasmid were noted (Kovalic et al. 2012). The collected reads were also aligned against the genomic sequence collection of the host genome in order to remove junction reads sourced from the plant endogenous homologues. Custom developed Perl script "junctions_by_bn.pl" was used to identify the junction position on the transformation plasmid and their supporting junction reads. For each junction position, all supporting junction reads were aligned at the 30 plasmid bases proximal to the junction position. The remaining bases of these reads were sorted to show the alignment and the consensus of the flanking junction sequences past the junction point.

Effective Sequencing Depth Determination

A single copy locus (Glycine max lectin (Le1), GenBank accession version: K00821.1) was selected from the Glycine max genome and used to determine the effective sequence depth coverage. All reads with at least 30 bases match and 96.7% identity were considered as reads sourced from this locus. A custom developed Perl script "farm_match_reads.pl" was used to perform such alignment and calculate the actual depth distribution at this locus. The analysis showed that *Le1* was covered by 100-mers at 75× or greater for each sample, as listed in Table B-1.

Table B-1. Sequencing (NGS) Conducted for MON 87751 and Control Genomic DNA

Sample	Total Nucleotides (Gb)	Effective Median Depth of Coverage (x-fold)
A3555	167.1	126×
R3	104.4	75×
R4	111.3	88×
R5	116.3	83×
R6	141.7	105×
R7	122.2	89×

For each sample the raw data produced are presented in terms of total nucleotide number. Effective depth of coverage is determined by mapping and alignment of all raw data to a well known single copy locus within the soybean genome (*Le1*). The median effective depths of coverage are shown for all samples.

Positive Spike-in Controls and Experimental Limit of Detection

To produce "spike-in" positive control samples for sequencing, plasmid DNA libraries were created as described above and then diluted to 1 and 1/10 soybean genome equivalents (representation of the plasmid DNA at concentrations equivalent to single copy or 1/10 copy per genome) before pooling with samples produced from the control materials (as described above). At 1 genome equivalent, 100% nucleotide identity was observed over 100% of PV-GMIR13196 (Table B-2). This result demonstrates that all nucleotides of the transformation plasmid are observed by the sequencing and bioinformatic assessments performed. Also, observed coverage was adequate (Clarke and Carbon 1976) at a level of at least 1/10th genomic equivalent (100% coverage at 100% identity for the 1/10th genome equivalent spiked control sample) and, hence, a detection level of at most 1/10th genome equivalent was achieved for the plasmid DNA sequence assessment.

Table B-2. Summary of NGS Data for the Conventional Control DNA Sample Spiked with PV-GMIR13196

	$1/10^{th}$ copy Spike	1 copy Spike
Extent of coverage[1] of PV-GMIR13196	100%	100%
Percent identity of coverage[2] of PV-GMIR13196	100%	100%

[1] Extent of coverage is calculated as the percent of all PV-GMIR13196 bases observed in the sequencing of the spike-in samples:

$$\text{Extent of coverage} = \frac{\text{number of spike in bases detected}}{\text{total length (bp) of spike in plasmid}} \times 100$$

[2] Percent identity of coverage is calculated as the percent of all PV-GMIR13196 bases observed in the sequencing of the spike-in samples:

$$\text{Percent identity of coverage} = \frac{\text{number of identical bases (spike in vs. plasmid sequence) detected}}{\text{total length (bp) of spike in plasmid detected}} \times 100$$

B.2.13 PCR and DNA Sequence Analyses to Examine the Insert and Flanking Sequences in MON 87751

Overlapping PCR products, denoted as Product A, Product B, Product C, and Product D were generated that span the insert and adjacent 5′ and 3′ flanking DNA sequences in MON 87751. For each fragment generation experimental conditions were chosen to successfully produce on-target amplifications. These products were analyzed to determine the nucleotide sequence of the insert in MON 87751, as well as that of the DNA flanking the 5′ and 3′ ends of the insert.

The PCR analyses for Product A was conducted using 200 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer and 1x concentration of PrimeSTAR® MAX DNA Polymerase Premix (Takara Bio Inc., Shiga, Japan).

The PCR analyses for Product B, Product C and Product D were each conducted using 200 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.1 µM of each primer and 0.02 units/rxn of Phusion High Fidelity PCR Master mix (Thermo Scientific, Waltham, MA).

The amplification of Product A was performed under the following cycling conditions: 38 cycles at 98°C for 10 seconds; 55°C for 5 seconds; 72°C for 16 seconds.

The amplification of Product B, Product C, and Product D were performed under the following cycling conditions: 1 cycle at 98°C for 30 seconds; 30 cycles at 98°C for 12 seconds, 60°C for 30 seconds; 72°C for 2 minutes; 1 cycle at 72°C for 8 minutes.

Aliquots of each PCR product were separated on a 0.8% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the products were the expected size. Prior to sequencing, each verified PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions and quantified using a Nanodrop spectrophotometer according to the manufacturer's instructions. The purified PCR products were sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by the TGAC using BigDye terminator chemistry (Applied Biosystems, Foster City, CA).

A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the overlapping PCR products. This consensus sequence was aligned to the PV-GMIR13196 sequence to determine the integrity and organization of the integrated DNA and the 5′ and 3′ insert-to-flank DNA junctions in MON 87751.

B.2.14 PCR and DNA Sequence Analyses to Examine the Integrity of the DNA Insertion Site in MON 87751

To examine the MON 87751 DNA insertion site in conventional control, PCR and sequence analyses were performed on genomic DNA from both MON 87751 and the conventional control A3555.

The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 87751. A forward primer specific to the DNA sequence flanking the 5′ end of the insert was paired with a reverse primer specific to the DNA sequence flanking the 3′ end of the insert.

The PCR reactions were conducted using 200 ng of genomic DNA template in a 50 µl reaction volume. The reaction contained a final concentration of 0.2 µM of each primer and 1x concentration of PrimeSTAR® MAX DNA Polymerase Premix (Takara). The amplification was performed under the following cycling conditions: 38 cycles at 98°C for 10 seconds; 55°C for 5 seconds; 72°C for 16 seconds.

A small aliquot of each PCR product was separated on a 0.8% (w/v) agarose gel and visualized by ethidium bromide staining to verify that the PCR products were the expected size prior to sequencing. Only the verified PCR product from the conventional control A3555 was purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions and quantified using the Nanodrop spectrophotometer according to the manufacturer's instructions. The purified PCR product was sequenced using primers used for PCR amplification. All sequencing was performed by TGAC using BigDye terminator chemistry (Applied Biosystems).

A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the verified PCR product. This consensus sequence was aligned to the 5′ and 3′ sequences flanking the MON 87751 insert to determine the integrity and organization of the insertion site.

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Appendix C: Materials, Methods and Results for Characterization of Cry1A.105 Protein and Cry2Ab2 Protein Produced in MON 87751

C.1. Characterization of Cry1A.105 Protein in MON 87751

C.1.1. Materials for Cry1A.105 Characterization

The Cry1A.105 protein was purified from defatted enzyme active seed flour (lot# 11333897) of MON 87751. The MON 87751-produced Cry1A.105 protein was stored in a -80 ºC freezer in a buffer solution containing 25 mM CAPS, pH 10.3, 0.5 mM EDTA, 1 mM dithiothreitol (DTT).

The *E. coli*-produced Cry1A.105 protein (lot 11349124) was used as the reference substance. The Cry1A.105 protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON149852 expression plasmid. The coding sequence for *cry1A.105* contained on the expression plasmid (pMON149852) was confirmed prior to and after fermentation. The *E. coli*-produced Cry1A.105 protein was characterized previously.

C.1.2. Cry1A.105 Protein Purification

The Cry1A.105 protein was purified from defatted enzyme active seed flour (lot# 11333897) of MON 87751. The purification procedure was not performed under a GLP protocol, however, all procedures were documented on worksheets and, where applicable, Standard Operation Procedures were followed. The Cry1A.105 was purified from an extract of defatted enzyme active seed flour using a combination of techniques, including ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography. The purification procedure is briefly described below.

The Cry1A.105 protein was purified from a total of four ~400 g aliquots of defatted enzyme active seed flour of MON 87751 seed in four separate runs that were pooled to generate the final MON 87751-produced Cry1A.105 protein sample. Each batch was process as follows. Seed flour was stirred in PBS pH 7.0, 5 mM DTT, 1 mM benzamidine HCl, 5 mM $MgCl_2$, 16.7 U/ml Benzonase and 1% PVPP at 7.5 ml/g flour for 1-2h. The Cry1A.105-containing washed flour was collected by centrifugation. Cry1A.105 protein was extracted from the washed flour with CAPS Extraction buffer (100 mM CAPS, pH 10.8, 10 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF, and 1 mM EDTA,) added at 7.5 ml/g of starting flour. The suspension was stirred for 1-2 h, and solubilized proteins, including Cry1A.105, were separated from insoluble material by centrifugation. An ammonium sulfate precipitate was prepared by the addition of ammonium sulfate salt to the CAPS extraction supernatant to a final saturation of 40%. After mixing, the precipitated proteins were collected by centrifugation, and were re-solubilized in Resuspension Buffer (50 mM Bis-Tris propane, pH 9.0, 150 mM NaCl, 1 mM EDTA, 2 mM benzamidine HCl, 1 mM PMSF, 5 mM DTT) at 3.75 ml per starting ml of CAPS supernatant. Insoluble material was removed by centrifugation, and the supernatant was diluted with Resuspension Buffer as well as QSFF Buffer A (50 mM Bis-Tris propane,

pH 9.0, 1 mM EDTA, 2 mM benzamidine HCl, 2 mM DTT) to bring the NaCl concentration to ~100 mM.

The diluted Cry1A.105-containing protein solution was loaded using a FPLC system (GE Healthcare, Piscataway, NJ) Q-Sepharose Fast Flow (GE Healthcare) anion exchange column equilibrated with QSFF Buffer A with 150 mM NaCl. After loading, the column was washed with the following buffers, QSFF Buffer A with 150 mM NaCl, QSFF Buffer A with 250 mM NaCl, and QSFF Buffer A with 300 mM NaCl. Proteins were then eluted with QSFF Buffer A with 600 mM NaCl.

For immunoaffinity chromatography, resin was prepared by chemically cross-linking a monoclonal anti-Cry1A.105 antibody to protein A-agarose (Sigma, St. Louis, MO). The Cry1A.105-containing fraction from the anion exchange column was loaded on to the immunoaffinity column in Binding buffer (50 mM Bis-Tris propane, pH 9.0, 600 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, and 1 mM PMSF). Following the loading, the column was washed with Binding buffer, Low salt wash buffer (50 mM Bis-Tris propane, pH 9.0, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl and 1 mM PMSF), and High salt wash buffer (50 mM Bis-Tris propane, pH 9.0, 800 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl and1 mM PMSF). Cry1A.105 was eluted with Elution Buffer (50 mM Bis-Tris propane, pH 9.0, 800 mM NaCl, 30% (v/v) ethylene glycol, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl and 1 mM PMSF).

Following the immunoaffinity chromatography runs, Cry1A.105 protein was concentrated using Amicon UF centrifilters with 10 kDa molecular weight cutoff (Millipore, Billerica, MA), and buffer exchanged into 25 mM CAPS, pH 10.3, 0.5 mM EDTA and 1 mM DTT. The final buffer composition of the sample was 25 mM CAPS, pH 10.3, 0.5 mM EDTA and 1 mM DTT. The purified MON 87751-produced Cry1A.105 protein was aliquoted, assigned lot #11355082 and stored in a -80°C freezer.

C.1.3. N-Terminal Sequencing

C.1.3.1. Methods

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 87751-produced Cry1A.105 protein. MON 87751-produced Cry1A.105 was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Blue R-250 and then destained with 1x Coomassie Brilliant Blue R-250 Destaining Solution. The band at 133 kDa containing MON 87751-produced Cry1A.105 was excised from the blot and used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al. 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and a Perkin Elmer Series 200 UV/VIS Absorbance Detector and Procise™ Control Software (version 2.1) were used. Chromatographic data were collected using SequencePro (Applied Biosystems, Foster City, CA; version 2.1) software. PTH-AA Standard Solution (Applied Biosystems) was used to chromatographically calibrate the instrument for the analysis. A

control protein (10 picomole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the MON 87751-produced Cry1A.105 protein.

C.1.3.2. Results of the N-terminal Sequence Analysis

Fifteen cycles of N-terminal sequencing was performed on the MON 87751-produced Cry1A.105 protein. The sequence obtained corresponded to the predicted N-terminal sequence for Cry1A.105 containing four amino acids derived from the CTP (Figure C-1). Cysteine is shown in the predicted sequence at position one based on the coding sequence of the Cry1A.105 construct in MON 87751, but it was not observed in the experimental analysis. However, cysteine is unstable during the acid hydrolysis reaction used for N-terminal sequencing, and is usually not explicitly observed (Speicher, et. al., 2009) The clear identification of amino acids in subsequent cycles of the sequencing analysis confirmed that an unidentified amino acid was present at position one. The N-terminal sequencing results for MON 87751-produced Cry1A.105 protein were consistent with the sequencing results for the *E. coli*-produced Cry1A.105 protein, which was engineered to contain a cysteine as the first amino acid. Hence, the sequence information confirms the identity of the Cry1A.105 protein isolated from the seed of MON 87751

Amino acid residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Expected Sequence →	C	M	Q	A	M	D	N	N	P	N	I	N	E	C	I
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence →	X	M	Q	A	M	D	N	N	(P)	X	X	X	X	X	X

Figure C-1. N-Terminal Sequence of MON 87751-Produced Cry1A.105 Protein

The experimental sequence obtained from the MON 87751-produced Cry1A.105 was compared to the expected sequence. The single letter IUPAC-IUB amino acid code is C, cysteine; M, methonine; Q, glutamine; A, alanine; D, aspatic acid; N, asparagine; P, proline; E, glutamic acid; I, isoleucine;. X indicates that the residue was not identified.
( )'s denote tenuous designations.

C.1.4. MALDI-TOF Tryptic Mass Map Analysis

C.1.4.1. Methods

MALDI-TOF MS tryptic mass fingerprint analysis confirmed the identity of the MON 87751-produced Cry1A.105 protein. A MON 87751-produced Cry1A.105 protein sample was separated by SDS-PAGE and the gel was stained using Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). The major band at ~130 kDa was excised, transferred to a microcentrifuge tube and destained. The excised band was washed in 100 mM ammonium bicarbonate and treated with 10 mM dithiothreitol (DTT) at 37°C for 2.5 h followed by incubation for 20 minutes with 10 mM iodoacetic acid in the dark. The excised band was then washed with 25 mM ammonium bicarbonate, dried using vacuum centrifugation and rehydrated with 20 µl of 20 µg/ml trypsin (Promega, Madison, WI). After 1 h, excess liquid was removed and the excised band was incubated at 38°C overnight in 40 µl of 10% (v/v) acetonitrile in 25 mM ammonium bicarbonate. The excised band was sonicated for 5 min and the resulting extract was transferred to a new microcentrifuge tube and dried using vacuum centrifugation. The excised band was then extracted two more times, each with 30 µl of a 60% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, 0.1% (v/w) β-octyl-glucopyranoside solution and sonicated for 5 min. These two extracts were pooled into a new tube and dried using vacuum centrifugation. The separate extracts were then treated with 0.1% trifluoroacetic acid (TFA) and dried. The extracts were solubilized in 50% (v/v) acetonitrile, 0.1% TFA and sonicated for 5 min. Extracts were spotted to wells on an analysis plate and mixed with either 2,5-dihydroxybenzoic acid (DHB, Thermo Fisher Scientific Inc. Waltham, MA), α-cyano-4-hydroxycinnamic acid (α-Cyano, Thermo Fisher Scientific Inc.), or 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid, Thermo Fisher Scientific Inc.). The samples in DHB, α-Cyano, and Sinapinic acid matrix were analyzed in the 300 to 5000 Da, the 500 to 5000 Da, and 500 to 7000 Da range, respectively. CalMix 2™ was used as the external calibrant (Sequazyme™ Peptide Mass Standards kit, AB SciEx, Foster City, CA). The analysis was performed on a Voyager™ DE Pro Biospectrometry™ workstation (Applied Biosystems) using Voyager Instrument Control Panel software (version 5.10.2) and Data Explorer data analysis software (version 4.0.0.0). Protonated peptide masses were isotopically resolved in reflector mode (Aebersold 1993; Billeci and Stults 1993). GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate an *in silico* digest of the Cry1A.105 protein sequence. Masses within 1 Da of the monoisotopic mass were matched against the *in silico* digest of the MON 87751-produced Cry1A.105 sequence. All matching masses were tallied and a coverage map was generated for the mass fingerprint.

C.1.4.2. Results of MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF MS analysis of peptide fragments produced by the trypsin digestion of the MON 87751-produced Cry1A.105 protein confirmed the identity of the MON 87751-produced Cry1A.105 protein. Sixty unique peptides corresponded to the expected masses (Table C-1) and were used to assemble a coverage map of the protein (Figure C-2). The experimentally determined coverage was 53.5% (632 out of 1181 amino acids). This analysis further confirms the identity of the MON 87751-produced Cry1A.105 protein.

Table C-1. Summary of the Tryptic Masses Identified for MON 87751-Produced Cry1A.105 Protein Using MALDI-TOF MS

Observed Mass	Expected Mass	Diff. [2]	Fragment	Sequence[3]
360.95	361.20	0.25	890-891	WR
515.34	515.34	0.00	529-532	ARIR
579.32	579.33	0.01	753-756	YQLR
589.29	589.31	0.02	229-232	DWIR*
	589.28	0.00	1027-1031	VCPGR*
621.35	621.37	0.02	1032-1036	GYILR
649.37	649.37	0.00	258-262	TYPIR
727.35	727.35	0.00	233-237	YNQFR
731.35	731.36	0.01	428-433	QGFSHR
764.40	764.39	0.01	92-97	IEEFAR
781.39	781.38	0.01	197-202	YNDLTR
804.46	804.46	0.00	263-269	TVSQLTR
816.39	816.40	0.01	222-228	VWGPDSR
832.34	832.48	0.14	670-676	ELSEKVK*
	832.48	0.14	742-748	IDESKLK*
854.40	854.41	0.01	1118-1124	SYTDGRR
907.46	907.46	0.00	178-185	DVSVFGQR
924.49	924.49	0.00	550-557	IFAGQFNK
940.51	940.51	0.00	365-372	TLSSTLYR
976.49	976.50	0.01	434-441	LSHVSMFR
1007.56	1007.55	0.01	541-549	IYVTVAGER
1038.50	1038.50	0.00	214-221	WYNTGLER
1066.44	1066.43	0.01	1125-1132	ENPCEFNR
1074.55	1074.55	0.00	286-296	GSAQ…GSIR
1089.57	1089.57	0.00	495-505	GPGF…DILR
1144.58	1144.57	0.01	454-462	APMFSWIHR
1203.69	1203.68	0.01	354-364	IVAQ…GVYR
1237.60	1237.60	0.00	186-196	WGFD…INSR
1253.65	1253.65	0.00	442-453	SGFS…SIIR
1258.65	1258.65	0.00	203-213	LIGN…HAVR
1269.70	1269.69	0.01	483-494	AHTL..TVVR
1303.67	1303.67	0.00	969-979	IFTA…YDAR
1352.73	1352.71	0.02	1136-1147	DYTP…YVTK
1398.68	1398.67	0.01	120-131	EWEA…PALR
1424.67	1424.65	0.02	998-1009	GHVD…NNQR
1551.83	1551.81	0.02	895-906	EKLE…IVYK
1576.87	1576.81	0.04	686-698	NLLQ…DINR*
	1576.87	0.00	627-641	AVNA…LGLK*
1598.87	1598.71	0.16	1124-1135	RENP…RGYR
1625.77	1625.70	0.07	984-997	NGDF…WNVK
1794.92	1794.87	0.05	703-720	GWGG…DVFK
1800.88	1800.87	0.01	757-771	GYIE…YSIR
1900.92	1900.91	0.01	270-285	EIYT…GSFR
1902.99	1902.96	0.03	104-119	LEGL…ESFR
1956.02	1956.01	0.01	1010-1026	SVLVV…QEVR
2088.97	2088.94	0.03	1099-1117	GYNE…YEEK
2098.17	2098.15	0.02	864-882	LGNL…ALAR
2108.08	2108.09	0.01	606-623	FELI…NLER
2125.16	2125.18	0.02	354-372	IVAQ…TLYR
2133.12	2133.11	0.01	507-526	TSGG…LPQR
2149.04	2149.05	0.01	408-427	SGTV…VPPR
2160.14	2160.16	0.02	463-482	SAEF…PLVK

Table C-1. Summary of the Tryptic Masses[1] Identified for MON 87751-Produced Cry1A.105 Protein Using MALDI-TOF MS (continued)

Observed Mass	Expected Mass	Diff. [2]	Fragment	Sequence[3]
2195.15	2195.16	0.01	239-257	ELTL…YDSR
2197.10	2197.11	0.01	297-315	SPHL…DAHR
2277.25	2277.10	0.15	757-775	GYIE…YNAK*
	2277.15	0.10	407-427	KSGT…VPPR*
2375.30	2375.24	0.06	776-798	HETV…PIGK
2616.39	2616.36	0.03	945-968	EAYL…LEGR
3363.75	3363.63	0.12	910-939	ESVD…ADKR
3729.07	3728.87	0.20	373-406	RPFN…AVYR

[1]The observed mass was collected from at least one of three matrices including a-cyano, DHB and sinapinic acid. The observed mass shown is the mass closest to the expected mass.

[2]The data represent the calculated difference between the expected mass and the observed mass

*The expected peptide masses are nearly identical (< 1 dalton). Because this analysis did not determine with certainty which expected peptide was actually observed, the peptides with an asterisk (*) were not included in determining sequence coverage (Figure C-1).

[3]For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are show separated by three dots (…)

```
   1  CMQAMDNNPN INECIPYNCL SNPEVEVLGG ERIETGYTPI DISLSLTQFL
  51  LSEFVPGAGF VLGLVDIIWG IFGPSQWDAF LVQIEQLINQ RIEEFARNQA
 101  ISRLEGLSNL YQIYAESFRE WEADPTNPAL REEMRIQFND MNSALTTAIP
 151  LFAVQNYQVP LLSVYVQAAN LHLSVLRDVS VFGQRWGFDA ATINSRYNDL
 201  TRLIGNYTDH AVRWYNTGLE RVWGPDSRDW IRYNQFRREL TLTVLDIVSL
 251  FPNYDSRTYP IRTVSQLTRE IYTNPVLENF DGSFRGSAQG IEGSIRSPHL
 301  MDILNSITIY TDAHRGEYYW SGHQIMASPV GFSGPEFTFP LYGTMGNAAP
 351  QQRIVAQLGQ GVYRTLSSTL YRRPFNIGIN NQQLSVLDGT EFAYGTSSNL
 401  PSAVYRKSGT VDSLDEIPPQ NNNVPPRQGF SHRLSHVSMF RSGFSNSSVS
 451  IIRAPMFSWI HRSAEFNNII ASDSITQIPL VKAHTLQSGT TVVRGPGFTG
 501  GDILRRTSGG PFAYTIVNIN GQLPQRYRAR IRYASTTNLR IYVTVAGERI
 551  FAGQFNKTMD TGDPLTFQSF SYATINTAFT FPMSQSSFTV GADTFSSGNE
 601  VYIDRFELIP VTATLEAEYN LERAQKAVNA LFTSTNQLGL KTNVTDYHID
 651  QVSNLVTYLS DEFCLDEKRE LSEKVKHAKR LSDERNLLQD SNFKDINRQP
 701  ERGWGGSTGI TIQGGDDVFK ENYVTLSGTF DECYPTYLYQ KIDESKLKAF
 751  TRYQLRGYIE DSQDLEIYSI RYNAKHETVN VPGTGSLWPL SAQSPIGKCG
 801  EPNRCAPHLE WNPDLDCSCR DGEKCAHHSH HFSLDIDVGC TDLNEDLGVW
 851  VIFKIKTQDG HARLGNLEFL EEKPLVGEAL ARVKRAEKKW RDKREKLEWE
 901  TNIVYKEAKE SVDALFVNSQ YDQLQADTNI AMIHAADKRV HSIREAYLPE
 951  LSVIPGVNAA IFEELEGRIF TAFSLYDARN VIKNGDFNNG LSCWNVKGHV
1001  DVEEQNNQRS VLVVPEWEAE VSQEVRVCPG RGYILRVTAY KEGYGEGCVT
1051  IHEIENNTDE LKFSNCVEEE IYPNNTVTCN DYTVNQEEYG GAYTSRNRGY
1101  NEAPSVPADY ASVYEEKSYT DGRRENPCEF NRGYRDYTPL PVGYVTKELE
1151  YFPETDKVWI EIGETEGTFI VDSVELLLME E
```

Figure C-2. MALDI-TOF MS Coverage Map of MON 87751-Produced Cry1A.105 Protein

The amino acid sequence of the MON 87751-produced Cry1A.105 protein was deduced from the *cry1A.105* gene present in MON 87751. Boxed regions correspond to peptides that were identified from the MON 87751-produced Cry1A.105 protein sample using MALDI-TOF MS. In total, 53.5% (632 out of 1181 amino acids) of the expected protein sequence was identified.

C.1.5. Western Blot Analysis-Immunoreactivity

C.1.5.1. Methods

Western blot analysis was performed as follows to confirm the identity of the MON 87751-produced Cry1A.105 protein and to compare the immunoreactivity of the MON 87751-produced and *E. coli*-produced Cry1A.105 proteins. MON 87751-produced and *E. coli*-produced Cry1A.105 protein were diluted in 1× LB and heated to 99 °C for 5 min. Three amounts (1, 2, and 3 ng) of the intact test substance (total protein concentration × purity of the intact Cry1A.105 protein) and the intact reference substance (total protein concentration × purity of the intact Cry1A.105 protein) were loaded in duplicate onto a pre-cast 4-20% polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight standards (Precision Plus, Bio-Rad) were loaded on the gel for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

The membrane was blocked with 5% non-fat dry milk (NFDM) in 1× phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST) and incubated with a goat anti-Cry1A.105 antibody (lot G839056) at a dilution of 1:4000 in 2% NFDM in PBST. After washing with PBST, the membrane was next incubated with horseradish peroxidase (HRP) -conjugated goat horse anti-goat IgG (H+L) (Vector Lab, Burlingame CA) at a dilution of 1:10,000 in 2% NFDM in PBST and washed again with PBST. Immunoreactive bands were detected using the ECL™ detection system (GE Healthcare) and Amersham Hyperfilm® (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Quantification of the bands on the blot was performed on a GS-800 densitometer with the supplied Quantity One® software (version 4.4.0, Bio-Rad) using the lane selection and contour tool. The signal intensities of the immunoreactive bands migrating at the expected position for the Cry1A.105 protein were quantified as "contour quantity" values. The immunoreactivity was reported in OD × mm^2.

C.1.5.2. Results of Cry1A.105 Protein Immunoreactivity Equivalence

Western blot analysis was conducted using goat anti-Cry1A.105 polyclonal antibody as additional means to confirm the identity of the Cry1A.105 protein isolated from the seed of MON 87751 and to assess the equivalence of the immunoreactivity of the MON 87751-produced and *E. coli*-produced Cry1A.105 proteins.

The results showed that immunoreactive bands migrating at the expected apparent MW were present in all lanes loaded with the MON 87751-produced (Figure C-3, lanes 2-7) or *E. Coli*-produced (Figure C-3, lanes 9-14) Cry1A.105 proteins. For each amount loaded, comparable signal intensity was observed between the MON 87751- and *E. coli*-produced Cry1A.105 protein bands. As expected, the signal intensity increased with increasing

load amounts of the MON 87751- produced and *E. coli*-produced Cry1A.105 proteins, thus, supporting identification of MON 87751-produced Cry1A.105 protein.

To compare the immunoreactivity of the MON 87751-produced and the *E. coli*-produced Cry1A.105 proteins, densitometric analysis was conducted on bands that migrated to the expected apparent MW for Cry1A.105 proteins (~130 kDa). The signal intensity (reported in $OD \times mm^2$) of the band of interest in lanes loaded with MON 87751-produced and the *E. coli*-produced Cry1A.105 protein was measured (Table C-2). Because the mean signal intensity of the MON 87751-produced Cry1A.105 protein band was within 35% of the mean signal of the *E. coli*-produced Cry1A.105 protein, the MON 87751-produced and *E. coli*-produced Cry1A.105 proteins were determined to have equivalent immunoreactivity.

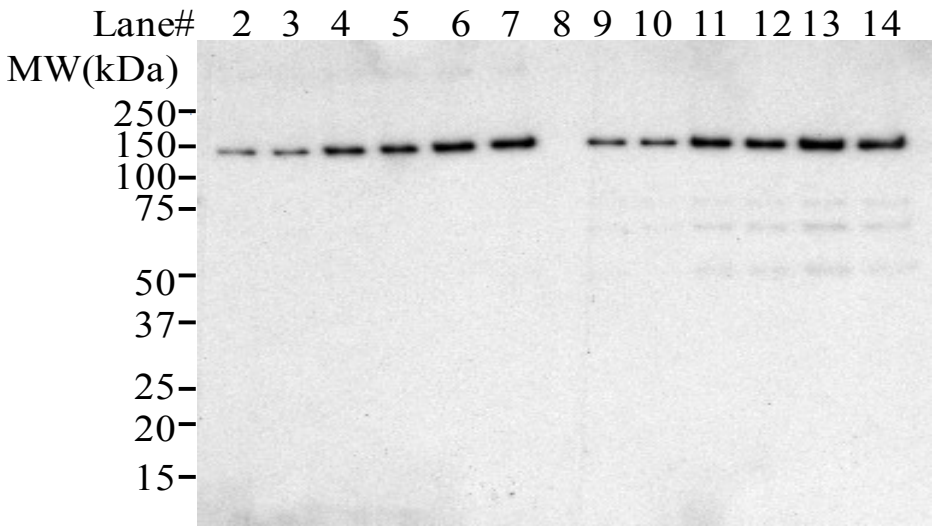

Figure C-3. Western Blot Analysis of MON 87751- and *E. coli*-Produced Cry1A.105 Proteins

Aliquots of the MON 87751-produced Cry1A.105 protein and the *E. coli*-produced Cry1A.105 protein were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. Proteins were detected using anti-Cry1A.105 antibodies as the primary antibodies. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. The molecular weights (kDa) of the standards are shown on the left. The 4 min exposure is shown. Lane 1 and 15 were cropped from the image. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color	-
2	MON 87751-produced Cry1A.105	1
3	MON 87751-produced Cry1A.105	1
4	MON 87751-produced Cry1A.105	2
5	MON 87751-produced Cry1A.105	2
6	MON 87751-produced Cry1A.105	3
7	MON 87751-produced Cry1A.105	3
8	Blank	-
9	*E. coli*-Produced Cry1A.105	1
10	*E. coli*-Produced Cry1A.105	1
11	*E. coli*-Produced Cry1A.105	2
12	*E. coli*-Produced Cry1A.105	2
13	*E. coli*-Produced Cry1A.105	3
14	*E. coli*-Produced Cry1A.105	3
15	Blank	-

Table C-2. Comparison of Immunoreactive Signal Between MON 87751- and *E. coli*-Produced Cry1A.105 Proteins

Mean signal intensity from MON 87751-produced Cry1A.105[1] (OD x mm^2)	Mean signal intensity from *E. coli* -produced Cry1A.105[1] (OD x mm^2)	Acceptance limits[2] (OD x mm^2)
2.67	3.33	2.16 – 4.50

[1]Each value represents the mean of six values (n=6)

[2] The acceptance limits are for the MON 87751-produced Cry1A.105 protein and are based on the interval between −35% (3.33 × 0.65 = 2.16) and +35% (3.33× 1.35 = 4.50) of the mean of the *E. coli*-produced Cry1A.105 signal intensity across all loads.

C.1.6. Molecular Weight Estimation of Cry1A.105 using SDS-PAGE

C.1.6.1. Methods

MON 87751-produced and *E. coli*-produced Cry1A.105 protein were diluted in 1× loading buffer and heated to 95°C for 5 min. The MON 87751 -produced Cry1A.105 protein was loaded in duplicate at ~0.5, ~1.0, and ~1.5 µg based on total protein concentration, onto a pre-cast 8 - 16% polyacrylamide mini-gel (Invitrogen). The *E. coli*-produced Cry1A.105 protein was loaded at 1.0 µg total protein in a single lane. Broad Range Molecular Weight Standards (Bio-Rad) were prepared and loaded on the gel in parallel. Following electrophoresis at a constant voltage, proteins were briefly fixed in 40% (v/v) methanol, 7% (v/v) acetic acid and stained for 18 ± 2 h with Brilliant Blue G-Colloidal stain (Sigma-Aldrich). Gels were briefly destained in 10% (v/v) acetic acid, 25% (v/v) methanol followed by 8 ± 2 h in 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer supplied with Quantity One software (version 4.4.0). Apparent MW and purity were reported as an average of all six lanes containing the MON 87751-produced Cry1A.105 protein. The acceptance limits are the 95% prediction interval derived from data obtained for the apparent MW of the *E. coli*-produced Cry1A.105 protein.

C.1.6.2. Results of Cry1A.105 Protein Molecular Weight Equivalence

The intact MON 87751-produced Cry1A.105 protein (Figure C-4, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced Cry1A.105 protein (Figure C-4, lane 2) and the apparent MW was calculated to be 132.9 kDa (Table C-3). Because the experimentally determined apparent MW of the MON 87751-produced Cry1A.105 protein was within the acceptance limits for equivalence (Table C-3), the MON 87751- and *E. coli*-produced Cry1A.105 proteins were determined to have equivalent apparent molecular weights.

Table C-3. Molecular Weight Comparison Between MON 87751- and *E. coli*-Produced Cry1A.105 Proteins

Apparent MW of MON 87751-produced Cry1A.105 Protein (kDa)	Apparent MW of *E. coli*-produced Cry1A.105 Protein[1] (kDa)	Acceptance Limits (kDa)
132.9	130.8	126.5 – 135.1

[1]As reported on the Certificate of Analysis for lot 11349124.

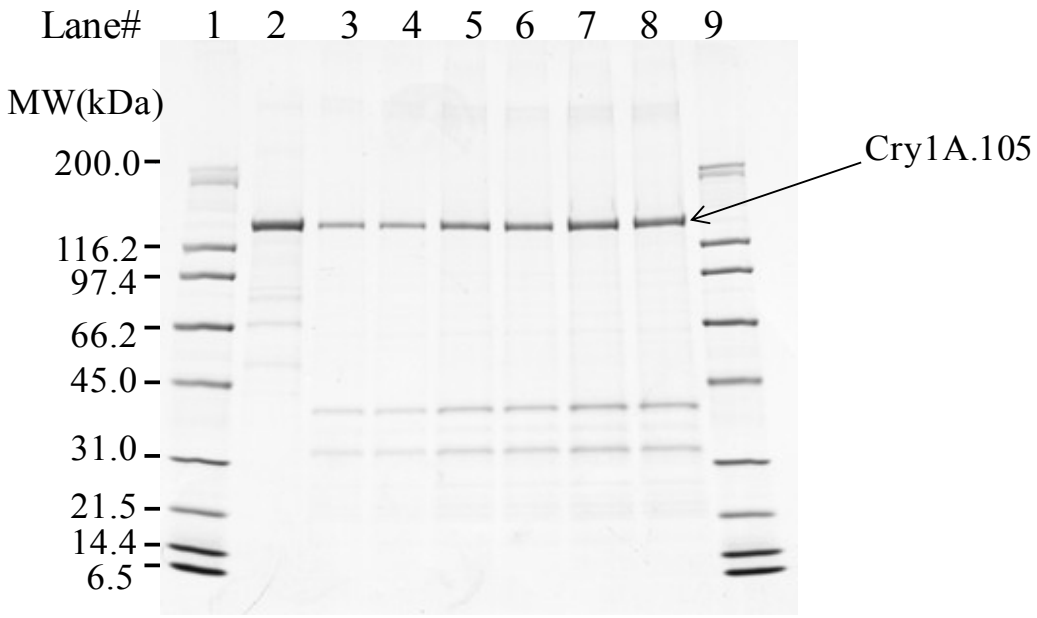

Figure C-4. Molecular Weight and Purity Analysis of MON 87751-Produced Cry1A.105 Protein

Aliquots of the MON 87751-produced Cry1A.105 and the *E. coli*-produced Cry1A.105 proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-Colloidal stain. The molecular weights (kDa) are shown on the left and correspond to the standards loaded in Lanes 1 and 9. Lane 10 was cropped from the image. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standards	4.5
2	*E. coli*-produced Cry1A.105	1.0
3	MON 87751-produced Cry1A.105	0.5
4	MON 87751-produced Cry1A.105	0.5
5	MON 87751-produced Cry1A.105	1.0
6	MON 87751-produced Cry1A.105	1.0
7	MON 87751-produced Cry1A.105	1.5
8	MON 87751-produced Cry1A.105	1.5
9	Broad Range MW Standards	4.5
10	Blank	-

C.1.7. Glycosylation Analysis

C.1.7.1. Methods

An ECL™ Glycoprotein Detection Kit (GE Healthcare) was used for glycoprotein detection. The MON 87751-produced Cry1A.105 protein, the *E. coli*-produced Cry1A.105 protein, and a positive control, transferrin (Sigma), were diluted in 1× LB and heated to 99 °C for 5 min. Two amounts (~100 and ~200 ng) of the intact MON 87751-produced Cry1A.105 protein (purity corrected) and the *E. coli*-produced Cry1A.105 protein (purity corrected) were loaded onto a pre-cast Tris-glycine 4 - 20% polyacrylamide gradient mini-gel (Invitrogen). Four amounts (~50, ~100, ~150, and ~200 ng) of the positive control were loaded on the gel. Protein MW Standards (Precision Plus ProteinTM Standards Dual color, Bio-Rad) were also loaded for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the Amersham ECL™ glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. Glycosylated proteins were detected using ECL™ reagents (GE Healthcare) and Amersham Hyperfilm® (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) and then destained with 1× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using Bio-Rad GS-800 densitometer.

C.1.7.2. Results of Glycosylation Analysis

Eukaryotic proteins can be post-translationally modified with carbohydrate moieties (Rademacher et al. 1988). To test whether Cry1A.105 protein was glycosylated when expressed in the seed of MON 87751, the MON 87751-produced Cry1A.105 protein was analyzed using an ECL™ Glycoprotein Detection Module (GE Healthcare). To assess equivalence of the MON 87751- and *E. coli*-produced Cry1A.105 proteins, the *E. coli*-produced Cry1A.105 protein was also analyzed.

No glycosylation signals was observed in the molecular weight range of 130 kDa in the lanes containing the MON 87751-produced Cry1A.105 protein (Figure C-5, panel A lanes 6 and 7) or the *E. coli*-produced Cry1A.105 protein (Figure C-5, panel A lanes 8 and 9). In contrast, clear glycosylation signals were observed at the expected MW (~80 kDa) in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure C-5, lanes 2-5). There is a signal of ~40 kDa in the lanes of MON 87751-produced Cry1A.105 protein, which is not likely derived from Cry1.A105 since no immunoreactive signal was observed at this molecular weight range in the western blot analysis. This low molecular weight signal is likely from glycosylated soy protein that was co-purified with Cry1A.105 protein.

To confirm that MON 87751-produced and *E. coli*-produced Cry1A.105 proteins were appropriately loaded for glycosylation analysis, a second membrane with identical loadings and transfer time was stained with Coomassie Blue R250 for protein detection (Figure C-5 Panel B). Both the MON 87751-produced Cry1A.105 (Figure C-5 panel B, lanes 6 and 7) and *E. coli*-produced Cry1A.105 (Figure C-5 panel B, lanes 8 and 9) proteins were detected. These data indicate that the glycosylation status of MON 87751-produced Cry1A.105 protein is equivalent to that of the *E. coli*-produced Cry1A.105 protein and that neither is glycosylated.

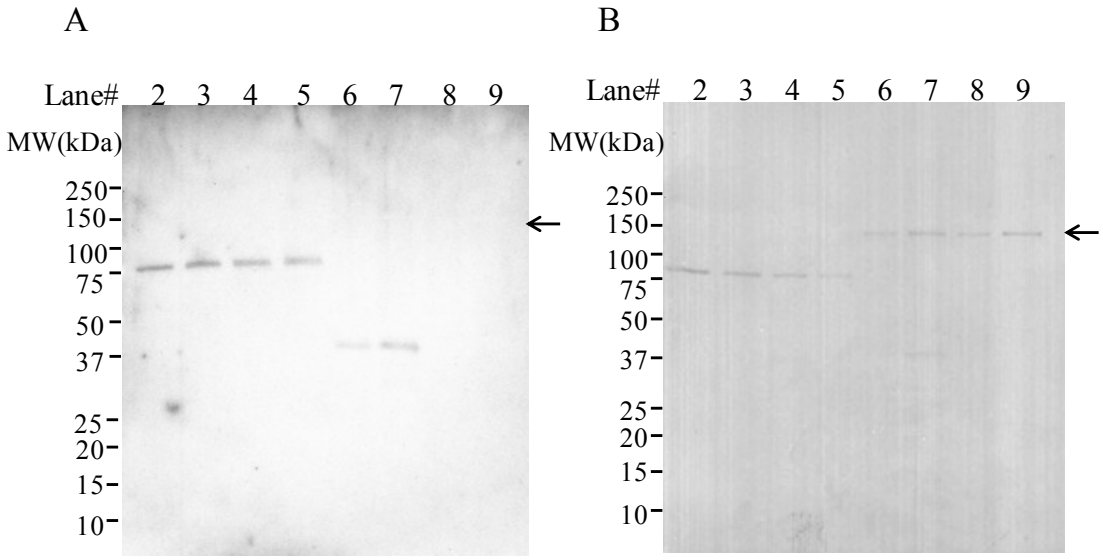

Figure C-5. Glycosylation Analysis of MON 87751-Produced Cry1A.105 Protein

Aliquots of the transferrin (positive control), MON 87751-produced Cry1A.105 and *E. coli* -produced Cry1A.105 were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The molecular weights (kDa) of the standards are shown on the left. The arrows show the expected migration of the MON 87751- and *E. coli*-produced Cry1A.105 protein. (A) Where present, the labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based detection using ECL reagents and exposure to Hyperfilm®. The 45 seconds exposure is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lanes 1 & 10 were cropped from both images. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	
2	Transferrin (positive control)	200
3	Transferrin (positive control)	150
4	Transferrin (positive control)	100
5	Transferrin (positive control)	50
6	MON 87751-produced Cry1A.105	100
7	MON 87751-produced Cry1A.105	200
8	*E. coli*-produced Cry1A.105	100
9	*E. coli*-produced Cry1A.105	200
10	Blank	

C.1.8. Functional Activity Analysis

C.1.8.1. Methods

Insects. Corn Ear Worm (CEW) eggs were obtained from Benzon Research, Inc. (Carlisle, PA). The eggs were incubated at temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

Bioassays. CEW larvae (≤ 30 hours old) were used to measure biological activity of the MON 87751-produced and *E. coli*-produced Cry1A.105 protein samples in accordance with the current version of Monsanto SOP BR-ME-1088. The bioassay was replicated three times on separate days, each with a separate batch of insects. The MON 87751-produced and *E. coli*-produced substances were run in parallel during each bioassay. Each bioassay replicate consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.00080 – 0.025 µg Cry1A.105 protein/ml diet for the *E. coli*-produced and MON 87751-produced Cry1A.105 and a single buffer control. The Cry1A.105 protein dosing solutions were prepared by diluting the respective protein with purified water and incorporating the dilution into a multiple species diet (Southland Products, Inc.; Lake Village, AR). This dose series in diet was chosen to adequately characterize the dose-effect relationship of CEW growth inhibition for the *E. coli*-produced and MON 87751-produced Cry1A.105 proteins. The diet mixture was then dispensed in 1.0 ml aliquots into a 128 well tray (#BAW128, Bio-Serv, Frenchtown, NJ). Individual insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BACV16, Bio-Serv, Frenchtown, NJ) and the insects were allowed to feed for a period of approximately 7 days in an environmental chamber programmed at 27° C, 60% relative humidity and a lighting regime of 14 h light: 10 h dark. The number of the surviving insects and their combined weight was recorded at each treatment level at the end of the seven-day incubation period. For a bioassay to be accepted, control mortality cannot be >20%.

C.1.8.2. Results of Functional Activity

The functional activity of the MON 87751- produced and *E. coli*-produced Cry1A.105 protein was determined by corn earworm diet incorporation assay. The MON 87751- and *E. coli*-produced Cry1A.105 proteins were considered functionally equivalent if the EC_{50}, of both were within acceptance limits of 0.0028 µg/ml diet to 0.0091 µg/ml diet; which is derived from the 95% prediction interval calculated from data obtained for the *E. coli*-produced Cry1A.105 protein activity. The EC_{50} of the MON 87751-produced and *E. coli*-produced Cry1A.105 proteins were determined to be 0.0035 and 0.0032 µg Cry1A.105/ml diet respectively (Table C-4). Because the EC_{50} of MON 87751-produced and *E. coli*-produced Cry1A.105 proteins were within the acceptance limits (Table C-4), the proteins were determined to have equivalent functional activity.

Table C-4. Cry1A.105 Functional Activity Assay

MON 87751-produced Cry1A.105[1] EC_{50} (µg /ml)	*E. coli*-produced Cry1A.105[1] EC_{50} (µg /ml)	Acceptance Limits[2] EC_{50} (µg /ml)
0.0035	0.0032	0.0028 – 0.0091

[1]Value refers to mean based on n = 3. Values in these columns determined from proteins having the same amino acid sequence.

[2] Data obtained for the *E. coli*-produced protein was used to generate the acceptance limits. Values in this column represent a 95% prediction interval developed from a series of 11 assays with *E. coli* produced Cry1A.105 protein. Eight assays relied on a protein with an amino acid sequence identical to the MON 89034-produced Cry1A.105 protein and three relied on a protein with an amino acid sequence identical to the MON 87751-produced protein.

C.2. Characterization of Cry2Ab2 Protein in MON 87751

C.2.1. Materials for Cry2Ab2 Characterization

The Cry2Ab2 protein was purified from defatted enzyme active seed flour (lot# 11333897) of MON 87751. The MON 87751-produced Cry2Ab2 protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM CAPS, pH11.2, 2 mM dithiothreitol (DTT).

The *E. coli*-produced Cry2Ab2 protein (lot 11351673) was used as the reference substance. The Cry2Ab2 protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON149850 expression plasmid. The coding sequence for *cry2Ab2* contained on the expression plasmid (pMON149850) was confirmed prior to and after fermentation The *E. coli*-produced Cry2Ab2 protein was characterized previously.

C.2.2. Cry2Ab2 Protein Purification

The Cry2Ab2 protein was purified from flour (lot# 11333897) of MON 87751. The purification procedure was not performed under a GLP protocol, however, all procedures were documented and, where applicable, Standard Operation Procedures were followed. The Cry2Ab2 was purified from an extract of defatted enzyme active seed flour using a combination of techniques, including anionic exchange and immunoaffinity chromatography.

Cry2Ab2 protein was purified from four batches (1 batch from ~1.5 kg and 3 batches from 0.8 kg) of flour of MON 87751. All operations were performed at ~4 °C. The flour was first stirred in cold phosphate buffer (10 mM Na_2PO_4, pH 7.0 2 mM $MgCl_2$ 25,000 U/1 Benzonase) at a 1: 10 (w/v) seed to buffer ratio. The Cry2Ab2-containing pellets were collected by centrifugation and the Cry2Ab2 protein was extracted from the washed flour pellet with CAPS solubilization buffer (50 mM CAPS, pH ~11.0, 1 mM EDTA, 2 mM benzamidine HCl, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1% polyvinylpolypyrroleidone) added at 10 ml per gram of starting flour. The

suspension was stirred, and solubilized proteins, including Cry2Ab2, were separated from insoluble material by centrifugation. The Cry2Ab2 protein was then precipitated by the addition of ammonium sulfate salt to the CAPS supernatant to a final saturation of 35%. After mixing, precipitated proteins were collected by centrifugation, and were re-solubilized in 50 mM CAPS, pH ~11.0, 1 mM EDTA, 2 mM benzamidine HCl, 10 mM DTT (Buffer A) at ~1 ml per starting ml of CAPS supernatant. After solubilization, solid $CaCl_2$ was added to 100 mM and the extract was mixed. Insoluble material was removed by centrifugation and the extract was filtered (GE/Whatman PolyCap HD75) and dialyzed against Buffer D containing 20 mM CAPS pH ~11.0, 1 mM EDTA, 2 mM benzamidine HCl, 2 mM DTT. The dialyzed extract was loaded onto a Q Sepharose FF (GE Healthcare, Piscataway, NJ) anion exchange column that was pre-equilibrated with Buffer A. The column was washed with Buffer A and the Cry2Ab2 protein was eluted with 20-30% Buffer B (50 mM CAPS, pH ~11.0, 1 M NaCl, 1 mM EDTA, 2 mM benzamidine HCl, 2 mM DTT (Buffer B). Pooled Cry2Ab2-containing fractions were dialyzed against 25 mM Bis-Tris propane, pH 9, 1 mM EDTA, 1 mM Benzamidine HCl, 2 mM DTT (Capto Q Buffer A) and loaded onto a Capto Q (GE Healthcare) anion exchange column equilibrated with Capto Q Buffer A. The flow through containing the Cry2Ab2 protein was collected. As a final purification step, the Cry2Ab2-containing Capto Q flow through was loaded onto an immunoaffinity column equilibrated with Capto Q Buffer A. The column consisted of Protein G sepharose cross-linked to an anti-Cry2Ab2 monoclonal antibody. The column was washed with Capto Q Buffer A, and with wash buffer containing 50 mM CAPS, pH 10, 30% polypropylene glycol, 1 mM EDTA, 2 mM benzamidine HCl, 2 mM DTT. Cry2Ab2 protein was eluted with 50 mM CAPS, pH 10, 0.8 M NaCl, 1 mM EDTA, 2 mM benzamidine HCl, 2 mM DTT. Cry2Ab2-containing fractions were pooled and buffer exchanged/concentrated in 50 mM CAPS, pH 11.2, 2 mM DTT using Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Billarice, MA). The final buffer composition of the sample was 50 mM CAPS, pH 11.2, 2 mM DTT. The purified MON 87751-produced Cry2Ab2 protein was aliquoted, assigned lot #11355400 and stored at in a -80°C freezer.

C.2.3. N-Terminal Sequencing

C.2.3.1. Methods

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 87751-produced Cry2Ab2 protein. MON 87751-produced Cry2Ab2 was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Blue R-250 and then destained with 1x Coomassie Brilliant Blue R-250 Destaining Solution. The band at 61 kDa containing MON 87751-produced Cry2Ab2 was excised from the blot and used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al. 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and a Perkin Elmer Series 200 UV/VIS Absorbance Detector and Procise™ Control Software (version 2.1) were used. Chromatographic data were collected using SequencePro (Applied Biosystems, Foster City, CA; version 2.1) software. PTH-AA Standard Solution (Applied Biosystems) was used to chromatographically calibrate the instrument for the analysis. A control protein

(10 picomole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the MON 87751-produced Cry1A.105 protein.

C.2.3.2. Results of the N-terminal Sequence Analysis

Fifteen cycles of N-terminal sequencing was performed on the MON 87751-produced Cry2Ab2 protein. The sequence obtained corresponded to the N-terminal sequence for Cry2Ab2 starting at position 16 relative to the first methionine of the predicted Cry2Ab2. The N-terminal sequencing results for MON 87751-produced Cry2Ab2 protein were consistent with the sequencing results for the *E. coli*-produced Cry2Ab2 protein, which was engineered to start at the same amino acid position as the MON 87751-produced Cry2Ab2 protein (Figure C-6). Hence, the sequence information confirms the identity of the Cry2Ab2 protein isolated from the seed of MON 87751.

Amino acid residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Expected Sequence →	A	Y	N	V	A	A	H	D	P	F	S	F	Q	H	K
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence →	A	Y	N	V	A	A	H	D	P	F	S	X	Q	X	X

Figure C-6. N-Terminal Sequence of MON 87751-Produced Cry2Ab2 Protein
The experimental sequence obtained from the MON 87751-produced Cry2Ab2 was compared to the expected sequence deduced from the *cry2Ab2* gene present in MON 87751. The amino acid at position 16 of the deduced sequence is shown as position 1 of the observed protein. The single letter IUPAC-IUB amino acid code is; A, alanine; D, aspatic acid; F, phenylalanine; H, histidine; K, lysine; N, asparagine; P, proline; Q, glutamine; S, serine; V, valine, Y tyrosine. X indicates that the residue was not identified.

C.2.4. MALDI-TOF Tryptic Mass Map Analysis

C.2.4.1. Methods

MALDI-TOF MS tryptic mass fingerprint analysis was used to confirm the identity of the MON 87751-produced Cry2Ab2 protein. A MON 87751-produced Cry2Ab2 protein sample was separated by SDS-PAGE and the gel was stained using Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). The major band at ~60 kDa band was excised, transferred to a microcentrifuge tube, and destained. The excised band were washed in 100 mM ammonium bicarbonate and treated with 10 mM dithiothreitol (DTT) at 37°C for 2 h followed by incubation for 30 minutes with 10 mM iodoacetic acid in the dark. The excised bands were then washed with 25 mM ammonium bicarbonate, dried using vacuum centrifugation and rehydrated with 20 µl of 20 µg/ml trypsin (Promega, Madison, WI). After 1.5 h, excess liquid was removed and the excised bands were incubated at 37°C overnight in 40 µl of 10% (v/v) acetonitrile in 25 mM ammonium bicarbonate. The excised bands were sonicated for 5 min and the resulting extract was transferred to new microcentrifuge tube labeled Extract 1 and dried using vacuum centrifugation. The excised bands were then extracted two more times, each with 30 µl of a 60% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, 0.1% (v/w) β-octyl-glucopyranoside solution and sonicated for 5 min. These two extracts were pooled into a new tube labeled Extract 2 and dried using vacuum centrifugation. Extract 1 and 2 were then treated with 0.1% trifluoroacetic acid (TFA) and dried. The extracts were solubilized in 50% (v/v) acetonitrile, 0.1% TFA and sonicated for 5 min. Extracts were spotted to wells on an analysis plate and mixed with either 2,5-dihydroxybenzoic acid (DHB, Thermo Fisher Scientific Inc. Waltham, MA), α-cyano-4-hydroxycinnamic acid (α-Cyano, Thermo Fisher Scientific Inc.), or 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid, Thermo Fisher Scientific Inc.). The samples in DHB, α-Cyano, and Sinapinic acid matrix were analyzed in the 300 to 5000 Da, the 500 to 5000 Da, and 500 to 7000 Da range, respectively. CalMix 2TM was used as the external calibrant (SequazymeTM Peptide Mass Standards kit, AB SciEx, Foster City, CA). The analysis was performed on a VoyagerTM DE Pro BiospectrometryTM workstation (Applied Biosystems) using Voyager Instrument Control Panel software (version 5.10.2) and Data Explorer data analysis software (version 4.0.0.0). Protonated peptide masses were isotopically resolved in reflector mode (Aebersold 1993; Billeci and Stults 1993). GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate an *in silico* digest of the Cry2Ab2 protein sequence. Masses within 1 Da of the monoisotopic mass were matched against the *in silico* digest of the MON 87751-produced Cry1A.105 sequence. All matching masses were tallied and a coverage map was generated for the mass fingerprint.

C.2.4.2. Results of MALDI-TOF Tryptic Mass Map Analysis

Peptide mass fingerprint analysis is a standard technique used for confirming the identity of proteins. The identity of the MON 87751-produced Cry2Ab2 protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced by the trypsin digestion of the MON 87751-produced Cry2Ab2 protein. There were 37 unique peptides identified that corresponded to the expected masses (Table C-5). The identified masses were used

to assemble a coverage map of the Cry2Ab2 protein (Figure C-7). The experimentally determined coverage of the Cry2Ab2 protein was 66% (406 out of 619 amino acids). Both acetylated and non-acetylated N-terminal peptides were present in the sample (Table C-5)

Table C-5. Summary of the Tryptic Masses Identified for MON 87751-Produced Cry2Ab2 Protein Using MALDI-TOF MS

Observed Mass[1]	Expected Mass	Diff.[2]	Fragment	Sequence[3]
439.05	439.23	0.18	193-195	TYR
506.22	506.25	0.03	82-85	ETEK
552.39	552.31	0.08	520-523	YTLR
560.39	560.32	0.07	219-223	GLNTR
646.32	646.32	0.00	421-425	NEDLR
677.38	677.37	0.01	86-90	FLNQR
709.38	709.36	0.02	392-398	SGAFTAR
724.39	724.39	0.00	496-501	TFISDEK
730.46	730.45	0.01	59-64	ILSELR
815.62	815.51	0.11	51-58	VGSLVGKR
878.44	878.40	0.04	23-28	EWTEWK
886.66	886.55	0.11	58-64	RILSELR
903.52	903.49	0.03	91-98	LNTDTLAR
958.55	958.50	0.05	193-199	TYRDYLK
993.50	993.48	0.02	502-510	FGNQGDSLR
1006.52	1006.50	0.02	23-29	EWTEWKK
1022.46	1022.45	0.01	362-370	SWLDSGSDR
1033.70	1033.56	0.14	535-544	VSSIGNSTIR
1053.65	1053.64	0.01	411-420	NISGVPLVVR
1060.59	1060.52	0.07	224-231	LHDMLEFR
1076.57	1076.53	0.04	448-456	AYMVSVHNR
1080.57	1080.51	0.06	511-519	FEQNNTTAR
1184.67	1184.60	0.07	435-447	NIAS...GGAR
1197.71	1197.65	0.06	426-434	RPLHYNEIR
1216.65	1216.61	0.04	116-125	QVDNFLNPNR
1270.71	1270.62	0.09	524-534	GNGN...LYLR
1492.74	1492.69	0.05	399-410	GNSN...YFIR**
	1492.75	0.01	193-203	TYRD...NYTR
1732.40	1731.82	0.58	1-15	AYNV….FQHK
1773.96	1773.82	0.14	1-15	[4]Ac-AYNV….FHQK
1844.05	1843.96	0.09	176-192	DVIL...ATLR
1904.09	1903.95	0.14	99-115	VNAE...EFNR
1919.11	1919.01	0.10	65-81	NLIF...DILR
2201.31	2201.20	0.11	30-49	NNHS...FLLK
2311.58	2311.06	0.52	551-572	VYTA...NGAR
2333.40	2333.23	0.17	126-146	NAVP...FLNR
2339.31	2339.15	0.16	371-391	EGVA...LGLR
2451.44	2451.34	0.10	302-325	LSNT...LAAR
4212.98	4212.04	0.94	458-495	NNIH...NQTR

[1]The observed mass was collected from at least one of three matrices including a-cyano, DHB and sinapinic acid. The observed mass shown is the mass closest to the expected mass.

[2]The data represent the calculated difference between the expected mass and the observed mass

**The expected peptide masses are nearly identical (< 1 dalton). Because this analysis did not determine with certainty which expected peptide was actually observed, the peptides with an asterisk (*) were not included in determining sequence coverage (Figure C-5).

[3]For peptide matches greater than nine amino acids in length the first 4 residues and last 4 residues are show separated by three dots (…). Fragment numbering is based on the observed N-terminus of the protein.

[4]AC is the abbreviation for acetylation.

001 AYNVAAHDPF SFQHKSLDTV QKEWTEWKKN NHSLYLDPIV GTVASFLLKK

051 VGSLVGKRIL SELRNLIFPS GSTNLMQDIL RETEKFLNQR LNTDTLARVN

101 AELTGLQANV EEFNRQVDNF LNPNRNAVPL SITSSVNTMQ QLFLNRLPQF

151 QMQGYQLLLL PLFAQAANLH LSFIRDVILN ADEWGISAAT LRTYRDYLKN

201 YTRDYSNYCI NTYQSAFKGL NTRLHDMLEF RTYMFLNVFE YVSIWSLFKY

251 QSLLVSSGAN LYASGSGPQQ TQSFTSQDWP FLYSLFQVNS NYVLNGFSGA

301 RLSNTFPNIV GLPGSTTTHA LLAARVNYSG GISSGDIGAS PFNQNFNCST

351 FLPPLLTPFV RSWLDSGSDR EGVATVTNWQ TESFETTLGL RSGAFTARGN

401 SNYFPDYFIR NISGVPLVVR NEDLRRPLHY NEIRNIASPS GTPGGARAYM

451 VSVHNRKNNI HAVHENGSMI HLAPNDYTGF TISPIHATQV NNQTRTFISE

501 KFGNQGDSLR FEQNNTTARY TLRGNGNSYN LYLRVSSIGN STIRVTINGR

651 VYTATNVNTT TNNDGVNDNG ARFSDINIGN VVASSNSDVP LDINVTLNSG

601 TQFDLMNIML VPTNISPLY

Figure C-7. MALDI-TOF MS Coverage Map of MON 87751-Produced Cry2Ab2 Protein

The amino acid sequence of the MON 87751-produced Cry2Ab2 protein was deduced from the *cry2Ab2* gene present in MON 87751, with the amino acid at position 16 of the deduced sequence shown as position 1 of the observed protein. Boxed regions correspond to peptides that were identified from the MON 87751-produced Cry2Ab2 protein sample using MALDI-TOF MS. In total, 66% (406 out of 619 amino acids) of the expected protein sequence was identified.

C.2.5. Western Blot Analysis-Immunoreactivity

C.2.5.1. Methods

Western blot analysis was performed as follows to confirm the identity of the MON 87751-produced Cry2Ab2 protein and to compare the immunoreactivity of the MON 87751-produced and *E. coli*-produced Cry2Ab2 protein. MON 87751-produced and *E. coli*-produced Cry2Ab2 protein were diluted in 1× LB and heated to 99 °C for 5 min. Three amounts (1, 2, and 3 ng) of the intact test substance (total protein concentration × purity of the intact Cry2Ab2 protein) and the intact reference substance (total protein concentration × purity of the intact Cry2Ab2 protein) were loaded in duplicate onto a pre-cast (4-20%) polyacrylamide mini-gel (Invitrogen). Pre-stained molecular weight standards (Precision Plus, Bio-Rad) were loaded on the gel for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a nitrocellulose membrane (Invitrogen).

The membrane was blocked with 5% non-fat dry milk (NFDM) in 1× phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST) and incubated with a monoclonal anti-Cry2Ab2 antibody (lot 11260969) at a dilution of 1:1000 in 2% NFDM in PBST. After washing with PBST, the membrane was next incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) IgG (Vector Lab, Burlingame CA) at a dilution of 1:10,000 in 2% NFDM in PBST and washed again, with PBST. Immunoreactive bands were detected using the ECL™ detection system (GE Healthcare) and Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Quantification of the bands on the blot was performed on a GS-800 densitometer with the supplied Quantity One® software (version 4.4.0, Bio-Rad) using the lane selection and contour tool. The signal intensities of the immunoreactive bands migrating at the expected position for the Cry2Ab2 protein were quantified as "contour quantity" values. The immunoreactivity was reported in OD × mm^2

C.2.5.2. Results of Cry2Ab2 Protein Immunoreactivity Equivalence

Western blot analysis was conducted using anti-Cry2Ab2 monoclonal antibody as additional means to confirm the identity of the Cry2Ab2 protein isolated from the seed of MON 87751and to assess the equivalence of the immunoreactivity of the MON 87751-produced and *E. coli*-produced Cry2Ab2 proteins.

The results showed that immunoreactive bands migrating at the expected apparent MW were present in all lanes loaded with the MON 87751-produced (Figure C-8, lanes 9-14) or *E. coli*-produced (Figure C-8, lanes 2-7) Cry2Ab2 proteins. For each amount loaded, comparable signal intensity was observed between the MON 87751- and *E. coli*-produced Cry2Ab2 protein bands. As expected, the signal intensity increased with increasing load

amounts of the MON 87751- produced and *E. coli*-produced Cry2Ab2 proteins, thus, supporting identification of MON 87751-produced Cry2Ab2 protein.

To compare the immunoreactivity of the MON 87751-produced and the *E. coli*-produced Cry2Ab2 proteins, densitometric analysis was conducted on bands that migrated to the expected apparent MW for Cry2Ab2 proteins (~60 kDa). The signal intensity (reported in OD × mm^2) of the band of interest in lanes loaded with MON 87751-produced and the *E. coli*-produced Cry2Ab2 protein was measured (Table C-6). Because the mean signal intensity of the MON 87751-produced Cry2Ab2 protein band was within 35% of the mean signal of the *E. coli*-produced Cry2Ab2 protein, the MON 87751-produced and *E. coli*-produced Cry2Ab2 proteins were determined to have equivalent immunoreactivity.

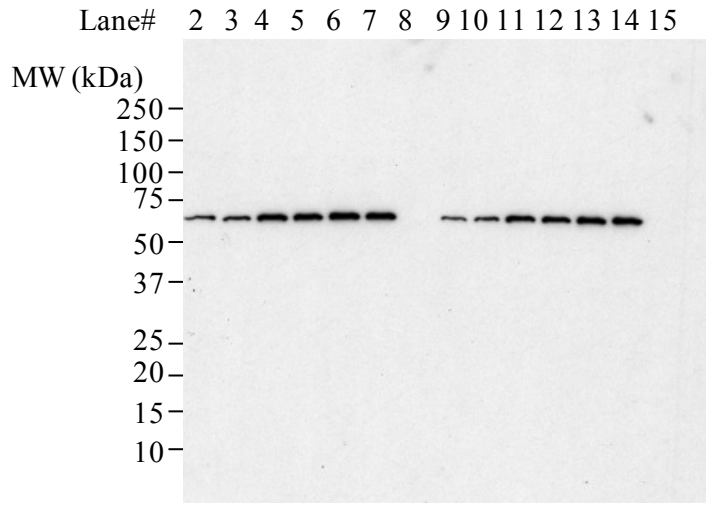

Figure C-8. Western Blot Analysis of MON 87751- and *E. coli* -Produced Cry2Ab2 Proteins

Aliquots of the MON 87751-produced Cry2Ab2 protein and the *E. coli*-produced Cry2Ab2 protein were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. Proteins were detected using anti-Cry2Ab2 antibodies as the primary antibodies. Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and an ECL system. The molecular weights (kDa) of the standards are shown on the left. The 2 min exposure is shown. Lane 1 was cropped from the image. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color	
2	*E. coli*-Produced Cry2Ab2	1
3	*E. coli*-Produced Cry2Ab2	1
4	*E. coli*-Produced Cry2Ab2	2
5	*E. coli*-Produced Cry2Ab2	2
6	*E. coli*-Produced Cry2Ab2	3
7	*E. coli*-Produced Cry2Ab2	3
8	Blank	-
9	MON 87751-produced Cry2Ab2	1
10	MON 87751-produced Cry2Ab2	1
11	MON 87751-produced Cry2Ab2	2
12	MON 87751-produced Cry2Ab2	2
13	MON 87751-produced Cry2Ab2	3
14	MON 87751-produced Cry2Ab2	3
15	Blank	

Table C-6. Comparison of Immunoreactive Signal Between MON 87751- and *E. coli*-Produced Cry2Ab2 Proteins

Mean signal intensity from MON 87751-produced Cry2Ab2[1] (OD x mm^2)	Mean signal intensity from *E. coli*-produced Cry2Ab2[1] (OD x mm^2)	Acceptance limits[2] (OD x mm^2)
5.04	5.46	3.55-7.37

[1]Each value represents the mean of six values (n=6)
[2] The acceptance limits are for the MON 87751-produced Cry2Ab2 protein and are based on the interval between +35% (5.46 × 1.35 = 7.37) and –35% (5.46 × 0.65 = 3.55) of the mean of the *E. coli* -produced Cry2Ab2 signal intensity across all loads.

C.2.6. Molecular Weight Estimation using SDS-PAGE

C.2.6.1. Methods

MON 87751-produced and *E. coli*-produced Cry2Ab2 protein were diluted in 1× loading buffer and heated to 95 °C for 5 min. The MON 87751-produced Cry2Ab2 protein was loaded in duplicate at ~1, ~ 2, and ~ 3 µg based on total protein concentration, onto a pre-cast (4-20%) polyacrylamide mini-gel (Invitrogen, Carlsbad, CA). The *E. coli*-produced Cry2Ab2 protein was loaded at 1 µg total protein in a single lane. Broad Range Molecular Weight Standards (Bio-Rad) were prepared and loaded on the gel in parallel. Following electrophoresis at a constant voltage, proteins were briefly fixed in 40% (v/v) methanol, 7% (v/v) acetic acid and stained for 17 h with Brilliant Blue G-Colloidal stain (Sigma-Aldrich). Gels were briefly destained in 10% (v/v) acetic acid, 25% (v/v) methanol followed by 6 h in 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer supplied with Quantity One software (Quant one version 4.4.0). Apparent MW and purity were reported as an average of all six lanes containing the MON 87751-produced Cry2Ab2 protein. The acceptance limits are the 95% prediction interval derived from data obtained for the apparent MW of the *E. coli*-produced Cry2Ab2 protein.

C.2.6.2. Results of Cry2Ab2 Protein Molecular Weight Equivalence

The intact MON 87751-produced Cry2Ab2 protein (Figure C-9, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced Cry2Ab2 protein (Figure C-9, lane 2) and the apparent MW was calculated to be 61.4 kDa (Table C-7). Because the experimentally determined apparent MW of the MON 87751-produced Cry2Ab2 protein was within the acceptance limits for equivalence (Table C-7), the MON 87751- and *E. coli*-produced Cry2Ab2 proteins were determined to have equivalent apparent molecular weights.

Table C-7. Molecular Weight Comparison Between MON 87751- and *E. coli*-Produced Cry2Ab2 Proteins

Apparent MW of MON 87751-produced Cry2Ab2Protein (kDa)	Apparent MW of *E. coli*-produced Cry2Ab2 Protein[1] (kDa)	Acceptance Limits (kDa)
61.4	60.1	58.7 - 61.5

[1]As reported on the Certificate of Analysis for lot 11351673

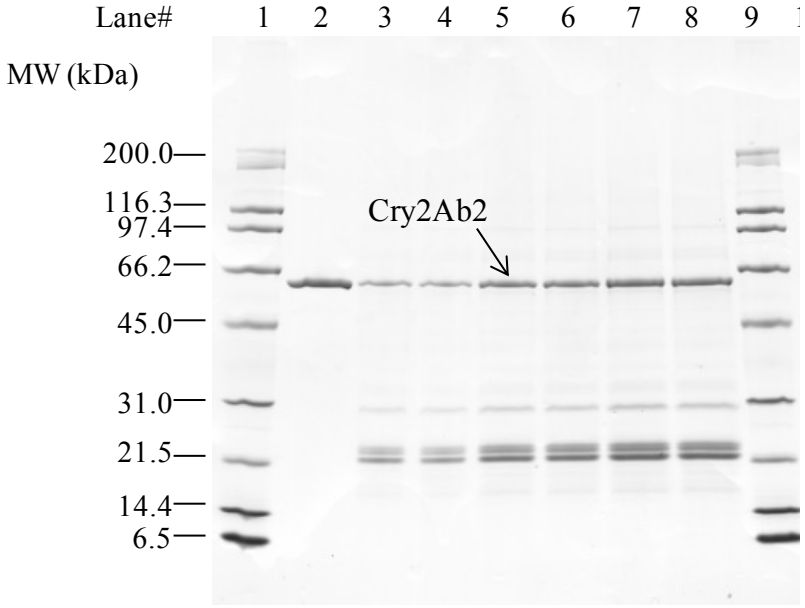

Figure C-9. Molecular Weight and Purity Analysis of MON 87751-Produced Cry2Ab2 Protein

Aliquots of the MON 87751-produced Cry2Ab2 and the *E. coli*-produced Cry2Ab2 proteins were subjected to SDS-PAGE and the gel was stained with Brilliant Blue G-Colloidal stain. The molecular weights (kDa) of the standards are shown on the left. Lane 10 was cropped from the image. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range MW Standards	4.5
2	*E. coli*-produced Cry2Ab2	1
3	MON 87751-produced Cry2Ab2	1
4	MON 87751-produced Cry2Ab2	1
5	MON 87751-produced Cry2Ab2	2
6	MON 87751-produced Cry2Ab2	2
7	MON 87751-produced Cry2Ab2	3
8	MON 87751-produced Cry2Ab2	3
9	Broad Range MW Standards	4.5
10	Blank	-

C.2.7. Glycosylation Analysis

C.2.7.1. Methods

An ECL Glycoprotein Detection Kit (GE Healthcare) was used for glycoprotein detection. The MON 87751-produced Cry2Ab2 protein, the *E. coli*-produced Cry2Ab2 protein, and a positive control, transferrin (Sigma, St. Louis, MO), were diluted in 1× LB and heated to 95 °C for 5 min. Two amounts (~100 and ~200 ng) of the intact MON 87751-produced Cry2Ab2 protein (purity corrected) and the *E. coli*-produced Cry2Ab2protein (purity corrected) were loaded onto a pre-cast Tris-glycine 4 - 20% polyacrylamide gradient mini-gel (Invitrogen). Four amounts (~50, ~100, ~150, and ~200 ng) of the positive control were loaded on the gel. Protein MW Standards (Precision Plus Protein™ Standards Dual color, Bio-Rad) were also loaded for molecular weight reference and to verify electrotransfer of the proteins to the membrane. Following electrophoresis at a constant voltage, proteins were electrotransferred to a PVDF membrane (Invitrogen).

Glycosylation analysis was performed on the PVDF membrane at room temperature using the ECL™ glycoprotein Detection Module (GE Healthcare) as directed by the manufacturer. Glycosylated proteins were detected using ECL™ reagents (GE Healthcare) and Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta). An identical gel was run and electrotransferred to a PVDF membrane in parallel. Proteins were stained with Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) and then destained with 1× Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad). After washing with water, the blot was scanned using Bio-Rad GS-800 densitometer.

C.2.7.2. Results of Glycosylation Analysis

Eukaryotic proteins can be post-translationally modified with carbohydrate moieties (Rademacher et al. 1988). To test whether Cry2Ab2 protein was glycosylated when expressed in the seed of MON 87751, the MON 87751-produced Cry2Ab2 protein was analyzed using an ECL ™ Glycoprotein Detection Module (GE Healthcare). To assess equivalence of the MON 87751- and *E. coli*-produced Cry2Ab2 proteins, the *E. coli*-produced Cry2Ab2 protein was also analyzed.

A clear glycosylation signal was observed at the expected (~80 kDa) in the lanes containing the positive control (transferrin) and the band intensity increased with increasing concentration (Figure C-10, panel A, lanes 1-4). In contrast, no glycosylation signals was observed in the lanes containing the MON 87751-produced Cry2Ab2 protein (Figure C-10, panel A, lanes 9 and 10) or *E. coli*-produced Cry2Ab2 protein (Figure C-10, panel A, lanes 7 and 8).

To confirm that MON 87751-produced and *E. coli*-produced Cry2Ab2 proteins were appropriately loaded for glycosylation analysis, a second membrane with identical loadings and transfer time was stained with Coomassie Blue R250 for protein detection (Figure C-10 Panel B). Both the MON 87751-produced Cry2Ab2 (Figure C-10 panel B,

lanes 9 and 10) and *E. coli*-produced Cry2Ab2 (Figure C-10 panel B, lanes 7 and 8) proteins were detected. These data indicate that the glycosylation status of MON 87751-produced Cry2Ab2 protein is equivalent to that of the *E. coli*-produced Cry2Ab2 protein and that neither is glycosylated.

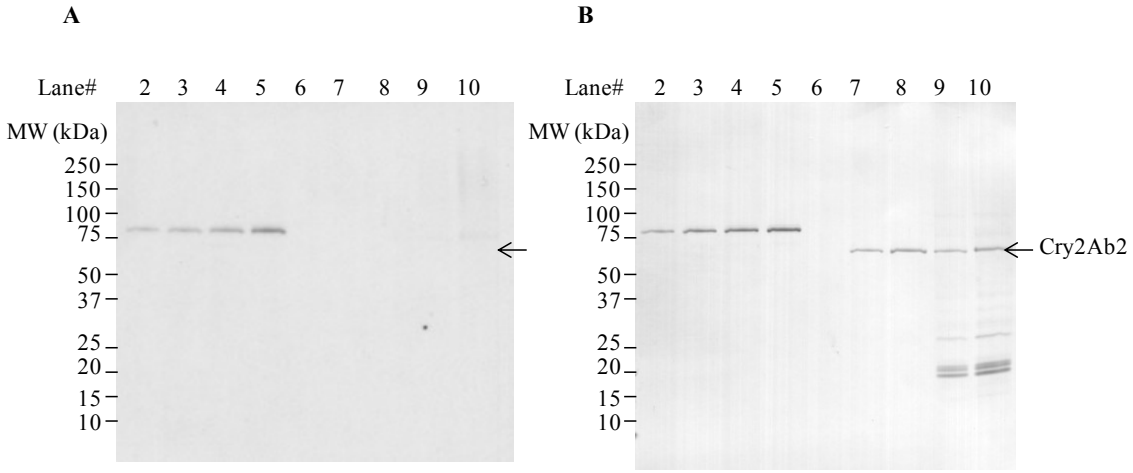

Figure C-10. Glycosylation Analysis of MON 87751-Produced Cry2Ab2 Protein

Aliquots of the transferrin (positive control), *E. coli*-produced Cry2Ab2 and MON 87751-produced Cry2Ab2 were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The MWs (kDa) correspond to the Precision Plus Protein™ Standards. Lane 1 in both images was loaded with MW standards and were cropped. The arrows show the expected migration of the MON 87751- and *E. coli*-produced Cry2Ab2 protein. (A) Where present, the labeled carbohydrate moieties were detected by addition of streptavidin conjugated to HRP followed by a luminol-based the detection using ECL reagents and exposure to Hyperfilm®. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Precision Plus Protein™ Standards	
2	Transferrin (positive control)	50
3	Transferrin (positive control)	100
4	Transferrin (positive control)	150
5	Transferrin (positive control)	200
6	Blank	
7	*E. coli* -produced Cry2Ab2	100
8	*E. coli* -produced Cry2Ab2	200
9	MON 87751-produced Cry2Ab2	100
10	MON 87751-produced Cry2Ab2	200

C.2.8. Functional Activity Analysis

C.2.8.1. Methods

Insects. CEW eggs were obtained from Benzon Research, Inc. (Carlisle, PA). The eggs were incubated at temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

Bioassays. CEW larvae (≤ 30 hours old) were used to measure biological activity of the MON 87751-produced and *E. coli*-produced Cry2Ab2 protein samples in accordance with the current version of Monsanto SOP BR-ME-1088. The bioassay was replicated twice on separate days, each with a separate batch of insects. The MON 87751-produced and *E. coli* -produced substances were run in parallel during each bioassay. Each bioassay replicate consisted of a series of seven dilutions yielding a dose series with a two-fold separation factor ranging from 0.0016 – 1.000 µg Cry2Ab2 protein/ml diet for the *E. coli* -produced and MON 87751-produced Cry2Ab2 and a single buffer control. The Cry2Ab2 protein dosing solutions were prepared by diluting the respective protein with purified water and incorporating the dilution into a multiple species diet (Southland Products, Inc.; Lake Village, AR). This dose series in diet was chosen to adequately characterize the dose-effect relationship of CEW growth inhibition for the *E. coli* -produced and MON 87751-produced Cry2Ab2 proteins. The diet mixture was then dispensed in 1.0 mL aliquots into a 128 well tray (#BAW128, Bio-Serv, Frenchtown, NJ). Individual insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BACV16, Bio-Serv, Frenchtown, NJ) and the insects were allowed to feed for a period of approximately 7-days in an environmental chamber programmed at 27° C, 60% relative humidity and a lighting regime of 14 light: 10 dark. The number of the surviving insects and their combined weight was recorded at each treatment level at the end of the seven-day incubation period. For a bioassay to be accepted, control mortality could not be >20%.

C.2.8.2. Results of Functional Activity

The functional activity of the MON 87751- produced and *E. coli*-produced Cry2Ab2 protein was determined by corn earworm diet incorporation assay. The MON 87751- and *E. coli*-produced Cry2Ab2 proteins were considered functionally equivalent if the EC_{50}, of both were within acceptance limits of 0.049 µg/ml diet to 0.204 µg/ml diet; the 95% prediction interval calculated from data obtained for the *E. coli*-produced Cry2Ab2 protein activity. The EC_{50} of the MON 87751-produced and *E. coli*-produced Cry2Ab2 proteins were determined to be 0.0734 and 0.115 µg Cry2Ab2/ml diet respectively (Table C-8). Because the EC_{50} of MON 87751-produced and *E. coli*-produced Cry2Ab2 proteins were within the acceptance limits (Table C-8), the proteins were determined to have equivalent functional activity.

Table C-8. Cry2Ab2 Functional Activity Assay

MON 87751-produced Cry2Ab2[1] EC_{50} (µg Cry2Ab2/ml diet)	*E. coli*-produced Cry2Ab2[1] EC_{50} (µg Cry2Ab2/ml diet)	Acceptance Limits[2] EC_{50} (µg Cry2Ab2/ml diet)
0.0734	0.1145	0.049 – 0.204

[1]Value refers to mean calculated based on n = 2. Values in these columns determined from proteins having the same amino acid sequence.

[2]Data obtained with *E. coli*-produced Cry2Ab2 was used to generate the acceptance limits. Values in this column represent a 95% prediction interval developed from a series of 11 assays with *E. coli*-produced Cry2Ab2 protein. Eight assays relied on a protein with an amino acid sequence identical to the MON 89034-produced Cry2Ab2 protein and three relied on a protein with an amino acid sequence identical to the MON 87751-produced protein.

References for Appendix C

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Appendix D: Materials and Methods Used for the Analysis of the Levels of Cry1A.105 and Cry2Ab2 Proteins in MON 87751

D.1. Materials

Over season leaf (OSL1-4), forage, root, and seed tissue samples from MON 87751 were harvested from five field sites in United States during the 2012 growing season from starting seed lot 11332614. Pollen/anther tissue of MON 87751 was also harvested from one field site in the United Sates during the 2012 growing season from starting seed lot 11332614. *Escherichia coli*-produced Cry1A.105 (lot 10000776) and *Bacillus thuringiensis*-produced Cry2Ab2 proteins (lot 10000747) were used as the analytical reference standards.

D.2. Characterization of the Materials

The identity of MON 87751 was confirmed by conducting MON 87751 event-specific polymerase chain reaction (PCR) analyses on the starting seed.

D.3. Field Design and Tissue Collection

Field trials were initiated during the 2012 planting season to generate MON 87751 at various soybean growing locations in The United States. OSL1-4, forage, root, and seed tissue samples from the following field sites were analyzed: Jackson County, Arkansas (site code ARNE); Jefferson County, Iowa (site code IARL); Pawnee County, Kansas (site code KSLA); Perquimans County, North Carolina (site code NCBD); and Lehigh County, Pennsylvania (site code PAGR). At each site, four replicated plots of plants containing MON 87751 were planted using a randomized complete-block field design. OSL1-4, forage, root, and seed tissue samples were collected from each replicated plot at all five field sites. Flowers for the extraction of pollen/anther tissue of MON 87751 was also collected during the 2012 United States growing season from a field site in Champaign County, Illinois (site code ILTH). At this site, tissue was collected from one non-randomized plot. See Tables V-1 and V-2 for detailed descriptions of when the samples were collected.

D.4. Tissue Processing and Protein Extraction

All tissue samples harvested were shipped to Monsanto's processing facility, and were ground, except flowers, by the Monsanto Sample Management Team to facilitate protein extraction. The ground tissue samples, and flowers, were stored in a -80 °C freezer until transferred on dry ice to the analytical facility. Once the flowers were received by the analytical facility, pollen/anther tissue was extracted from the flowers using forceps. Flowers and pollen/anther tissue was kept frozen on dry ice while extracting the tissue.

D.4.1. Cry1A.105 Protein

The Cry1A.105 protein was extracted from soybean tissues as described in Table D-1. The protein extracts were aliquoted and stored frozen in a -80 °C freezer until analysis.

Table D-1. Protein Extraction Methods for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[2]/Forage/(Pollen/Anther)	1:100	50 mM sodium carbonate + 10 mM DTT[3]
Root	1:25	50 mM sodium carbonate + 10 mM DTT
Seed	1:40	50 mM sodium carbonate + 10 mM DTT

[1]Cry1A.105 protein was extracted from each tissue by adding the appropriate volume of extraction buffer and chrome steel beads, and shaking in a Harbil mixer (Harbil Industries Inc., Compton, CA). The extracted samples were clarified using a serum filter, except root, which was clarified by centrifugation.
[2]Over season leaf (OSL1, OSL2, OSL3, and OSL4).
[3] 0.05 M Na_2CO_3, 0.07 % (v/v) Tween 20, and 0.01 M DL-dithiothreitol (DTT).

D.4.2. Cry2Ab2 Protein

The Cry2Ab2 protein was extracted from soybean tissues as described in Table D-2. The protein extracts were aliquoted and stored frozen in a -80 °C freezer until analysis.

Table D-2. Cry2Ab2 Protein Extraction Methods[1] for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[2]/Root/Seed/Forage /(Pollen/Anther)	1:100	1 × Tris-borate buffer[3]

[1]Cry2Ab2 protein was extracted from each tissue by adding the appropriate volume of extraction buffer and chrome steel beads, and shaking in a Harbil mixer (Harbil Industries Inc., Compton, CA). The extracted samples were clarified using a serum filter.
[2]Over season leaf (OSL1, OSL2, OSL3, and OSL4).
[3]0.1 M Tris, 0.1 M $Na_2B_4O_7$, 0.005 M $MgCl_2$, 0.05 % (v/v) Tween 20, pH 7.8.

D.5. Cry1A.105 and Cry2Ab2 Antibodies

D.5.1. Cry1A.105 Antibodies

Mouse monoclonal antibody (lot G-876133) specific for the Cry1A.105 protein was purified using Protein G affinity chromatography. The concentration of the purified antibody was determined to be 7.6 mg/ml by spectrophotometric methods. The purified antibody was stored in phosphate buffered saline (0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, 0.0027 M KCl, and 0.05 % NaN_3).

Goat polyclonal antibodies specific for the Cry1A.105 protein were purified using Protein G affinity chromatography. The purified antibodies were coupled with biotin (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and assigned lot G-848052. The detection reagent was NeutrAvidin conjugated to horseradish peroxidase (Thermo Fisher Scientific).

D.5.2. Cry2Ab2 Antibodies

Mouse monoclonal antibody (lot G-848028) specific for the Cry2Ab2 protein was purified using Protein G affinity chromatography. The concentration of the purified antibody was determined to be 1.57 mg/ml by spectrophotometric methods. The purified antibody was stored in phosphate buffered saline (0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, 0.0027 M KCl and 0.015 M NaN_3).

Goat polyclonal antibodies specific for the Cry2Ab2 protein were purified using Protein G affinity chromatography. The purified Cry2Ab2 antibodies were coupled with biotin (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and assigned lot G-842887. The detection reagent was NeutrAvidin conjugated to horseradish peroxidase (Thermo Fisher Scientific).

D.6. Cry1A.105 and Cry2Ab2 ELISA Methods

D.6.1. Cry1A.105 Protein

Mouse anti-Cry1A.105 capture antibody was diluted in a coating buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$) and immobilized onto 96-well microtiter plates at 2 µg/ml followed by incubation in a 4 °C refrigerator for ≥12 hours. Prior to each step in the assay, plates were washed with 1 × phosphate buffered saline containing 0.05 % (v/v) Tween 20. Plates were blocked with the addition of 150 µl per well of blocking buffer, 5 % (w/v) bovine serum albumin in 1 × phosphate buffered saline containing 0.05 % (v/v) Tween 20 for 60 to 70 minutes at 37 °C. Cry1A.105 protein standard or sample extract was added at 100 µl per well and incubated for 60 to 65 minutes at 37 °C. Biotinylated goat anti-Cry1A.105 antibodies were added at 100 µl per well and incubated for 60 to 65 minutes at 37 °C. NeutraAvidin-horseradish peroxidase conjugate was added at 100 µl per well and incubated for 30 to 35 minutes at 37 °C. Plates were developed by adding 100 µl per well of horseradish peroxidase substrate, 3,3',5,5'-tetramethyl-benzidine (Kirkegaard & Perry, Gaithersburg, MD). The enzymatic

reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantification of the Cry1A.105 protein was accomplished by interpolation on a Cry1A.105 protein standard curve that ranged from 0 − 100 ng/ml.

D.6.2. Cry2Ab2 Protein

Mouse anti-Cry2Ab2 capture antibody were diluted in a coating buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$) and immobilized onto 96-well microtiter plates at 2 µg/ml followed by incubation in a 4 °C refrigerator for ≥12 hours. Prior to each step in the assay, plates were washed with 1 × phosphate buffered saline containing 0.05 % (v/v) Tween 20. Plates were blocked with the addition of 200 µl per well of 5 % (w/v) non-fat dry milk in 1 × phosphate buffered saline containing 0.05 % (v/v) Tween 20 for 60 to 90 minutes at 37 °C. Cry2Ab2 protein standard or sample extract was added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. Biotinylated goat anti-Cry2Ab2 antibodies were added at 100 µl per well and incubated for 60 to 70 minutes at 37 °C. NeutraAvidin- horseradish peroxidase conjugate was added at 100 µl per well and incubated for 30 to 35 minutes at 37 °C. Plates were developed by adding 100 µl per well of horseradish peroxidase substrate, 3,3',5,5'-tetramethyl-benzidine (Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H3PO4. Quantification of the Cry2Ab2 protein was accomplished by interpolation on a Cry2Ab2 protein standard curve that ranged from 0 − 1000 ng/ml.

D.7. Moisture Analysis

Moisture content was determined in all tissues, except pollen/anther, using a Mettler Toledo HR83 Moisture Analyzer System (Mettler-Toledo, Inc. Columbus, OH). A homogeneous tissue-specific site pool (TSSP) was prepared consisting of samples of a given tissue type grown at a specific site. The mean percent moisture for each TSSP was calculated from triplicate analyses. A TSSP dry weight conversion factor (DWCF) was calculated using Moisture Direct version 4.0 software as follows:

$$\text{DWCF} = 1 - \left(\frac{\text{Mean\% TSSP Moisture}}{100}\right)$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fw) basis into levels reported on a µg/g dry weight (dw) basis using the following calculation:

$$\text{Protein Level in Dry Weight} = \frac{\text{Protein Level Fresh Weight}}{\text{DWCF}}$$

The protein levels (ng/ml) that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

Due to a limited amount of tissue, pollen/anther was not analyzed for moisture content. Therefore, no dry weight calculation was performed and pollen/anther was reported on a µg/g fw basis only.

D.8. Data Analyses

ELISA plates were analyzed on a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. Protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GxP version 5.4 software. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit for Cry1A.105 protein. Absorbance readings and protein standard concentrations were fitted with a five-parameter logistic curve fit for Cry2Ab2 protein. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a "µg/g fw" basis for data that were greater than or equal to the LOQ. This conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values in "µg/g fw" were also converted to "µg/g dw" by applying the DWCF, except for expression levels in pollen/anther. Microsoft Excel 2007 (Microsoft, Redmond, WA) was used to calculate the protein levels in tissues. The sample means, standard deviations (SDs), and ranges were also calculated using Microsoft Excel 2007. All protein expression levels were rounded to two significant figures.

A test substance extract that resulted in unexpectedly negative result by ELISA analysis was re-extracted twice for the protein of interest and re-analyzed by ELISA to confirm the result. The sample with the confirmed unexpected result was omitted from all calculations.

Appendix E: Materials and Methods for Compositional Analysis of MON 87751 Seed and Forage

Compositional comparisons between MON 87751 and the conventional control soybean variety were performed using the principles and analytes outlined in the OECD consensus documents for soybean composition (OECD 2012). These principles are accepted globally and have been employed previously in assessments of soybean products derived through biotechnology. The compositional assessment was conducted on seed and forage samples harvested from a single growing season conducted in the United States during 2012 under normal agronomic practices.

E.1. Materials

Harvested seed and forage from MON 87751, a conventional control that has similar genetic background to that of MON 87751, and conventional, commercial reference soybean varieties were compositionally assessed. The reference varieties are listed in Table E-1.

Table E-1. Commercial Reference Soybean Varieties

Material	Orion ID	Field Site Code(s)
DWIGHT	10001434	IARL, PAGR
Crows C2804	11242902	IAHU, IARL
Garst 3585N	11242913	IAHU, IARL, PAGR,KSLA
Midland 363	11243106	IAHU, IARL
Crows C3908	10001074	NCBD
NuPride 3202	11226938	NECC
C3211N	11226860	PAGR, KSLA
Midwest Genetics G2712	11242900	IAHU, PAGR
A3244	11212038	KSLA, NECC
Stewart SB3819	11226928	KSLA, ARNE
Wilken 3316	11242907	ILTH
Hoffman HS387	11225760	ILTH
LG C3540	11226858	NECC
Stine 3300-0	10001134	ILTH, ARNE
A3525	10001257	ILTH, ARNE
Lewis 391	10001125	ARNE, NCBD
WILLIAMS 82	11225762	NCBD
Stewart SB3454	10000887	NCBD
Crows C37003N	10001508	NECC

E.2. Characterization of the Materials

The starting seed for MON 87751 and the control were characterized by event-specific polymerase chain reaction (PCR) analysis for the presence or absence of the events included in the field production for producing starting seed. The reference substances were characterized via the manufacturer label. Chain-of-custody documentation supplied with the harvested seed and forage samples of the test, control and reference (T/C/R) substances from the field plots were used to confirm sample identity prior to use in the compositional assessment.

E.3. Field Production of the Samples

Seed and forage samples from MON 87751, the control, and the reference varieties were collected from eight replicated sites in the United States during the 2012 growing season. The field sites were located in: Jackson County, Arkansas (ARNE); Story County, Iowa (IAHU); Jefferson County, Iowa (IARL); Champaign County, Illinois (ILTH); Pawnee County, Kansas (KSLA); Perquimans County, North Carolina (NCBD); Merrick County, Nebraska (NECC) and Lehigh County, Pennsylvania (PAGR). Starting seeds were planted in a randomized complete block design with four plots for each of MON 87751, the control, and the reference varieties. The production was conducted under normal agronomic field conditions for their respective geographic regions that are typical areas for soybean production in the United States.

Forage was collected at the R6 growth state and seed was collected at physiological maturity. Forage samples were shipped on dry ice and seed was shipped at ambient temperature from the field sites to Monsanto Company (Saint Louis, Missouri). Subsamples were ground to a powder, stored in a freezer set to maintain -20°C located at Monsanto Company (Saint Louis, Missouri). Subsamples were shipped on dry ice to Covance Laboratories Inc. (Madison, Wisconsin) for compositional analysis.

E.4. Summary of Analytical Methods

Nutrients analyzed in this study included moisture, ash, protein, total fat, carbohydrates by calculation, acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids (18 components), fatty acids (22 components), minerals (calcium and phosphorus), vitamin E (α-tocopherol) and vitamin K1 (phylloquinone), in the seed, and moisture, ash, protein, total fat, carbohydrates by calculation, ADF and NDF in the forage. The anti-nutrients assessed in seed included lectin, phytic acid, raffinose, stachyose and trypsin inhibitor. Other components assessed in seed included isoflavones.

All compositional analyses were performed at Covance Laboratories, Inc. (Madison, Wisconsin). Methods for analysis were based on internationally-recognized procedures and literature publications. Brief descriptions of the methods utilized for the analyses are described below.

E.4.1 Acid Detergent Fiber

The ANKOM2000 Fiber Analyzer automated the process of removal of proteins, carbohydrates, and ash. Fats and pigments were removed with an acetone wash prior to analysis. The fibrous residue that was primarily cellulose and lignin and insoluble protein complexes remained in the Ankom filter bag, and was determined gravimetrically (Komarek et al. 1993; USDA 1970). The limit of quantitation was 0.100%.

E.4.2. Amino Acid Composition

The following 18 amino acids were analyzed:

- Total alanine
- Total arginine
- Total aspartic acid (including asparagine)
- Total cystine (including cysteine)
- Total glutamic acid (including glutamine)
- Total glycine
- Total histidine
- Total isoleucine
- Total leucine
- Total lysine
- Total methionine
- Total phenylalanine
- Total proline
- Total serine
- Total threonine
- Total tryptophan
- Total tyrosine
- Total valine

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

The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection (AOAC 2012e; Barkholt and Jensen 1989; Henderson et al. 2000; Schuster 1988). The limit of quantitation was 0.100 mg/g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
L-Alanine	Sigma-Aldrich	BCBC5470	99.8
L-Arginine Monohydrochloride	Sigma-Aldrich	1361811	100
L-Aspartic Acid	Sigma-Aldrich	BCBB9274	100.6
L-Cystine	Sigma-Aldrich	1451329	100
L-Glutamic Acid	Sigma-Aldrich	1423805	100.2
Glycine	Sigma-Aldrich	1119375	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	BCBB1348	99.9
L-Isoleucine	Sigma-Aldrich	1423806	100
L-Leucine	Sigma-Aldrich	BCBC6907	99.9
L-Lysine Monohydrochloride	Sigma-Aldrich	1362380	100.2
L-Methionine	Sigma-Aldrich	1423807	99.9
L-Phenylalanine	Sigma-Aldrich	BCBC5774	100
L-Proline	Sigma-Aldrich	1414414	99.7
L-Serine	Sigma-Aldrich	1336081	99.9
L-Threonine	Sigma-Aldrich	1402329	100
L-Tyrosine	Sigma-Aldrich	BCBC2417	100
L-Valine	Sigma-Aldrich	1352709	100
L-Tryptophan	Sigma-Aldrich	BCBC1685	>99

E.4.3 Ash

All organic matter was driven off when the samples were ignited at approximately 550ºC in a muffle furnace for at least 5 hours. The remaining inorganic material was determined gravimetrically and referred to as ash (AOAC 2012g). The limit of quantitation was 0.100%.

E.4.4 Carbohydrate

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation (USDA 1973):

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

The limit of quantitation was calculated as 0.100%.

E.4.5 Fat by Acid Hydrolysis

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 (AOAC 2012a; h). The limit of quantitation was 0.100%.

E.4.6 Fat by Soxhlet Extraction

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 (AOAC 2012f; j). The limit of quantitation was 0.100%.

E.4.7 Fatty Acids

The following 22 fatty acids were analyzed:

- 8:0 Caprylic
- 10:0 Capric
- 12:0 Lauric
- 14:0 Myristic
- 14:1 Myristoleic
- 15:0 Pentadecanoic
- 15:1 Pentadecenoic
- 16:0 Palmitic
- 16:1 Palmitoleic
- 17:0 Heptadecanoic
- 17:1 Heptadecenoic
- 18:0 Stearic
- 18:1 Oleic
- 18:2 Linoleic
- 18:3 gamma-Linolenic
- 18:3 Linolenic
- 20:0 Arachidic
- 20:1 Eicosenoic
- 20:2 Eicosadienoic
- 20:3 Eicosatrienoic
- 20:4 Arachidonic
- 22:0 Behenic

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 (AOCS 2009a; b). The limit of quantitation was 0.0200%.

Reference Standards:

Manufacturer	Lot No.	Component	Weight (%)		Purity (%)
			JY10-W	MA7-W	
Nu-Chek Prep GLC Reference Standard Covance 1 Covance 2	JY10-W MA7-W	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	15.75	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	14.00	99.8
		Methyl Oleate	10.0	15.75	99.8
		Methyl Linoleate	10.0	15.75	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 Eicosatrienoate	1.0	1.25	99.5
		Methyl Arachidonate	1.0	1.25	99.4
		Methyl Behenate	1.0	1.25	99.8

E.4.8 Minerals/ ICP Emission Spectrometry

The following two minerals were analyzed:

- Calcium
- Phosphorus

The samples were dried, precharred, and ashed overnight in a muffle furnace set to maintain 500°C. The ashed samples were re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown samples, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions (AOAC 2012c; d).

Reference Standards:

Inorganic Ventures Reference Standards and Limits of Quantitation:

Mineral	Lot Numbers	Concentration (µg/mL)	LOQ (ppm)
Calcium	F2-MEB417082MCA, F2-MEB417084	200, 1000	20.0
Phosphorus	F2-MEB417082MCA, F2-MEB417084	200, 1000	20.0

E.4.9 Isoflavones

The samples were extracted using a solution of hydrochloric acid and reagent alcohol heated on hot plates. The extracts were brought to volume, diluted, and centrifuged. An aliquot of the supernatants were placed onto a C18 solid-phase extraction column. Unwanted components of the matrix were rinsed off with 20% methanol and then the isoflavones were eluted with 80% methanol. The samples were analyzed on a highperformance liquid chromatography system with ultraviolet detection and were compared to an external standard curve of known standards for quantitation (Pettersson and Kiessling 1984; Seo and Morr 1984). The limit of quantitation was calculated as 10.0 µg/g.

Reference Standards:

Manufacturer	Component	Lot No.	Purity %
LC Laboratories	Daidzein	DA-121	99.7
Indofine Chemical Co.	Glycitein	0803103	96.08
LC Laboratories	Genistein	CH-148	99.7

E.4.10 Lectins

The samples were suspended in phosphate buffered saline (PBS), shaken, and filtered. An aliquot of the resulting extract was serially diluted in 10 cuvettes containing PBS. A 10% hematocrit of lyophilized rabbit blood in PBS was added to each dilution. After 2.5 hours, the absorbance of each dilution of the samples and lectin controls were measured on a spectrophotometer at 620 nm, using PBS to zero the instrument. One hemagglutinating unit (H.U.) was defined as the level that caused 50% of the standard cell suspension to sediment in 2.5 hours (Klurfeld and Kritchevsky 1987; Liener 1955). The limit of quantitation was calculated as 0.10 H.U./mg.

E.4.11 Moisture

The samples were dried in a vacuum oven at approximately 100°C. The moisture weight loss was determined and converted to percent moisture (AOAC 2012b; i). The limit of quantitation was 0.100%.

E.4.12 Neutral Detergent Fiber

The ANKOM2000 Fiber Analyzer automated the process of the removal of protein, carbohydrate, and ash. Fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin and insoluble protein fraction were left in the filter bag and determined gravimetrically (AACC 1998; Komarek et al. 1994; USDA 1970). The limit of quantitation was 0.100%.

E.4.13 Phytic acid

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 (Lehrfeld 1989; Lehrfeld 1994). The limit of quantitation was 0.125%.

Reference Standard:

Component	Manufacturer	Lot No.	Purity (%)
Phytic Acid Sodium Salt Hydrate	Sigma-Aldrich	BCBH8701V	97.9

E.4.14 Protein

The protein and other organic nitrogen in the samples were converted to ammonia by digesting the samples with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. Instrumentation was used to automate the digestion, distillation and titration processes. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOAC 2012l; k). The limit of quantitation was 0.100%.

E.4.15 Raffinose/Stachyose

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 (Brobst 1972; Mason and Slover 1971). The limit of quantitation was 0.0500%.

Reference Standards:

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

E.4.16 Trypsin Inhibitor

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. Absorbance was determined at 410 nm. Trypsin inhibitor activity was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DL-arginine~p~nitroanilide hydrochloride (AOCS 1997; Kakade et al. 1974). The limit of quantitation was calculated as 1.00 Trypsin Inhibitor Units (TIU)/mg.

E.4.17 Vitamin E

The samples were saponified to break down any fat and release vitamin E. The saponified mixtures were extracted with ethyl ether and then quantitated by high-performance liquid chromatography using a silica column (Cort et al. 1983; McMurray et al. 1980; Speek et al. 1985). The limit of quantitation was 0.500 mg/100g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
alpha-Tocopherol	USP	O0K291	98.5

E.4.18 Vitamin K

The samples were extracted with organic solvents and injected on a reverse phase high-performance liquid chromatography system with post-column reduction and fluorescence detection. Quantitation was achieved with linear regression analysis using a laboratory automation system (USP 1995; Woollard et al. 2002). The limit of quantitation was calculated as 0.0800 µg/g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
Phytonadione	USP	O0H310	99.7

E.5. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company for review. Data were then transferred to Certus International, Inc., where they were converted into the appropriate units and statistically analyzed. Means, standard errors, and a range of component values were determined for the test substance and the conventional control substance across all sites. The following formulas were used for re-expression of composition data for statistical analysis (Table E-2):

Table E-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates (excluding Moisture), Fiber, Phytic Acid, Raffinose, Stachyose	% fwt	% dwt	X/d
Isoflavones, Vitamin K	µg/g fwt	µg/g dwt	X/d
Lectin	H.U./mg fwt	H.U./mg dwt	X/d
Trypsin Inhibitor	TIU/mg fwt	TIU/mg dwt	X/d
Vitamin E	mg/100g fwt	mg/100g dwt	X/d
Minerals	ppm fwt	g/100g dwt	$X/(10^4 d)$
Amino Acids (AA)	mg/g fwt	% dwt	X/(10d)
Fatty Acids (FA)	% fwt	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA

[1] 'X' is the individual sample value; 'd' is the fraction of the sample that is dry matter.

In order to complete a statistical analysis for a compositional constituent in this study, at least 50% of all the values for an analyte in seed or forage had to be greater than the assay limit of quantitation (LOQ). Analytes with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following 14 analytes in seed, with more than 50% of observations below the assay LOQ, were excluded from statistical analysis: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic acid. 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid and 20:4 arachidonic acid.

The data were assessed for potential outliers by screening studentized PRESS residuals. A PRESS residual is the difference between any value and its value predicted from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual ranges are considered for exclusion, as outliers, from the final analyses. Four components had PRESS residual values outside the ± 6 range. Of the four flagged values, only the seed total fat and vitamin E values were removed from further analysis as outliers. The remaining values were not removed because they were not an extreme value or they were deemed sufficiently close to neighboring values and lacked evidence for removal.

The outlier test procedure was reapplied to the remaining seed total fat and vitamin E data to detect potential outliers that were masked in the first analysis. One vitamin E value from a commercial reference was identified as an outlier, but the value was not an extreme value and was not removed as an outlier.

Soybean compositional components were statistically analyzed using a mixed model analysis of variance with the SAS MIXED procedure.

Analyses of the combined replicated sites were performed using the following model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk,}$$

where Y_{ijk} = unique individual observation, U = overall mean, T_i = substance effect, L_j = random site effect, $B(L)_{jk}$ = random block within site effect, LT_{ij} = random site by substance interaction effect, and e_{ijk} = residual error.

For each component analysis, individual mean comparison tests of the test substance vs. conventional control were conducted.

A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured.

For each compositional component analyzed, two-sided 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of commercial substances. Each estimate was based upon the average of all observations per unique reference substance. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

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Appendix F: Materials, Methods, and Individual Site Results for Seed Dormancy and Germination Assessment of MON 87751

F.1. Materials

Seed of MON 87751, the conventional control A3555, and 12 commercial reference varieties were produced in Merrick County, NE (NECC site); Jefferson County, IA (IARL site); and Champaign County, IL (ILTH site) in 2012. The seed from each entry was harvested from four replications of each field trial site and pooled for use as starting seed in this study (Table F-1). Standardized germination assay was used to assess the dormancy and germination characteristics of the harvested seed.

F.2. Characterization of the Materials

The identities of the MON 87751 and the conventional control were verified by event-specific polymerase chain reaction (PCR) analyses prior to planting the three sites (NECC, IARL and ILTH). During the growing season, the field planting order of MON 87751 and the conventional control plots was confirmed by event-specific PCR analyses. Chain-of-custody documentation for all starting seed for dormancy and germination study was maintained from harvest through shipment to the performing laboratory with the use of packaging labels and plant sample transfer forms.

F.3. Germination Testing Facility and Experimental Methods

Seed dormancy and germination evaluations were conducted at BioDiagnostics, Inc. in River Falls, WI. The Principal Investigator was qualified to conduct seed dormancy and germination testing consistent with the standards established by the Association of Official Seed Analysts (AOSA, 2012a; b; AOSA/SCST, 2010). The performing laboratory employs appropriately educated, trained, and experienced personnel and maintains laboratory equipments of appropriate design and capacity to conduct this study.

Seed lots of test, control and reference varieties were produced from each of three sites and tested under six different temperature regimes of 10°C, 20°C, 30°C, 10°C/20°C, 10°C/30°C and 20°C/30°C. For each seed lot, four replicated paper germination towels were prepared per facility SOPs for each temperature regime. Wax coated paper was placed on a large tray followed by a water-moistened germination towel. A target of 100 seeds per seed lot were placed on the germination towel (*i.e.*, one seed lot per towel) using a vacuum planting system. Rolled germination towels were placed into appropriately labeled buckets that were arranged in the germination chambers in a split-plot design where the whole-plot was the seed production location and the sub-plot was the seed material (*i.e.*, test, control, or reference).

Each rolled germination towel in the AOSA-recommended temperature regime (*i.e.*, 20°C/30°C) was evaluated periodically during the study for normal germinated, abnormal germinated, viable hard, dead, and viable firm-swollen seed as defined by AOSA guidelines (AOSA, 2012a; b). Each rolled germination towel in the additional temperature regimes (*i.e.*, 10°C, 20°C, 30°C, 10°C/20°C, and 10°C/30°C) was evaluated for germinated, viable hard, dead, and viable firm-swollen seed. Emergence and/or

development of essential structures of seedlings that otherwise would be categorized as "normal germinated" under optimal temperature conditions may not be so at non-optimal temperatures. Therefore, for the additional temperature regimes, no distinction was made between normal and abnormal germinated seed.

F.4. Statistical Analysis

An analysis of variance was conducted according to a split-plot design using SAS® (SAS, 2012). MON 87751 was compared to the conventional soybean control for dormancy and germination characteristics of seed produced within each site (*i.e.*, individual-site analysis) and in a combined-site analysis in which the data were pooled across seed production sites. The seed dormancy and germination characteristics analyzed included percent germinated (categorized as percent normal germinated and percent abnormal germinated for the AOSA temperature regime), percent viable hard seed, percent dead seed, and percent viable firm swollen seed. The level of statistical significance was predetermined ($\alpha = 0.05$). MON 87751 was not statistically compared to the reference materials, nor were comparisons made across temperature regimes. The minimum and maximum mean values (reference range) were determined from the reference materials across the seed production sites.

F.5. Individual-Site Seed Dormancy and Germination Analysis

A total of 68 comparisons were evaluated in the individual-site analysis. Fifty nine out of 68 comparisons showed no difference between MON 87751 and the conventional control. Furthermore, no statistical comparisons could be made for seven additional comparisons due to lack of variability in the data. For these data, the values for MON 87751 and the control were identical, indicating no biological differences. Nine statistically significant differences were detected between MON 87751 and the conventional control for seed produced at the NECC, IARL, and ILTH sites (Table F-2). Percent viable hard seed was higher for MON 87751 than the conventional control at 20°C for seed produced at the IARL site (0.5% vs. 0.0%). MON 87751 had higher percent germinated seed than the conventional control at 10°C/30°C for seed produced at the ILTH site (99.5% vs. 98.3%). MON 87751 had lower percent dead seed than the conventional control at 10°C/30°C for seed produced at the ILTH site (0.5% vs. 1.5%). MON 87751 had higher percent normal germinated seed than the conventional control at 20°C/30°C for seed produced at the NECC site (85.5% vs. 78.3%). MON 87751 had lower percent abnormal germinated seed than the conventional control at 20°C/30°C for seed produced at the NECC site (14.5% vs. 21.0%). MON 87751 had lower percent viable hard seed than the conventional control at 20°C/30°C for seed produced at the NECC site (0.0% vs. 0.8%). MON 87751 had lower percent abnormal germinated seed than the conventional control at 20°C/30°C for seed produced at the ILTH site (1.5% vs. 4.0%). MON 87751 had higher percent viable hard seed than the conventional control at 20°C/30°C for seed produced at the ILTH site (0.5% vs. 0.0%). MON 87751 had lower percent viable firm swollen seed than the conventional control at 20°C/30°C for seed produced at the ILTH site (0.0% vs. 0.3%). These differences detected between MON 87751 and the conventional control for dormancy and germination characteristics in the individual-site analysis were not consistently detected across seed production sites or temperature regimes. Therefore, the

results indicate that there is no increased weed potential for MON 87751 compared to conventional soybean.

Table F-1. Starting Seed of MON 87751, Control and Commercial Soybean Reference Varieties Used in Dormancy Assessment

Site[1]	Material Type	Material Name	Phenotype	Material ID
NECC	Control	A3555	Conventional	11350793
NECC	Reference	LG C3540	Conventional	11350794
NECC	Reference	Crows C37003N	Conventional	11350795
NECC	Reference	A3244	Conventional	11350796
NECC	Reference	NuPride 3202	Conventional	11350797
NECC	Test	MON 87751	Insect protected	11350798
IARL	Control	A3555	Conventional	11350781
IARL	Reference	DWIGHT	Conventional	11350782
IARL	Reference	Crows C2804	Conventional	11350783
IARL	Reference	Garst 3585N	Conventional	11350784
IARL	Reference	Midland 363	Conventional	11350785
IARL	Test	MON 87751	Insect protected	11350786
ILTH	Control	A3555	Conventional	11350787
ILTH	Reference	Stine 3300-0	Conventional	11350788
ILTH	Reference	A3525	Conventional	11350789
ILTH	Reference	Hoffman HS387	Conventional	11350790
ILTH	Reference	Wilken 3316	Conventional	11350791
ILTH	Test	MON 87751	Insect protected	11350792

[1]NECC = Merrick County, NE; IARL = Jefferson County, IA; ILTH = Champaign County, IL.

Table F-2. Dormancy and Germination Characteristics of MON 87751 and the Conventional Control Seed Produced at each of the Three Field Sites

Temperature Regime	Germination Category	NECC[1] Mean %[2] (S.E.)[3]		IARL[1] Mean %[2] (S.E.)[3]		ILTH[1] Mean %[2] (S.E.)[3]	
		MON 87751	Control	MON 87751	Control	MON 87751	Control
10°C	Germinated	99.3 (0.48)	99.5 (0.29)	99.0 (0.41)	98.0 (0.41)	97.5 (0.29)	96.0 (0.82)
	Viable Hard	0.0 (0.00)	0.0 (0.00)	1.0 (0.41)	0.8 (0.25)	1.3 (0.25)	1.8 (0.85)
	Dead	0.8 (0.48)	0.3 (0.25)	0.0 (0.00)	0.8 (0.25)	0.8 (0.48)	1.8 (0.85)
	Viable Firm Swollen	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)	0.5 (0.29)	0.5 (0.29)	0.5 (0.50)
20°C	Germinated	99.8 (0.25)	100.0 (0.00)	99.3 (0.25)	99.5 (0.29)	98.5 (0.65)	97.8 (1.31)
	Viable Hard	0.0 (0.00)	0.0 (0.00)	0.5 (0.29)*	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)
	Dead	0.3 (0.25)	0.0 (0.00)	0.3 (0.25)	0.5 (0.29)	1.5 (0.65)	2.0 (1.08)
	Viable Firm Swollen	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.3 (0.25)
30°C	Germinated	99.5 (0.29)	99.8 (0.25)	100.0 (0.00)	99.3 (0.48)	99.3 (0.48)	99.0 (0.41)
	Viable Hard	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
	Dead	0.5 (0.29)	0.3 (0.25)	0.0 (0.00)	0.8 (0.48)	0.8 (0.48)	1.0 (0.41)
	Viable Firm Swollen	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)

Table F-2 (continued). Dormancy and Germination Characteristics of MON 87751 and the Conventional Control Seed Produced at each of the Three Field Sites

Temperature Regime	Germination Category	NECC [1] Mean %[2] (S.E.)[3]		IARL [1] Mean %[2] (S.E.)[3]		ILTH [1] Mean %[2] (S.E.)[3]	
		MON 87751	Control	MON 87751	Control	MON 87751	Control
10°C/20°C	Germinated	99.8 (0.25)	99.5 (0.29)	99.5 (0.29)	99.5 (0.29)	98.0 (1.08)	97.5 (0.65)
	Viable Hard	0.0 (0.00)	0.5 (0.29)	0.5 (0.29)	0.3 (0.25)	1.0 (0.41)	1.5 (0.65)
	Dead	0.3 (0.25)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	1.0 (0.71)	1.0 (0.00)
	Viable Firm Swollen	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)	0.0 (0.00)
10°C/30°C	Germinated	99.3 (0.48)	99.8 (0.25)	99.5 (0.29)	99.3 (0.25)	99.5 (0.50)*	98.3 (0.48)
	Viable Hard	0.0 (0.00)	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)	0.0 (0.00)	0.3 (0.25)
	Dead	0.8 (0.48)	0.3 (0.25)	0.3 (0.25)	0.8 (0.25)	0.5 (0.50)*	1.5 (0.50)
	Viable Firm Swollen	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)†	0.0 (0.00)
20°C/30°C	Normal Germinated	85.5 (1.26)*	78.3 (1.31)	91.3 (2.95)	93.0 (1.22)	96.8 (1.11)	93.8 (0.85)
(AOSA)	Abnormal Germinated	14.5 (1.26)*	21.0 (1.35)	8.3 (2.90)	6.8 (1.44)	1.5 (0.65)*	4.0 (0.82)
	Viable Hard	0.0 (0.00)*	0.8 (0.25)	0.3 (0.25)	0.3 (0.25)	0.5 (0.29)*	0.0 (0.00)
	Dead	0.0 (0.00)†	0.0 (0.00)	0.3 (0.25)	0.0 (0.00)	1.3 (0.75)	2.0 (0.00)
	Viable Firm Swollen	0.0 (0.00)†	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)*	0.3 (0.25)

Note: The experimental design was a split-plot with four replications and statistical analysis consisted of an analysis of variance (ANOVA).
*Statistically significant differences detected ($\alpha = 0.05$) between MON 87751 and the conventional soybean control.
[1] Site codes are as follows: NECC = Merrick County, NE; IARL = Jefferson County, IA; ILTH = Champaign County, IL.
[2] In some instances, the total percentage of MON 87751 or the control did not equal 100% due to numerical rounding of the means.
[3] S.E. = Standard Error.
†No statistical comparison could be made due to lack of variability in the data.

References for Appendix F

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AOSA. 2012b. Rules for testing seeds. Volume 4. Seedling evaluation. Association of Official Seed Analysts, Ithaca, New York.

AOSA/SCST. 2010. Tetrazolium testing handbook. Association of Official Seed Analysts and the Society of Commercial Seed Technologists, Ithaca, New York.

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Appendix G: Materials, Methods, and Individual Site Results from Phenotypic, Agronomic, and Environmental Interaction Assessment of MON 87751 under Field Conditions

G.1. Materials

Agronomic, phenotypic, and environmental interaction characteristics were assessed for MON 87751, conventional control, and 34 reference varieties grown under similar agronomic conditions. Four reference varieties were planted per site (Table G-1).

G.2. Characterization of the Materials

The presence or absence of the MON 87751 event in the starting seed of MON 87751 and the conventional control was verified by event-specific polymerase chain reaction (PCR) analyses. No molecular analyses were performed on the reference starting seed.

G.3. Field Sites and Plot Design

Field trials were grown in 2012 at 17 sites that provided a range of environmental and agronomic conditions representative of U.S. soybean growing regions (Section VII, Table VII-3). The Principal Investigator at each site was familiar with soybean growth and production, and evaluation of soybean characteristics.

At all sites, seed of MON 87751, the conventional control, and four conventional reference varieties were planted in a randomized complete block design with four replications. At 12 of the study sites (ARNE, IAHU, IARL, ILCY, ILMN, ILTH, KSLA, MOFI, NCBD, NECC, OHTR, and PAGR) each replicated plot consisted of 6 rows of soybean spaced approximately 0.76m apart and approximately 6m long (Table G-2). The plots were separated by two rows of conventional soybean along their length. The entire trial area was surrounded by a border of conventional soybean approximately 3m wide. Phenotypic and qualitative environmental interaction data were collected from rows 4 and 5.

At five sites (GACH, IABG, ILAG, LACH, and SCEK) each replicated plot consisted of 16 rows of soybean spaced approximately 0.76 – 1.00m apart and approximately 6.1m long (Table G-2). The entire trial area was surrounded by a border of conventional soybean approximately 3m wide. Phenotypic and qualitative environmental interaction data were collected from rows 2 and 3 with the exception of the following: rows 4 and 5 were used at GACH site in plots 101, 201, 301, and 401. Additionally, rows 1 and 2 were utilized in all plots at ILAG site. Rows 6, 8, 10, and 12 were used to collect arthropod samples using vertical beat sheets at all sites. Rows 13 and 14 were used to assess plant damage caused by bean leaf beetle and stink bug at all sites except LACH where rows 2 and 3 were utilized.

G.4. Planting and Field Operations

Planting information, soil description, and cropping history of the trial area are listed in Table G-2. Prior to planting, the Principal Investigator at each site prepared the plot area

with a proper seed bed according to local agronomic practices, including tillage, fertilization, and pest management. During the growing season, all plots were assessed for agronomic conditions and pest populations, including pest arthropods, diseases and weeds. Fertilizer, irrigation, agricultural chemicals, and other management practices were applied as necessary. Maintenance operations were performed uniformly across all plots.

Table G-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interaction Assessment

Site Code[1]	Material Name	Monsanto Lot Number	Phenotype[2]	T/C/R[3]
All	MON 87751	11332614	IP	T
All	A3555	11332613	Conventional	C
ARNE	Lewis 391	10001125	Conventional	R
	Stine 3300-0	10001134	Conventional	R
	A3525	10001257	Conventional	R
	Stewart SB3819	11226928	Conventional	R
GACH	Stewart SB3454	10000887	Conventional	R
	Pioneer 93M52	11266955	Conventional	R
	FS Seed HiSoy HS3846	11233959	RR	R
	Midwest Genetics G2712	11242900	Conventional	R
IABG	Croplan HT3596STS	10001450	Conventional	R
	Crows C2804	11242902	Conventional	R
	Stewart SB2772R	11226922	RR	R
	NK S30-D4	11226843	RR	R
IAHU	Garst 3585N	11242913	Conventional	R
	Midwest Genetics G2712	11242900	Conventional	R
	Crows C2804	11242902	Conventional	R
	Midland 363	11243106	Conventional	R
IARL	DWIGHT	10001434	Conventional	R
	Crows C2804	11242902	Conventional	R
	Garst 3585N	11242913	Conventional	R
	Midland 363	11243106	Conventional	R
ILAG	FS 3591	10001448	Conventional	R
	Wilken 3316	11242907	Conventional	R
	Midland Phillips 299NR	11226699	RR	R
	A2553	11242899	Conventional	R
ILCY	LG C3540	11226858	Conventional	R
	NuPride 2954	11213020	Conventional	R
	C3211N	11226860	Conventional	R
	Lewis 372	11242918	Conventional	R
ILMN	NuPride 2954	11213020	Conventional	R
	Wilken 3316	11242907	Conventional	R
	Lewis 372	11242918	Conventional	R
	Hoffman HS387	11225760	Conventional	R
ILTH	Stine 3300-0	10001134	Conventional	R
	A3525	10001257	Conventional	R
	Hoffman HS387	11225760	Conventional	R
	Wilken 3316	11242907	Conventional	R

Table G-1. Starting Seed for Phenotypic, Agronomic, and Environmental Interaction Assessment (continued)

Site Code[1]	Material Name	Monsanto Lot Number	Phenotype[2]	T/C/R[3]
KSLA	Garst 3585N	11242913	Conventional	R
	A3244	11212038	Conventional	R
	C3211N	11226860	Conventional	R
	Stewart SB3819	11226928	Conventional	R
LACH	Midland 363	11243106	Conventional	R
	A3244	11212038	Conventional	R
	eMerge 348TC	11266960	Conventional	R
	SB3888R	11226926	RR	R
MOFI	Stewart SB3454	10000887	Conventional	R
	Crows C3908	10001074	Conventional	R
	WILLIAMS 82	11225762	Conventional	R
	Hoffman H419	11273007	Conventional	R
NCBD	Stewart SB3454	10000887	Conventional	R
	Crows C3908	10001074	Conventional	R
	Lewis 391	10001125	Conventional	R
	WILLIAMS 82	11225762	Conventional	R
NECC	LG C3540	11226858	Conventional	R
	Crows C37003N	10001508	Conventional	R
	A3244	11212038	Conventional	R
	NuPride 3202	11226938	Conventional	R
OHTR	Crows C3908	10001074	Conventional	R
	NuPride 3202	11226938	Conventional	R
	C3211N	11226860	Conventional	R
	Hoffman H419	11273007	Conventional	R
PAGR	DWIGHT	10001434	Conventional	R
	Garst 3585N	11242913	Conventional	R
	Midwest Genetics G2712	11242900	Conventional	R
	C3211N	11226860	Conventional	R
SCEK	Stewart SB3819	11226928	Conventional	R
	A3244	11212038	Conventional	R
	Pioneer 93M62	11226582	Conventional	R
	Stewart SB3993R	11226927	RR	R

[1] Site code: ARNE = Newport, AR; GACH = Chula, GA; IABG = Bagley, IA; IAHU = Huxley, IA; IARL = Richland, IA; ILAG = Thomasboro, IL; ILCY = Carlyle, IL; ILMN = Monmouth, IL; ILTH = Thomasboro, IL; KSLA = Larned, KS; LACH = Cheneyville, LA; MOFI = Fisk, MO; NCBD = Belvidere, NC; NECC = Waco, NE; OHTR = Troy, OH; PAGR = Germansville, PA; SCEK = Elko, SC.
[2] Phenotype: IP = Insect-Protected; RR = Roundup Ready® (glyphosate-tolerant).
[3] T/C/R = test (T), control (C), or reference (R) starting material.

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Table G-2. Field and Planting Information

Site[1]	Planting Date[2]	Harvest Date[2]	Approximate Planting Rate (seeds/m)	Approximate Plot Size[3] (m × m)	Rows per Plot	Soil Type	% OM[4]	Previous Crop 2011
ARNE	05/23/2012	09/27/2012	29.5	4.6 × 6.0	6	Silt Loam	1.8	Cotton
GACH	05/02/2012	09/07/2012	29.5	13.7 × 6.1	16	Sandy Loam	<1.0	Watermelon
IABG	05/16/2012	10/16/2012	29.5	11.4 × 6.1	16	Loam	4.0	Maize
IAHU	05/22/2012	10/11/2012	29.5	4.6 × 6.0	6	Loam	5.5	Maize
IARL	05/15/2012	10/09/2012	29.5	4.6 × 6.0	6	Silt Clay Loam	2.8	Sorghum
ILAG	05/10/2012	10/11/2012	29.5	11.4 × 6.1	16	Elliot Silt Loam	3.5	Maize
ILCY	06/14/2012	10/29/2012	29.5	4.6 × 6.0	6	Silt Loam	2.5	Milo
ILMN	05/18/2012	11/01/2012	29.5	4.6 × 6.0	6	Silt Clay Loam	4.5	Maize
ILTH	05/11/2012	10/12/2012	29.5	4.6 × 6.0	6	Silt Clay Loam	3.2	Maize
KSLA	05/15/2012	10/05/2012	29.5	4.6 × 6.0	6	Silt Loam	3.1	Sorghum
LACH	05/16/2012	09/26/2012	29.5	15.2 × 6.1	16	Silt	1.3	Soybean
MOFI	06/06/2012	10/08/2012	29.5	4.6 × 6.0	6	Sandy Loam	2.1	Rice
NCBD	05/21/2012	10/12/2012	26.2	4.6 × 6.0	6	Sandy Loam	23.9	Cotton
NECC	05/16/2012	10/09/2012	29.5	4.6 × 6.0	6	Loam	2.0	Maize
OHTR	05/15/2012	10/24/2012	29.5	4.6 × 6.0	6	Silt Clay Loam	3.1	Maize
PAGR	06/07/2012	10/22/2012	29.5	4.6 × 6.0	6	Loam	3.2	Soybean
SCEK	05/19/2012	09/25/2012	14.8	13.7 × 6.1	16	Sandy Loam	1.5	Cotton

[1] Site code: ARNE = Newport, AR; GACH = Chula, GA; IABG = Bagley, IA; IAHU = Huxley, IA; IARL = Richland, IA; ILAG = Thomasboro, IL; ILCY = Carlyle, IL; ILMN = Monmouth, IL; ILTH = Thomasboro, IL; KSLA = Larned, KS; LACH = Cheneyville, LA; MOFI = Fisk, MO; NCBD = Belvidere, NC; NECC = Waco, NE; OHTR = Troy, OH; PAGR = Germansville, PA; SCEK = Elko, SC.
[2] Planting and Harvest Date = mm/dd/yyyy.
[3] Width × length in meters.
[4] % OM = Percent Organic Matter.

G.5. Phenotypic Observations

The description of the characteristics measured and the designated developmental stages when observations occurred are listed in Section VII, Table VII-1.

G.6. Environmental Observations

Environmental interactions (*i.e.*, interactions between the crop plants and their receiving environment) were used to characterize MON 87751 by evaluating plant response to abiotic stressors, disease damage, and arthropod-related damage using qualitative methods described in Section G.7. In addition, specific arthropod damage from bean leaf beetle and stink bug (Section G.7.) and arthropod abundance were evaluated using the quantitative methods (Section G.8.).

G.7. Plant Response to Abiotic Stress, Disease Damage, and Arthropod-Related Damage

MON 87751, control, and reference varieties were evaluated at 17 sites for plant response to abiotic stressors, disease damage, and arthropod damage. A target of three abiotic stressors, three diseases, and three arthropod pests were evaluated four times during the following four crop developmental stages: late vegetative – R1, R2 – R3, R4 – R5, and R6 – R8.

Abiotic stressors, disease damage and arthropod damage observations were collected from each plot using the categorical scale of increasing severity listed below:

Category	Severity of plant damage
None	No symptoms observed
Slight	Symptoms not damaging to plant development (*e.g.*, minor feeding or minor lesions); mitigation likely not required
Moderate	Intermediate between slight and severe; likely requires mitigation
Severe	Symptoms damaging to plant development (*e.g.*, stunting or death); mitigation unlikely to be effective

Prior to each data collection, soybean was surveyed in proximity to the study area or the border rows of the study for abiotic stressors (*e.g.*, drought), diseases (*e.g.*, Asian rust), and arthropod damage (*e.g.*, aphids). The Principal Investigator chose three abiotic stressors, three diseases, and three arthropod species that are actively causing damage for subsequent evaluation in the study plots. The Principal Investigators were requested to select additional stressors if present. If fewer than three abiotic stressors, diseases, or arthropod species were present, the cooperator chose additional abiotic stressors, diseases, and arthropod species that are known to commonly occur in that geographical region and cause damage at the study site at that time. All plots at a site were rated for the same abiotic stressors, diseases, and arthropod pests at a given observation, even if that selected stressor was not present in some or all of the plots. If a selected stressor was not present, the cooperator recorded the rating as "none". The type of abiotic stressors,

diseases, and arthropod pests assessed varied between observations at a site and between sites.

Specific arthropod (bean leaf beetle and stink bug) damage was evaluated quantitatively from observations performed at five sites (*i.e.*, GACH, IABG, ILAG, LACH, and SCEK sites). Bean leaf beetle damage was assessed by examining ten plants (five consecutive plants per designated row) in each plot. The first assessment date was conducted two to three weeks following emergence. The second assessment was conducted approximately two weeks later. At each assessment time, each plant was rated on a scale of zero to five adapted from Koch et al. (Koch et al. 2005) as described below:

Rating	Rating Description[1]
0	No symptoms observed
1	≤ 5 shot-holes per leaf
2	6-10 shot-holes per leaf
3	≥ 11 shot holes per leaf (less than 50% defoliation, leaf tissues/veins still present)
4	≥ 11 shot holes per leaf (more than 50% defoliation, leaf tissues/veins still present)
5	Leaves completely removed; plant is desiccated and dead

[1] "Shot-hole" caused by bean leaf beetle is roughly circular and approximately 2–3 mm in diameter.

Stink bug damage was evaluated once at the R6-R8 growth stage by counting and recording the total number of pods and number of damaged pods (presence of shrunken seed) on 10 plants (five consecutive plants per designated row) in each plot. Only pods from the upper 6 nodes on each plant were evaluated. Stink bug damage was calculated as percentage of the total number of damaged pods divided by the total number of evaluated pods.

G.8. Arthropod Abundance

Arthropod abundance was assessed quantitatively from collections performed at five sites (*i.e.*, GACH, IABG, ILAG, LACH, and SCEK). Arthropods were collected using a vertical beat sheet method (Drees and Rice 1985) five times during the growing season starting at approximately R1 growth stage and then approximately every two weeks for a total of five collection times.

The vertical beat sheet consists of a roughly square metal sheet, approximately 0.91 × 0.91m, with a collecting trough at the bottom. A sample was taken by placing the trough at the base of the soybean plants and shaking the plants against the upper portion of the sheet to dislodge arthropods from the plants. This process was repeated for a total of four sub-samples combined into one sample per plot from the designated rows at each collection time. Samples were then sent to SynTech Research, Sanger, CA for arthropod identification and enumeration.

A maximum of twelve arthropods were enumerated for each collection. Six pre-selected pest and beneficial arthropods (or arthropod groups), namely bean leaf beetles, stink bugs, and Japanese beetles for the pests, and big-eyed bugs, ladybird beetles, and damsel bugs for the beneficial arthropods, were enumerated at all sites for each collection time. Additionally, for each individual collection (*e.g.*, Collection 1, IABG site), four non-systematically selected samples were examined to determine the most abundant arthropods to obtain an additional six arthropods for a total of up to twelve arthropods to be enumerated for that particular collection and site. Thus, the suite of arthropods assessed often varied among collections at a site and among sites due to differences in temporal activity and geographical distribution of arthropod taxa.

G.9. Data Assessment

Experienced scientists familiar with the experimental design and evaluation criteria were involved in all components of data collection, summarization, and analysis. Study personnel ensured that measurements were taken properly, data were consistent with expectations based on experience with the crop, and the study was carefully monitored. Prior to analysis, the overall dataset was evaluated for evidence of biologically relevant changes and for possible evidence of unexpected plant responses. Any unexpected observations or issues identified during the study that might impact the study objectives were noted. Data were then subjected to statistical analysis as indicated in Section G.10.

G.10. Statistical Analysis

G.10.1. Agronomic and Phenotypic Data

Plant growth stage, plant vigor and flower color data are categorical and were not subjected to an analysis of variance (ANOVA). For plant growth stage and plant vigor, MON 87751 and the control were considered different from each other if the range observed for MON87751 did not overlap with the range observed for the control across all replications. Any observed differences between the MON 87751 and control were further assessed in the context of the range of the commercial reference materials, and for consistency at other sites. For flower color, MON 87751 and the control were considered different from each other if the flower color did not match across all replications and sites.

An ANOVA was conducted according to a randomized complete block design using SAS® (2010) to compare MON 87751 and the control for nine phenotypic characteristics listed in Table VII-1 (early stand count, days to 50% flowering, plant lodging, pod shattering, plant height, final stand count, grain moisture, 100 seed weight, and yield). The level of statistical significance was predetermined ($\alpha = 0.05$). Comparisons of MON 87751 and the control were conducted within sites (individual-site analysis) and in a combined-site analysis, in which the data were pooled across sites. MON 87751 and the

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control material were not statistically compared to the commercial reference materials. The reference range for each measured phenotypic characteristic was determined from the minimum and maximum mean values from the 34 commercial soybean reference varieties grown across the 17 sites.

Data excluded from the study and the reasons for excluding these data are listed in Table G-3.

G.10.2. Environmental Interaction Data

The environmental interaction data (*i.e.*, plant response to abiotic stressors, disease damage, and arthropod damage) are categorical and were not subjected to ANOVA. MON 87751 and conventional control were considered different in susceptibility or tolerance if the range of injury symptoms of each did not overlap across all four replications. Any observed differences were further assessed in the context of the range of the reference materials, and for consistency of response observed at other sites.

An ANOVA was conducted according to a randomized complete block design (SAS 2010) for bean leaf beetle damage, stink bug damage, and arthropod abundance. The level of statistical significance was predetermined ($\alpha = 0.05$). MON 87751 was compared to the control at each site (individual-site analysis) for bean leaf beetle, stink bug damage, and the arthropod abundance. The reference range for the arthropod abundance evaluated from a given collection and site was determined from the minimum and maximum mean values collected from the commercial soybean reference varieties at the site. Due to the lack of variability, a combined-site analysis was not done for the bean leaf beetle data. Stink bug damage data were pooled across sites (combined-site analysis) for a statistical comparison between MON 87751 and the control. Minimum and maximum mean values for stink bug damage were calculated from the 19 commercial soybean reference varieties that were included at the GACH, IABG, ILAG, LACH, and SCEK sites.

For the arthropod abundance data, statistical analyses and significance testing of the differences between MON 87751 and the control were only performed for the arthropods present in sufficient numbers in order to estimate the mean insect counts as well as the variation of the means. An inclusion criterion was established where a given taxa must have an average count per plot per sampling time across all materials of ≥ 1.

Data excluded from the study and the reasons for excluding these data are listed in Table G-3.

G.11. Individual Field Site Analysis for Agronomic and Phenotypic Characteristics: Results and Discussion

In the individual-site analysis, a total of 21 statistically significant differences were detected out of 134 comparisons made between MON 87751 and the control (Table G-4). Lack of variability in the data precluded statistical comparisons between MON 87751 and control for 19 additional comparisons; however, the means for MON 87751 and the control had the same value for these comparisons, indicating no biological differences. The 21 observed differences were distributed among all nine phenotypic characteristics.

MON 87751 had a lower early stand count than the control at IABG (19.4 vs. 23.7 plants per linear meter) and SCEK (13.8 vs. 16.1 plants per linear meter). MON 87751 had more days to 50% flowering than the control at NCBD (37.8 vs. 35.5 days) and NECC (52.3 vs. 49.0 days). MON 87751 exhibited lower plant lodging than the control at NCBD (2.8 vs. 3.5 rating). MON 87751 had lower pod shattering than the control at LACH (1.0 vs. 2.0 rating) and SCEK (2.8 vs. 6.0 rating). MON 87751 had shorter plants than the control at ILMN (107.3 vs. 115.8 cm); however, MON 87751 had taller plants than the control at PAGR site (83.7 vs. 78.3 cm). Final stand count was lower for MON 87751 than the control at IAHU (24.4 vs. 27.3 plants per linear meter). Grain moisture percentage was lower for MON 87751 than the control at ARNE (12.5 vs. 12.9 %), NCBD (15.6 vs. 16.1%), and OHTR (10.9 vs. 12.4 %). The 100 seed weight was higher for MON 87751 than the control at IABG (19.6 vs. 18.6 g), ILMN (20.0 vs. 19.0 g), KSLA (16.4 vs. 15.3 g), OHTR (16.3 vs. 15.0 g), and SCEK (16.0 vs. 14.1 g). MON 87751 had a lower yield than the control at IAHU (3.8 vs. 4.6 t/ha), ILAG (2.2 vs. 2.7 t/ha), and NCBD (4.3 vs. 4.6 t/ha). The statistical differences between MON 87751 and the control detected in the individual-site analysis for early stand count, days to 50% flowering, plant lodging, pod shattering, plant height, final stand count, grain moisture percentage, 100 seed weight, and yield were not detected in the combined-site analysis. Thus, the differences detected for these phenotypic characteristics do not indicate a consistent response associated with the trait and are unlikely to be biologically meaningful in terms of increased pest/weed potential of MON 87751 compared to the conventional soybean (See Figure VII-1, Step 2, “no” answer).

In individual-site assessments of plant vigor, MON 87751 and the control were considered different if the range of values did not overlap across all four replications. There were no differences observed between MON 87751 and the control in plant vigor at any of the sites (Table G-4).

In individual-site assessments of flower color, MON 87751 and the control were considered different if the flower color was not the same across all four replications. There were no differences observed between MON 87751 and the control in flower color as flowers were purple as expected at all sites (Table G-4).

In individual-site assessments of plant growth stage, MON 87751 and the control were considered different if the range of values did not overlap across all four replications. There were no differences observed between MON 87751 and the control in growth stage at any of the sites (Table G-5).

G.12. Individual Field Site Analysis for Environmental Interactions: Results and Discussion

Plant Response to Abiotic Stressor, Disease Damage, and Arthropod-related Damage:

In the individual-site assessment, no differences were observed between MON 87751 and the control for any of the 193 comparisons for the assessed abiotic stressors, including drought, frost, hail injury, heat, mineral toxicity, nutrient deficiency, soil compaction, sun scald, wet soil, and wind (Table G-6).

In the individual-site assessment, no differences were observed between MON 87751 and the control for any of the 191 comparisons for the assessed diseases, including *Alternaria* leaf spot, Asian rust, bacterial blight, bacterial leaf spot, bean pod mottle, soybean brown spot, brown stem rot, *Cercospora* leaf disease, charcoal rot, damping-off, downy mildew, frogeye leaf spot, leaf bacterial pustule, Phytophthora root rot, pod and stem blight, powdery mildew, *Pythium sp*., *Rhizoctonia sp*., root knot nematode, soybean rust, *Septoria sp*., soybean mosaic virus, soybean stem cankers, sudden death, and white mold (Table G-7).

In the individual-site assessment, no differences were observed between MON 87751 and the control for any of the 154 comparisons for the assessed arthropods, including aphids, bean leaf beetles, blister beetles, corn rootworm beetles, grape colaspis, grasshoppers, Japanese beetles, kudzu bugs, Mexican bean beetles, soybean stem borers, spider mites, stink bugs, striped flea beetles, three-cornered alfalfa hoppers, thrips, and whiteflies (Table G-8).

Bean Leaf Beetle and Stink Bug Damage:

In the individual-site analysis, no statistically significant differences were detected for any of the comparisons between MON 87751 and the control for bean leaf beetle and stink bug damage among all observations at five sites (Table G-9).

Arthropod Abundance:

A total of 170 statistical comparisons were made between MON 87751 and the control for arthropod abundance involving the following pest and beneficial arthropods: aphids, bean leaf beetles, corn rootworm beetles, Japanese beetles, kudzu bugs, minute brown scavenger beetles, leaf beetles, leafhoppers, tarnished plant bugs, plant bugs, spider mites, stink bugs, thrips, treehoppers, whiteflies, ant-like flower beetles, spiders, assassin bugs, big-eyed bugs, brown lacewings, green lacewings, damsel bugs, ladybird beetles, micro-Hymenoptera, minute pirate bugs, and predatory mites (Table G-10). Lack of sufficient arthropod abundance precluded statistical comparisons between MON 87751 and the control for 127 additional comparisons; however, descriptive statistics were provided for these comparisons.

No statistically significant differences were detected between MON 87751 and the control for 157 out of 170 comparisons (Table G-10). The abundance of spiders was higher in MON 87751 than the control in Collection 3 (5.5 vs. 2.3 per plot) at IABG and Collection 5 at SCEK (10.0 vs. 5.0 per plot). The abundance of big-eyed bugs was higher in MON 87751 than the control in Collection 2 at GACH (10.3 vs. 5.3 per plot) and in Collection 5 at SCEK (39.5 vs. 26.8 per plot). The abundance of damsel bugs was higher in MON 87751 than the control in Collection 4 at SCEK (2.5 vs. 0.3 per plot). The abundance of predatory mites was lower in MON 87751 than the control in Collection 1 at ILAG (0.5 vs. 3.8 per plot). Bean leaf beetle abundance was higher in MON 87751 than the control in Collection 4 at ILAG site (1.0 vs. 0.5 per plot). The abundance of kudzu bugs was higher in MON 87751 than the control in Collection 4 at SCEK (126.3 vs. 48.8 per plot). Plant bug abundance was higher in MON 87751 than the control

in Collection 5 at GACH (2.3 vs. 0.0 per plot) and in Collection 3 at LACH (9.5 vs. 2.8 per plot). The abundance of stink bugs was lower in MON 87751 than the control in Collection 4 at SCEK (5.30 vs. 15.5 per plot). Thrips abundance was higher in MON 87751 than the control in Collection 2 at GACH site (65.0 vs. 32.3 per plot) and lower abundance than the control in Collection 3 at SCEK (41.8 vs. 84.0 per plot). The mean abundance values for MON 87751 were within the reference ranges for all differences detected in arthropod abundance with the exception of the difference detected for big-eyed bug abundance in Collection 5 at SCEK (MON 87751 mean = 39.5 per plot; reference range = 26.3 – 37.3 per plot), predatory mite abundance in Collection 1 at ILAG (MON 87751 mean = 0.5 per plot; reference range = 2.0 – 7.8 per plot), spider abundance in Collection 5 at SCEK (MON 87751 mean = 10.0 per plot; reference range = 6.0 – 8.5 per plot), kudzu bug abundance in Collection 4 at SCEK (MON 87751 mean = 126.3 per plot; reference range = 74.3 – 107.0 per plot), plant bug abundance in Collection 3 at LACH (MON 87751 mean = 9.5 per plot; reference range = 0.8 – 6.5 per plot), thrips abundance in Collection 2 at GACH (MON 87751 mean = 65.0 per plot; reference range = 32.3 – 59.3 per plot), and thrips abundance in Collection 3 at SCEK (MON 87751 mean = 41.8 per plot; reference range = 56.0 – 106.0 per plot). These differences were not consistently detected across collections or sites.

Thus, the difference in abundance for big-eyed bugs, bean leaf beetles, damsel bugs, kudzu bugs, plant bugs, predatory mites, spiders, stink bugs, and thrips were not indicative of a consistent response associated with the trait and are not considered biologically meaningful in terms of increased pest potential of MON 87751 compared to the control soybean (See Section VII.B.2).

Table G-3. Data Missing or Excluded from Analysis

Site Code[1]	Material Name	Material Type	Plots	Characteristics	Reason for Exclusion
IABG	Stewart SB2772R NK S30-D4	Reference Reference	105, 203, 204, 303, 305, 403, 406	Flower color	Rating error
ILCY	LG C3540	Reference	204	Early stand count	Missing data
ILMN	NuPride 2954	Reference	205, 302, 404	Flower color	Missing data
ILMN	Hoffman HS387	Reference	102	Plant height	Missing data
KSLA	A3555 MON 87751 Stewart SB3819 Garst 3585N	Control Test Reference Reference	101, 102, 103, 104	100 seed weight	Missing data
INKI	All	All	All	Phenotypic and environmental interactions	Extreme weather (drought) caused extensive damage across the plots.
NECC	A3244	Reference	103	Harvest weight	Missing data
ARNE, GACH, IARL, ILAG, ILCY, KSLA, LACH, MOFI, NCBD, PAGR, SCEK	All	All	All	Environmental interaction data	In some instances, potential target arthropod damage data were included in the stressor assessments. This study was not designed to collect efficacy data on target insect pests as stressor assessments.
IARL	All	All	All	Environmental interaction evaluation #1 (grey leaf spot) (Goss's bacterial wilt)	Improper selection of stressor
ILAG, ILTH, OHTR	All	All	All	Environmental interaction evaluation #1, # 2, #3 (Animal damage) (Herbicide injury)	Improper selection of stressor
ILTH	All	All	All	Environmental interactions evaluation#2, #3 (arthropod) (abiotic)	Improper selection of stressor
KSLA	All	All	All	Environmental interaction all evaluations (other stressor) (other disease #3)	Improper selection of stressor

Table G-3. Data Missing or Excluded from Study (continued)

Site Code[1]	Material Name	Material Type	Plots	Characteristics	Reason for Exclusion
OHTR	All	All	All	Environmental interaction evaluation (Maize dwarf mosaic virus #2)	Improper selection of stressor
IABG, ILAG	All	All	All	Environmental interaction all evaluations (Bean leaf beetle)	Improper selection of stressor
GACH, LACH	All	All	All	Environmental interaction evaluation # 1 (all stressors)	Missing data
ILAG	All	All	All	Environmental interaction evaluation # 1 (black stem)	Improper selection of stressor
ILAG	All	All	All	Environmental interaction evaluation # 2 (abiotic stressor)	Improper selection of stressor
LACH	All	All	All	Environmental interaction evaluation # 3 (extra rating at R5, all stressors)	Not required by the protocol
NCBD	All	All	All	Environmental interaction evaluation # 2 (extra rating at R3, all stressors)	Not required by the protocol
SCEK	All	All	All	Environmental interaction evaluation # 4 (abiotic stressor)	Improper selection of stressor
SCEK	All	All	All	Environmental interactions evaluation # 2 (disease stressor)	Missing data
SCEK	All	All	All	Environmental interactions (extra rating at R5, all stressors)	Not required by the protocol
SCEK	All	All	All	Environmental interactions all evaluations(other stressor)	Improper selection of stressor
GACH	Pioneer 93M52	Reference	206	Arthropod abundance collection #1 (beneficial 5 count)	Missing data
IABG	Crows C2804	Reference	404	Arthropod abundance collection #1 (beneficial 3 count)	Missing data
LACH	All	All	All	Bean leaf beetle assessment #2	Incorrect damage assessment

[1] Site code: ARNE = Newport, AR; GACH = Chula, GA; IABG = Bagley, IA; IAHU = Huxley, IA; IARL = Richland, IA; ILAG = Thomasboro, IL; ILCY = Carlyle, IL; ILMN = Monmouth, IL; ILTH = Thomasboro, IL; INKI = Sheridan, Indiana; KSLA = Larned, KS; LACH = Cheneyville, LA; MOFI = Fisk, MO; NCBD = Belvidere, NC; NECC = Waco, NE; OHTR = Troy, OH; PAGR = Germansville, PA; SCEK = Elko, SC.

Table G-4. Individual Site Analysis of Phenotypic Characteristics of MON 87751 Compared to the A3555 Control

	Phenotypic Characteristics (units)					
	Early stand count (#/linear meter)		Days to 50% flowering		Plant vigor (1-9 rating)[4]	
	Mean (S.E.)[2]		Mean (S.E.)[2]		Range[3]	
Site Code[1]	MON 87751	Control	MON 87751	Control	MON 87751	Control
ARNE	26.7 (1.45)	24.8 (0.91)	37.0 (0.00)†	37.0 (0.00)	1-3	2-4
GACH	25.0 (0.76)	26.6 (0.44)	27.5 (0.29)	27.5 (0.29)	1-3	1-3
IABG	19.4 (2.33)*	23.7 (1.11)	63.0 (0.00)†	63.0 (0.00)	1-3	3
IAHU	27.3 (1.93)	28.7 (0.82)	47.0 (0.58)	46.5 (0.50)	2-4	1-2
IARL	20.6 (1.99)	22.2 (0.40)	49.0 (0.00)	49.5 (0.50)	2	1-3
ILAG	21.4 (0.60)	22.3 (0.42)	54.0 (0.00)†	54.0 (0.00)	1†	1
ILCY	26.1 (0.81)	24.1 (1.40)	33.5 (0.29)	33.0 (0.00)	1†	1
ILMN	24.2 (0.22)	24.8 (1.10)	49.5 (0.50)	48.5 (0.50)	1-2	1-2
ILTH	22.3 (1.10)	21.4 (0.37)	53.0 (0.00)†	53.0 (0.00)	1†	1
KSLA	17.4 (1.86)	20.1 (3.39)	45.0 (0.00)†	45.0 (0.00)	3-4	3-4
LACH	19.3 (1.19)	20.2 (0.83)	29.0 (0.00)†	29.0 (0.00)	2-3	2
MOFI	25.8 (0.22)	26.7 (1.10)	36.0 (0.00)	36.0 (0.00)	1-2	1-2
NCBD	26.7 (0.65)	26.3 (0.76)	37.8 (0.48)*	35.5 (0.29)	3†	3
NECC	31.1 (2.39)	34.8 (0.44)	52.3 (1.03)*	49.0 (0.58)	2-3	2
OHTR	24.8 (2.02)	23.6 (1.26)	52.3 (0.25)	53.0 (0.41)	4-6	3-7
PAGR	27.3 (0.89)	26.2 (0.89)	48.0 (0.00)	47.5 (0.50)	1-2	1-2
SCEK	13.8 (0.65)*	16.1 (0.32)	34.0 (0.00)†	34.0 (0.00)	1†	1

Table G-4. Individual Site Analysis of Phenotypic Characteristics of MON 87751 Compared to the A3555 Control (continued)

Site Code[1]	Phenotypic Characteristics (units)					
	Flower color		Plant lodging (1-9 rating)[5]		Pod shattering (1-9 rating)[6]	
	Category[3]		Mean (SE)		Mean (SE)	
	MON 87751	Control	MON 87751	Control	MON 87751	Control
ARNE	Purple	Purple	2.5 (0.29)	2.3 (0.25)	1.0 (0.00)†	1.0 (0.00)
GACH	Purple	Purple	1.0 (0.00)†	1.0 (0.00)	2.5 (0.50)	2.5 (0.50)
IABG	Purple	Purple	1.5 (0.29)	1.5 (0.29)	1.3 (0.25)	1.0 (0.00)
IAHU	Purple	Purple	3.3 (0.48)	2.5 (0.29)	1.0 (0.00)†	1.0 (0.00)
IARL	Purple	Purple	3.8 (0.48)	3.0 (0.41)	2.0 (0.00)	1.8 (0.25)
ILAG	Purple	Purple	2.3 (0.25)	2.5 (0.29)	1.0 (0.00)†	1.0 (0.00)
ILCY	Purple	Purple	1.0 (0.00)†	1.0 (0.00)	1.0 (0.00)†	1.0 (0.00)
ILMN	Purple	Purple	5.0 (0.41)	4.3 (0.48)	2.3 (0.25)	2.0 (0.00)
ILTH	Purple	Purple	1.0 (0.00)	1.3 (0.25)	1.3 (0.25)	1.0 (0.00)
KSLA	Purple	Purple	1.0 (0.00)†	1.0 (0.00)	1.0 (0.00)†	1.0 (0.00)
LACH	Purple	Purple	1.8 (0.25)	1.8 (0.25)	1.0 (0.00)*	2.0 (0.41)
MOFI	Purple	Purple	5.0 (0.41)	5.5 (0.29)	3.0 (0.00)	3.0 (0.00)
NCBD	Purple	Purple	2.8 (0.25)*	3.5 (0.29)	1.3 (0.25)	1.3 (0.25)
NECC	Purple	Purple	4.0 (0.91)	2.8 (0.25)	1.0 (0.00)†	1.0 (0.00)
OHTR	Purple	Purple	1.8 (0.25)	1.3 (0.25)	1.0 (0.00)†	1.0 (0.00)
PAGR	Purple	Purple	2.5 (0.29)	1.8 (0.48)	1.0 (0.00)†	1.0 (0.00)
SCEK	Purple	Purple	1.0 (0.00)†	1.0 (0.00)	2.8 (0.63)*	6.0 (0.41)

Table G-4. Individual Site Analysis of Phenotypic Characteristics of MON 87751 Compared to the A3555 Control (continued)

Site Code[1]	Phenotypic Characteristics (units)					
	Plant height (cm)		Final stand count (#/linear meter)		Grain moisture (%)	
	Mean (S.E.)[2]		Mean (S.E.)[2]		Mean (S.E.)[2]	
	MON 87751	Control	MON 87751	Control	MON 87751	Control
ARNE	87.1 (1.25)	83.3 (1.14)	23.6 (1.25)	23.0 (0.62)	12.5 (0.09)*	12.9 (0.10)
GACH	69.7 (3.45)	67.8 (2.07)	22.3 (0.82)	22.7 (0.59)	14.2 (0.55)	14.4 (0.60)
IABG	79.0 (3.06)	81.0 (2.82)	23.7 (0.30)	23.1 (1.18)	11.9 (0.22)	11.7 (0.17)
IAHU	102.1 (5.05)	101.7 (2.84)	24.4 (1.42)*	27.3 (0.41)	8.3 (0.21)	8.2 (0.09)
IARL	80.6 (2.45)	77.7 (2.58)	20.8 (1.00)	20.4 (0.37)	8.5 (0.17)	8.2 (0.06)
ILAG	74.1 (3.70)	77.5 (7.02)	18.8 (1.12)	19.0 (1.13)	11.6 (2.02)	14.5 (2.19)
ILCY	63.9 (1.86)	61.6 (2.50)	24.2 (0.74)	24.2 (0.65)	11.4 (0.26)	12.0 (0.21)
ILMN	107.3 (2.79)*	115.8 (1.65)	21.2 (1.55)	22.2 (0.89)	11.6 (0.09)	11.7 (0.06)
ILTH	79.7 (2.87)	73.0 (5.22)	22.0 (0.59)	23.0 (0.68)	12.4 (0.10)	12.7 (0.20)
KSLA	90.3 (2.69)	90.2 (0.79)	15.6 (1.42)	14.1 (0.75)	7.6 (0.03)	7.6 (0.08)
LACH	73.7 (2.48)	71.0 (3.32)	15.9 (0.55)	15.4 (0.79)	12.7 (0.14)	12.7 (0.13)
MOFI	101.8 (2.11)	97.0 (3.11)	23.8 (0.89)	25.7 (0.83)	11.8 (0.04)	11.8 (0.06)
NCBD	107.7 (0.92)	105.2 (0.46)	22.6 (0.42)	23.2 (0.52)	15.6 (0.14)*	16.1 (0.05)
NECC	108.4 (7.56)	106.3 (4.25)	20.5 (1.23)	22.5 (0.88)	8.9 (0.17)	8.6 (0.18)
OHTR	89.0 (2.60)	86.6 (4.90)	21.9 (1.60)	21.5 (1.11)	10.9 (0.09)*	12.4 (0.15)
PAGR	83.7 (0.70)*	78.3 (1.55)	25.0 (1.01)	25.9 (0.63)	17.7 (0.17)	17.9 (0.18)
SCEK	54.9 (3.28)	52.5 (3.69)	13.5 (0.57)	14.5 (0.63)	12.0 (0.39)	11.5 (0.12)

Table G-4. Individual Site Analysis of Phenotypic Characteristics of MON 87751 Compared to the A3555 Control (continued)

Site Code[1]	Phenotypic Characteristics (units)			
	100 seed weight (g)		Yield (t/ha)	
	Mean (S.E.)[2]		Mean (S.E.)[2]	
	MON 87751	Control	MON 87751	Control
ARNE	17.3 (0.28)	17.0 (0.42)	3.6 (0.06)	3.3 (0.04)
GACH	19.7 (0.24)	19.1 (0.63)	1.9 (0.35)	2.0 (0.10)
IABG	19.6 (0.29)*	18.6 (0.28)	2.4 (0.08)	2.6 (0.38)
IAHU	17.4 (0.13)	17.8 (0.50)	3.8 (0.25)*	4.6 (0.08)
IARL	19.0 (0.71)	19.5 (0.50)	3.0 (0.17)	3.5 (0.35)
ILAG	20.1 (0.71)	19.2 (0.91)	2.2 (0.12)*	2.7 (0.26)
ILCY	18.5 (0.60)	17.9 (0.20)	3.3 (0.11)	3.0 (0.09)
ILMN	20.0 (0.00)*	19.0 (0.58)	4.5 (0.13)	4.8 (0.02)
ILTH	19.5 (0.42)	19.5 (0.15)	3.2 (0.21)	3.0 (0.25)
KSLA	16.4 (0.43)*	15.3 (0.64)	4.9 (0.26)	4.8 (0.54)
LACH	20.2 (0.54)	19.3 (0.33)	3.0 (0.07)	2.9 (0.11)
MOFI	17.5 (0.14)	17.3 (0.20)	4.3 (0.13)	4.3 (0.11)
NCBD	17.4 (0.25)	17.4 (0.23)	4.3 (0.04)*	4.6 (0.09)
NECC	17.3 (0.26)	18.9 (0.24)	3.9 (0.32)	4.1 (0.15)
OHTR	16.3 (0.25)*	15.0 (0.71)	4.2 (0.19)	4.1 (0.29)
PAGR	17.2 (0.29)	17.1 (0.28)	3.9 (0.09)	3.9 (0.12)
SCEK	16.0 (0.55)*	14.1 (0.73)	2.7 (0.21)	2.2 (0.24)

Note: The experimental design was a randomized complete block with four replications (n = 4 except where noted in Table G-3).
* Indicates statistically significant difference between MON 87751 and the A3555 control (α =0.05) using ANOVA.
[†]No statistical comparisons were made or no range is reported due to the lack of variability in the data.

[1] Site code: ARNE = Newport, AR; GACH = Chula, GA; IABG = Bagley, IA; IAHU = Huxley, IA; IARL = Richland, IA; ILAG = Thomasboro, IL; ILCY = Carlyle, IL; ILMN = Monmouth, IL; ILTH = Thomasboro, IL; KSLA = Larned, KS; LACH = Cheneyville, LA; MOFI = Fisk, MO; NCBD = Belvidere, NC; NECC = Waco, NE; OHTR = Troy, OH; PAGR = Germansville, PA; SCEK = Elko, SC.
[2] MON 87751 and control values represent means with standard error in parentheses.
[3] Data were not subjected to statistical analysis.
[4] Plant vigor rating range (minimum - maximum); the range of plant vigor ratings for the references is as follows: ARNE 1–4; GACH 1–4; IABG 1–3; IAHU 1–4; IARL 2–4; ILAG 1; ILCY 1; ILMN 1–4; ILTH 1–4; KSLA 3–4; LACH 2–5; MOFI 1–2; NCBD 3–4; NECC 2–3; OHTR 4–7; PAGR 1–4; SCEK 1.
[5] Plant lodging was rated on a 1-9 scale, where 1 = completely upright plants and 9 = completely lodged plants.
[6] Pod shattering was rated on a 1-9 scale, where 1 = no shattering and 9 = completely shattered pods.

Table G-5. Growth Stage Monitoring of MON 87751, Control, and Reference Varieties

Site[1]	Material	Assessment Date and Range of Growth Stages Observed[1]			
		Obs. 1	Obs. 2	Obs. 3	Obs. 4
ARNE		6/7/12	7/2/12	7/24/12	8/27/12
	MON 87751	VC-V1	R2	R4	R6
	Control	VC-V1	R2	R4	R6
	References	VC	R2	R3-R4	R6
GACH		5/17/12	6/11/12	7/2/12	8/2/12
	MON 87751	V1	R3	R5	R7
	Control	VC-V1	R3	R5	R7
	References	VC-V1	R3	R5-R6	R7
IABG		5/30/12	6/29/12	7/25/12	8/15/12
	MON 87751	V1	V5-V7	R2-R3	R4-R5
	Control	VC-V1	V5-V7	R2-R3	R5
	References	VC-V1	V4-R1	R2-R3	R3-R5
IAHU		6/11/12	7/2/12	7/23/12	8/28/12
	MON 87751	V1	V6	R3	R6
	Control	V1	V6-V7	R3	R6
	References	V1	V6-V7	R3	R6
IARL		6/2/12	6/29/12	7/26/12	8/24/12
	MON 87751	V2	V5	R4	R6
	Control	V2	V5	R4	R6
	References	V2	V5	R4	R6
ILAG		6/1/12	6/14/12	7/10/12	8/9/12
	MON 87751	V1	V3	R2	R5
	Control	V1	V3-V4	R2	R5
	References	V1	V3-V4	R2	R5
ILCY		7/6/12	7/25/12	8/14/12	9/26/12
	MON 87751	V2-V3	R1	R3-R4	R7
	Control	V2-V3	R1	R3	R7
	References	V2-V3	R1	R3-R4	R7
ILMN		6/4/12	6/27/12	7/18/12	8/20/12
	MON 87751	V1	V6	R3	R5
	Control	V1	V6-V7	R3	R5
	References	VC-V1	V6-V7	R2-R3	R5
ILTH		5/30/12	6/14/12	7/10/12	8/9/12
	MON 87751	V1	V3-V4	R2	R5
	Control	V1	V3	R2	R5
	References	V1	V3-V4	R2	R5

Table G-5. Growth Stage Monitoring of MON 87751, Control, and Reference Varieties (continued)

		Assessment Date and Range of Growth Stages Observed[2]			
Site[1]	Material	Obs. 1	Obs. 2	Obs. 3	Obs. 4
KSLA		6/15/12	6/29/12	7/20/12	8/13/12
	MON 87751	VC-V1	R1	R3	R5
	Control	VC-V1	R1	R3	R5
	References	VC-V1	R1	R3	R5-R6
LACH		6/5/12	6/26/12	7/20/12	8/21/12
	MON 87751	V3	R3	R5	R7
	Control	V3	R3	R5	R7
	References	V3	R3	R5	R6-R7
MOFI		6/27/12	7/20/12	8/17/12	9/19/12
	MON 87751	V2	R2	R5	R7
	Control	V2	R2	R5	R7
	References	V2	R2	R5	R6-R7
NCBD		6/6/12	6/29/12	7/23/12	8/23/12
	MON 87751	V2	R1	R3	R5
	Control	V2	R1	R3	R5
	References	V1-V2	R1	R3	R5
NECC		6/5/12	6/29/12	7/20/12	9/12/12
	MON 87751	VC	V5-V6	R3	R7
	Control	VC	V6	R2-R3	R7
	References	VC	V5-V6	R2-R3	R7
OHTR		6/5/12	6/25/12	7/27/12	8/22/12
	MON 87751	V1	V3-V4	R2-R3	R6
	Control	V1	V3-V4	R3	R6
	References	V1	V3-V4	R2-R3	R5-R6
PAGR		6/22/12	7/18/12	8/6/12	9/6/12
	MON 87751	VC	V5	R3	R6
	Control	VC	V4-V5	R3	R5-R6
	References	VC	V4-V5	R3	R5-R6
SCEK		6/6/12	7/12/12	8/7/12	8/20/12
	MON 87751	V2	R3	R6	R6-R7
	Control	V2	R3	R6	R7
	References	V1-V2	R3	R6	R6-R7

[1] Site code: ARNE = Newport, AR; GACH = Chula, GA; IABG = Bagley, IA; IAHU = Huxley, IA; IARL = Richland, IA; ILAG = Thomasboro, IL; ILCY = Carlyle, IL; ILMN = Monmouth, IL; ILTH = Thomasboro, IL; INKI = Sheridan, Indiana; KSLA = Larned, KS; LACH = Cheneyville, LA; MOFI = Fisk, MO; NCBD = Belvidere, NC; NECC = Waco, NE; OHTR = Troy, OH; PAGR = Germansville, PA; SCEK = Elko, SC.

[2] Month-day-year

Obs. = Observation number

Table G-6. Abiotic Stressor Evaluations for MON 87751 and the A3555 Control

Abiotic Stressor	Number of Observations across Sites	Number of Observations where No Differences were Observed between MON 87751 and the Conventional Control
Total	193	193
Drought	38	38
Frost	4	4
Hail injury	12	12
Heat	33	33
Mineral toxicity	4	4
Nutrient deficiency	24	24
Soil compaction	8	8
Sun scald	18	18
Wet soil[1]	19	19
Wind	33	33

No differences were observed between MON 87751 and the control during any observation for damage caused by any of the assessed abiotic stressors.
Note: The experimental design was a randomized complete block with four replicates.
Data were not subjected to statistical analysis.
Observational data collected at four crop development stages: approximately late vegetative – R1; R2 – R3; R4 – R5; and R6 – R8 except for the third stressor rating at SCEK site which was taken at R6.
[1] Includes flood

Table G-7. Disease Damage Evaluations for MON 87751 and the A3555 Control

Disease	Number of Observations across Sites	Number of Observations where No Differences were Observed between MON 87751 and the Conventional Control
Total	191	191
Alternaria leaf spot[1]	2	2
Asian rust	7	7
Bacterial blight	20	20
Bacterial leaf spot	2	2
Bean pod mottle	5	5
Soybean brown spot	20	20
Brown stem rot	1	1
Cercospora leaf disease	10	10
Charcoal rot	3	3
Damping-off	2	2
Downy mildew	22	22
Frogeye leaf spot	43	43
Leaf bacterial pustule	4	4
Phytophthora root rot	1	1
Pod and stem blight	1	1
Powdery mildew	2	2
Pythium sp.	1	1
Rhizoctonia sp.	2	2
Root knot nematode	2	2
Soybean rust	9	9
Septoria sp.	12	12
Soybean mosaic virus	3	3
Soybean stem cankers	2	2
Sudden death	7	7
White mold	8	8

No differences were observed between MON 87751 and the control during any observation for damage caused by any of the assessed disease stressors.
Note: The experimental design was a randomized complete block with four replicates.
Data were not subjected to statistical analysis.
Observational data collected at four crop development stages: approximately late vegetative – R1; R2 – R3; R4 – R5; and R6 – R8 except for the third stressor rating at SCEK site which was taken at R6.
[1] includes leaf spot (common, Stemphylium)

Table G-8. Arthropod Damage Evaluations for MON 87751 and the A3555 Control

Arthropod	Number of Observations across Sites	Number of Observations where No Differences were Observed between MON 87751 and the Conventional Control
Total	154	154
Aphids (Aphididae)[1]	22	22
Bean leaf beetles (*Cerotoma trifurcacta*)	24	24
Blister beetles (Meloidae)	2	2
Corn rootworm beetles (*Diabrotica* sp.)	4	4
Grape Colaspis (*Colaspis brunnea*)	3	3
Grasshoppers (Acrididae)	29	29
Japanese beetles (*Popillia japonica*)	20	20
Kudzu bugs (*Megacopta cribraria*)	3	3
Mexican bean beetles (*Epilachna* sp.)	4	4
Soybean stem borers (*Dectes texanus*)	3	3
Spider mites (*Tetranychus* sp.)	8	8
Stink bugs (Pentatomidae)	18	18
Striped flea beetles (*Phyllotreta* sp.)	1	1
Three-cornered alfalfa hoppers (*Spissistilus festinus*)	6	6
Thrips (Thysanoptera)	2	2
Whiteflies (Aleyrodidae)	5	5

No differences were observed between MON 87751 and the control during any observation for damage caused by any of the assessed arthropod stressors.
Note: The experimental design was a randomized complete block with four replicates except for bean leaf beetles which were only observed in three replicates.
Data were not subjected to statistical analysis.
Observational data collected at four crop development stages: approximately late vegetative – R1; R2 – R3; R4 – R5; and R6 – R8 except for the third stressor rating at SCEK site which was taken at R6.
[1] includes soybean aphids

Table G-9. Individual-site Analysis: Quantitative Assessment of Bean Leaf Beetle and Stink Bug Damage to MON 87751 Compared to the A3555 Control

				Mean (S.E.)[1]	
Pest Arthropod	Damage assessment	Site[2]	Timing	MON 87751	Control
Bean leaf beetle (*Cerotoma trifurcata*)[3]	Damage on 10 plants per plot (0-5 rating)	GACH	1	0.0 (0.00)†	0.0 (0.00)
		IABG	1	1.6 (0.21)	1.9 (0.19)
		ILAG	1	0.0 (0.00)†	0.0 (0.00)
		LACH	1	0.0 (0.00)†	0.0 (0.00)
		SCEK	1	0.0 (0.00)†	0.0 (0.00)
		GACH	2	0.0 (0.00)†	0.0 (0.00)
		IABG	2	1.2 (0.11)	1.2 (0.05)
		ILAG	2	0.4 (0.19)	0.4 (0.14)
		LACH	2	—	—
		SCEK	2	0.0 (0.00)†	0.0 (0.00)
Stink bug (Pentatomidae)[4]	% of damaged pods per plot	GACH	1	27.9 (1.83)	30.5 (3.36)
		IABG	1	2.8 (1.24)	2.4 (1.61)
		ILAG	1	3.2 (1.38)	4.4 (1.75)
		LACH	1	65.8 (3.84)	62.7 (10.14)
		SCEK	1	6.2 (1.46)	7.3 (1.60)

Note: The experimental design was a randomized complete block with four replications (n = 4 except where noted in Table G-3). No statistically significant differences were detected between MON 87751 and the control (α=0.05) using ANOVA.
†p-values could not be generated due to the lack of variability in the data.
[1] MON 87751 and control values represent means with standard error in parentheses.
[2] Site code: GACH = Chula, GA; IABG = Bagley, IA; ILAG = Thomasboro, IL; LACH = Cheneyville, LA; SCEK = Elko, SC.
[3] Bean leaf beetle damage assessments were conducted twice, once two to three weeks after emergence and then two weeks later.
[4] Damage assessments for stink bugs were conducted once at R6-R8 growth stage.
A dash (—) indicates data not available.

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control

		Aphids (Aphididae)			Bean leaf beetles (*Cerotoma* sp.)			Corn rootworm beetles (*Diabrotica* sp.)		
		Pest Arthropod			Pest Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	IABG	—	—	—	1.8 (0.48)	4.5 (1.94)	1.5-2.0	0.5 (0.29) †	0.3 (0.25)	0.3-1.0
	ILAG	—	—	—	0.5 (0.50) †	0.0 (0.00)	0.0-0.3	—	—	—
	LACH	3.0 (0.91)	1.5 (0.50)	0.8-1.5	5.8 (0.75)	3.3 (0.85)	2.8-6.8	—	—	—
	SCEK	1.8 (0.48)	4.5 (1.32)	1.5-4.3	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
2	GACH	1.8 (1.44)	2.3 (1.60)	0.8-4.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	—	—	—
	IABG	—	—	—	7.0 (2.48)	8.3 (1.25)	5.0-8.0	—	—	—
	ILAG	—	—	—	1.3 (0.63)	1.0 (0.41)	0.5-1.8	—	—	—
	LACH	—	—	—	8.8 (1.75)	6.5 (2.50)	3.0-9.3	—	—	—
	SCEK	8.5 (4.63)	5.8 (2.66)	5.3-12	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
3	GACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	IABG	—	—	—	9.8 (3.12)	10.5 (5.12)	7.0-17.8	—	—	—
	ILAG	—	—	—	2.8 (0.75)	1.3 (0.75)	0.8-2.3	—	—	—
	LACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	SCEK	7.3 (1.18)	23.8 (13.60)	4.3-7.8	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
4	GACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	IABG	—	—	—	9.8 (2.59)	11.3(1.11)	5.8-10.3	—	—	—
	ILAG	—	—	—	1.0 (0.00) *	0.5 (0.50)	0.5-2.3	—	—	—
	LACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	SCEK	—	—	—	0.3 (0.25) †	0.0 (0.00)	0.0-0.8	—	—	—
5	GACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	IABG	—	—	—	0.3 (0.25) †	0.5 (0.29)	0.0-1.3	—	—	—
	ILAG	—	—	—	5.0 (0.41)	7.5 (1.94)	3.3-17.5	—	—	—
	LACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	SCEK	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Japanese beetles (*Popillia japonica*)			Kudzu bugs (*Megacopta cribraria*)			Minute brown scavenger beetles (Latridiidae)		
		Pest Arthropod			Pest Arthropod			Fungi Feeder		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	9.5 (3.01)	6.0 (1.35)	0.8-23.8	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	ILAG	0.3 (0.25) †	0.5 (0.29)	0.0-0.0	—	—	—	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	46.8 (5.79)	41.5 (15.78)	31.5-57.0	—	—	—
2	GACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	7.3 (2.14)	5.0 (3.34)	1.3-5.0	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	ILAG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	6.8 (3.82)	5.0 (1.35)	3.3-7.8	—	—	—
3	GACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	10.8 (2.43)	23.8 (10.46)	4.3-26.0	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	ILAG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	91.3 (4.77)	49.5 (8.61)	48.5-157.5	—	—	—
4	GACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	42.5 (9.72)	41.0 (8.11)	22.5-73.0	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	0.8 (0.25) †	1.0 (0.41)	0.0-0.8
	ILAG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	126.3 (33.61) *	48.8 (8.77)	74.3-107.0	—	—	—
5	GACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	0.5 (0.29) †	0.8 (0.48)	0.0-0.8
	ILAG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	258.8 (105.95)	71.3 (28.52)	138.5-190.8	—	—	—

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Leaf beetles (Chrysomelidae)			Leafhoppers (Cicadellidae)			Tarnished plant bugs (*Lygus* spp.)		
		Pest Arthropod			Pest Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	—	—	—	—	—	—	—	—	—
	IABG	—	—	—	1.0 (1.00)	1.3 (0.63)	1.3-6.5	—	—	—
	ILAG	1.8 (0.25)	1.0 (0.41)	0.5-5.0	29.5 (3.23)	19.8 (3.57)	28.5-38.5	—	—	—
	LACH	—	—	—	—	—	—	—	—	—
	SCEK	—	—	—	—	—	—	—	—	—
2	GACH	—	—	—	—	—	—	—	—	—
	IABG	4.0 (1.47)	1.8 (0.25)	2.5-4.8	—	—	—	—	—	—
	ILAG	1.0 (0.71)	1.3 (0.48)	0.5-5.8	0.5 (0.29)	1.3 (0.48)	1.0-3.0	—	—	—
	LACH	—	—	—	—	—	—	—	—	—
	SCEK	—	—	—	—	—	—	—	—	—
3	GACH	2.8 (1.89)	2.3 (1.31)	0.8-4.8	—	—	—	—	—	—
	IABG	6.8 (0.85)	5.5 (1.66)	6.3-8.5	0.0 (0.00) †	0.0 (0.00)	0.0-0.8	—	—	—
	ILAG	2.5 (1.19)	3.5 (2.18)	2.8-22.0	0.3 (0.25) †	0.5 (0.29)	0.8-1.5	0.8 (0.75) †	0.0 (0.00)	0.0-3.3
	LACH	—	—	—	—	—	—	—	—	—
	SCEK	—	—	—	—	—	—	—	—	—
4	GACH	—	—	—	—	—	—	—	—	—
	IABG	0.3 (0.25)	2.0 (0.71)	1.5-3.5	0.3 (0.25) †	0.0 (0.00)	0.0-0.8	—	—	—
	ILAG	2.0 (0.91)	4.3 (2.36)	1.3-27.0	—	—	—	—	—	—
	LACH	4.8 (0.25)	4.8 (1.11)	4.0-5.0	—	—	—	—	—	—
	SCEK	—	—	—	—	—	—	—	—	—
5	GACH	—	—	—	—	—	—	—	—	—
	IABG	6.8 (1.75)	4.8 (1.03)	2.8-8.3	0.3 (0.25) †	0.3 (0.25)	0.0-0.8	—	—	—
	ILAG	—	—	—	—	—	—	—	—	—
	LACH	1.0 (0.71) †	0.8 (0.25)	0.5-1.5	0.5 (0.29) †	0.8 (0.75)	0.0-0.8	—	—	—
	SCEK	—	—	—	—	—	—	—	—	—

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Plant bugs (Miridae)			Spider mites (*Tetranychus* sp.)			Stink bugs (Pentatomidae)		
		Pest Arthropod			Pest Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	—	—	—	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.3
	IABG	—	—	—	—	—	—	0.3 (0.25) †	0.0 (0.00)	0.0-2.5
	ILAG	—	—	—	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-1.3
	LACH	—	—	—	41.8 (20.79)	40.8 (14.85)	14.8-45.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.0
	SCEK	—	—	—	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.0
2	GACH	—	—	—	—	—	—	1.8 (1.11) †	0.8 (0.25)	0.0-1.8
	IABG	—	—	—	—	—	—	1.8 (1.03)	1.3 (0.75)	0.8-2.0
	ILAG	—	—	—	—	—	—	0.5 (0.29) †	0.0 (0.00)	0.0-0.8
	LACH	11.0 (2.45)	12.0 (4.95)	11.8-19.5	43.3 (18.09)	32.3 (9.94)	18.5-86.0	0.8 (0.75) †	0.0 (0.00)	0.0-0.0
	SCEK	—	—	—	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-0.3
3	GACH	—	—	—	—	—	—	3.8 (1.11)	1.8 (0.85)	1.0-3.8
	IABG	—	—	—	—	—	—	1.8 (1.11)	1.3 (0.48)	0.0-2.5
	ILAG	—	—	—	—	—	—	2.8 (1.18)	1.3 (0.63)	0.8-1.5
	LACH	9.5 (5.19) *	2.8 (2.14)	0.8-6.5	—	—	—	3.0 (0.82)	3.5 (1.19)	0.3-1.3
	SCEK	—	—	—	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.0-3.3
4	GACH	6.3 (1.38)	11.5 (7.23)	10.3-15.5	—	—	—	24.5 (7.33)	18.3 (8.67)	16.8-55.8
	IABG	—	—	—	—	—	—	3.8 (1.55)	1.3 (0.95)	1.3-3.0
	ILAG	—	—	—	3.0 (1.47)	1.3 (0.75)	0.5-2.5	2.3 (0.75)	1.8 (0.85)	2.5-3.8
	LACH	—	—	—	—	—	—	7.0 (0.91)	4.8 (2.14)	2.8-7.0
	SCEK	—	—	—	—	—	—	5.3 (3.04) *	15.5 (5.45)	2.0-7.3
5	GACH	2.3 (0.85) *	0.0 (0.00)	2.3-4.8	—	—	—	20.5 (6.98)	12.0 (3.16)	3.5-19.0
	IABG	—	—	—	—	—	—	2.8 (0.48)	6.3 (1.38)	2.5-5.5
	ILAG	—	—	—	—	—	—	1.5 (0.50)	3.3 (0.95)	0.8-4.3
	LACH	—	—	—	—	—	—	4.0 (1.22)	6.3 (3.61)	3.5-9.8
	SCEK	—	—	—	—	—	—	5.0 (2.20)	5.3 (2.50)	6.3-16.0

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Thrips (Thysanoptera)			Treehoppers			Whiteflies (Aleyrodidae)		
		Pest Arthropod			Pest Arthropod			Pest Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	14.5 (5.42)	13.0 (6.39)	13.5-22.3	1.3 (0.75)	2.3 (0.63)	0.3-2.3	—	—	—
	IABG	78.0 (33.37)	95.3 (41.68)	69.0-126.0	—	—	—	—	—	—
	ILAG	71.3 (18.06)	62.3 (11.56)	52.8-68.3	—	—	—	—	—	—
	LACH	55.5 (13.58)	56.8 (7.23)	37.0-62.8	—	—	—	—	—	—
	SCEK	17.8 (3.04)	25.3 (3.82)	15.8-27.3	—	—	—	—	—	—
2	GACH	65.0 (7.13)*	32.3 (10.55)	32.3-59.3	—	—	—	—	—	—
	IABG	105.0 (52.87)	66.5 (26.55)	67.0-121.0	—	—	—	1.0 (0.58)	0.3 (0.25)	0.8-4.8
	ILAG	59.0 (23.57)	48.0 (12.44)	46.5-68.0	—	—	—	—	—	—
	LACH	44.0 (7.15)	50.0 (7.01)	38.0-95.3	—	—	—	—	—	—
	SCEK	268.8 (51.33)	173.8 (48.51)	210.3-276.0	—	—	—	—	—	—
3	GACH	208.3 (26.88)	157.5 (52.60)	102.3-187.5	—	—	—	—	—	—
	IABG	0.3 (0.25)†	0.0 (0.00)	0.0-0.3	—	—	—	—	—	—
	ILAG	—	—	—	—	—	—	—	—	—
	LACH	16.0 (1.63)	15.0 (1.91)	10.0-22.0	2.5 (0.65)	3.3 (1.65)	3.3-9.3	—	—	—
	SCEK	41.8 (17.35)*	84.0 (6.93)	56.0-106.0	—	—	—	—	—	—
4	GACH	31.8 (14.85)	17.0 (6.19)	32.5-49.0	—	—	—	—	—	—
	IABG	—	—	—	—	—	—	—	—	—
	ILAG	0.3 (0.25)†	0.5 (0.50)	0.0-1.3	—	—	—	—	—	—
	LACH	—	—	—	4.8 (0.95)	4.3 (0.85)	7.8-9.3	—	—	—
	SCEK	—	—	—	2.5 (0.65)	1.8 (0.75)	1.5-3.0	—	—	—
5	GACH	—	—	—	0.0 (0.00) †	0.0 (0.00)	0.3-0.5	—	—	—
	IABG	—	—	—	—	—	—	—	—	—
	ILAG	23.3 (8.29)	24.0 (7.72)	20.8-41.8	2.8 (0.48)	3.5 (1.71)	1.3-4.0	1.8 (0.48)	4.0 (2.74)	4.5-18.3
	LACH	—	—	—	2.3 (0.25)	2.8 (0.95)	2.0-8.5	—	—	—
	SCEK	23.3 (3.61)	37.0 (7.71)	28.5-34.3	3.0 (0.91)	3.3 (1.11)	3.5-5.0	—	—	—

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Ant-like flower beetles (Anthicidae)			Spiders (Araneae)			Assassin bugs (Reduviidae)		
		Pollen feeder			Beneficial Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	0.3 (0.25) †	0.3 (0.25)	0.5-0.8	1.3 (0.75)	1.5 (0.65)	0.3-1.5	—	—	—
	IABG	—	—	—	1.3 (0.48)	2.3 (1.31)	0.8-3.5	—	—	—
	ILAG	1.0 (0.58)	2.0 (0.91)	0.3-7.5	—	—	—	—	—	—
	LACH	—	—	—	4.8 (0.85)	4.0 (1.47)	2.0-3.5	—	—	—
	SCEK	—	—	—	1.8 (0.85)	1.3 (0.48)	1.3-2.3	—	—	—
2	GACH	2.8(0.48)	2.0 (0.41)	2.0-5.0	2.5 (0.87)	2.5 (0.87)	1.0-2.0	—	—	—
	IABG	—	—	—	3.3 (0.48)	2.8 (1.11)	0.5-4.5	—	—	—
	ILAG	—	—	—	—	—	—	—	—	—
	LACH	7.8 (1.75)	8.8 (2.29)	1.8-3.8	13.5 (3.66)	12.0 (1.68)	7.8-12.8	—	—	—
	SCEK	—	—	—	1.3 (0.48)	1.8 (0.85)	0.3-2.3	—	—	—
3	GACH	5.0 (2.80)	4.3 (2.29)	1.3-5.8	—	—	—	—	—	—
	IABG	3.3 (1.65)	1.0 (0.71)	1.3-2.8	5.5 (1.26)*	2.3 (0.63)	2.5-6.0	—	—	—
	ILAG	1.3 (0.75)	1.0 (0.41)	1-3-3.0	—	—	—	—	—	—
	LACH	2.0 (1.68)	0.8 (0.48)	0.3-3.5	5.5 (1.32)	6.3 (1.11)	6.3-7.5	—	—	—
	SCEK	—	—	—	3.0 (0.71)	2.8 (0.95)	2.3-4.8	—	—	—
4	GACH	3.5 (0.65)	5.8 (4.44)	2.3-4.8	—	—	—	—	—	—
	IABG	2.8 (1.44)	3.8 (1.25)	1.3-6.0	9.3 (3.75)	3.5 (0.87)	3.0-6.5	—	—	—
	ILAG	—	—	—	1.5 (0.87)	1.0 (0.58)	0.8-2.3	0.5 (0.29)	0.5 (0.50)	1.0-2.5
	LACH	1.3 (0.48) †	0.0 (0.00)	0.3-0.8	13.5 (3.77)	8.0 (2.42)	7.3-8.0	0.8 (0.48) †	0.3 (0.25)	0.0-1.0
	SCEK	1.3 (0.63)	2.5 (1.19)	0.3-3.8	8.8 (2.50)	5.5 (0.65)	4.8-9.5	3.3 (1.11)	4.5 (1.85)	1.8-3.8
5	GACH	4.3 (2.21)	1.5 (0.87)	0.3-4.0	0.5 (0.29) †	0.3 (0.25)	0.0-0.8	0.0 (0.00) †	0.3 (0.25)	0.0-0.8
	IABG	—	—	—	1.8 (0.75)	4.3 (0.85)	1.5-2.5	0.8 (0.48) †	0.8 (0.25)	0.3-0.8
	ILAG	8.8 (0.63)	10.0 (0.71)	3.8-11.5	4.3 (1.70)	1.5 (1.19)	1.3-4.0	—	—	—
	LACH	0.3 (0.25) †	0.3 (0.25)	0.5-1.0	10.5 (1.94)	9.8 (2.21)	8.5-11.5	—	—	—
	SCEK	5.3 (1.49)	3.8 (1.60)	5.5-9.5	10.0 (1.08)*	5.0 (2.12)	6.0-8.5	—	—	—

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Big-eyed bugs (Geocoridae)			Brown lacewings (Hemerobiidae)			Green lacewings (Chrysopidae)		
		Beneficial Arthropod			Beneficial Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	6.0 (1.91)	2.0 (0.41)	2.5-3.3	—	—	—	—	—	—
	IABG	0.0 (0.00) †	0.3 (0.25)	0.0-0.3	—	—	—	—	—	—
	ILAG	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—	—	—	—
	LACH	4.5 (1.85)	2.0 (0.71)	3.5-5.3	—	—	—	—	—	—
	SCEK	1.0 (0.00)	1.3 (0.48)	0.0-2.5	—	—	—	—	—	—
2	GACH	10.3 (1.80)*	5.3 (1.80)	5.8-12.3	—	—	—	—	—	—
	IABG	1.5 (0.65) †	0.5 (0.29)	0.3-1.0	2.0 (1.08)	1.0 (0.58)	0.8-5.8	—	—	—
	ILAG	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	—	—	—	0.0 (0.00)	0.5 (0.50)	0.0-3.5
	LACH	6.5 (0.29)	6.5 (1.26)	5.5-9.3	—	—	—	—	—	—
	SCEK	4.5 (0.65)	2.0 (0.58)	2.5-4.0	—	—	—	—	—	—
3	GACH	8.0 (1.08)	10.8 (3.33)	6.3-9.3	—	—	—	—	—	—
	IABG	0.0 (0.00) †	1.0 (0.71)	0.5-2.8	—	—	—	—	—	—
	ILAG	0.0 (0.00) †	0.3 (0.25)	0.0-0.8	—	—	—	0.0 (0.00) †	0.3 (0.25)	0.3-2.0
	LACH	20.0 (3.81)	16.0 (5.28)	16.3-29	—	—	—	—	—	—
	SCEK	8.3 (1.44)	5.0 (0.58)	2.0-4.3	—	—	—	—	—	—
4	GACH	16.5 (3.97)	10.0 (6.15)	15.3-22.8	—	—	—	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-1.3	—	—	—	—	—	—
	ILAG	0.0 (0.00) †	0.3 (0.25)	0.0-1.3	—	—	—	—	—	—
	LACH	18.3 (4.13)	24.3 (4.96)	17.5-38.5	—	—	—	—	—	—
	SCEK	15.5 (3.40)	17.8 (1.89)	14.0-19.8	—	—	—	—	—	—
5	GACH	10.8 (2.90)	10.3 (2.02)	8.8-16.8	—	—	—	—	—	—
	IABG	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	—	—	—	—	—	—
	ILAG	0.0 (0.00) †	0.5 (0.50)	0.0-0.5	—	—	—	—	—	—
	LACH	25.3 (3.28)	21.3 (1.11)	16.8-27.5	—	—	—	—	—	—
	SCEK	39.5 (3.97)*	26.8 (4.05)	26.3-37.3	—	—	—	—	—	—

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Damsel bugs (Nabidae)			Ladybird beetles (Coccinellidae)			Micro-Hymenoptera		
		Beneficial Arthropod			Beneficial Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	IABG	0.3 (0.25) †	1.5 (0.87)	0.3-0.8	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	0.3 (0.25) †	0.3 (0.25)	0.0-0.8
	ILAG	0.5 (0.50) †	0.3 (0.25)	0.5-0.8	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	2.5 (1.32)	3.0 (0.91)	1.8-7.5
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	0.0 (0.00) †	0.5 (0.50)	0.0-0.0	0.5 (0.50) †	0.5 (0.29)	0.3-0.8
2	GACH	0.0 (0.00) †	0.3 (0.25)	0.0-0.5	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	IABG	0.8 (0.48)	0.5 (0.29)	0.0-4.0	0.3 (0.25) †	0.0 (0.00)	0.0-0.3	—	—	—
	ILAG	0.8 (0.25)	1.0 (0.71)	1.3-5.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	0.0 (0.00) †	0.3 (0.25)	0.0-0.5
	LACH	0.3 (0.25) †	0.5 (0.29)	0.0-0.5	0.5 (0.50) †	0.0 (0.00)	0.0-0.3	—	—	—
	SCEK	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	2.0 (0.91)	0.8 (0.48)	0.8-2.0
3	GACH	0.0 (0.00) †	0.3 (0.25)	0.0-0.0	1.0 (1.00)	0.8 (0.48)	0.3-4.0	1.8 (1.18)	1.3 (0.48)	0.8-2.0
	IABG	0.5 (0.29)	1.3 (0.48)	1.0-2.5	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	ILAG	0.5 (0.29)	1.8 (0.75)	1.5-8.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.5	0.0 (0.00) †	0.0 (0.00)	0.0-0.8	0.8 (0.75)	0.5 (0.50)	1.3-1.8
	SCEK	1.3 (0.75) †	1.0 (0.41)	0.3-1.5	0.0 (0.00) †	0.0 (0.00)	0.0-0.3	1.8 (0.75)	0.8 (0.48)	1.0-2.0
4	GACH	0.0 (0.00) †	0.3 (0.25)	0.5-2.5	0.0 (0.00) †	0.3 (0.25)	0.0-0.5	1.0 (0.71)	2.8 (1.60)	2.0-6.8
	IABG	0.8 (0.25)	1.3 (0.25)	0.5-2.3	0.0 (0.00) †	0.3 (0.25)	0.0-0.0	—	—	—
	ILAG	0.3 (0.25) †	0.3 (0.25)	0.0-1.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-1.3	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	SCEK	2.5 (0.50)*	0.3 (0.25)	0.8-2.8	0.8 (0.75) †	1.0 (1.00)	0.3-1.0	—	—	—
5	GACH	0.3 (0.25) †	0.0 (0.00)	0.0-0.8	0.0 (0.00) †	0.3 (0.25)	0.0-0.5	—	—	—
	IABG	0.3 (0.25)	0.3 (0.25)	0.0-2.5	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	—	—	—
	ILAG	0.8 (0.48)	2.5 (0.87)	0.5-3.3	0.0 (0.00) †	0.3 (0.25)	0.0-0.0	—	—	—
	LACH	0.0 (0.00) †	0.0 (0.00)	0.0-0.8	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	0.0 (0.00) †	0.3 (0.25)	0.0-0.8
	SCEK	2.5 (0.50)	2.3 (1.31)	1.0-2.0	0.0 (0.00) †	0.0 (0.00)	0.0-0.0	2.5 (1.19)	2.3 (0.63)	1.5-4.3

Table G-10. Individual-site Analysis: Arthropod Abundance in Vertical Beat Sheet Samples Collected from MON 87751 Compared to the A3555 Control (continued)

		Minute Pirate bugs (*Orius* sp.)			Predatory mites (Phytoseiidae)		
		Beneficial Arthropod			Beneficial Arthropod		
		Mean (S.E.)[3]		Reference	Mean (S.E.)[3]		Reference
Coll.[1]	Site[2]	MON 87751	Control	range[4]	MON 87751	Control	range[4]
1	GACH	1.5 (0.87) †	0.0 (0.00)	0.0-0.7	—	—	—
	IABG	1.0 (0.41)	1.0 (1.00)	1.0-2.3	—	—	—
	ILAG	9.0 (0.91)	10.3 (2.29)	6.0-20.3	0.5 (0.29)*	3.8 (0.85)	2.0-7.8
	LACH	13.5 (3.38)	8.5 (2.47)	5.0-9.8	—	—	—
	SCEK	0.3 (0.25) †	0.8 (0.48)	0.3-1.3	—	—	—
2	GACH	2.5 (0.96)	1.3 (0.25)	1.0-3.0	—	—	—
	IABG	8.8 (5.79)	4.5 (0.87)	4.8-12	—	—	—
	ILAG	4.5 (1.66)	3.8 (1.65)	5.0-7.3	—	—	—
	LACH	2.0 (0.71)	3.3 (0.75)	2.3-3.0	—	—	—
	SCEK	7.0 (2.38)	6.0 (1.78)	7.0-8.5	—	—	—
3	GACH	2.3 (0.85)	3.3 (0.63)	1.3-4.3	—	—	—
	IABG	3.0 (1.47)	1.8 (0.85)	1.8-4.0	—	—	—
	ILAG	3.8 (1.38)	3.8 (1.25)	1.3-20.0	—	—	—
	LACH	—	—	—	—	—	—
	SCEK	32.5 (3.28)	18.3 (5.20)	17.0-62.0	—	—	—
4	GACH	4.5 (1.26)	9.0 (5.24)	4.0-13.3	—	—	—
	IABG	9.5 (3.07)	5.0 (0.91)	5.8-10.3	—	—	—
	ILAG	2.3 (0.63)	6.8 (4.77)	3.3-21.8	—	—	—
	LACH	—	—	—	—	—	—
	SCEK	—	—	—	—	—	—
5	GACH	—	—	—	—	—	—
	IABG	6.3 (0.48)	3.5 (2.25)	2.0-14.0	—	—	—
	ILAG	4.0 (1.22)	4.3 (2.36)	3.3-6.8	—	—	—
	LACH	—	—	—	—	—	—
	SCEK	—	—	—	—	—	—

Note: The experimental design was a randomized complete block with four replications (n = 4 except where noted in Table G-3).
* Indicates a statistically significant difference between MON 87751 and the control ($\alpha = 0.05$) using ANOVA.

† Indicates p-values could not be generated where the taxa did not meet inclusion criteria (see Appendix G.10.2).
A dash (—) indicates data not available.
[1] Arthropods were enumerated at five crop development stages beginning at approximately R1 and then every two weeks for a total of five collections.
[2] Site code: GACH = Chula, GA; IABG = Bagley, IA; ILAG = Thomasboro, IL; LACH = Cheneyville, LA; SCEK = Elko, SC
[3] MON 87751 and control values represent means with standard error in parentheses.
[4] Reference range is calculated from the minimum and maximum mean values from among reference materials at each site.

References for Appendix G

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Appendix H: Materials and Methods for Pollen Morphology and Viability Assessment

H.1. Plant Production

MON 87751, a conventional soybean control A3555, and four commercial reference varieties (Table H-1) were grown under similar agronomic conditions in a growth chamber in St. Louis County, MO. The trial was arranged in a randomized complete block design with four replications and seven plants per entry per replication.

H.2. Flower Collection

Soybean flowers were collected from each entry and each replication. Sampled flowers from each entry and each replication were placed in a labeled container and stored on wet ice until the pollen was prepared and stained.

H.3. Pollen Sample Preparation

Pollen samples were prepared in a laboratory. Clean microscope slides were labeled with the plot number. Tweezers were used to open each of the collected flowers from a replication and brush the pollen into the circle on the slide. The tweezers were cleaned between extractions of the pollen from the flowers of another entry or replication. Approximately 20-30 µl of Alexander's stain (Alexander 1980) was added to the center of the circle containing the pollen. The pollen was stained at ambient temperature for at least ten minutes prior to examination. Pollen samples from all entries within a replication were stained and evaluated on the same day.

H.4. Data Collection

Pollen characteristics were assessed by viewing samples under an Olympus© BX53 light microscope equipped with an Olympus© DP72 digital color camera. The microscope and camera were connected to a computer running Microsoft Windows XP® and installed with a Olympus© cellSens (version 1.4.1) imaging software.

H.4.1. Pollen Viability

When exposed to the staining solution, viable pollen grains stained red to purple due to the presence of living cytoplasmic content. Non-viable pollen grains stained light blue to green or are colorless and may have appeared round to collapsed in shape, depending on

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the degree of hydration. For each pollen sample, the number of viable and non-viable pollen grains was counted from a random field of view under the microscope. A minimum of 75 pollen grains were evaluated for each sample. Dense clusters of pollen or pollen grains adhering to flower parts were not considered for evaluation because they may not have absorbed the staining solution uniformly.

H.4.2. Pollen Diameter

Micrographs of 10 representative, viable pollen grains from each replication were taken at 200X magnification and imported into the imaging software. The software was used to measure pollen grain diameter along two perpendicular axes for each selected pollen grain. Mean pollen diameter for each replication was calculated from the total of 20 measurements.

H.4.3. General Pollen Morphology

General pollen morphology was observed from micrographs of MON 87751, the conventional control A3555, and reference materials (Figure H-1). The same micrographs were used for pollen diameter measurements.

H.5. Statistical Analysis

An analysis of variance was conducted according to a randomized complete block design using SAS® (SAS 2012). The level of statistical significance was predetermined (α=0.05). MON 87751 was compared to the control material for percent viable pollen and pollen grain diameter. The test material was not statistically compared to the reference materials. A reference range for each measured characteristic was determined from the minimum and maximum mean values associated with the four reference soybean varieties. General pollen morphology was qualitative characteristic; therefore, no statistical analysis was conducted on these observations.

Table H-1. Starting Seed for Pollen Morphology and Viability Assessment

Material Name[1]	Material Type	Phenotype	Monsanto Lot Number
A3555	Control	Conventional	11332613
Garst 3585N	Reference	Conventional	11242913
FS 3591	Reference	Conventional	10001448
eMerge 348TC	Reference	Conventional	11266960
Midland 363	Reference	Conventional	10001570
MON 87751	Test	Insect Protected	11332614

[1]The test material name is Monsanto Regulatory designations; the control and reference material names are commercial names.

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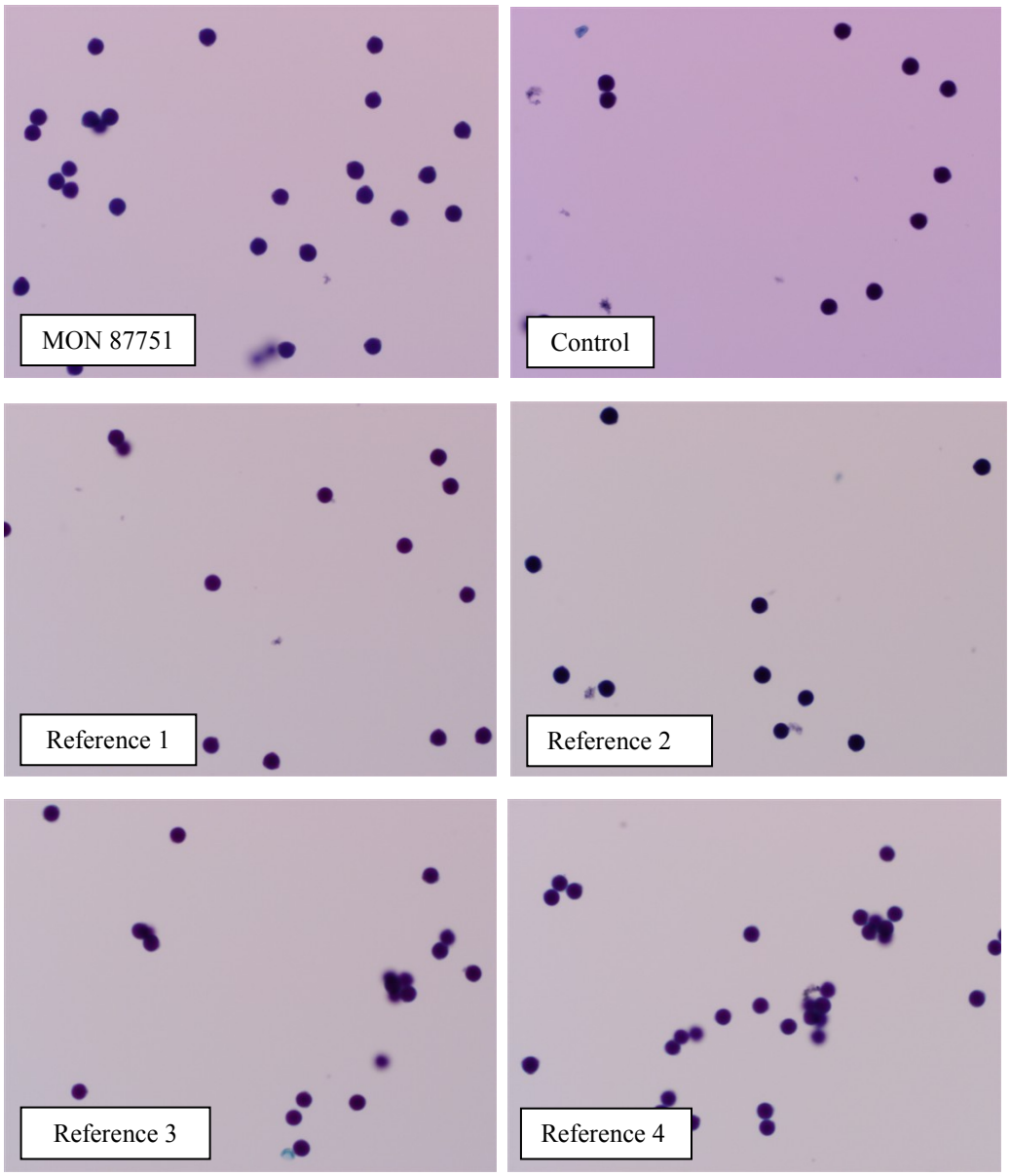

Figure H-1. General Morphology of Pollen from MON 87751, the Conventional Control, and Reference Varieties under 200X Magnification

References for Appendix H

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Appendix I: Materials and Methods for Symbiont Assessment

I.1. Materials

The soybean materials for the symbiont interaction assessment included MON 87751, the conventional control A3555, and six commercial references varieties (Table I-1). Nodule, root tissue, and shoot tissue collected from MON 87751, the conventional control A3555, and the commercial reference varieties were evaluated.

I.2. Characterization of the Methods

The identities of MON 87751 and the conventional control A3555 seed were verified by event-specific polymerase chain reaction analyses.

I.3. Greenhouse Phase and Experimental Design

MON 87751, the conventional control A3555, and the commercial reference varieties starting seed were planted in 6-inch pots containing nitrogen-deficient potting medium (Sunshine® Mix #2 Basic/LB2) composed of primarily peat, vermiculite, and perlite. Plants from MON 87751, the conventional control A3555, and commercial reference varieties starting seed were grown in a greenhouse where actual temperatures ranged from approximately 17 to 35°C. Eight replicate pots were planted with three seeds per pot for each of MON 87751, the conventional control A3555, and commercial reference varieties. At planting, each seed was inoculated with approximately 4×10^7 cells of Bradyrhizobium japonicum (VAULT® NP, Becker, Underwood, Ames, IA) in phosphate-buffered saline. Pots were arranged in eight replicated blocks for the 6-week sampling period using a randomized complete block design.

The starting seeds for replicates 1, 2, and 3 were planted on September 11, 2012, replicates 4, 5, and 6 were planted on September 12, 2012, and replicates 7 and 8 were planted on September 13, 2012. In all cases, replicate pots had a minimum of one plant emerge within one week. A solution of nitrogen-free nutrient solution (approximately 250 ml) was added weekly after plant emergence.

I.4. Plant Harvesting/Data Collection

Six weeks after emergence, plants were excised at the surface of the potting medium and shoot and root plus nodule material were removed from pots. The shoot material was cut into smaller pieces and placed in labeled bags. The plant roots with nodules were separated from the potting medium by washing with water. Excess moisture was removed using absorbent paper towels and the roots plus nodules were placed in labeled bags. The same day that plants were harvested, nodules were removed by hand from the roots of each plant, enumerated, and weighed to determine the fresh weight (fwt) of the nodules.

The remaining root and shoot fresh weight were determined for each plant. Nodules as well as root and shoot material were placed in a drying oven on the same day as collected. The plant material was dried for at least 72 hours at approximately 60-61°C to

determine dry weight (dwt). The shoot tissue was ground using a Harbil 5G high-speed paint shaker prior to total nitrogen analysis. Shoot total nitrogen was determined by combustion using a nitrogen analyzer (Rapid N Cube, Elementar Americas, Inc).

I.5. Statistical Analysis

The data consisted of six measurements taken at the six week sampling period: nodule number, nodule dwt (g), shoot dwt (g), root dwt (g), shoot total nitrogen (%), and shoot total nitrogen (g). An analysis of variance was conducted using a randomized complete block design with eight replications. Data were analyzed using SAS®. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$). No statistical comparisons were made between MON 87751 and the commercial reference varieties. Instead, a reference range for each measured characteristic was determined from the minimum and maximum mean values from among the six commercial reference varieties.

Table I-1. Starting Seed of MON 87751, Conventional Control, and Commercial Reference Varieties Used in the Symbiont Assessment

Material Name	Phenotype	Monsanto Lot Number
MON 87751	Insect Protected	11332614
A3555	Conventional Control	11332613
NuPride 2954	Conventional Reference	11213020
Crows C2804	Conventional Reference	11242902
Midland 363	Conventional Reference	11243106
LG C3540	Conventional Reference	11226858
Hoffman H419	Conventional Reference	11273007
Stewart SB3819	Conventional Reference	11226928

References for Appendix I

SAS. 2012. SAS/STAT software version 9.3. SAS Institute, Inc., Cary, North Carolina.