MONSANTO

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July 1, 2009

Dr. Michael Watson
Biotechnology Regulatory Services, APHIS
4700 River Road, Unit 147
Riverdale, MD 20737–1236

RE: Request for the Determination of Nonregulated Status for MON 87769

Dear Dr. Watson:

Monsanto Company requests that the USDA/APHIS review the enclosed petition for the determination of nonregulated status in whole for MON 87769.

Monsanto has developed biotechnology-derived soybean MON 87769 that produces stearidonic acid (SDA) a sustainable alternative omega-3 fatty acid source to help meet the needed dietary intake of long chain omega-3 fatty acids. SDA is a metabolic intermediate in the mammalian production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from alpha linolenic acid (ALA), a common dietary constituent. The development of MON 87769 involved the introduction of two desaturase genes, *Primula juliae* Δ6 desaturase (*Pj.D6D)* and *Neurospora crassa* Δ15 desaturase (*Nc.Fad3)* that encode for the PjΔ6D and NcΔ15D proteins, respectively. The PjΔ6D and NcΔ15D proteins share sequence similarity to other desaturase proteins that are ubiquitous in the human diet and animal feed. In MON 87769, NcΔ15D converts linoleic acid (LA) to ALA and PjΔ6D converts ALA to SDA and LA to gamma linolenic acid (GLA).

Compositional analyses have confirmed that MON 87769 seed contains approximately 20-30% SDA (wt% of total fatty acids) and approximately 7% GLA. Other components in MON 87769 seed and forage were compositionally equivalent to conventional soybean. Publically available scientific data on the safety of SDA and GLA, including results from Monsanto's toxicological studies on SDA soybean oil derived from MON 87769, confirm that MON 87769 seed and processed products (e.g., oil, meal, protein isolate, and lecithin) are safe for food and feed use.

The data in this petition support the conclusion that MON 87769 is not likely to present an increased plant pest risk or to have a significant environmental impact compared to

conventional soybean based on reasonably foreseeable actions. Monsanto requests a determination from APHIS that MON 87769, and any progeny derived from crosses between MON 87769 and conventional soybean or deregulated biotechnology-derived soybean be granted nonregulated status under 7 CFR Part 340.

The enclosed "Petition for Determination of Nonregulated Status for MON 87769" contains relevant information upon which to make a determination. This package contains three printed copies and one electronic copy of the petition.

Monsanto will provide similar information to the government of Canada as well as other regulatory agencies in major soybean importing countries. A food and feed safety assessment for MON 87769 was submitted to FDA on March 23, 2009.

We would be pleased to meet with you and other USDA officials and scientists to respond to any questions you may have, or to provide you with additional information that you may request. Should you have any questions concerning this letter or the enclosed petition, or wish to set up a meeting to further discuss MON 87769, please contact (b) (6) [redacted]
Washington DC, (b) (6) [redacted]

Sincerely,

(b) (6) [redacted]

cc: (b) (6) [redacted]
Monsanto Regulatory Files

MONSANTO

Petition for the Determination of Nonregulated Status for MON 87769

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

July 1, 2009

OECD Unique Identifier: MON-87769-7
Monsanto Petition Number: 09-SY-200U

Submitted by

(b) (6) [redacted]

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Prepared by

(b) (6) [redacted]

Contributors and/or Principal Investigators

(b) (6) [redacted]

RELEASE OF INFORMATION

Monsanto is submitting the information in this petition for review by the USDA as part of the regulatory process. By submitting this information, Monsanto does not authorize its release to any third party. In the event the USDA receives a Freedom of Information Act request, pursuant to 5 U.S.C. § 552, and 7 CFR Part 1, covering all or some of this information, 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 expects that no information that has been identified as CBI (confidential business information), will be provided to any third party. Monsanto understands that a CBI-deleted copy of this information may be made available to the public in a reading room and by individual request, as part of a public comment period. Except in accordance with the foregoing, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

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CERTIFICATION

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

(b) (6)

[redacted]

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EXECUTIVE SUMMARY

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility under the Plant Protection Act (7 USC § 7701-7772) to prevent the introduction and dissemination of plant pests into the United States. APHIS 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 no longer should 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.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status in whole for the new biotechnology-derived soybean product, MON 87769, any progeny derived from crosses between MON 87769 and conventional soybean, and any progeny derived from crosses of MON 87769 with other biotechnology-derived soybean that has been granted nonregulated status under 7 CFR Part 340.

Product Description

Monsanto has developed biotechnology-derived soybean MON 87769 that contains stearidonic acid (SDA; 18:4), a sustainable alternate source of an omega-3 fatty acid to help meet the needed dietary intake of long chain omega-3 fatty acids. The production of SDA in MON 87769 is accomplished through the introduction of two desaturase genes, *Primula juliae* Δ6 desaturase (*Pj.D6D)* and *Neurospora crassa* Δ15 desaturase (*Nc.Fad3)* into conventional soybean, A3525. The two introduced genes encode for the PjΔ6D and NcΔ15D proteins, respectively. SDA is an 18-carbon fatty acid with four double bonds (18:4) and is found in plants, fish and fish/algal oil products. In mammals, SDA is a metabolic intermediate in the production of eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) from alpha linolenic acid (ALA; 18:3), a common dietary constituent. In addition to SDA, MON 87769 contains approximately 7% γ-linolenic acid (GLA; 18:3), which is an *in vivo* metabolite in the conversion of linoleic acid (LA: 18:2) to arachidonic acid (20:4) in mammals.

Refined oil produced from MON 87769 contains approximately 20 to 30% SDA (wt% of total fatty acids)[1], and can be used for the production of margarine, mayonnaise, shortenings, salad dressings, ready-to-eat foods, and other food products. Since SDA has fewer double bonds than the omega-3 fatty acids, EPA (20:5) or DHA (22:6), SDA soybean oil is more stable to oxidation (i.e., less prone to fishy or rancid odors and taste) than fish oils, thereby expanding the potential formulation options for food companies and food products for consumers. Fish and plant oils rich in omega-3 fatty acids also are used in feed applications such as aquaculture and poultry feeds. SDA soybean oil from MON 87769 may eventually be used in aquaculture and feed applications as an alternative to fish oil and other omega-3 rich feed components. The meal derived from

[1] Referred to as SDA soybean oil.

MON 87769 is compositionally similar to other commodity soybean meal and will be used in a manner similar to conventional soybean meal.

Data and Information Presented to Assess Plant Pest Potential of MON 87769

The data and information presented in this petition demonstrate that MON 87769 is agronomically, phenotypically, and biochemically comparable to conventional soybean and that MON 87769 is not likely to pose an increased plant pest potential, including weediness or adverse environmental impact, compared to conventional soybean. The data presented in this petition to support the overall safety and plant pest risk potential of MON 87769 include the following:

1. A detailed molecular characterization of the inserted DNA, that confirmed the presence of intact *Pj.D6D* and *Nc.Fad3* gene cassettes stably integrated at a single locus of the soybean genome,
2. An extensive set of biochemical characterizations that demonstrate the identity of the PjΔ6D and NcΔ15D proteins produced in MON 87769,
3. A comprehensive safety assessment of the PjΔ6D and NcΔ15D proteins, that shows no allergenic potential, acute toxicity or dietary risk to humans and animals,
4. A detailed compositional and nutritional assessment demonstrating that MON 87769 is equivalent in composition to conventional soybean, except for the intended fatty acid change (i.e., the presence of SDA and GLA),
5. The confirmation by a scientific panel that SDA soybean oil from MON 87769 containing approximately 20-30% SDA and 7% GLA (wt% of total fatty acids), is generally recognized as safe (GRAS),
6. The expected lack of adverse environmental impact from exposure to SDA and GLA produced in MON 87769 due to the presence of these fatty acids in a number of other plant species,
7. An extensive evaluation of phenotypic and agronomic characteristics and environmental interactions that demonstrate MON 87769 is not likely to have increased plant pest potential compared to conventional soybean,
8. An assessment of the potential impact to non-target organisms (NTOs) and endangered species concludes that MON 87769 is unlikely to have adverse effects on these organisms under normal agricultural practices, and
9. An evaluation of the impact on agronomic practices and land use patterns demonstrating that MON 87769 is not likely to change existing cultivation practices or impact land use patterns.

Molecular Characterization of Inserted DNA

The development of MON 87769 involved the introduction of two desaturase genes, *Primula juliae* Δ6 desaturase (*Pj.D6D)* and *Neurospora crassa* Δ15 desaturase (*Nc.Fad3)* that encode for the PjΔ6D and NcΔ15D proteins. The genetic modification in MON 87769 was extensively characterized. The results confirm that MON 87769 contains a single insert with the intended sequence, where the insert is stably maintained over multiple generations, and that the insert will not result in unintended gene products with similarity to known allergens or toxins. The methodology used to characterize the

genetic modification included: 1) Southern blot analyses to assay the entire soybean genome for the presence of DNA derived from the transformation plasmid, PV-GMPQ1972, to confirm that a single copy was inserted at a single site in the genome and that the insert was stably inherited; 2) DNA sequencing analyses to determine the exact sequence of the inserted DNA and allow a comparison to the transfer DNA (T-DNA) sequence of the transformation vector to confirm that only the expected sequences were integrated; and 3) a segregation analysis to confirm that the introduced trait is inherited according to Mendelian laws of genetics. Taken together, the characterization data demonstrate that a single copy of the T-DNA was inserted at a single locus in the genome of MON 87769. The stability of the integrated DNA and absence of the backbone sequences in multiple generations of MON 87769 was also confirmed. These results are consistent with a single site of insertion that segregates in subsequent progeny according to the Mendelian laws of genetics.

Characterization of the PjΔ6D and NcΔ15D Proteins

A multistep approach was used to characterize the PjΔ6D and NcΔ15D proteins expressed in MON 87769 resulting from the genetic modification. This detailed characterization confirms that both proteins are safe for human and animal consumption. The assessment involved: 1) characterizing the physicochemical and functional properties of both proteins; 2) quantifying protein levels in plant tissues; 3) examining the similarity of each of these proteins to known allergens, toxins and other biologically active proteins known to have adverse effects on mammals; 4) evaluating the digestibility of both proteins in simulated gastrointestinal fluids; 5) documenting the history of safe consumption of these proteins or their structural/functional homologues that lack documented adverse affects on human or animal health; and 6) investigating potential mammalian toxicity through an acute oral gavage assay. The PjΔ6D and NcΔ15D proteins share sequence similarity to several proteins that are ubiquitous in the human diet and are directly consumed in many common foods. The PjΔ6D and NcΔ15D proteins are expressed at low levels in the seed and not detectable in other tissues analysed. Both proteins lack biologically relevant amino acid sequence similarities to known allergens, toxins and antinutritional proteins known to have adverse effects on mammals. Additionally, both proteins are rapidly digested in simulated gastric and intestinal fluids and do not exhibit any signs of toxicity when administered to mice via oral gavage. The safety assessment supports the conclusion that dietary exposure to the PjΔ6D and NcΔ15D proteins derived from MON 87769 poses no meaningful risk to human or animal health.

Composition and Nutrition of Forage and Grain

Detailed compositional and nutritional comparisons of MON 87769, a conventional soybean control, and ten commercially available soybean varieties were conducted. These compositional comparisons were made by analyzing the seed and forage harvested from five replicated field sites across the United States during the 2006 field season. The analysis conducted in accordance with OECD guidelines, included protein, fat, carbohydrates, fiber, ash, moisture, amino acids, fatty acids, vitamin E, and antinutrients. The compositional analyses confirmed that MON 87769 had the intended change in fatty

acid composition, while the other components analyzed in MON 87769 were compositionally equivalent to conventional soybean. As intended, MON 87769 seed contained SDA (26%) and GLA (7.1%) and low levels of two minor fatty acids, trans-SDA (0.18 %) and trans-ALA (0.44%). Statistical analysis was conducted on the composition data for other components for evidence of biologically-relevant changes. Statistical analysis was conducted on data from a combination of all sites (combined-site) and data from each of five individual sites (individual-site). Considering LA is the starting material from which SDA and GLA are produced, the LA levels were significantly different in MON 87769 compared to conventional soybean. As anticipated, the LA values were also outside the 99% tolerance interval for the population of conventional references as well as the range of values found in the published literature and the International Life Science Institute Crop Composition Database (ILSI-CCD). In addition to LA, combined-site statistical differences were found in five other fatty acid levels (palmitic, oleic, linolenic, arachidic, and behenic acid). The difference in the level of each of these five fatty acids was relatively small in absolute magnitude (<4 wt% of total fatty acids) and/or their mean values and ranges in MON 87769 seed were within the 99% tolerance interval for the population of the conventional reference varieties. Given the intended shift in the fatty acid metabolism toward an increase in SDA content in MON 87769, differences in fatty acid levels were expected.

Statistical comparisons between MON 87769 and the conventional control for the presence of other components showed that 22 non-fatty acid analytes in soybean seed were significantly different ($p<0.05$) in the combined-site analysis. For the 22 non-fatty acid analytes, most of the statistical differences between MON 87769 and conventional soybean control were seen in amino acids (17 out of the 22 significant differences) and the magnitude of these differences were small (<10%). The mean and range values of these amino acids were within the calculated 99% tolerance interval for the population of commercial conventional soybean varieties and also within the range of values found in the published literature and the ILSI-CCD. The mean level of three isoflavones, genistein, daidzein, and glycitein, were lower in MON 87769 harvested seed compared to the values in the conventional soybean control. However, the mean and range of values for these three analytes in MON 87769 were within the 99% tolerance interval for the population of conventional reference varieties, and were also within the range of values found in the published literature and the ILSI-CCD. It is well documented that the soybean isoflavone levels are greatly influenced by many factors, ranging from environmental conditions, variety, and agronomic practices. The remaining two statistical differences observed were for carbohydrates and protein, and their magnitude of differences between MON 87769 and conventional soybean control were small (<6%). Furthermore, the mean and range values of carbohydrates and protein were within the calculated 99% tolerance interval for the population of commercial conventional soybean varieties and also within the range of values found in the published literature and the ILSI-CCD. Therefore, these differences were not considered to be biologically meaningful from a food and feed safety or nutritional perspective. Combined-site analysis of forage showed no significant differences ($p>0.05$) between MON 87769 and the conventional soybean control.

In addition to the compositional analysis of seed and forage, four soybean processed fractions (oil, meal, lecithin, and protein isolate) were produced from MON 87769 seed and subjected to compositional analysis in accordance with OECD guidelines. As expected, apart from the intended fatty acid change, the composition of the soybean processed fractions from MON 87769 is equivalent to the composition of soybean processed fractions from the conventional soybean control. Thus, the processed fractions from MON 87769 are concluded to be as safe and nutritious as the processed fractions prepared from conventional soybean.

Collectively, the compositional data support the conclusion that MON 87769, with the exception of the expected changes in fatty acid composition, does not have biologically meaningful differences from conventional soybean from a food/feed safety and/or nutritional perspective.

History of Environmental Exposure to SDA and GLA

Environmental exposure to SDA and GLA from MON 87769 is expected to be limited primarily to soybean pests and animals that feed on seed. These fatty acids are already present in the environment. SDA, GLA, and other polyunsaturated fatty acids (PUFAs) exist in many sources in the environment without known adverse ecological effects. Many fish species, particularly marine fish, contain SDA. SDA and GLA are produced in many plant seed oils. More than 230 species from among 17 plant families are documented to produce SDA in seed oil at concentrations greater than 1% of their total fatty acid composition. Similarly, GLA is present in a number of plant species at levels greater than 1% of their total fatty acid composition. As natural components of the plant and animal world, SDA, GLA and other PUFAs are not expected to accumulate, persist or be detrimental to the environment.

Phenotypic and Agronomic Characteristics and Environmental Interactions

The commercial soybean species cultivated 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 scientific literature, nor is it present on the lists of noxious weed species compiled by the Federal government. Soybean does not possess any of the attributes commonly associated with weeds, such as long persistence of seed in the soil, the ability to disperse, invade, and become a dominant species in new or diverse landscapes, or the ability to compete well with native vegetation. Due to a pronounced lack of dormancy, soybean seed can germinate quickly under adequate temperature and moisture and potentially can grow as a volunteer plant. However, a volunteer plant likely would be killed by frost during autumn or winter of the year it was produced. If it did become established, a volunteer plant would not compete well with the succeeding crop, and could be controlled readily by either mechanical or chemical means. In addition, since wild populations of *Glycine* species are not known to exist in the U.S., the potential does not exist for MON 87769 to outcross to wild or weedy relatives and alter their weediness potential.

A phenotypic and agronomic assessment was conducted to determine whether MON 87769 has altered soybean characteristics that may result in increased weediness or have impact on cultivation practices. Additionally, the environmental interactions of MON 87769 were assessed relative to conventional soybean. The overall assessment indicates that MON 87769 is not fundamentally different from conventional soybean and is unlikely to have an increased plant pest risk. An important element in assessing plant pest potential and the environmental impact of MON 87769 is to compare MON 87769 to conventional soybean. The assessment is based initially on the concept of familiarity, which USDA recognizes, plays an important role in these assessments. Familiarity is based on the fact that the biotechnology-derived plant is developed from a conventional plant variety 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 interactions among these factors, and provides a basis for comparative risk assessment between a biotechnology-derived plant and its conventional counterpart. The MON 87769 characteristics assessed included: seed dormancy and germination, pollen morphology, symbiont interactions conducted in the laboratory and greenhouse, and plant phenotypic and environmental interaction evaluations conducted in the field.

Seed dormancy and germination characterization indicated that MON 87769 seed had germination characteristics similar to that of the conventional soybean control. In particular, the lack of increased hard seed, a well-accepted characteristic of weediness affecting seed germination rate and viability, supports a conclusion of no increased weediness potential of MON 87769 compared to conventional soybean for germination and dormancy characteristics. In an assessment of pollen characteristics, there were no significant differences ($p>0.05$) observed for any of the parameters measured, including pollen viability and diameter. Furthermore, MON 87769 was not altered in its symbiotic relationship with *Bradyrhizobium japonicum* compared to conventional soybean based on assessments of nodule number, shoot nitrogen, and biomass of nodules, shoot material, or root material. Collectively, these results support the conclusion that MON 87769 is not likely to exhibit increased weed potential compared to conventional soybean.

The field evaluation of phenotypic, agronomic, and ecological characteristics of MON 87769 also supports the conclusion that MON 87769 is not likely to pose an increased plant pest potential compared to conventional soybean. These evaluations were conducted at 21 field locations across U.S. soybean production regions during the 2006 and 2007 growing seasons. Fourteen phenotypic characteristics were assessed including: early stand count, seedling vigor, plant growth stage, days to 50% flowering, flower color, plant pubescence, plant height, lodging, pod shattering, final stand count, seed moisture, 100 seed weight, test weight, and yield. The observed phenotypic characteristics of MON 87769 were not different from the conventional soybean control. No significant differences ($p<0.05$) were observed in either year in a combined-site analysis of the data for any of the phenotypic characteristics measured. The results from the 2006 and 2007 field trials demonstrate that the assessed phenotypic and agronomic characteristics of MON 87769 were not altered, compared to the conventional control, as a result of the introduced genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D) and the intended fatty acid change. These results are

indicative of no increased plant pest or weediness potential of MON 87769 compared to conventional soybean.

Similarly, an assessment of the plant response to abiotic stressors (e.g., drought, nutrient deficiency, wind, etc.), disease damage, and arthropod damage, indicates no increased plant pest potential or adverse ecological interactions of MON 87769 relative to conventional soybean. No biologically meaningful differences were detected between MON 87769 and the conventional control for the 703 comparative observations of plant response to abiotic stressors, disease damage, and arthropod damage and 260 comparative observations of the abundance of various pest and beneficial arthropods in field trials over two consecutive years.

Nontarget Organisms and Threatened or Endangered Species

An environmental assessment of MON 87769 indicates that MON 87769 would not have a significant impact (either positive or negative) on NTOs, including threatened or endangered species under normal agricultural practices. The assessment considered the expected differences between MON 87769 and conventional soybean, including the presence of the inserted genes, the expression of the PjΔ6D and NcΔ15D proteins, and the production of the expected fatty acids (SDA and GLA) in MON 87769 seed. Since MON 87769 is a source of omega-3 enriched soybean oil with no pesticidal activity, all exposed organisms are considered to be NTOs. The assessment considered pertinent information from product characterization and protein safety assessment, history of environmental exposure to the additional fatty acids in MON 87769 seed, and results from the ecological interaction assessment. Based on the weight of evidence of this information, there is no indication that MON 87769 would have an adverse impact on NTOs including threatened or endangered species under normal agricultural practices.

The potential for MON 87769 to outcross with sexually compatible species in the U.S., including threatened or endangered plant species, is unlikely, since no known wild *Glycine* species related to cultivated soybean are known to be present in North America. In those world areas where sexually compatible species do exist, the potential for outcrossing is low because soybean is a highly self-pollinated species, with cross-pollination to adjacent plants of other soybean varieties occurring at very low frequencies (0.3 to 3.62%) (Beard and Knowles, 1971). Furthermore, in the rare event when cross-pollination may occur, MON 87769 and its progeny are not expected to have a significant environmental impact. The evaluations have shown that the introduced genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change is not likely to enhance the weediness or plant pest potential of MON 87769. Therefore, the environmental consequence of pollen transfer from MON 87769 to other *Glycine* species is considered negligible.

Soybean Agronomic Practices and Land Use

Soybean is one of the largest U.S. crops in terms of acreage planted and quantity of seed harvested. In 2008, soybean was planted on 75.7 million acres in the U.S., where the harvested soybean seed had an average yield of 36.9 bushels per acre and total productivity was 2.96 billion bushels, resulting in a net value greater than $27.4 billion.

Specialty soybean varieties are produced on approximately 12% of the U.S. soybean acreage and according to the Midwest Shippers Association this acreage could grow to over 25% of the crop acreage in certain states within the next decade.

Soybean fields are typically highly managed agricultural areas that can be expected to be dedicated to crop production for many years. No significant impact would be expected following the introduction of MON 87769 on current cultivation and management practices for soybean. MON 87769 has been shown to be no different from conventional soybean in its agronomic, phenotypic, and ecological characteristics and has the same levels of resistance to insects and diseases as current commercial soybean. Cultivation of MON 87769 would not be expected to differ from typical speciality soybean cultivation. Based on these considerations, there is no apparent potential for significant impact on land use or agronomic practices.

Conclusion

Based on the data and information presented in this petition, it is concluded that MON 87769 is not likely to be a plant pest. Therefore, Monsanto Company requests a determination from APHIS that MON 87769 and any progeny derived from crosses between MON 87769 and conventional soybean or deregulated biotechnology-derived soybean be granted nonregulated status under 7 CFR Part 340.

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ABBREVIATION AND DEFINITIONS*

~	Approximately
2T-DNA	Plasmid vector containing two separate T-DNA regions each surrounded by left and right borders of the Ti plasmid
35S	The promoter and leader from the cauliflower mosaic virus (CaMV) 35S RNA
A3525	Conventional Soybean Control
aadA	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
AA	Amino Acid
AAA	Amino Acid Analysis
ADF	Acid Detergent Fiber
AD8	Allergen Database; www.allergenonline.com, released in January 2008
ALA	Alpha Linolenic Acid
AN(C)OVA	Analysis of (Co)Variance
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
AOCS	American Oil Chemists Society
AOSA	Association of Official Seed Analysts
AOSCA	Association of Official Seed Certifying Agencies
APS	Analytical Protein Standard
ASA	America Soybean Association
B-Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
B-Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
BSA	Bovine Serum Albumin
CaMV	Cauliflower Mosaic Virus
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CAST	Council for Agricultural Science and Technology, USDA
CBI	Confidential Business Information
CEQ	The Council on Environmental Quality

* Note: Standard abbreviations, e.g., units of measure, are used according to the format described in 'Instructions to Authors' in the *Journal of Biological Chemistry*.

CEW	Corn Earworm [*Helicoverpa zea* (Boddie)]
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
COA	Certificate of Analysis
CPB	Cartagena Protocol on Biosafety
CSFII	Continuing Survey of Food Intakes by Individuals
CS-*cp4 epsps*	Coding Sequence (codon-modified) of the *aroA* gene from *Agrobacterium sp*. strain CP4 encoding the CP4 EPSPS protein
CS-*Nc.Fad3*	Coding Sequence (codon-optimized) of the gene from *Neurospora crassa* encoding Δ15 desaturase
CS-*Pj.D6D*	Coding sequence for the fatty acid Δ6 desaturase from *Primula juliae*
CS-*rop*	Coding Sequence for repressor of primer protein used for maintenance of plasmid copy number in *E. coli*
CTAB	Hexadecyltrimethylammonium Bromide
CTP	Chloroplast Transit Peptide
CV	Coefficient of Variation
Da	Dalton
DAP	Days After Planting
dCTP	Deoxycytidine Triphosphate
DDI	Daily Dietary Intake
DHA	Docosahexaenoic Acid (22:6)
DHB	Dihydroxybenzoic Acid
DI	De-Ionozed
DEEM-FCID	Dietary Exposure Evaluation Model-Food Commodity Intake Database
dNTP	Deoxynucleotide Triphosphate
DT	Defatted and Toasted (in reference to soybean meal)
DTT	Dithiothreitol
DW	Dry Weight
DWCF	Dry Weight Conversion Factor
dwt	Dry Weight of Tissue
E. coli	*Escherichia coli*
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ELSD	Evaporative Light Scattering Detector
EPA	Eicosapentaenoic Acid

EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
EU	European Union
FA	Fatty Acid
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMV	Figwort mosaic virus 35S promoter
FONSI	Finding of No Significant Impact
FW	Fresh Weight
fwt	Fresh Weight of Tissue
GLP	Good Laboratory Practice
GE	Genetically Engineered
GMO	Genetically Modified Organism
GLA	Gamma Linolenic Acid (18:3)
GOI	Gene of Interest
GRAS	Generally Recognized as Safe
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HOSFO	High Oleic Sunflower Seed Oil
IA	Immunoaffinity
IABW	Immunoaffinity Binding and Washing
IDP	Identity Preserved
IFIC	International Food Information Council
IgG	Immunoglobulin G
ILDIS	International Legume Database and Information Service
ILSI	International Life Science Institute
ILSI-CCD	International Life Sciences Institute Crop Composition Database
IRM	Insect resistance Management
ISO	International Organization for Standardization
IUPAC-IUB	International Union of Pure and Applied Chemistry-International Union of Biochemists
kDa	Kilo Daltons
LB	Loading Buffer
Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
LOD	Limit of Detection
LOQ	Limit of Quantitation

LA	Linoleic Acid
LC	Long Chain (i.e., long chain fatty acids)
L-*ShkG*	5' UTR from the Arabidopsis *ShkG* gene encoding EPSPS
MAFF	Ministry of Agriculture, Forestry and Fisheries, Japan
MALDI-TOF MS	Matrix-Assisted laser Desorption/ionization Time-of-Flight Mass Spectrometry
MEEC	Maximum Expected Environmental Concentration
MES	2-(N-morpholino)ethanesulfonic Acid
MH^+	Protonated Mass Ion
MHLW	Ministry of Health, Labor and Welfare, Japan
MMT	Million Metric Tones
MOE	Margin of Exposure
MW	Molecular Weight
MWM	Molecular Weight Marker
N/A	Not Applicable
NcΔ15D	*Neurospora crassa* delta-15 desaturase
NDF	Neutral Detergent Fiber
NEPA	National Environmental Policy Act
NFDM	Non-Fat Dried Milk
NI	Novel Ingredient
NIST	National Institute of Standards and Technology
NLT	Not Less Than
NMT	Not More Than
NMWC	Nominal Molecular Weight Cut-Off
NOAEL	No Observable Adverse Effect Level
NOEC	No Observable Effect Concentration
NOEL	No Observable Effect Level
NOP	National Organic Program
NTO	Nontarget Organism
OECD	Organization for Economic Co-operation and Development
OR	Origin of replication
ORF	Open Reading Frame
OR-*ori-pBR322*	Origin of replication from *pBR322* necessary for maintenance of plasmid in *E. coli*
OR–*ori V*	Origin of replication from the broad host range plasmid RK2 necessary for maintenance of plasmid in *Agrobacterium*
OSL	Overseason Leaf
PjΔ6D	*Primula juliae* delta-6 desaturase
P-7Sα′	Promoter and leader from the *Sphas1* gene of soybean

P-7Sα	Promoter and leader from the *Sphas2* gene from soybean
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Containing 0.05% (v/v) Tween-20
PC	Phosphotidyl Choline
PCR	Polymerase Chain Reaction
PE	Phosphotidyl Ethanolamine
PEG	Polyethylene Glycol
P-FMV	Promoter for the 35S RNA from Figwort Mosaic Virus (FMV)
PMSF	Phenylmethanesulfonyl fluoride
POI	Protein of Interest
PPA	Plant Protection Act
ppm	Parts Per Million
PTH	Phenylthiohydantoin
PRESS	Predicted Residual Sums of Squares
PVDF	Polyvinylidene Difluoride
PVPP	Polyvinylpolypyrrolidone
PUFA(s)	Polyunsaturated Fatty Acid(s)
PV-GMPQ1972	Plasmid Vector used to develop MON 87769
RbcS4	Promoter, leader, and 5' nontranslated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1,5-bisphosphate carboxylase small subunit 1A
RBD	Refined, Bleached, and Deodorized (in reference to oils)
Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA
SAS	Statistical Analysis System
SBO	Soybean Oil (non-SDA)
SBM	Soybean Meal
SCN	Soybean Cyst Nematode
SD	Standard Deviation
SDA	Stearidonic Acid
SDA-TAG	Stearidonic acid - Triacylglycerol
SDA-EE	Stearidonic acid – Ethyl ester
SDA-FA	Steridonic acid – Fatty acid
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard Error
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid

SMIC	Soybean Meal Information Center
SOP	Standard Operating Procedure
Strep	Streptavidin
TDF	Total Dietary Fiber
TBST	Tris-Buffered Saline with 0.1% (v/v) Tween-20
T-DNA	Transfer DNA
TES	Threatened or Endangered Species
TIU	Trypsin Inhibitor Unit
TFA	Trans Fatty Acid
TMB	3,3',5,5'-Tetramethylbenzidene
Tris	Tris (hydroxymethyl) aminomethane
TSSP	Tissue-Specific Site Pool
Tween-20	Polyoxyethylenesorbitan Monolaurate
TAG	Triacylglycerol, i.e., a Triglyceride
T/C/R	Test, Control and Reference
TDF	Total Dietary Fiber
T-DNA I	Transferred DNA I
T-DNA II	Transferred DNA II
T-*E9*	3′ nontranslated region of the pea *rbcS2* gene that functions to direct polyadenylation of the mRNA
TG	Triglyceride
TS-*CTP2*	Transit peptide regions of *Arabidopsis thaliana* EPSPS used to direct proteins into chloroplasts
T-*tml*	3′ nontranslated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid that functions to direct polyadenylation of the mRNA
USDA	U.S. Department of Agriculture
USDA-APHIS	United States Department of Agriculture – Animal and Plant Health Inspection Service
USDA-ARS	United State Department of Agriculture – Agricultural Research Service
USDA-ERS	United States Department of Agriculture – Economic Research Service
USDA-GRIN	United State Department of Agriculture – Germplasm Resources Information Network
USDA-NASS	United States Department of Agriculture – National Agricultural Statistics Service
USDA-NSHS	United States Department of Agriculture – National Seed Health System
USFWS	United States Fish and Wildlife Service

v/v	Volume Per Volume
w/v	Weight Per Volume

I. RATIONALE FOR THE DEVELOPMENT OF MON 87769

I.A. Basis for the Request for a Determination of Nonregulated Status

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772) to prevent the introduction and dissemination of plant pests into the U.S. APHIS 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 no longer should 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.

Monsanto Company is submitting this request to APHIS for a determination of nonregulated status in whole for the new biotechnology-derived soybean product, MON 87769, any progeny derived from crosses between MON 87769 and conventional soybean, and any progeny derived from crosses of MON 87769 with other biotechnology-derived soybean that has been granted nonregulated status under 7 CFR Part 340.

I.B. Rationale for the Development of Soybean MON 87769

Monsanto has developed biotechnology-derived soybean MON 87769 that contains stearidonic acid (SDA; 18:4), a sustainable alternate source of an omega-3 fatty acid to help meet the needed dietary intake of long chain omega-3 fatty acids. The production of SDA in MON 87769 is accomplished through the introduction of two desaturase genes, *Primula juliae* Δ6 desaturase (*Pj.D6D)* and *Neurospora crassa* Δ15 desaturase (*Nc.Fad3)* into conventional soybean, A3525. The two introduced genes encode for the PjΔ6D and NcΔ15D proteins, respectively. SDA is an eighteen-carbon fatty acid with four double bonds (18:4) and as such is found in plants, fish and fish/algal oil products. In mammals, SDA is a metabolic intermediate in the production of eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) from alpha linolenic acid (ALA; 18:3), a common dietary constituent. In addition to SDA, MON 87769 contains approximately 7% GLA, which is an *in vivo* metabolite in the conversion of linoleic acid (LA; 18:2) to arachidonic acid (20:4) in mammals (see Figure VII-1).

Although the benefits of omega-3 fatty acid consumption are widely recognized, typical Western diets contain very little fish, and it is not practical to expect the general population to take fish oil supplements on a regular basis. An alternative approach to increase omega-3 fatty acid intake is to provide a wider range of foods that are enriched in omega-3 fatty acids so that people can choose foods that suit their usual dietary habits. Human and animal studies have shown that 1 g dietary SDA is approximately equivalent to 200 – 300 mg dietary EPA in terms of increasing tissue concentrations of EPA (James et al., 2003; Yamazaki et al., 1992). Thus, MON 87769 can serve as an alternate source to help meet the needed dietary intake of long chain omega-3 fatty acids in food and feed.

The oil from MON 87769 contains approximately 20 to 30% SDA (wt% of total fatty acids). SDA soybean oil can be used for the production of margarine, mayonnaise, shortenings, salad dressings, ready-to-eat foods, and other food categories. The use of SDA soybean oil in selected food categories could provide a wide range of dietary alternatives for increasing the omega-3 fatty acid intake. Because SDA has fewer double bonds than EPA and DHA, SDA soybean oil is more stable to oxidation (e.g., less prone to fishy or rancid odors and taste), thereby expanding the potential food formulation options for food companies and food products for consumers, and serving as an alternative to fish oils where they currently are used in food production. Fish oil and plant oils rich in omega-3 fatty acids are used in feed applications, especially in aquaculture and poultry feeds. SDA soybean oil may eventually be used in aquaculture and feed applications as an alternative to fish oil and other omega-3 rich feed components.

Given the targeted commercial applications of SDA soybean oil from MON 87769 as an alternate source of omega-3 fatty acids in food and feed, it is anticipated that MON 87769 will be a low acreage (<5% of total U.S. soybean acreage, i.e. up to 3 – 3.5 million acres) product planned initially for production in North America. In order to derive commercial value from this product, the SDA soybean crop will be grown and processed in an identity-preserved manner (IDP) in the Northern U.S. soybean growing region. The oil will be used in food applications and may be used in feed where omega-3 products are currently being used. The coproduct, soybean meal, has been shown to be compositionally comparable to other commodity soybean meal, and it will be used in a manner similar to conventional soybean meal.

I.C. Submissions to Other Regulatory Agencies

Under the Coordinated Framework for Regulation of Biotechnology, the responsibility for regulatory oversight of biotechnology-derived crops falls on two federal agencies: FDA and USDA (USDA, 1986). Deregulation of MON 87769 by USDA constitutes only one component of the overall regulatory oversight and review of this product. As a practical matter, MON 87769 cannot be released and marketed until FDA and USDA have completed their reviews and assessments under their respective jurisdictions.

Submission to FDA

MON 87769 falls within the scope of the 1992 U.S. Food and Drug Administration policy statement concerning regulation of products derived from new plant varieties, including those developed through biotechnology (FDA, 1992). In compliance with this policy, on March 23, 2009, Monsanto initiated consultation with the FDA on the food and feed safety and nutritional assessment for MON 87769 (FDA BNF 00117).

Submissions to Foreign Government Agencies

To support commercial introduction of MON 87769 in the U.S., regulatory submissions will be made to countries that import significant quantities of soybean or its processed fractions from the U.S. and have established regulatory approval processes in place. These will include submissions to a number of foreign government regulatory authorities,

including: Ministry of Agriculture, People's Republic of China; Japan's Ministry of Agriculture, Forestry, and Fisheries (MAFF) and the Ministry of Health, Labor, and Welfare (MHLW); the Canadian Food Inspection Agency (CFIA) and Health Canada; the Intersectoral Commission for Biosafety of Genetically Modified Organisms (CIBIOGEM), Mexico; the European Food Safety Authority (EFSA); and the regulatory authorities in other soybean importing countries with functioning regulatory systems. As appropriate, notifications of importation will be made to importing countries that do not have a formal approval process.

II. THE SOYBEAN FAMILY

This section summarizes the taxonomy, biology, and use of soybean based on: 1) the consensus document for *Glycine max* (L.) Merr. prepared by the Organization for Economic Co-operation and Development (OECD, 2000; OECD, 2001), and 2) a summary prepared by USDA-APHIS (2006) and a biology document published by Canadian Food Inspection Agency-Plant Biosafety Office (CFIA, 1996),

II.A. Soybean as a Crop

Soybean is the most prevalently grown oilseed in the world, with approximately 222.1 million metric tons of harvested seed (MMT) produced in 2007, which represented 56% of world oilseed seed production that year (ASA, 2008; Soyatech, 2008). Soybean is grown as a commercial crop in over 35 countries. The major producers of soybean are the U.S., Brazil, Argentina, China, and India, which accounted for approximately 91% of the global soybean production in 2007 (Soyatech, 2008); also see Table II-1. Approximately one-third of the 2007 world soybean production was produced in the U.S. (Soyatech, 2008). The soybean produced in China and India are primarily for domestic use, while a significant portion of that produced in U.S., Brazil, and Argentina is traded globally in the form of soybean harvested seed, soybean meal or soybean oil. Globally, the U.S. was the largest soybean seed export country, while Argentina led the soybean meal and soybean oil export markets in 2007 (ASA, 2008; Soyatech, 2008).

Table II-1. World Soybean Production in 2007/2008

Country	Production (million metric tons)
U.S.	71.4
Brazil	61.0
Argentina	47.0
China	15.6
Other	8.9
India	7.9
Paraguay	6.2
Canada	3.1
EU	1.0

Source: Soyatech (2008).

Approximately 50% of the world soybean seed supply was crushed to produce soybean meal and oil in 2007 (ASA, 2008; Soyatech, 2008), and the majority was used to supply the feed industry for livestock use or the food industry for edible vegetable oil and soybean protein isolates. Another 34% of the world soybean seed supply was traded to other geographies, with China, EU, Japan, and Mexico being the top soybean seed import geographies (ASA, 2008). The remainder of the soybean seed produced was used as certified seed, feed, or stocks.

Soybean is used in various food products, including tofu, soybean 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 generally has a smaller contribution to soybean's overall value compared to soybean meal because the oil constitutes just 18 to 19% of the soybean's weight. Nonetheless, soybean oil accounted for approximately 30% of all the vegetable oils consumed globally, and was the second largest source of vegetable oil worldwide, slightly behind palm oil at approximately 32% share (Soyatech, 2008).

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. By far, soybean meal is the world's most important protein feed, accounting for nearly 69% of world protein meal supplies (ASA, 2008). 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 Cahoon (Cahoon, 2003) and the American Soybean Association (ASA, 2008).

Global soybean plantings reached 90.8 million hectares in 2007/08, an 8.9% increase over the previous four years with an average of 82.3 million hectares planted from 2002/03 – 2007/08 (Soyatech, 2008). Soybean production has realized, on average, a 6.2% annual growth between 1995/96 to 2006/07. Increased planting flexibility, increased yield from narrow-row seeding practices, a higher rate of corn-soybean rotations, and low production costs favored expansion of soybean areas in the mid-1990s, and the expanded areas tended to be concentrated where soybean yields were highest.

II.B. History of Soybean

Domestication of soybean is thought to have taken place in China during the Shang dynasty (approximately 1500 to 1027 B.C.) or earlier (Hymowitz, 1970). However, historical and geographical evidence only could be traced back to the Zhou dynasty (1027 to 221 B.C.) where the soybean was utilized as a domesticated crop in the northeastern part of China. By the first century A.D., the soybean probably reached Central and Southern China as well as peninsular Korea. The movement of soybean germplasms was probably associated with the development and consolidation of territories and the degeneration of Chinese dynasties (Ho, 1969; Hymowitz, 1970).

From the first century A.D. to the 15th and 16th centuries, soybean was introduced into several countries, with land races eventually developing in Japan, Indonesia, Philippines, Vietnam, Thailand, Malaysia, Myanmar, Nepal, and Northern India. The movement of soybean throughout this period was due to the establishment of sea and land trade routes, the migration of certain tribes from China, and the rapid acceptance of seeds as a staple food by other cultures (Hymowitz and Newell, 1981; Hymowitz et al., 1990).

Starting in the late 16th century and through the 17th century, soybean was used by the Europeans, and in the 17th century, soybean sauce was a common item of trade from the East to the West.

Soybean was introduced into North America in the 18th century. In 1851, soybean was introduced in Illinois and subsequently throughout the Corn Belt. In 1853, soybean seed were deposited at the New York State Agricultural Society, the Massachusetts Horticultural Society, and the Commissioner of Patents. The two societies and the Commissioner of Patents sent soybean seed to dozens of growers throughout the U.S. Soybean has been cultivated extensively and improved through conventional breeding programs following its introduction in the U.S. and subsequently has become a key source of nutrients for food and feed use in the U.S. (Hymowitz and Singh, 1987).

II.C. Taxonomy and Phylogenetics of Soybean

Cultivated soybean, *Glycine max* (L.) Merr., is a diploidized tetraploid (2n=40), which belongs to the family Leguminosae, the subfamily Papilionoideae, the tribe Phaseoleae, the genus *Glycine* Willd., and the subgenus *Soja* (Moench) F.J. Herm.

Family: Leguminosae
Subfamily: Papilionoideae
Tribe: Phaseoleae
Genus: *Glycine*
Subgenus: *Soja* (Moench) F.J. Herm.
Species: *max*

The genus *Glycine* Willd. is of Asian and Australian origin and is divided into two subgenera, *Glycine* and *Soja* (Moench) F.J. Herm. The subgenus *Glycine* consists of 22 wild perennial species, which are indigenous to Australia, West, Central and South Pacific Islands, China, Russia, Japan, Indonesia, Korea, Papua New Guinea, the Philippines, and Taiwan (Hymowitz, 2004). The subgenus *Soja* includes the cultivated soybean, *G. max* (L.) Merr. and its wild annual relatives from Asia, *G. soja* Sieb. and Zucc. The list of species in the genus *Glycine* Willd. is presented in Table II-2.

Table II-2. List of Species in the Genus *Glycine* Willd., 2n Chromosome Number, Genome Symbol, and Distribution

Genus		2n	Genome[1]	Distribution
Subgenus *Glycine*				
1.	*G. albicans* Tind. & Craven	40	I1	Australia
2.	*G. aphyonota* B. Pfeil	40	--[2]	Australia
3.	*G. arenaria* Tind.	40	HH	Australia
4.	*G. argyrea* Tind.	40	A2A2	Australia
5.	*G. canescens* F.J. Herm.	40	AA	Australia
6.	*G. clandestina* Wendl.	40	A1A1	Australia
7.	*G. curvata* Tind.	40	C1C1	Australia
8.	*G. cyrtoloba* Tind.	40	CC	Australia
9.	*G. dolichocarpa* Tateishi and Ohashi	80	--	(Taiwan)
10.	*G. falcate* Benth.	40	FF	Australia
11.	*G. hirticaulis* Tind. & Craven	40	H1H1	Australia
		80	--	Australia
12.	*G. lactovirens* Tind. & Craven.	40	I1I1	Australia
13.	*G. latifolia* (Benth.) Newell & Hymowitz	40	B1B1	Australia
14.	*G. latrobeana* (meissn.) Benth.	40	A3A3	Australia
15.	*G. microphylla* (Benth.) Tind.	40	BB	Australia
16.	*G. peratosa* B. Pfeil & Tind.	40	--	Australia
17.	*G. pindanica* Tind. & Craven	40	H3H2	Australia
18.	*G. pullenii* B. Pfeil, Tind. & Craven	40	--	Australia
19.	*G. rubiginosa* Tind. & B. Pfeil	40	--	Australia
20.	*G. stenophita* B. Pfeil & Tind.	40	B3B3	Australia
21.	*G. tabacina* (Labill.) Benth.	40	B2B2	Australia
		80	Complex[3]	Australia, West Central and South Pacific Islands
22.	*G. tomentella* Hayata	38	EE	Australia
		40	DD	Australia, Papua New Guinea
		78	Complex[4]	Australia, Papua New Guinea
		80	Complex[5]	Australia, Papua New Guinea, Indonesia, Philippines, Taiwan
Subgenus *Soja* (Moench) F.J. Herm.				
23.	*G. soja* Sieb. & Zucc.	40	GG	China, Russia, Taiwan, Japan, Korea (Wild Soybean)
24.	*G. max* (L.) Merr.	40	GG	Cultigen (Soybean)

[1] Genomically similar species carry the same letter symbols.
[2] Genome designation has not been assigned to the species.
[3] Allopolyploids (A and B genomes) and segmental allopolyploids (B genomes).
[4] Allopolyploids (D and E, A and E, or any other unknown combination).
[5] Allopolyploids (A and D genomes, or any other unknown combination).
Note: Table is adapted from Hymowitz (2004).

Glycine soja grows wild in China, Japan, Korea, the Russian Far East, and Taiwan and is commonly found in fields, hedgerows, roadsides, and riverbanks (Lu, 2004). The plant is an annual, slender in build with narrow trifoliolate leaves. The purple or very rarely white flowers are inserted on short, slender racemes. The pods are short and tawny with hirsute pubescence, producing oval-oblong seeds (Hermann, 1962).

Glycine max (L.) Merr., the cultivated soybean, is an annual that generally exhibits an erect, sparsely branched, bush-type growth habit with trifoliolate leaves. The leaflets are broadly ovate, and the purple, pink, or white flowers are borne on short axillary racemes or reduced peduncles. The pods are either straight or slightly curved, and one to three ovoid to sub-spherical seeds are produced per pod.

A third and unofficial species named *G. gracilis* is also described within the context of the *Soja* subgenus in addition to *G. soja* and *G. max*. The *G. gracilis* is known only from Northeast China, is intermediate in morphology between *G. max* and *G. soja*, and is sometimes considered a variant of *G. max*. The three species in the *Soja* subgenus can cross-pollinate, and the hybrid seed can germinate normally and subsequently produce fertile pollen and seed (Singh and Hymowitz, 1989). The taxonomic position of *G. gracilis* has been an area of debate, and neither ILDIS (International Legume Database and Information Service) nor USDA-GRIN (USDA Germplasm Resources Information Network) recognizes *G. gracilis* as a distinct species. The wild and weedy relatives (*G. soja* and *G. gracilis*) of soybean do not occur in the U.S., and, therefore, are not likely to contribute to the potential for outcrossing (USDA-APHIS, 2006).

II.D. The Genetics of Soybean

Glycine is the only genus in the tribe Phaseoleae where species have diploid chromosome numbers of 40 and 80, but not 20 (Lackey, 1981). The unique chromosome number of *Glycine* is probably derived from diploid ancestors with a base number of 11. The ancestral species have undergone aneuploid reduction (loss of a specific chromosome), which is prevalent throughout the Papilionoideae, to a base number of 10 chromosomes (Lackey, 1981). Tetraploidization ($2n = 2x = 40$) through autopolyploidy or allopolyploidy of the progenitor species occurred either prior to or after dissemination from the ancestral region. The path of migration from a common progenitor is assumed by Singh et al. (2001) as: wild perennial ($2n = 4x = 40$, unknown or extinct) to wild annual ($2n = 4x = 40$; *G. soja*) to soybean ($2n = 4x = 40$; *G. max*). Soybean should be regarded as a stable tetraploid with diploidized genome (Gurley, 1979; Lee and Verma, 1984; Skorupska, 1989).

II.E. Pollination of Cultivated Soybean

Soybean is largely a self-pollinated species, propagated by seed (OECD, 2000). The papilionaceous flower consists of a tubular calyx of five sepals, a corolla of five petals, one pistil, and nine fused stamens with a single separate posterior stamen. The stamens form a ring at the base of the stigma and elongate one day before pollination, at which time the elevated anthers form a ring around the stigma (OECD, 2000). The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains

receptive for 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybean is considered to be a highly self-pollinated species, with cross-pollination to adjacent plants of other soybean varieties occurring at very low frequency (0.3 to 3.62%) (Beard and Knowles, 1971). Pollination typically takes place on the day the flower opens. The pollen naturally comes in contact with the stigma during the process of anthesis. Anthesis normally occurs in late morning, depending on the environmental conditions. The pollen usually remains viable for two to four hours, and no viable pollen can be detected by late afternoon. Natural or artificial cross-pollination can only take place during the short time when the pollen is viable.

II.F. Cultivated Soybean as a Volunteer

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). Soybean seed rarely exhibit any dormancy characteristics, and seed remaining in the field after harvest likely will readily imbibe water (Lersten and Carlson, 2004), germinate, and will be killed by frost or field cultivation. If they 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).

II.G. Characteristics of the Recipient Plant

The soybean variety used as the recipient for the DNA insertion to create MON 87769 was A3525, a nontransgenic conventional variety developed by Asgrow Seed Company. A3525 is a mid-maturity group III soybean variety with very high yield potential. It has superior yields relative to lines of similar maturity and has excellent agronomic characteristics (Steffen, 2004).

II.H. Soybean as a Test System in Product Safety Assessment

In developing the data to support the safety assessment of MON 87769, A3525 was used as the nontransgenic conventional soybean comparator. In general, the genetic background of MON 87769 was matched with that of the control so the effect of the genetic insertion and the presence of the PjΔ6D and NcΔ15D proteins could be assessed in an unbiased manner. Since MON 87769 was derived from the A3525 conventional variety, it was deemed appropriate to use the nontransformed A3525 as the control variety because its use would minimize the potential bias in subsequent comparative assessments. In addition, commercial conventional varieties were used as reference materials to establish ranges of responses or values representative of commercial soybean varieties. Reference varieties were selected based on prevalence and performance of the soybean varieties at each trial location. As a general principle, varieties that were well adapted to the local environments and could be used by the local producers were considered for use as reference soybean varieties.

III. DESCRIPTION OF THE TRANSFORMATION SYSTEM

MON 87769 was developed through *Agrobacterium*-mediated transformation of soybean meristem tissue using plasmid vector PV-GMPQ1972 (Section IV.A, Figure IV-1 and Table IV-1). *Agrobacterium*-mediated transformation is a well-documented process for the transfer and integration of exogenous DNA into a plant's nuclear genome (Bevan, 1984). PV-GMPQ1972 contains two separate T-DNAs (herein referred to as a 2T-DNA system). The first T-DNA, designated as T-DNA I, contains two expression cassettes: the *Pj.D6D* gene expression cassette and the *Nc.Fad3* gene expression cassette. The second T-DNA region (T-DNA II) contains the *cp4 epsps* gene expression cassette that encodes the CP4 EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4) that provides tolerance to the action of glyphosate, which is the active ingredient in Roundup® agricultural herbicides.

The use of the 2T-DNA system is the basis for an effective approach to generate marker-free plants. It allows for insertion of the T-DNA with the traits of interest (e.g., T-DNA I) and the T-DNA encoding the selectable marker (e.g., *cp4 epsps*, T-DNA II) into two independent loci within the genome of the plant. Following selection of the transformants that contain both T-DNAs, the inserted T-DNA encoding the selectable marker (e.g., T-DNA II) can be segregated in progeny through subsequent selfing of plants (self-pollination) and genetic selection, while progeny with the T-DNA containing the trait(s) of interest are maintained (e.g., T-DNA I). This 2T-DNA binary vector approach has been successfully used in tobacco (Komari et al., 1996), soybean (Xing et al., 2000), barley (Matthews et al., 2001), corn (Miller et al., 2002), and rice (Breitler et al., 2004; Komari et al., 1996). MON 87769 was developed using such 2T-DNA vector transformation and selection techniques.

The *Agrobacterium*-mediated soybean transformation to produce MON 87769 was based on published methods that generate transformed plants without utilization of callus (Armstrong et al., 2007; Martinell et al., 2002). *Agrobacterium tumefaciens* strain ABI contains a disarmed Ti plasmid that is incapable of inducing tumor formation due to the deletion of the phytohormone genes originally present in the *Agrobacterium* plasmid (Koncz and Schell, 1986). The transformation vector, PV-GMPQ1972, contains both the left and right border sequences flanking the transfer DNA (T-DNA) to facilitate transformation.

Embryos were excised from germinated A3525 seed and meristematic tissues were targeted for transformation. After co-culturing this tissue with the *Agrobacterium* carrying the vector, the meristems were placed on selection medium containing glyphosate to inhibit the growth of untransformed plant cells, and antibiotics carbenicilin and claforan to inhibit the growth of excess *Agrobacterium*, respectively. The meristems then were placed on media conducive to shoot and root development, and only rooted

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plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R0 plants generated through the aforementioned transformation system were self-pollinated, and the subsequent R1 plants were screened for the single insert present in MON 87769 using Invader (Third Wave Technologies, Inc.) and Southern blot analysis. Invader is a non-PCR based assay that can be used to accurately quantify transgene copy number in plant genomes (Gupta et al., 2008). The R1 plants containing the T-DNA I cassette for the *Pj.D6D* and *Nc.Fad3* genes of interest (GOI) and not having the T-DNA II cassette with the *cp4 epsps* gene cassette were advanced for further evaluation and development. Each R1 plant was self-pollinated to generate a population of R2 plants that were repeatedly self-pollinated through the R6 generation. These progeny were subjected to further molecular (Southern blot) and phenotypic assessments. MON 87769 was selected as the lead event based on superior phenotypic characteristics and molecular profile. Regulatory studies on MON 87769 were initiated to further characterize the genetic insertion and the expressed proteins, and to confirm the food, feed, and environmental safety relative to conventional soybean. The major steps involved in the development of MON 87769 are depicted in Figure III-1.

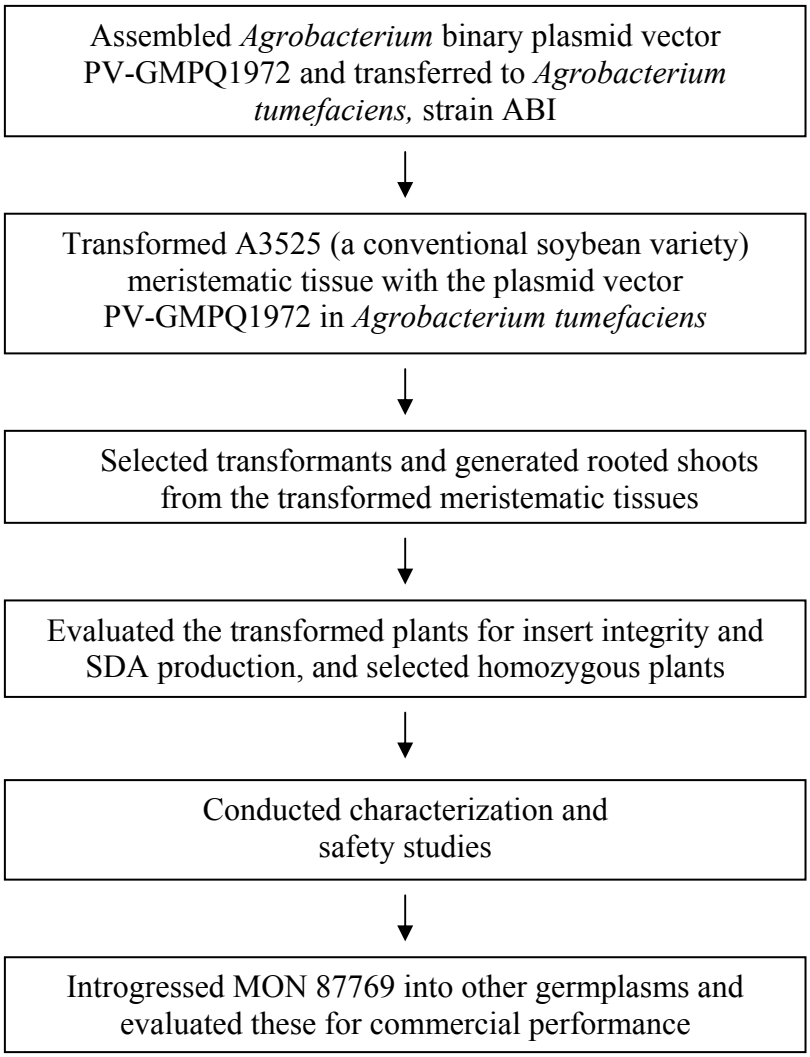


Figure III-1. Schematic of the Development of MON 87769

IV. GENETIC ELEMENTS

This section describes the vector, the donor genes and the regulatory sequences used in the development of MON 87769, the deduced amino acid sequence of the PjΔ6D and NcΔ15D proteins produced in MON 87769, and a description of the CP4 EPSPS protein selectable marker employed to produce MON 87769. In this section, T-DNA refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of a coding sequence and the regulatory elements necessary for the expression of the coding sequence.

IV.A. Plasmid PV-GMPQ1972

This section describes the donor genes and regulatory sequences used in the development of MON 87769 and the deduced amino acid sequences of the *Primula juliae* Δ6 desaturase (PjΔ6D) and *Neurospora crassa* Δ15 desaturase (NcΔ15D) proteins produced in MON 87769.

The plasmid vector PV-GMPQ1972 used to transform soybean meristems to produce MON 87769 is shown in Figure IV-1. The vector is an approximately 16.5 kb plasmid containing two T-DNA regions, each delineated by a left and right border region. The first T-DNA (T-DNA I) region contains two expression cassettes: the *Pj.D6D* gene expression cassette and the *Nc.Fad3* gene expression cassette. The second T-DNA region (T-DNA II) contains the *cp4 epsps* gene expression cassette that was used for early event selection and was segregated away from T-DNA I by conventional breeding (self-pollination). The T-DNA II elements are therefore not present in MON 87769 other than those common elements that are also present in T-DNA I. The T-DNA present in MON 87769 (T-DNA I) is approximately 7.4 kb, and the DNA backbone and T-DNA II region that is not incorporated into the soybean genome is approximately 9.1 kb.

The *Pj.D6D* expression cassette consists of the *Pj.D6D* coding sequence under the regulation of the *7Sα'* promoter and the *tml* 3' nontranslated sequence. The *Nc.Fad3* expression cassette consists of the *Nc.Fad3* coding sequence under the regulation of the *7Sα* promoter and the *E9* 3' nontranslated region. The *cp4 epsps* expression cassette in T-DNA II, which is not present in MON 87769, consists of the *cp4 epsps* coding region under the regulation of the *FMV* promoter and the *E9* 3′ nontranslated region.

The backbone region outside of the T-DNA contains two origins of replication for maintenance of plasmid in bacteria (OR-*ori V,* OR-*ori*-pBR322), as well as a bacterial selectable marker gene (*aadA*). A description of the genetic elements and their prefixes (e.g., P-, L-, I-, TS-, OR-, B-, CS-, and T-) in PV-GMPQ1972 is provided in Table IV-1.

IV.B. T-DNA I

This section describes the elements contained on T-DNA I that were integrated into the soybean genome to produce MON 87769.

IV.B.1. The *Pj.D6D* Coding Sequence and PjΔ6D Protein

The *Pj.D6D* gene was isolated from *Primula juliae* (Primrose) and encodes a single polypeptide, designated PjΔ6D, of 446 amino acids (Ursin et al., 2008). The deduced full-length amino acid sequence is shown in Figure IV-2. The PjΔ6D protein is a Δ6 desaturase which creates a double bond at the 6th position from the carboxyl end of a fatty acid.

IV.B.2. The *Pj.D6D* Regulatory Sequences

Adjacent to the right border region of plasmid PV-GMPQ1972 is the *Pj.D6D* expression cassette. The *Pj.D6D* coding sequence is under the regulatory control of the P-*7Sα'* transcriptional promoter. P-*7Sα'* is a seed-specific promoter and leader sequence from the *Sphas1* gene encoding the alpha prime subunit of the beta-conglycinin storage protein of *Glycine max* (Doyle et al., 1986). Following the *Pj.D6D* coding sequence is the 3' nontranslated region of the *tml* gene derived from the octopine-type Ti plasmid of *Agrobacterium tumefaciens* (T-*tml*) (Kemp et al., 2000) that directs transcriptional termination and polyadenylation of the *Pj.D6D* mRNA.

IV.B.3. The *Nc.Fad3* Coding Sequence and NcΔ15D Protein

The *Nc.Fad3* gene was isolated from *Neurospora crassa* and encodes a single polypeptide, designated NcΔ15D, of 429 amino acids (Ursin et al., 2006). The deduced full-length amino acid sequence is shown in Figure IV-3. The NcΔ15D protein is a Δ15 desaturase which creates a double bond at the 15th position from the carboxyl end of a fatty acid.

IV.B.4. The *Nc.Fad3* Regulatory Sequences

Adjacent to the *Pj.D6D* expression cassette is the *Nc.Fad3* expression cassette. The *Nc.Fad3* coding sequence is under the regulatory control of the P-*7Sα* promoter. P-*7Sα* is a seed-specific promoter and leader sequence from the *Sphas2* gene that encodes the alpha subunit of beta-conglycinin seed storage protein in *Glycine max* (Wang and Dubois, 2004). Following the *Nc.Fad3* coding sequence is the 3' nontranslated region of the pea (*Pisum sativum*) ribulose-1,5-bisphosphate carboxylase, small subunit (*rbcS2*) gene (T-*E9*) (Coruzzi et al., 1984) that directs transcriptional termination and polyadenylation of the *Nc.Fad3* mRNA.

IV.B.5. The T-DNA Borders

Plasmid vector PV-GMPQ1972 contains right border and left border regions that delineate the T-DNAs and are involved in their efficient transfer into soybean genome. These border regions (Figure IV-1; Table IV-1) were derived from *Agrobacterium tumefaciens* plasmids (Barker et al., 1983; Depicker et al., 1982).

IV.C. T-DNA II

This section describes the elements contained on T-DNA II that were segregated away in progeny, through subsequent selfing and genetic selection, to produce MON 87769.

IV.C.1. The *cp4 epsps* Coding Sequence and the CP4 EPSPS Protein

The *cp4 epsps* gene from *Agrobacterium* sp. strain CP4, a common soil-borne bacterium, has been sequenced and shown to encode a 47.6 kDa EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996).

IV.C.2. The *Arabidopsis thaliana* EPSPS Transit Peptide

The *cp4 epsps* coding sequence is preceded by a chloroplast transit peptide sequence, (TS-*CTP2)*, derived from the *Arabidopsis thaliana epsps* gene (Klee et al., 1987). This transit peptide directs the transport of the CP4 EPSPS protein to the chloroplast, which is where the plant EPSPS resides and the site of aromatic amino acid biosynthesis (Kishore et al., 1988; Klee et al., 1987). Transit peptides are typically cleaved from the translated polypeptide following delivery to the plastid (Della-Cioppa et al., 1986).

IV.C.3. The *cp4 epsps* Regulatory Sequences

The CTP2/*cp4 epsps* gene cassette that produces the CTP/CP4 EPSPS protein consists of the promoter (P-*FMV)* for the 35S RNA from figwort mosaic virus (FMV) (Rogers, 2000) that directs transcription in plant cells. Located between the P-*FMV* promoter and the *CTP2/cp4 epsps* coding sequence is the 5' nontranslated *ShkG* leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS (Klee et al., 1987). Following the *CTP2/cp4 epsps* coding sequence is the T-*E9* element, the 3' nontranslated region of the pea (*Pisum sativum*) ribulose-1,5-bisphosphate carboxylase, and the small subunit (*rbcS2*) *E9* gene (Coruzzi et al., 1984) that directs transcriptional termination and polyadenylation of the *CTP2/cp4 epsps* mRNA.

The unlinked T-DNA II insertion was eliminated as a result of segregation during breeding. T-DNA II elements are not present in MON 87769 other than those common elements that are also part of T-DNA I. This has been confirmed by Southern blot analyses, which are presented in Section V.C.

IV.C.4. The T-DNA Borders

The right and left T-DNA borders used in T-DNA II are the same as those described under Section IV.B.5.

IV.D. Genetic Elements Outside of the T-DNA Borders

Four genetic elements exist outside of the T-DNA borders that are essential for the maintenance and selection of the vector PV-GMPQ1972 in bacteria. They include: *OR-ori V,* origin of replication for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981); CS-*rop,* coding sequence for repressor of primer (ROP) protein for

maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989); *OR-ori-*pBR322*,* origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978); and *aadA*, a bacterial promoter and coding sequence of an enzyme from transposon Tn7 that confers spectinomycin and streptomycin resistance for molecular cloning and selection purposes (Fling et al., 1985).

Because these elements are outside of the border regions, they are not expected to be transferred into the soybean genome. The absence of the backbone sequence in MON 87769 has been confirmed by Southern blot analyses, which are presented in Section V.B.

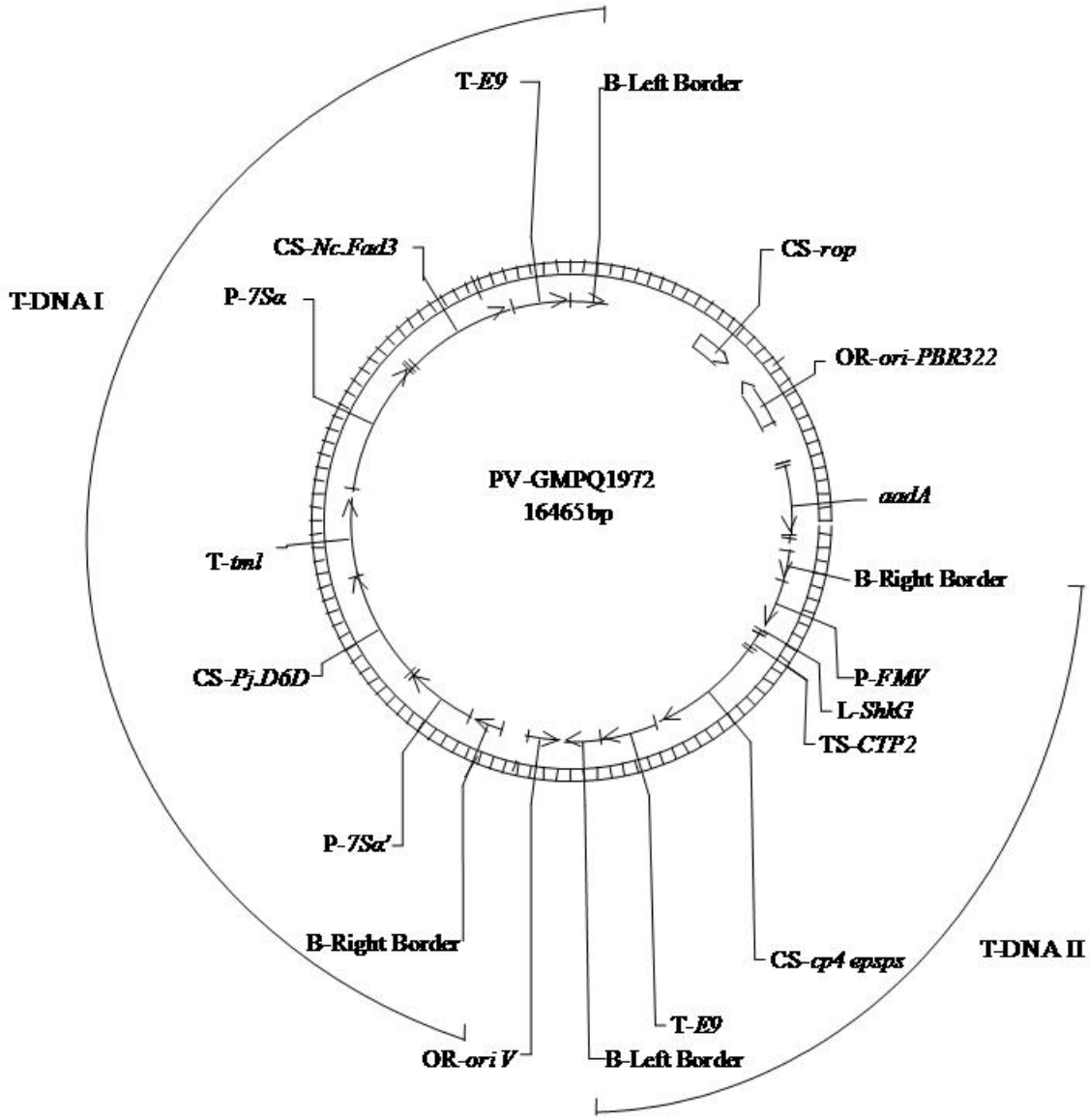


Figure IV-1. Plasmid Map of Vector PV-GMPQ1972
Plasmid PV-GMPQ1972 was used in *Agrobacterium*-mediated transformation to develop MON 87769. Approximate locations of the genetic elements (with approximate positions relative to the plasmid vector) are shown on the exterior of the map. PV-GMPQ1972 contains two T-DNA regions designated as T-DNA I and T-DNA II.

Table IV-1. Summary of Genetic Elements in Plasmid Vector PV-GMPQ1972

Genetic Element	Position in Plasmid	Function and Source (Reference)
T-DNA I (continued from bp 16465)		
Intervening Sequence	1–14	Sequence used in DNA cloning
B[1]-Left Border	15-456	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker et al., 1983)
Vector Backbone		
Intervening Sequence	457–1619	Sequence used in DNA cloning
CS[2]-*rop*	1620-2092	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	2093-2340	Sequence used in DNA cloning
OR[3]-*ori-pBR322*	2341-2969	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1978)
Intervening Sequence	2970-3469	Sequence used in DNA cloning
aadA	3470-4358	Bacterial promoter and 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
Intervening Sequence	4359-4494	Sequence used in DNA cloning
T-DNA II (not present in MON 87769)		
B-Right Border	4495-4851	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	4852-4884	Sequence used in DNA cloning
P[4]-*FMV*	4885-5448	Promoter for the 35S RNA from figwort mosaic virus (FMV) (Rogers, 2000) that directs transcription in plant cells
Intervening Sequence	5449-5491	Sequence used in DNA cloning
L[5]-*ShkG*	5492–5558	5' nontranslated leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS (Klee et al., 1987) that helps regulate gene expression
TS[6]–*CTP2*	5559–5786	Transit peptide region of *Arabidopsis thaliana* EPSPS (Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS-*cp4 epsps*	5787-7154	Codon modified coding sequence of the *aroA* gene from the *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Barry et al., 1997; Padgette et al., 1996)
Intervening Sequence	7155-7196	Sequence used in DNA cloning

[1]B – Border; [2]CS – Coding Sequence; [3]OR – Origin of Replication; [4]P – Promoter; [5]L-Leader; [6]TS – Targeting Sequence.

Table IV-1 (continued). Summary of Genetic Elements in Plasmid Vector PV-GMPQ1972

T-DNA II (cont.)		
T[7]-*E9*	7197-7839	A 3' nontranslated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	7840-7886	Sequence used in DNA cloning
B-Left Border	7887-8328	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker et al., 1983)
Vector Backbone		
Intervening Sequence	8329-8414	Sequence used in DNA cloning
OR-*ori V*	8415-8811	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	8812-9072	Sequence used in DNA cloning
T-DNA I		
B-Right Border	9073-9429	DNA area from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	9430-9480	Sequence used in DNA cloning
P-*7Sα'*	9481-10321	Promoter and leader from the *Sphas1* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'- bcsp) (Doyle et al., 1986) that directs mRNA transcription in seed
Intervening Sequence	10322-10337	Sequence used in DNA cloning
CS-*Pj.D6D*	10338-11678	Coding region for the Δ6 desaturase from *Primula juliae* (Ursin et al., 2008)
Intervening Sequence	11679-11686	Sequence used in DNA cloning
T-*tml*	11687-12636	3' nontranslated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid (Kemp et al., 2000) that directs polyadenylation of the mRNA
Intervening Sequence	12637-12737	Sequence used in DNA cloning
P-*7Sα*	12738-14417	Promoter and leader from the *Sphas2* gene from soybean encoding the alpha subunit of beta-conglycinin (Wang and Dubois, 2004) that directs mRNA transcription in seed
Intervening Sequence	14418-14445	Sequence used in DNA cloning
CS-*Nc.Fad3*	14446-15735	Codon optimized coding sequence for the gene from *Neurospora crassa* encoding Δ15 desaturase (Ursin et al., 2006)
Intervening Sequence	15736-15787	Sequence used in DNA cloning
T-*E9*	15788-16430	3' nontranslated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	16431-16465	Sequence used in DNA cloning

[7]T – Transcript Termination Sequence and polyadenylation signal sequences.

```
  1 MTKTIYITSS ELEKHNKPGD LWISIHGQVY DVSSWAALHP GGIAPLLALA GHDVTDAFLA
 61 YHPPSTSRLL PPFSTNLLLE KHSVSETSSD YRKLLDSFHK MGMFRARGHT AYATFVIMIL
121 MLVSSVTGVL CSENPWVHLV CGAAMGFAWI QCGWIGHDSG HYRIMTDRKW NRFAQILSSN
181 CLQGISIGWW KWNHNAHHIA CNSLEYDPDL QYIPLLVVSP KFFNSLTSRF YDKKLNFDGV
241 SRFLVQYQHW SFYPVMCVAR LNMLAQSFIL LFSRREVANR VQEILGLAVF WLWFPLLLSC
301 LPNWGERIMF LLASYSVTGI QHVQFSLNHF SSDVYVGPPV GNDWFKKQTA GTLNISCPAW
361 MDWFHGGLQF QVEHHLFPRM PRGQFRKISP FVRDLCKKHN LTYNIASFTK ANVLTLETLR
421 NTAIEARDLS NPIPKNMVWE AVKNVG
```

Figure IV-2. Deduced Amino Acid Sequence of *Primula juliae* Δ6 Desaturase from PV-GMPQ1972

The amino acid sequence of the PjΔ6D protein was deduced from the full-length *Pj.D6D* coding sequence present in PV-GMPQ1972.

```
  1 MAVTTRSHKA AAATEPEVVS TGVDAVSAAA PSSSSSSSSQ KSAEPIEYPD IKTIRDAIPD
 61 HCFRPRVWIS MAYFIRDFAM AFGLGYLAWQ YIPLIASTPL RYGAWALYGY LQGLVCTGIW
121 ILAHECGHGA FSRHTWFNNV MGWIGHSFLL VPYFSWKFSH HRHHRFTGHM EKDMAFVPAT
181 EADRNQRKLA NLYMDKETAE MFEDVPIVQL VKLIAHQLAG WQMYLLFNVS AGKGSKQWET
241 GKGGMGWLRV SHFEPSSAVF RNSEAIYIAL SDLGLMIMGY ILYQAAQVVG WQMVGLLYFQ
301 QYFWVHHWLV AITYLHHTHE EVHHFDADSW TFVKGALATV DRDFGFIGKH LFHNIIDHHV
361 VHHLFPRIPF YYAEEATNSI RPMLGPLYHR DDRSFMGQLW YNFTHCKWVV PDPQVPGALI
421 WAHTVQSTQ
```

Figure IV-3. Deduced Amino Acid Sequence of *Neurospora crassa* Δ15 Desaturase from PV-GMPQ1972

The amino acid sequence of the NcΔ15D protein was deduced from the full-length *Nc.Fad3* coding sequence present in PV-GMPQ1972.

V. GENETIC ANALYSIS

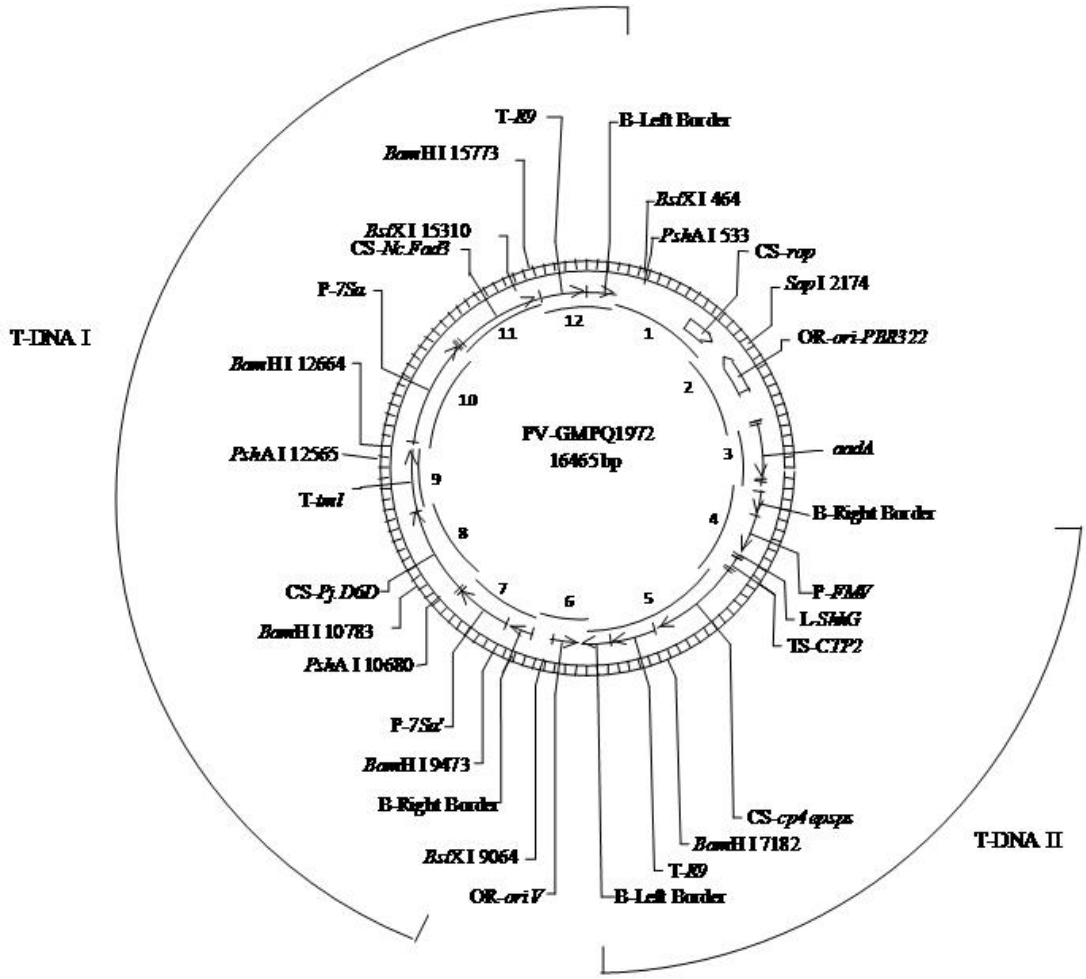
This section details the molecular analyses used to characterize the integrated DNA insert in MON 87769. Southern blot analysis confirmed the presence of a single insertion site for each genetic element present in T-DNA I, the absence of plasmid backbone and T-DNA II elements, and the insert stability across generations. In addition, DNA sequencing analyses confirmed the nucleotide sequence and the expected organization of the insert in MON 87769. A comparison of the soybean genomic DNA flanking the insert in MON 87769 to the sequence of the insertion site in conventional soybean demonstrated that no major rearrangements occurred at the insertion site during transformation. Furthermore, insert segregation analysis shows that the insert segregates as expected indicating that the *Pj.D6D* and *Nc.Fad3* gene cassettes segregate according to Mendel's laws of genetics. All these results indicate a single detectable chromosomal insertion of T-DNA I in MON 87769.

Genomic DNA from MON 87769 was digested with appropriate restriction enzymes and subjected to Southern blot analyses to characterize the T-DNA that was integrated into the soybean genome. Genomic DNA from conventional soybean (A3525) was used as a negative control to determine potential nonspecific hybridization signals and to establish hybridization signals to endogenous soybean DNA sequences since several genetic elements in T-DNA I were derived from soybean. There were two types of positive controls used. For all Southern blots, PV-GMPQ1972 plasmid DNA was digested with a restriction enzyme or enzyme combination to produce DNA banding patterns that were relevant to the molecular assessment of MON 87769. Additionally, in some analysis where multiple probes were used, probe templates were used as positive hybridization controls for Southern blots. DNA markers were included to provide size estimation of the hybridized bands on all Southern blots. The sizes of bands present in the short run lanes were estimated using the molecular weight markers on the right side of the figures, whereas size estimations for the long run lanes were obtained using the molecular weight markers on the left side. The genetic elements present in MON 87769 are listed in Table V-1 starting at partial Right Border and ending at partial Left Border. The probes used in the Southern analyses and the map of the plasmid (PV-GMPQ1972) used in the transformation to generate MON 87769 are shown in Figures V-1 and V-2, respectively. The information and results derived from the molecular sequence analysis were used to construct a linear map of the insert in MON 87769. This linear map depicts restriction sites identified in the insert and the soybean genomic DNA flanking the insert, and provides information on the expected banding patterns and sizes of the DNA fragments after restriction enzyme digestions. The linear map is shown in Figure V-3. A table based on the insert linear map and the plasmid map, summarizing the expected DNA fragments for Southern analyses, is presented in Table V-2. The materials and methods used in the analyses are presented in Appendix B.

Table V-1. Summary of Genetic Elements in MON 87769

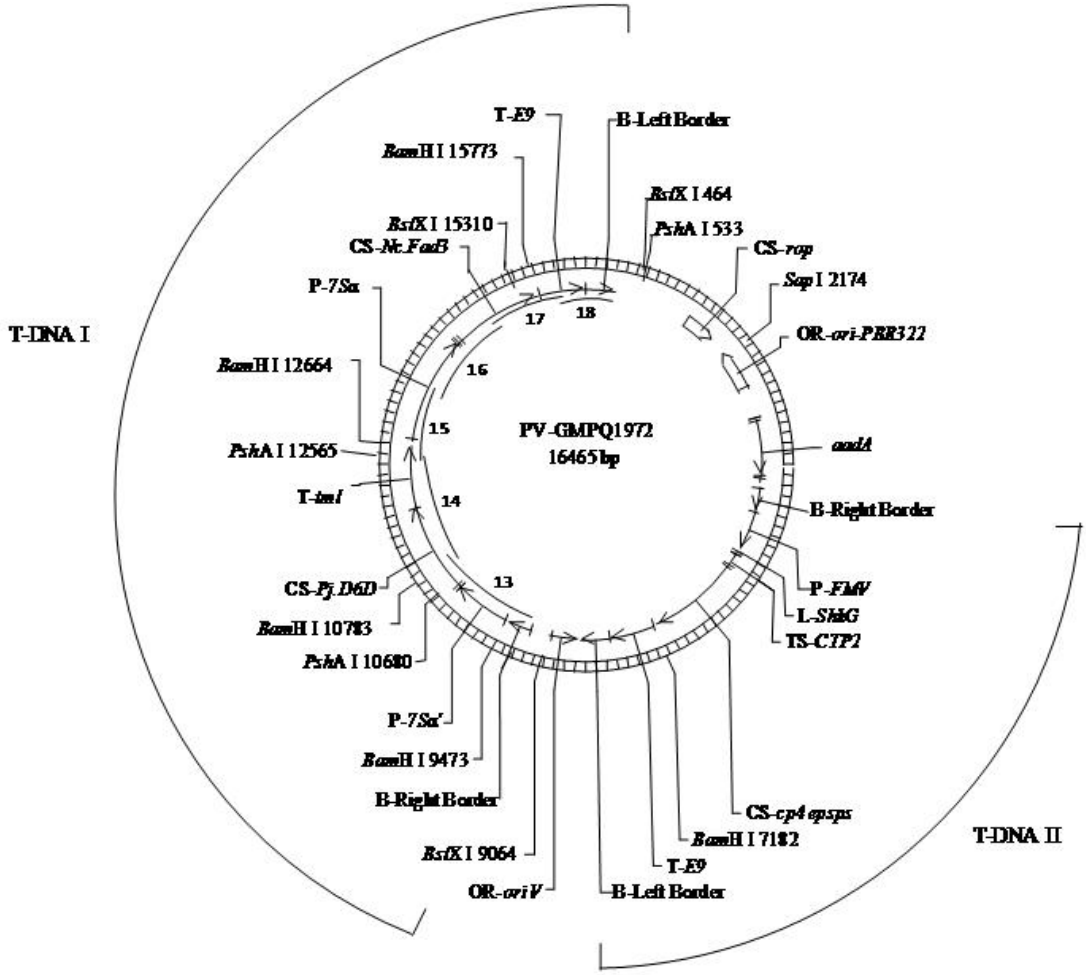
Genetic Element[1]	Location in Sequence[1]	Function (Reference)
Sequence flanking 5' end of the insert	1-933	Soybean genomic DNA
[2]**B-Right Border**	934-976	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	977-1027	Sequence used in DNA cloning
[3]**P-*7Sα'***	1028-1868	Promoter and leader from the *Sphas1* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle et al., 1986) that directs mRNA transcription in seed
Intervening Sequence	1869-1884	Sequence used in DNA cloning
[4]**CS-*Pj.D6D***	1885-3225	Coding region for the fatty acid Δ6 desaturase from *Primula juliae* (Ursin et al., 2008)
Intervening Sequence	3226-3233	Sequence used in DNA cloning
[5]**T-*tml***	3234-4183	3′ nontranslated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid (Kemp et al., 2000) that directs polyadenylation of the mRNA
Intervening Sequence	4184-4284	Sequence used in DNA cloning
P-*7Sα*	4285-5964	Promoter and leader from the *Sphas2* gene from soybean encoding the alpha subunit of beta-conglycinin (Wang and Dubois, 2004) that directs mRNA transcription in seed
Intervening Sequence	5965-5992	Sequence used in DNA cloning
CS-*Nc.Fad3*	5993-7282	Codon optimized coding sequence for the gene from *Neurospora crassa* encoding delta-15 desaturase (Ursin et al., 2006)
Intervening Sequence	7283-7334	Sequence used in DNA cloning
T-*E9*	7335-7977	3′ nontranslated region of the pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	7978-8026	Sequence used in DNA cloning
B-Left Border	8027-8300	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Sequence flanking 3' end of the insert	8301-9131	Soybean genomic DNA

[1]Numbering refers to the sequence that includes the insert in MON 87769 and adjacent genomic DNA starting with 933 bp flanking the 5' of the sequence and ending with 831 bp flanking 3' end of the insert.
[2]B – Border; [3]P – Promoter; [4]CS – Coding Sequence; [5]T – Transcript Termination Sequence and polyadenylation signal sequences.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	Backbone 1	457	2254	1.8
2	Backbone 2	2168	3656	1.5
3	Backbone 3	3553	4494	0.9
4	T-DNA II probe 1	4495	6512	2.0
5	T-DNA II probe 2	6436	8330	1.9
6	Backbone 4	8329	9072	0.7
7	B-Right Border/P-*7Sa'*	9073	10329	1.3
8	CS-*Pj.D6D*	10338	11680	1.3
9	T-*tml*	11687	12636	0.95
10	P-*7Sa*	12728	14431	1.7
11	CS-*Nc.Fad3*	14446	15736	1.3
12	T-*E9*/B-Left Border	15786	458	1.1

Figure V-1. Plasmid Map of Vector PV GMPQ1972 Showing Probes 1-12
PV-GMPQ1972 was used to develop MON 87769. Approximate locations of the genetic elements and in the Southern analyses (with approximate positions relative to the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are detailed in Table V-2. PV-GMPQ1972 contains two T-DNA regions designated as T-DNA I and T-DNA II.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
13	T-DNA I probe 1	9073	11046	2.0
14	T-DNA I probe 2	10966	12710	1.7
15	T-DNA I probe 3	12545	14151	1.6
16	T-DNA I probe 4	14012	15300	1.3
17	T-DNA I probe 5	15168	16205	1.0
18	T-DNA I probe 6	16116	458	0.8

Figure V-2. Plasmid Map of Vector PV-GMPQ1972 Showing Probes 13-18
Plasmid PV-GMPQ1972 was used to develop MON 87769. Genetic elements and restriction sites for enzymes used in the Southern analyses (with approximate positions relative to the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are detailed in the accompanying Table V-2. PV-GMPQ1972 contains two T-DNA regions designated as T-DNA I and T-DNA II.

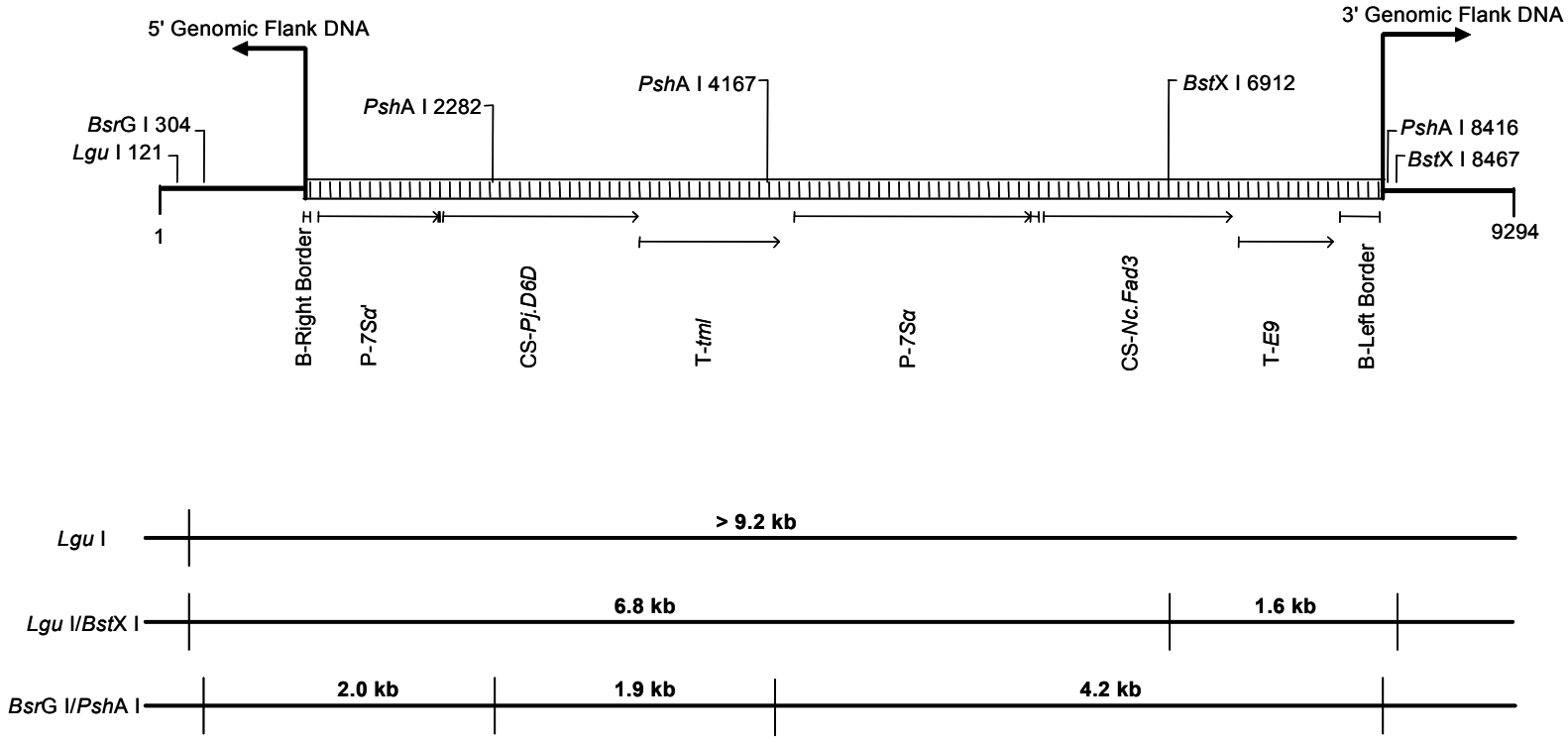


Figure V-3. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87769

A linear map of the insert and genomic DNA flanking the insert in MON 87769 is shown. The upper portion of the figure displays genetic elements within the insert (rectangular bar), as well as all restriction sites used in Southern blot analyses. The numbered designation of the restriction sites are consistent with the information presented in Table V-1. Arrows underneath the designated insert indicate the direction of transcription. Shown on the lower portion of the map are the expected sizes of the DNA fragments after digestions with respective restriction enzyme or combination of enzymes. The two large arrows above the designated insert indicate the end of the insert and the beginning of soybean genomic flanking sequence.

Table V-2. Summary Chart of the Expected Sizes of DNA Fragments Using Combinations of Restriction Enzymes and Probes

Probes used	**13 - 15**	**16 – 18**	**7**	**8**	**9**	**10**	**11**	**12**	**1-3, 6**	**4 and 5**
Southern blot in Figure	V-4a and V-14a*	V-4b and V-14b*	V-7	V-8	V-9	V-10	V-11	V-12	V-5 and IV-15*	V-6 and V-16*
Plasmid										
*Bam*HI	1.3, 1.9, 2.3, 3.1, and 7.9 kb	2.3, 3.1, and 7.9 kb	1.3, 2.3, and 7.9 kb	1.3 and 1.9 kb	1.9 kb	3.1 kb	3.1 kb	2.3 and 7.9 kb	2.3 and 7.9 kb	2.3 and 7.9 kb
Probe templates	1.6, 1.7, and 2.0 kb	0.8, 1.0, and 1.3 kb	--[1]	--	--	--	--	--	0.7, 0.9, 1.5, and 1.8 kb	2.0, and 1.9 kb
MON 87769										
Lgu I	> 9.2 kb	> 9.2 kb	--	--	--	--	--	--	--	
Lgu I and *Bst*X I	6.8 kb	6.8 and 1.6 kb	6.8 kb	6.8 kb	6.8 kb	6.8 kb	6.8 and 1.6 kb	1.6 kb	NE[2]	6.8 and 1.6 kb
*Bsr*G I and *Psh*A I	--	--	2.0 kb	1.9 and 2.0 kb	1.9 and 4.2 kb	4.2 kb	4.2 kb	4.2 kb	NE[2]	2.0 and 4.2 kb

[1] '--' indicates that the particular restriction enzyme or combination of enzymes was not used in the analysis.
[2] 'NE' indicates that no DNA band was expected or detected.
[*] Southern blots not conducted with material digested with *Bsr*G I *and Psh*A I.

Southern blot analysis confirmed the presence of a single insertion site for each genetic element present in T-DNA I, confirmed the lack of plasmid backbone elements and absence of T-DNA II, and the insert stability across generations. In addition, DNA sequencing analyses confirmed the expected organization and nucleotide sequence of the insert in MON 87769. Furthermore, insert segregation analysis confirmed that the *Pj.D6D* and *Nc.Fad3* gene cassettes segregate according to Mendel's laws of genetics. All these results indicate a single detectable chromosomal insertion of the T-DNA I in MON 87769.

V.A. Insert and Copy Number Determination

The number of T-DNA inserts (insert number) in the MON 87769 genome was determined by Southern blot analysis using MON 87769 and conventional soybean DNA that was digested with the restriction enzyme *Lgu* I. *Lgu* I does not cleave within T-DNA I and, therefore, releases a restriction fragment containing the entire T-DNA I and adjacent plant genomic DNA (Figure V-3). The number of restriction fragments detected should indicate the number of inserts present in MON 87769.

The number of copies of the T-DNA I (copy number) integrated at a single locus was determined by digesting the MON 87769 DNA with the combination of restriction enzymes *Lgu* I and *Bst*X I, which cleave once within the insert and within each flank (Figure V-3). If MON 87769 contains one copy of T-DNA I, Southern blots probed with the entire T-DNA I will result in two bands, each representing a portion of the T-DNA I, along with adjacent plant genomic DNA. The samples were subjected to Southern blot analysis using six overlapping ^{32}P-labeled T-DNA I probes (probes 13-18, Figure V-2) spanning the entire T-DNA I insert. This analysis was divided between two Southern blots. Each blot was probed with a combination of three probes. The results of this analysis are presented in Figure V-4a for probes 13-15 and Figure V-4b for probes 16-18. The expected DNA fragments are summarized in Table V-2.

To determine the endogenous background hybridization of various probes to MON 87769 DNA, conventional soybean DNA was used as a control. As shown in Figure V-4, conventional soybean DNA digested with *Lgu* I alone (lanes 3 and 7) or a combination of *Lgu* I and *Bst*X I (lanes 1 and 5) produced several hybridization signals. This is expected because several genetic elements within T-DNA I were derived from soybean. These hybridization signals result from the probes hybridizing to endogenous DNA sequences residing in the conventional soybean genome and are not specific to the inserted DNA. These signals were produced in all lanes, including those containing the conventional soybean DNA material and therefore, are considered to be endogenous background hybridization.

To ensure that each of the T-DNA probes hybridized to their intended target, plasmid vector DNA and probe template spikes were used as controls on the Southern blots. Plasmid PV-GMPQ1972 DNA controls digested with *Bam*H I, mixed with conventional soybean DNA predigested with *Lgu* I and *Bst*X I, and analyzed using probes 13-15 (lane 9, Figure V-4a) produced the expected bands at approximately 1.3, 1.9, 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the

conventional soybean DNA (refer to Table V-2). The probe template controls (probes 13-15) mixed with predigested conventional DNA and hybridized with probes 13-15 (lanes 10 and 11) produced the expected bands at 1.6, 1.7, and 2.0 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA

Plasmid PV-GMPQ1972 DNA digested with *Bam*H I, mixed with predigested conventional soybean DNA and analyzed with probes 16-18 (lane 9, Figure V-4b) produced the expected bands at approximately 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA (refer to Table V-2). Conventional soybean DNA spiked with probes 16-18 (lanes 10 and 11) produced expected bands at 0.8, 1.0, and 1.3 kb, in addition to the endogenous background hybridization. The 0.1 genomic equivalent of the probe templates resulted in a fairly faint hybridization signal (lane 11), but is detectable on the actual autoradiography film.

MON 87769 DNA was digested with *Lgu* I and hybridized with overlapping T-DNA probes (probes 13-15, Figure V-4a and probes 16-18, Figure V-4b). A single unique band of approximately 10.0 kb was detected in lanes 4 and 8 in addition to the endogenous background hybridization signals produced by the conventional soybean DNA. This result shows that MON 87769 contains one insert located on an approximately 10.0 kb *Lgu* I restriction fragment.

MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6, Figure V-4a) and hybridized with probes 13-15 produced a single unique band of approximately 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean controls (lanes 1 and 5). MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6, Figure V-4b) and hybridized with probes 16-18 produced two bands of approximately 1.6 and 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean control (lanes 1 and 5). These results confirm that a single copy of T-DNA I is present in MON 87769.

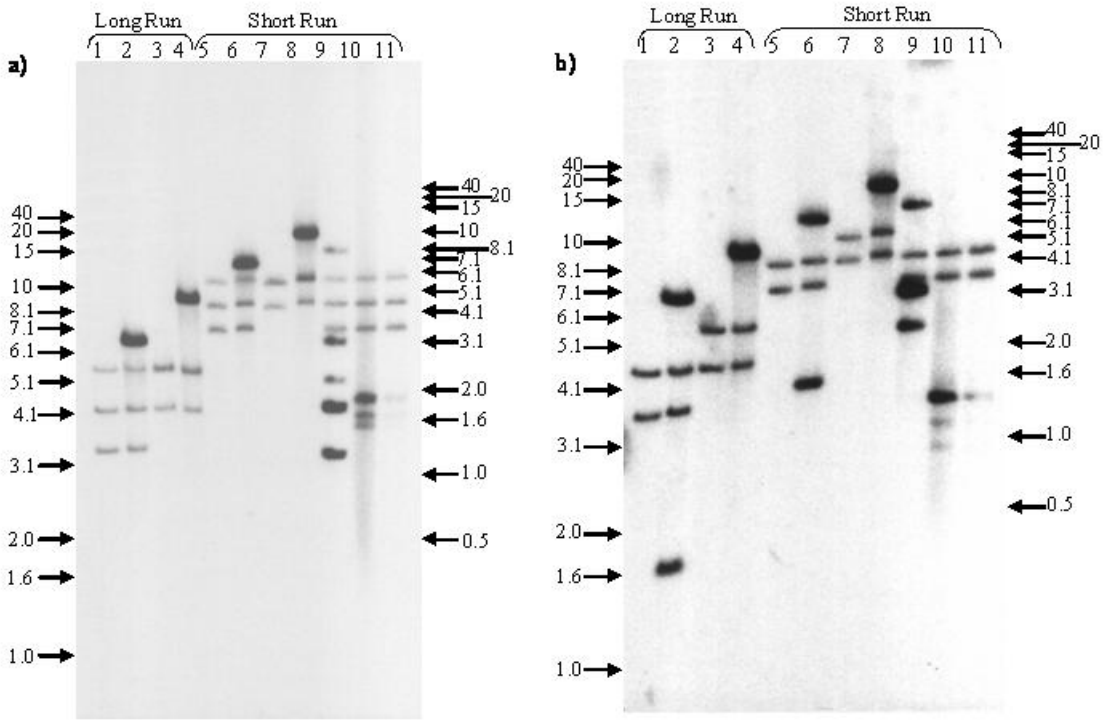


Figure V-4. Southern Blot Analysis of MON 87769 with Insert and Copy Number Probes

Each blot was hybridized simultaneously with three overlapping ^{32}P-labeled T-DNA probes (probes 13-18, Figure V-2). Figure V-4a was hybridized with probes 13-15 and Figure V-4b was hybridized with probes 16-18. Each lane contains ~10 µg of digested genomic DNA isolated from leaf tissue. Lane designations for each blot are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Lgu* I)
4: MON 87769 (*Lgu* I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean *(Lgu* I*)*
8: MON 87769 (*Lgu* I*)*
9: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe template [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe template [~0.1 genomic equivalent

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

V.B. Confirmation of the Absence of Plasmid PV-GMPQ1972 Backbone

To determine the presence or absence of PV-GMPQ1972 backbone sequence, MON 87769 and conventional soybean DNA were digested with either the combination of restriction enzymes *Lgu* I and *Bst*X I or restriction enzymes *Bsr*G I and *Psh*A I and analyzed by Southern blot using probes 1-3 and 6 (Figure V-1). These probes span the plasmid backbone sequences of PV-GMPQ1972 not contained in T-DNA I or T-DNA II. The results are summarized in Table V-2.

Conventional soybean DNA (A3525) was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the absence of plasmid backbone (Figure V-5). Conventional soybean control DNA digested with a combination of *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Psh*A I (lanes 3 and 7) and hybridized with the backbone probes (probes 1-3 and 6) (Figure V-5) simultaneously showed no hybridization bands. This indicates that no endogenous soybean DNA sequences hybridized to the plasmid backbone probes.

To ensure that each of the backbone probes were capable of hybridizing to their intended target, both plasmid PV-GMPQ1972 DNA digested with *Bam*H I and probe template spikes were mixed with conventional soybean DNA (predigested with *Lgu* I and *Bst*X I) and included on the Southern blot (Figure V-5). PV-GMPQ1972 DNA digested with *Bam*H I, mixed with predigested conventional soybean DNA, and hybridized simultaneously with probes 1-3 and 6 (Figure V-5) produced the expected size bands of approximately 2.3 and 7.9 kb (lane 9). The probe template controls, mixed with digested conventional soybean DNA, and hybridized with the backbone probes produced four expected bands at approximately 0.7, 0.9, 1.5, and 1.8 kb (lanes 10 and 11). Also, a nonspecific hybridization spot exists between lanes 5 and 6. There is no DNA present at the location of the hybridization spot since lanes 5 and 6 contain the "short run" analysis, and therefore does not affect the results of the Southern blot. These results indicate that all the backbone probes labelled properly and hybridized to their intended targets.

Southern blot analysis of MON 87769 DNA digested with either a combination of *Lgu* I and *Bst*X I (lanes 2 and 6) or *Bsr*GI and *Psh*A I (lanes 4 and 8) and hybridized simultaneously with probes 1-3, and 6 showed no detectable hybridization signal (Figure V-5). This indicates that MON 87769 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ1972.

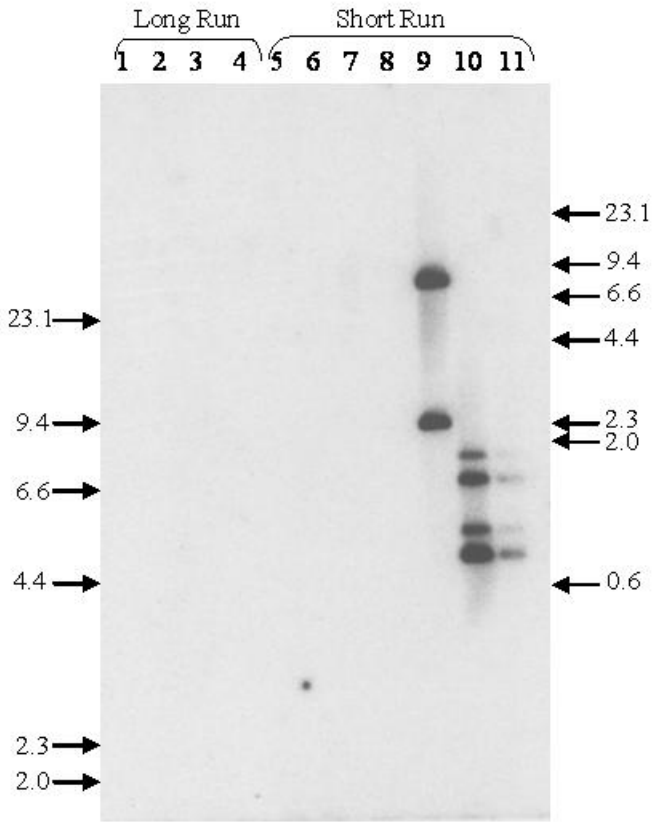


Figure V-5. Southern Blot Analysis of MON 87769 with PV-GMPQ1972 Backbone Probes

The blot was hybridized simultaneously with four overlapping ^{32}P-labeled probes that span the entire backbone sequence (probes 1-3 and 6, Figure V-1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
10: Conventional soybean (*Lgu* I and *Bst*XI) spiked with probe templates [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

V.C. Confirmation of the Absence of T-DNA II Sequence

The T-DNA II insert was segregated away from MON 87769 by traditional breeding after the initial transformation. To confirm the absence of T-DNA II region, MON 87769 and conventional soybean DNA were digested with either a combination of the restriction enzymes *Lgu* I and *Bst*X I or *Bsr*G I and *Psh*A I and analyzed by Southern blot (Figure V-6) using probes 4 and 5 (Figure V-1). The results are summarized in Table V-2.

Conventional soybean DNA was used as a control in order to determine the endogenous background of MON 87769 on the Southern blot used to confirm the absence of T-DNA II sequences (Figure V-6). Conventional soybean DNA digested with a combination of *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Psh*A I (lanes 3 and 7) and hybridized simultaneously with the probes 4 and 5 produced no detectable hybridization bands. This indicates that no endogenous soybean DNA sequences hybridized to the T-DNA II probes.

To ensure that both of the overlapping T-DNA II probes were capable of hybridizing to their intended target, both plasmid PV-GMPQ1972 DNA digested with *Bam*H I and probe template spikes were mixed with conventional soybean DNA (predigested with *Lgu* I and *Bst*X I) and used on the Southern blot (Figure V-6). PV-GMPQ1972 DNA digested with *Bam*H I and analyzed using the two overlapping T-DNA II probes produced the expected size bands of approximately 7.9 and 2.3 kb (lane 9). The probe template controls, mixed with conventional soybean, and analyzed using the T-DNA II probes produced two expected bands at approximately 2.0 and 1.9 kb (lanes 10 and 11). The 0.1 genomic equivalent of the probe templates resulted in a fairly faint hybridization signal (lane 11), but is detectable on the actual autoradiography film. These results indicate that the T-DNA II probes labelled properly and hybridized to their intended targets.

To confirm the absence of T-DNA II sequences not associated with T-DNA I, MON 87769 DNA was analyzed by Southern blot using probes 4 and 5 (Figure V-1) that span the T-DNA II sequence of PV-GMPQ1972. The overlapping probes spanning T-DNA II contain the right border sequence, the *E9* 3′ nontranslated region sequence, and the left border sequence that are also contained on T-DNA I. Therefore, given the common genetic elements shared between T-DNA I and T-DNA II, the T-DNA II probes are expected to hybridize to fragments derived from T-DNA I. Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) produced a single band of approximately 1.6 kb. This band is consistent with the 1.6 kb band detected with the *E*9 3′ nontranslated region + left border sequence (lanes 2 and 6, Figure V-12). Although a band at 6.8 kb, that represents the right border sequence in the insert, was also expected in MON 87769, this band was not visible in Figure V-6. This band was not observed in the reported exposure because, as revealed by sequence analyses, there are only 43 base pairs of the right border sequence in the MON 87769 insert. There were no additional fragments observed. Southern blot analysis of MON 87769 DNA digested with *Bsr*G I and *Psh*A I (lanes 4 and 8) produced a single band of approximately 4.2 kb. This band is consistent with the 4.2 kb band detected with the *E*9 3′ nontranslated region + left border sequence (lanes 4 and 8, Figure V-12). Although a band at 2.0 kb that represents the

right border sequence in the insert was also expected, this band was not visible in the Figure V-6. This band was not observed in the reported exposure because, as revealed by sequence analyses, there are only 43 base pairs of the right border sequence in the MON 87769 insert. No additional fragments were observed. These results indicate that MON 87769 does not contain any additional detectable T-DNA II elements other than those associated with T-DNA I.

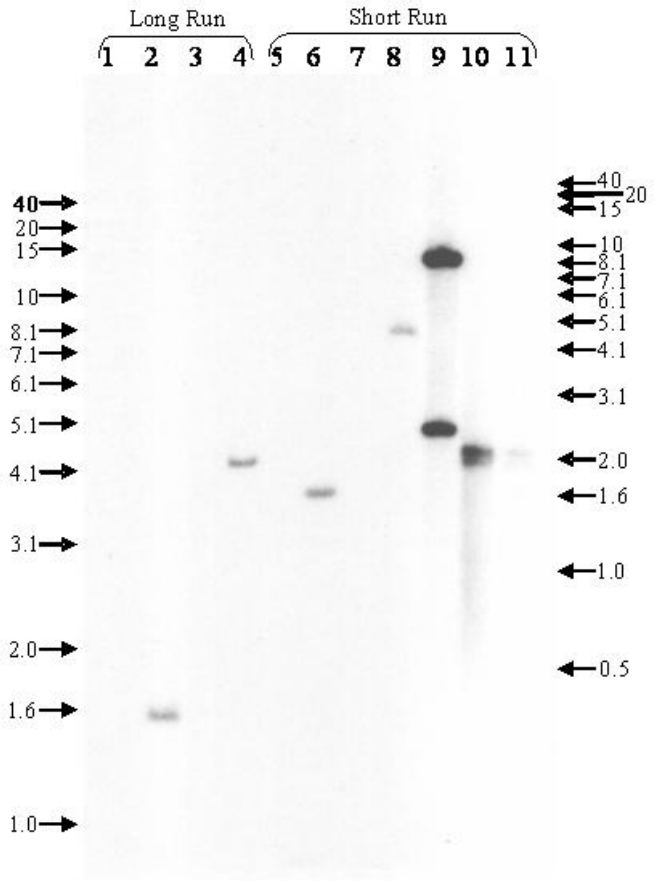


Figure V-6. Southern Blot Analysis of MON 87769 with T-DNA II Probes
The blot was hybridized simultaneously with two overlapping ^{32}P-labeled probes that span the entire T-DNA II sequence (probes 4 and 5, Figure V-1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Conventional soybean (*Bsr*G I and *Psh*A I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
10: Conventional soybean (*Bsr*G I and *Psh*A I) spiked with probe templates [~1 genomic equivalent]
11: Conventional soybean (*Bsr*G I and *Psh*A I) spiked with probe templates [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

V.D. *Pj.D6D* and *Nc.Fad3* Cassette Copy Number and Integrity

The copy number of the inserted *Pj.D6D* and *Nc.Fad3* coding sequences and their associated genetic elements was assessed by digesting MON 87769 DNA with a combination of *Lgu* I and *Bst*X I or a combination of *Bsr*G I and *Psh*A I and hybridizing the Southern blots with individual genetic elements from the inserted *Pj.D6D* and *Nc.Fad3* cassettes. The size of the genomic fragments and the T-DNA elements expected to be contained in each of those fragments is described below and summarized in Table V-2.

Digestion of MON 87769 genomic DNA with *Lgu* I and *Bst*X I was expected to generate two border fragments of approximately 6.8 and 1.6 kb (Figure V-3). The 6.8 kb fragment contains genomic DNA flanking the 5′ end of the insert, right border sequence, *7Sα′* promoter sequence, *Pj.D6D* coding sequence, *tml* 3′ nontranslated region, *7Sα* promoter sequence, and a portion of the *Nc.Fad3* coding sequence. The 1.6 kb fragment contains the remaining portion of the *Nc.Fad3* coding sequence, *E9* 3′ nontranslated region, left border sequence, and genomic DNA flanking the 3′ end of the insert.

Digestion of MON 87769 genomic DNA with *Bsr*G I and *Psh*A I was expected to generate one internal restriction fragment of approximately 1.9 kb and two border fragments of approximately 2.0 and 4.2 kb (Figure V-3). The 2.0 kb fragment contains genomic DNA flanking the 5′ end of the insert, right border sequence, *7Sα′* promoter sequence, and a portion of the *Pj.D6D* coding sequence. The 1.9 kb fragment contains the remaining portion of the *Pj.D6D* coding sequence and a portion of the *tml* 3′ nontranslated region. The 4.2 kb fragment contains the remaining portion of the *tml* 3′ nontranslated region, *7Sα* promoter sequence, *Nc.Fad3* coding sequence, *E9* 3′ nontranslated region, left border sequence, and genomic DNA flanking the 3′ end of the insert.

Individual Southern blots were examined with the following probes: the right border + *7Sα′* promoter probe, *Pj.D6D* coding region probe, *tml* 3′ nontranslated region probe, *7Sα* promoter probe, *Nc.Fad3* coding sequence probe, or *E9* 3’ nontranslated region + left border probe (probes 7-12, Figure V-1). The expected DNA fragments identified by probes 7-12 are summarized in Table V-2. To ensure that each of the probes was capable of hybridizing to the respective target, plasmid PV-GMPQ1972 DNA digested with *Bam*H I and combined with predigested conventional soybean DNA was used as a positive hybridization control.

V.D.1. Right Border/*7Sα′* Promotor

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to assess the copy number of the right border and *7Sα′* promoter (Figure V-7). Conventional soybean DNA digested with *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Ps*hA I (lanes 3 and 7) and hybridized with the right border + *7Sα′* promoter probe (probe 7, Figure V-1) produced a single hybridization signal. This hybridization signal results from the probe hybridizing to an endogenous soybean genomic sequence and is not specific to the inserted DNA.

This signal was produced in both test and control lanes and, therefore, the band is considered to be endogenous background.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA, and included on the Southern blot analysis that was hybridized with probe 7 (Figure V-7). The results of this experiment showed the expected size hybridization bands at approximately 1.3, 2.3 and 7.9 kb in addition to the endogenous hybridization band observed in the conventional soybean control (lanes 10 and 11).

Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) using probe 7 produced the expected single unique band of approximately 6.8 kb in addition to the endogenous hybridization band observed in the conventional soybean control (lanes 1 and 5; Figure V-7). Southern blot analysis of MON 87769 DNA digested with *Bsr*G I and *Psh*A I (lanes 4 and 8) produced the single expected unique band of approximately 2.0 kb in addition to the endogenous hybridization band observed in the conventional soybean control (lanes 3 and 7; Figure V-7). There were no unexpected bands present in the MON 87769 DNA samples. This indicates that MON 87769 contains no additional detectable Right Border or *7Sα′* promoter elements other than those endogenous to the soybean genome or associated with the *Pj.D6D* gene cassette.

V.D.2. *Pj.D6D* Coding Sequence

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the copy number of the *Pj.D6D* coding sequence (Figure V-8). Conventional soybean DNA digested with the enzyme combination *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Psh*A I (lanes 3 and 7) and hybridized with the *Pj.D6D* coding sequence probe (probe 8, Figure V-1) showed no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the *Pj.D6D* coding sequence probe.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA (predigested with *Lgu* I and *Bst*X I), and included on the Southern blot analysis that was hybridized with probe 8. The results of this experiment showed the expected size hybridization bands at approximately 1.3 and 1.9 kb (lanes 10 and 11).

Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) using probe 8 produced the expected single unique band of approximately 6.8 kb. Southern blot analysis of MON 87769 DNA digested with *Bsr*G I and *Psh*A I (lanes 4 and 8) produced the expected unique bands of approximately 1.9 and 2.0 kb. Although these two bands appear as one band in the short run (lane 8), the long run (lane 4) clearly resolves the 1.9 and 2.0 kb bands. There are no unexpected bands in the MON 87769 DNA. This indicates that MON 87769 contains no additional detectable *Pj.D6D* coding sequence elements other than those associated with the *Pj.D6D* cassette.

V.D.3. *tml* 3′ Nontranslated Sequence

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the copy number of the *tml* 3' nontranslated region (Figure V-9). Conventional soybean control DNA digested with *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Psh*A I (lanes 3 and 7) and hybridized with the *tml* 3' nontranslated region probe (probe 9, Figure V-1) showed no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the *tml* 3' nontranslated region probe.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA (predigested with *Lgu* I and *Bst*X I) and included on the *tml* 3' nontranslated region Southern blot. The results for this probe showed the expected size band at approximately 1.9 kb (lanes 10 and 11).

Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) using probe 9 produced the expected single unique band of approximately 6.8 kb. Southern blot analysis of MON 87769 DNA digested with *Bsr*G I and *Psh*A I (lanes 4 and 8) produced two expected unique bands of approximately 1.9 and 4.2 kb. There were no additional bands detected using the *tml* 3′ nontranslated region probe. This indicates that MON 87769 contains no additional, detectable *tml* 3′ elements other than those associated with the *Pj.D6D* gene cassette.

V.D.4. *7Sα* Promoter

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the copy number of the *7Sα* Promoter (Figure V-10). The conventional soybean DNA, digested with *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bs*rG I and *Psh*A I (lanes 3 and 7) and hybridized with the *7Sα* promoter probe (probe 10, Figure V-1), produced two hybridization signals. These hybridization signals result from the probe hybridizing to endogenous soybean genomic sequences and are not specific to the inserted DNA. These signals were produced in both test and control lanes and therefore the bands are considered to be endogenous background.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA (predigested with *Lgu* I and *Bst*X I), and included in the 7Sα promoter Southern blot analysis. The results of this experiment showed the expected size band at approximately 3.1 kb in addition to the endogenous hybridization bands observed in the conventional soybean control (lanes 10 and 11).

Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) produced the expected single unique band of approximately 6.8 kb in addition to the endogenous hybridization bands observed in the conventional soybean control (lanes 1 and 5). Southern blot analysis of MON 87769 DNA digested with *Bsr*G I and *Psh*A I (lanes 4 and 8) produced the expected single unique band of approximately 4.2 kb in addition to the endogenous hybridization bands observed in the conventional soybean

control (lanes 3 and 7). The migration of approximately 6.8 kb and 4.2 kb fragments is slightly higher than indicated by molecular weight marker band sizes. The altered migration may be due to the difference in salt concentrations between the DNA sample and the molecular weight marker (Sambrook and Russell, 2001). There were no unexpected bands detected in the MON 87769 DNA samples. This indicates that MON 87769 contains no additional detectable *7Sα* promoter elements other than those endogenous to the soybean genome or associated with the *Nc.Fad3* gene cassette.

V.D.5. *Nc.Fad3* Coding Sequence

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the copy number of the *Nc.Fad3* coding sequence (Figure V-11). Conventional soybean DNA digested with *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Psh*A I (lanes 3 and 7) and hybridized with the *Nc.Fad3* coding sequence probe (probe 11, Figure V-1) produced no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the *Nc.Fad3* coding sequence probe.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA (digested with *Lgu* I and *Bst*X I), and included on the *Nc.Fad3* coding sequence Southern blot analysis using probe 11. The results of this experiment showed the expected size hybridization band at approximately 3.1 kb (lanes 10 and 11).

Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) using probe 11 produced the two expected unique bands of approximately 1.6 and 6.8 kb. The migration of the approximately 1.6 kb fragment is slightly lower than indicated by the molecular weight marker band sizes. The altered migrations may be due to the difference in salt concentrations between the DNA sample and the molecular weight marker (Sambrook and Russell, 2001). Southern blot analysis of MON 87769 DNA digested with *Bsr*G I and *Psh*A I (lanes 4 and 8) produced the expected single unique band of approximately 4.2 kb. There were no unexpected bands observed on the Southern blot using MON 87769 DNA. This indicates that MON 87769 contains no additional detectable *Nc.Fad3* coding sequence elements other than those associated with the *Nc.Fad3* cassette.

V.D.6. *E9* 3′ Nontranslated Sequence/Left Border

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the copy number of the *E9* 3′ nontranslated region and left border sequence (Figure V-12). Conventional soybean DNA digested with *Lgu* I and *Bst*X I (lanes 1 and 5) or *Bsr*G I and *Psh*A I (lanes 3 and 7) and hybridized with the *E9* 3′ nontranslated region + left border probe (probe 12, Figure V-1) produced no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the *E9* 3′ nontranslated region and left border sequence.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA (digested with *Lgu* I and *Bst*X I), and included in the *E9* 3′ nontranslated region and + left border Southern blot analysis (Figure V-12). The results of this experiment showed the two expected size bands at approximately 2.3 and 7.9 kb (lanes 10 and 11).

Southern blot analysis of MON 87769 DNA digested with *Lgu* I and *Bst*X I (lanes 2 and 6) using probe 12 produced the expected single unique band of approximately 1.6 kb. Southern blot analysis of MON 87769 DNA digested with *Bs*rG I and *Psh*A I (lanes 4 and 8) produced the expected single unique band of approximately 4.2 kb. There were no additional bands detected using the *E9* 3′ nontranslated region + left border probe. This indicates that MON 87769 contains no additional detectable *E9* 3′ nontranslated region or left border elements other than those associated with the *Nc.Fad3* gene cassette.

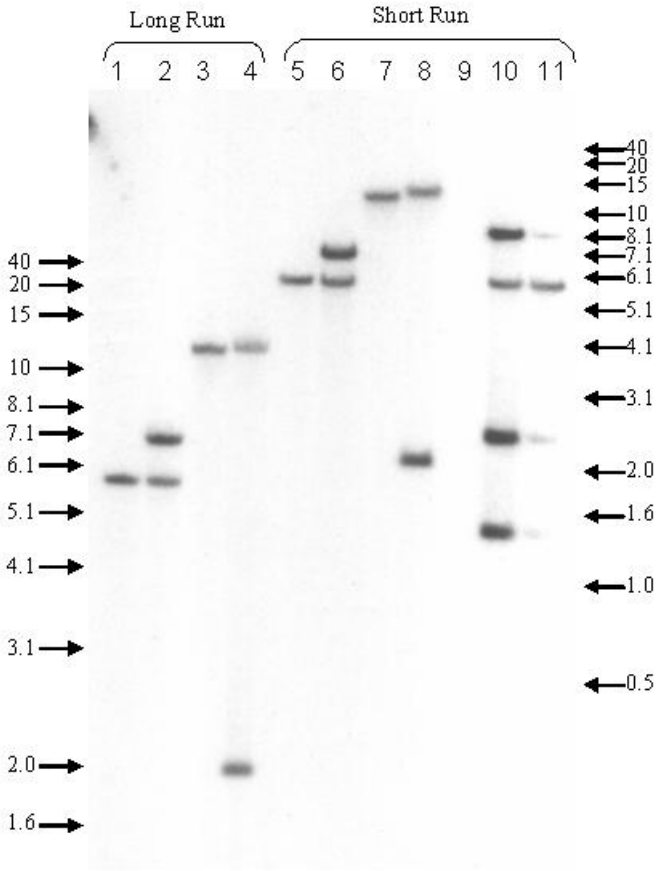


Figure V-7. Southern Blot Analysis of MON 87769 with Right Border/P-7sα′ Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the right border and *7sα′* promoter sequence (probe 7, Figure V-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8 MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

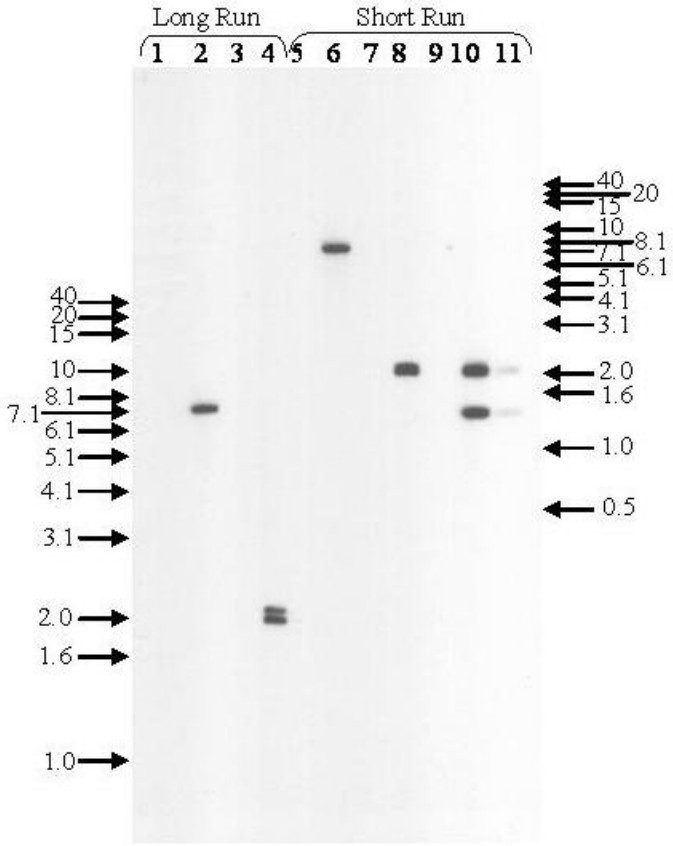


Figure V-8. Southern Blot Analysis of MON 87769 with CS-*Pj.D6D* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *Pj.D6D* coding sequence (probe 8, Figure V-1). Each lane contains approximately 10µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

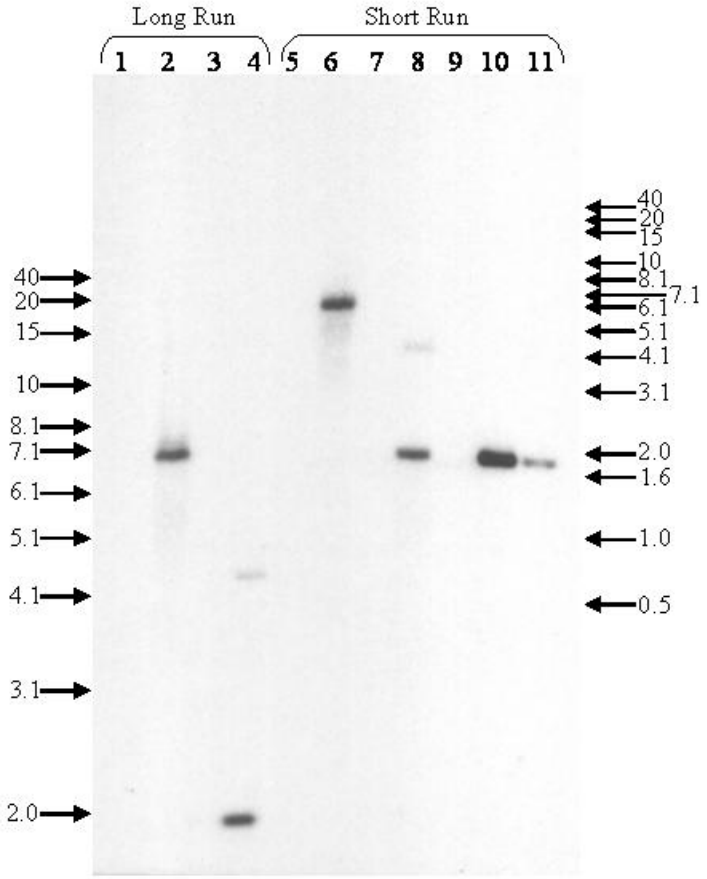


Figure V-9. Southern Blot Analysis of MON 87769 with T-*tml* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *tml* nontranslated region (probe 9, Figure V-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *PshA* I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

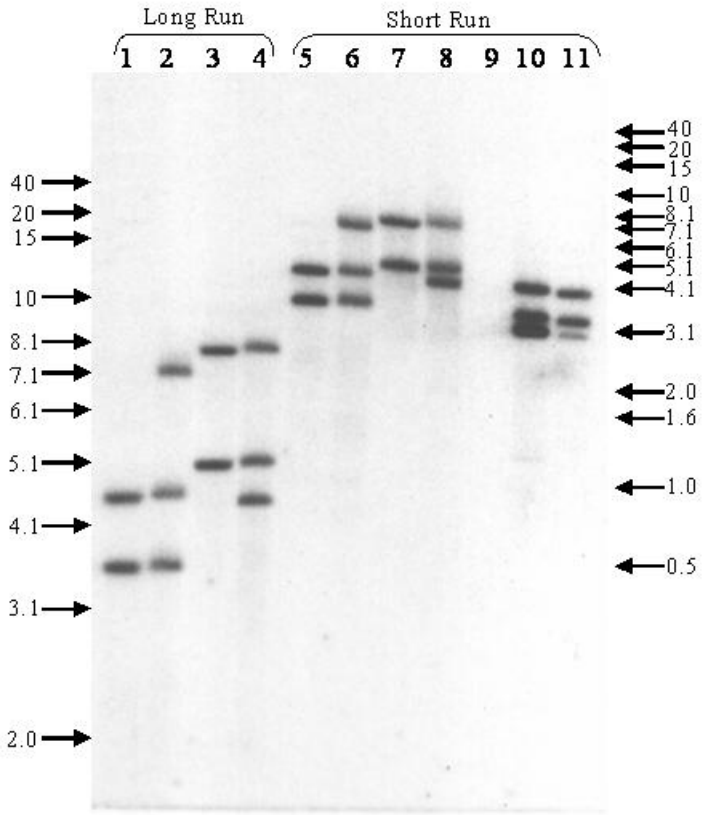


Figure V-10. Southern Blot Analysis of MON 87769 with P-*7sα* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *7sα* promoter sequence (probe 10, Figure V-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

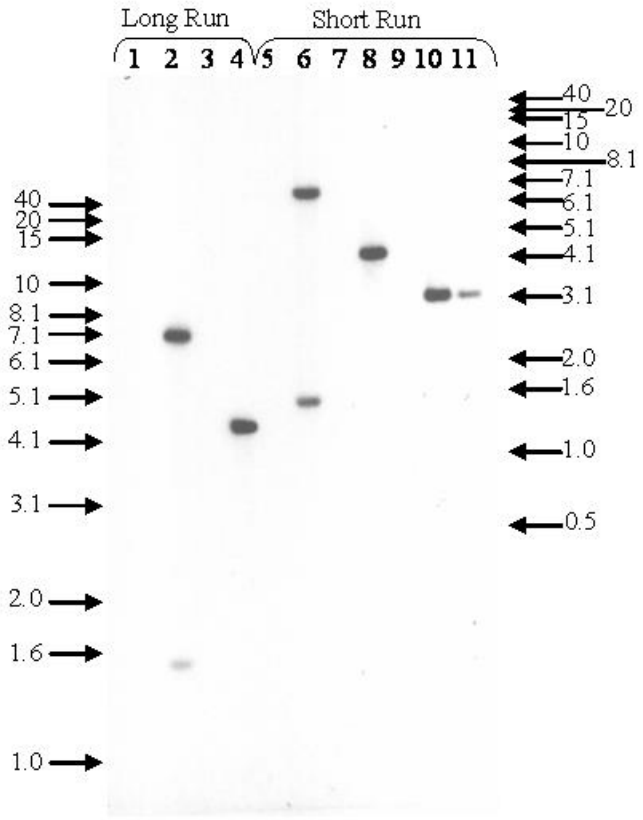


Figure V-11. Southern Blot Analysis of MON 87769 with CS-*Nc.Fad3* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *Nc.Fad3* coding sequence (probe 11, Figure V-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bsr*G I and *Psh*AI)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

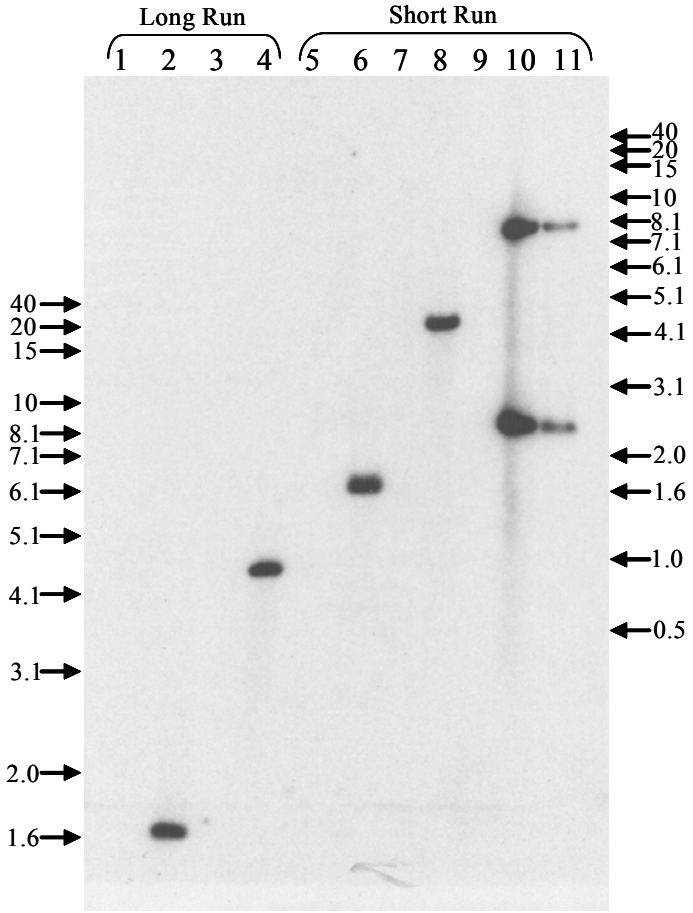


Figure V-12. Southern Blot Analysis of MON 87769 with T-*E9*/Left Border Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *E9* nontranslated region and left border (probe 12, Figure V-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I)
2: MON 87769 (*Lgu* I and *Bst*X I)
3: Conventional soybean (*Bsr*G I and *Psh*A I)
4: MON 87769 (*Bs*rG I and *Psh*A I)
5: Conventional soybean (*Lgu* I and *Bst*X I)
6: MON 87769 (*Lgu* I and *Bst*X I)
7: Conventional soybean (*Bsr*G I and *Psh*A I)
8: MON 87769 (*Bsr*G I and *Psh*A I)
9: Empty
10: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
11: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~0.1 genomic equivalent]

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

V.E. Southern Blot Analyses of MON 87769 Across Multiple Generations

In order to assess the stability of the T-DNA in MON 87769 across multiple generations, Southern blot analyses were performed using DNA obtained from multiple generations of MON 87769. DNA from four generations was analyzed by Southern blot using six overlapping probes that span the entire T-DNA I region of plasmid PV-GMPQ1972 (probes 13-18, Figure V-2). For reference, the breeding history of MON 87769 is presented in Figure V-13. The expected Southern hybridization DNA banding pattern for these analyses is summarized in Table V-2.

V.E.1. Generational Stability of the Insert

Conventional soybean DNA from A3525 was used as a control to determine the endogenous background hybridization of MON 87769. As shown in Figure V-14, conventional soybean DNA digested with a combination of *Lgu* I and *Bst*X I (lane 4) produced several hybridization signals. This is expected because several genetic elements within T-DNA I were derived from soybean. These hybridization signals result from the probes hybridizing to endogenous soybean gene sequences and are not specific to the inserted DNA. These signals were produced in all lanes, including those containing the conventional soybean DNA and, therefore, are considered to be endogenous background hybridization.

To ensure that each of the T-DNA probes was capable of hybridizing to their intended target, plasmid DNA and probe template spikes were used as controls on the Southern blots. Plasmid PV-GMPQ1972 DNA controls digested with *Bam*H I, mixed with conventional soybean DNA (digested with *Lgu* I and *Bst*X I), and analyzed using probes 13-15 (lane 1, Figure V-14a) produced the expected bands at approximately 1.3, 1.9, 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA (refer to Table V-2). Conventional soybean DNA spiked with probes 13-15 (lanes 2 and 3) produced the expected bands at 1.6, 1.7, and 2.0 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA.

Plasmid PV-GMPQ1972 DNA digested with *Bam*H I, mixed with conventional soybean DNA (digested with *Lgu* I and *Bst*X I) and analyzed with probes 16-18 (lane 1, Figure V-14b) produced the expected bands at approximately 2.3, 3.1, and 7.9 kb, in addition to the endogenous background hybridization produced by the conventional soybean DNA (refer to Table V-2). Conventional soybean DNA spiked with probes 16-18 (lanes 2 and 3) produced expected bands at 0.8, 1.0, and 1.3 kb, in addition to the endogenous background hybridization.

Southern blot analysis of MON 87769 DNA from four generations digested with *Lgu* I and *Bst*X I (lanes 5-9, Figure V-14a) and hybridized simultaneously with probes 13-15 produced the expected band of approximately 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean DNA controls (lanes 4). Southern blot analysis of MON 87769 DNA from all four generations digested with *Lgu* I and *Bst*X I (lanes 5-9, Figure V-14b) using probes 16-18 produced the two expected

bands of approximately 1.6 and 6.8 kb in addition to the endogenous background hybridization observed in the conventional soybean control DNA (lane 1). Also, several nonspecific hybridization spots exist in lanes 4, 8, and 9. These nonspecific hybridization spots are faint and do not affect the results of the Southern blot. The observed results are consistent with the results presented in Figures V-4a and V-4b, and confirm that the single copy of the insert in MON 87769 is stably maintained across multiple generations of its breeding history.

V.E.2. Confirmation of the Absence of PV-GMPQ1972 Backbone Sequence

The four generations of MON 87769 utilized to assess generational stability also were examined for the absence of backbone sequence by Southern blot. MON 87769 and control DNA samples were digested with *Lgu* I and *Bst*X I and the blot was hybridized simultaneously with four overlapping probes, which taken together, span the backbone sequence of plasmid PV-GMPQ1972 (probes 1-3 and 6, Figure V-1).

Conventional soybean DNA was used as a control in order to determine the endogenous background hybridization of MON 87769 on the Southern blot used to confirm the absence of plasmid backbone across multiple generations (Figure V-15). Conventional soybean DNA digested with a combination of *Lgu* I and *Bst*X I (lane 4) and hybridized with the backbone probes (probes 1-3 and 6, Figure V-1) showed no hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the plasmid backbone probes.

To ensure that each of the backbone probes was capable of hybridizing to their intended target, plasmid PV-GMPQ1972 DNA and probe template spikes were used as positive hybridization controls on the Southern blot (Figure V-15). PV-GMPQ1972 control DNA digested with *Bam*H I, mixed with conventional soybean, and analyzed with the backbone probes (probes 1-3 and 6; Figure V-1) produced the expected size bands of approximately 2.3 and 7.9 kb (lane 1; refer to Table V-2). The probe template controls, mixed with digested conventional soybean DNA and analyzed with the backbone probes, produced four expected bands at approximately 0.7, 0.9, 1.5, and 1.8 kb (lanes 2 and 3). These results indicate that all the backbone probes labelled properly and hybridized to their intended targets.

To confirm the absence of PV-GMPQ1972 backbone sequence, MON 87769 DNA was analyzed by Southern blot using probes 1-3 and 6 (Figure V-1) that span the entire backbone sequence of PV-GMPQ1972. Southern blot analysis of MON 87769 DNA digested with a combination of *Lgu* I and *Bst*X I (lanes 5-9) showed no detectable hybridization signal. These results are consistent with the data depicted in Figure V-5. This indicates that MON 87769 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ1972.

V.E.3. Confirmation of the Absence of T-DNA II Sequence

The four generations of MON 87769 material utilized to assess generational stability also were examined for the absence of T-DNA II sequence by Southern blot analysis.

MON 87769 and control DNA samples were digested with *Lgu* I and *Bst*X I and the blot was hybridized simultaneously with two overlapping probes, which span the T-DNA II sequence of plasmid PV-GMPQ1972 (probes 4 and 5, Figure V-1).

Conventional soybean DNA was used as a control in order to determine the endogenous background of MON 87769 on the Southern blot used to confirm the absence of T-DNA II sequences (Figure V-16). Conventional soybean DNA digested with a combination of *Lgu* I and *Bst*X I (lanes 4) and hybridized with the T-DNA II probes produced no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the T-DNA II probes.

To ensure that both of the overlapping T-DNA II probes were capable of hybridizing to their intended target, plasmid PV-GMPQ1972 DNA and probe template spikes were used as positive hybridization controls on the Southern blot (Figure V-16). PV-GMPQ1972 DNA digested with *Bam*H I, mixed with conventional soybean DNA, and analyzed using the two overlapping T-DNA II probes produced the expected size bands of approximately 7.9 and 2.3 kb (lane 1; refer to Table V-2). The probe template controls, mixed with conventional soybean DNA and analyzed using the T-DNA II probes, produced two expected bands at approximately 2.0 and 1.9 kb (lanes 2 and 3). Lanes 1 and 2 have an apparent smear throughout the lane due to background hybridization. These results indicate that the T-DNA II probes labelled properly and hybridized to their intended targets.

To confirm the absence of T-DNA II sequences not associated with T-DNA I, MON 87769 DNA from four generations was analyzed by Southern blot using probes 4 and 5 (Figure V-1) that span the T-DNA II sequence of PV-GMPQ1972. The overlapping probes spanning T-DNA II contain the right border sequence, the *E9* 3′ nontranslated region sequence, and the left border sequence that also are contained on T DNA I. Therefore, the T-DNA II probes are expected to hybridize to fragments derived from T-DNA I. Southern blot analysis of MON 87769 DNA from all four generations digested with *Lgu* I and *Bst*X I (lanes 5-9) produced two bands of approximately 1.6 and 6.8 kb. The 1.6 kb band is consistent with the 1.6 kb band detected with the *E*9 3′ nontranslated region + left border sequence (lanes 2 and 6, Figure V-12). The hybridization band at 6.8 kb that represents the right border sequence in the insert was faint in the reported exposure because there are only 43 base pairs of the right border sequence in the MON 87769 insert. No additional fragments were observed. The observed results are consistent with the results presented in Figure V-6, and confirm that all four generations of MON 87769 do not contain any additional detectable T-DNA II elements other than those associated with T-DNA I.

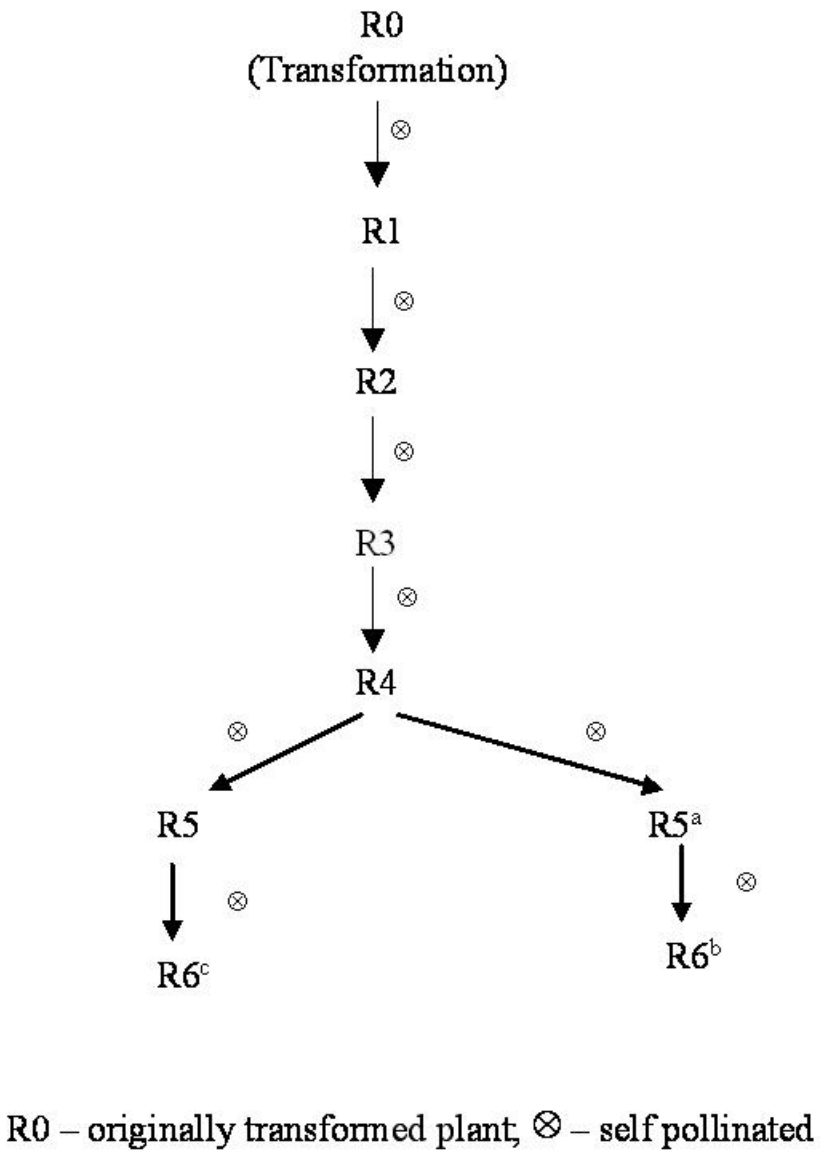


Figure V-13. MON 87769 Breeding Diagram
All generations shown were self-pollinated (⊗). The R1 generation was used for segregation analysis and the selection of plants homozygous for the T-DNA I insert (Section III). R5 seed material was used for commercial variety development. Generation R4 was used in the molecular characterization analyses. Generations R3, R4, $R5^a$, $R6^b$, and $R6^c$ were used in the molecular generation stability analyses.

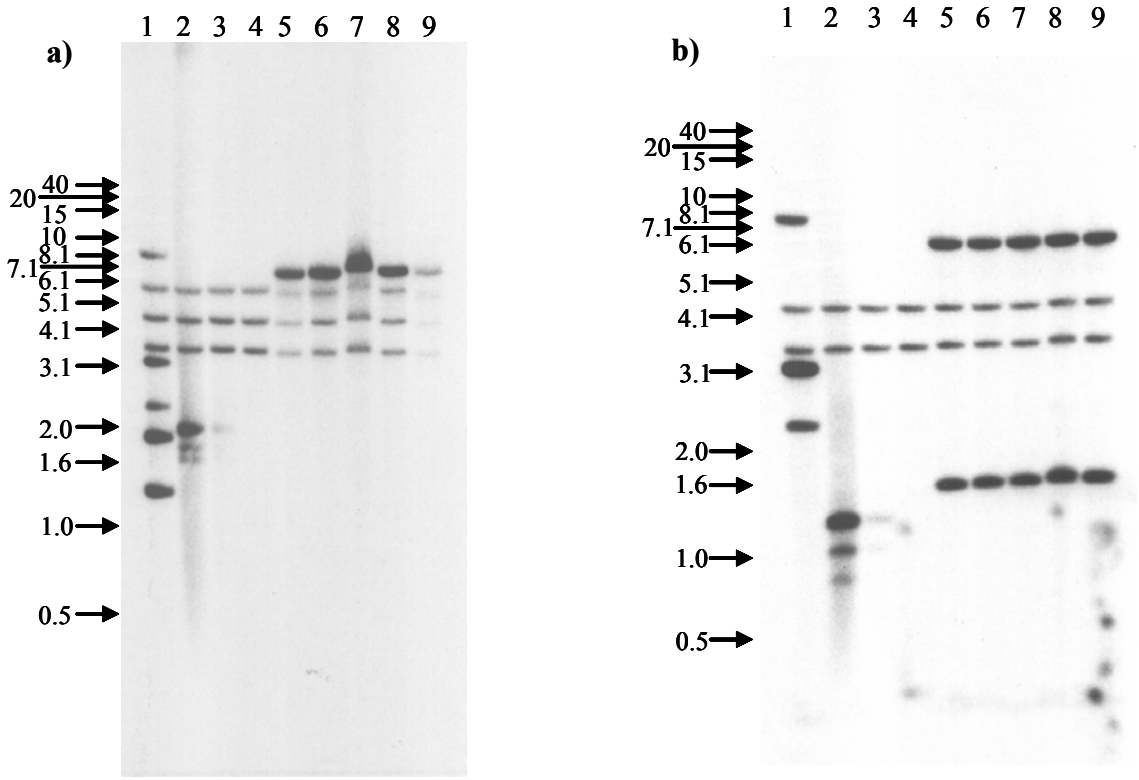


Figure V-14. Generational Stability of MON 87769 with T-DNA I Probes
The blot was hybridized with overlapping ^{32}P-labeled probes that spanned the T-DNA (probes 13-18, Figure V-2). Each blot was hybridized simultaneously with three of the overlapping probes. Figure V-14a was hybridized with probes 13-15 and Figure V-14b was hybridized with probes 16-18. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87769 is illustrated in Figure V-13. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
2: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]
4: Conventional soybean (*Lgu* I and *Bst*X I)
5: R3: MON 87769 (*Lgu* I and *Bst*X I)
6: R4: MON 87769 (*Lgu* I and *Bst*X I)
7: R5[a]: MON 87769 (*Lgu* I and *Bst*X I)
8: R6[c]: MON 87769 (*Lgu* I and *Bst*X I)
9: R6[b]: MON 87769 (*Lgu* I and *Bst*X I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

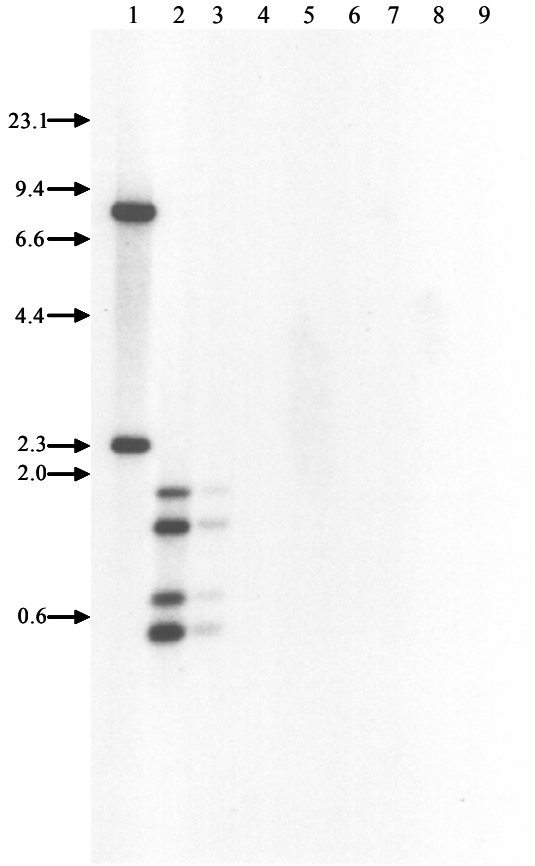


Figure V-15. Generational Stability of MON 87769 with PV-GMPQ1972 Backbone Probes

The blot was hybridized with four overlapping ^{32}P-labeled probes that span the entire backbone sequence (probes 1, 2, 3, and 6, Figure V-1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87769 is illustrated in Figure IV-17. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
2: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]
4: Conventional soybean (*Lgu* I and *Bst*X I)
5: R3: MON 87769 (*Lgu* I and *Bs*tX I)
6: R4: MON 87769 (*Lgu* I and *Bst*X I)
7: R5[a]: MON 87769 *(Lgu* I and *Bs*tX I)
8: R6[c]: MON 87769 *(Lgu* I and *Bst*X I)
9: R6[b]: MON 87769 *(Lgu* I and *Bst*X I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

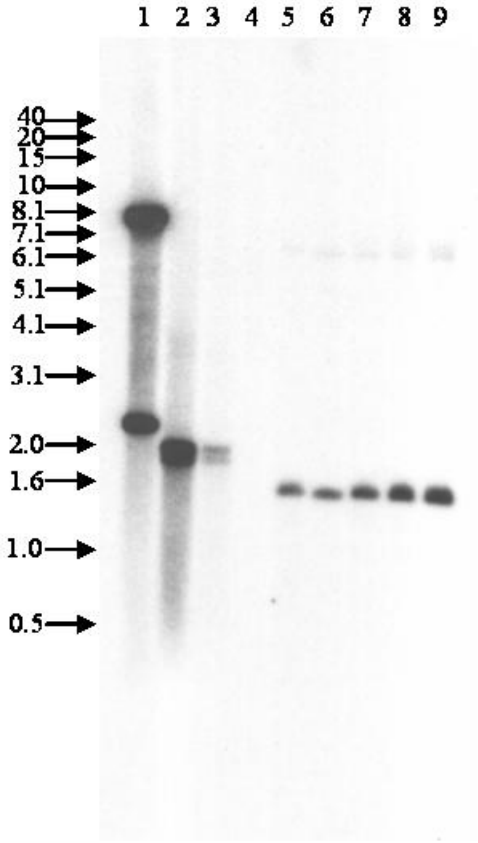


Figure V-16. Generational Stability of MON 87769 with T-DNA II Probes
The blot was hybridized with two overlapping ^{32}P-labeled probes that span the entire T-DNA II (probes 4 and 5, Figure V-1) of plasmid PV-GMPQ1972. Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. The breeding history of MON 87769 is illustrated in Figure V-13. Lane designations are as follows:

Lane 1: Conventional soybean (*Lgu* I and *Bst*X I) spiked with PV-GMPQ1972 (*Bam*H I) [~1 genomic equivalent]
2: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~1 genomic equivalent]
3: Conventional soybean (*Lgu* I and *Bst*X I) spiked with probe templates [~0.1 genomic equivalent]
4: Conventional soybean (*Lgu* I and *Bst*X I)
5: R3: MON 87769 (*Lgu* I and *Bst*X I)
6: R4: MON 87769 (*Lgu* I and *Bst*X I)
7: R5[a]: MON 87769 (*Lgu* I and *Bst*X I)
8: R6[c]: MON 87769 (*Lgu* I and *Bst*X I)
9: R6[b]: MON 87769 (*Lgu* I and *Bst*X I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium bromide stained gel.

V.F. Organization of the Genetic Elements in MON 87769

The organization of the genetic elements within the insert of MON 87769 was confirmed by PCR and DNA sequence analyses. Overlapping DNA fragments spanning the entire length of the insert and genomic DNA directly adjacent to the insert were amplified by PCR. The amplified DNA fragments were subjected to DNA sequence analyses. The DNA sequence of the insert contains 7367 base pairs beginning at base 9387 of PV-GMPQ1972 located in the right border region, and ending at base 288 in the left border region of PV-GMPQ1972. In addition, 933 base pairs of soybean genomic DNA flanking the 5' end of the insert and 831 base pairs of soybean genomic DNA flanking the 3' end of the insert were also determined. A comparison of the soybean genomic DNA flanking the insert in MON 87769 to the sequence of the insertion site in conventional soybean demonstrated that no major rearrangements occurred at the insertion site during transformation. Results confirm that the DNA sequence of the insert in MON 87769 is identical to that of plasmid PV-GMPQ1972, and the organization of the insert genetic elements is as depicted in Figure V-3.

V.G. Inheritance of the Introduced Traits

The genotypic stability and segregation of T-DNA I in MON 87769, initially demonstrated by Southern blot analysis (Section V.E), was confirmed by performing a Chi-square analysis on zygosity data generated for the T-*tml* 3' genetic element over three generations. The Chi-square analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles. The breeding path for these segregation data on MON 87769 is described in Figure V-17. The R0 plant was self-pollinated to produce R1 seed, which is expected to segregate 1:2:1 (1 homozygote:2 hemizygote:1 null segregant) for the insert. A homozygous selection (R1 plant) was identified from the segregating population by Invader and Southern blot analyses. The selected R1 plant was self-pollinated to produce R2 seed, which was expected to be fixed for the trait, meaning all seed are homozygous for T-DNA I. The homozygosity of subsequent generations was confirmed using the Invader assay (data not shown).

Homozygous R4 MON 87769 plants were crossed to a conventional soybean variety to produce hemizygous F1 seed. Seed from the resulting F1 plants were harvested to produce the F2 generation. The F2 plants are expected to segregate 1:2:1 (1 homozygote:2 hemizygote:1 null segregant) for the insert. Individual plants were tested for the presence and zygosity of the insert using an Invader assay for the T-*tml* 3' genetic element (Gupta et al., 2008). Hemizygous positive F2 plants were selected and self-pollinated to produce F3 seed. The F3 plants were tested for the presence and zygosity of the insert using the T-*tml* 3' Invader assay. Hemizygous F3 plants were selected and self-pollinated to produce F4 seed. Likewise, the F4 plants were tested for the presence and zygosity of the insert using the T-*tml* 3′ Invader assay. Like the F2 progeny, the F3 and F4 progeny each were predicted to segregate 1:2:1 (1 homozygote:2 hemizygote:1 null segregant) for the insert.

The Chi-square (χ 2) test for each generation was computed as:

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

where o = observed frequency of the genotype and e = expected frequency of the genotype. The level of statistical significance was predetermined to be 5% ($p \leq 0.05$). Table V-3 shows the segregation data obtained with Invader-based assay. The Chi-square value for the F2, F3, and F4 generations indicated no significant difference between the observed and expected segregation ratios. These results are consistent with molecular characterization data, indicating a single insertion site of the insert within MON 87769 and show the insert follows the expected Mendelian pattern of segregation.

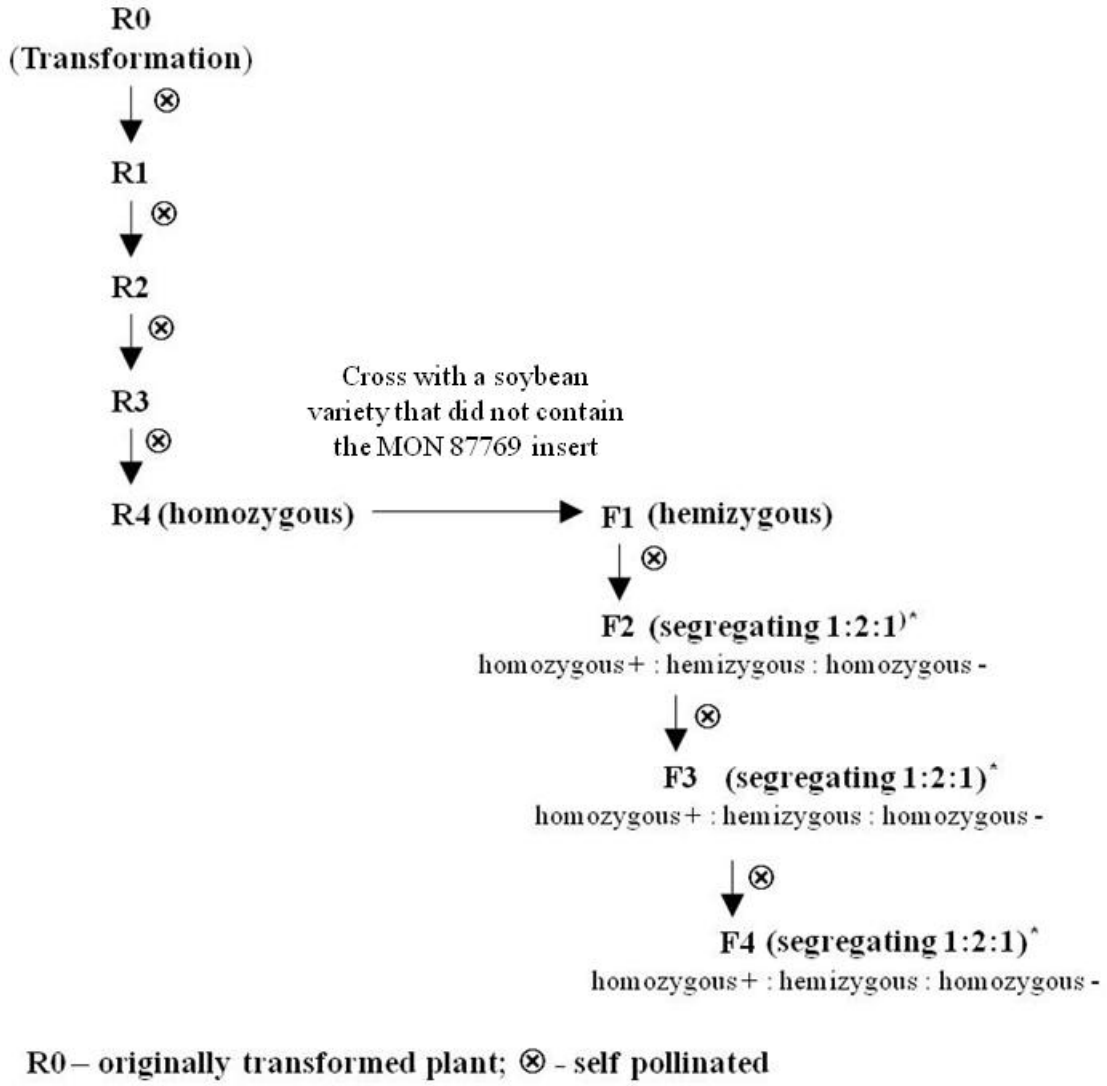


Figure V-17. Breeding Path for Generating Segregation Data on MON 87769

Table V-3. Chi-square (χ^2) analysis of MON 87769 Progeny

					1:2:1 Segregation				
Generation[1]	Total Plants Tested[2]	Observed # Plants Homozygous Positive	Observed # Plants Hemizygous Positive	Observed # Plants Homozygous Negative	Expected # Plants Homozygous Positive	Expected # Plants Hemizygous Positive	Expected # Plants Homozygous Negative	χ^2	Probability
F2	47	13	23	11	11.75	23.5	11.75	0.2	0.9087
F3	174	45	81	48	43.5	87	43.5	0.9	0.6278
F4	222	60	102	60	55.5	111	55.5	1.5	0.482

[1] F2 progeny were from the cross of homozygous MON 87769 with a soybean variety that did not contain the MON 87769 insert. F3 and F4 progeny were from self-pollinated F2 and F3 plants hemizygous positive for the MON 87769 insert.
[2] Plants were tested for the presence of the *tml* 3′ genetic element by Invader analysis.

V.H. Conclusions of Molecular Characterization

Molecular analyses were performed to characterize the integrated DNA insert in MON 87769. Southern blot analyses were used to determine the DNA insert number (number of integration sites within the soybean genome), copy number (the number of copies within one insert), intactness of the *Pj.D6D* and *Nc.Fad3* gene expression cassettes, and absence of plasmid backbone and T-DNA II sequences in the plant. The stability of the DNA insert across multiple generations also was demonstrated by Southern blot fingerprint analysis. In addition, DNA sequencing analyses were performed to confirm the organization of the elements within the MON 87769 DNA insert, and to confirm the sequence identity between the plasmid PV-GMPQ1972 T-DNA and the insert.

Data show that one intact copy of the *Pj.D6D* and *Nc.Fad3* expression cassettes was integrated at a single chromosomal locus contained within a ~10 kb *Lgu* I restriction fragment. No additional elements from the transformation vector PV-GMPQ1972, linked or unlinked to the intact DNA insert were detected in the genome of MON 87769. Additionally, backbone sequence from PV-GMPQ1972 was not detected and it also was confirmed that MON 87769 does not contain any additional detectable T-DNA II elements other than those associated with T-DNA I. Generational stability analysis demonstrated that the expected Southern blot fingerprint of MON 87769 has been maintained across four generations of breeding, thereby confirming the stability of the DNA insert over multiple generations. These generations were also shown not to contain any detectable T-DNA II or backbone sequence from plasmid PV-GMPQ1972. In addition, DNA sequence analyses confirmed the sequence identities between the MON 87769 insert and the portion of the T-DNA from PV-GMPQ1972 that was integrated into the soybean genome. These results also confirmed the organization of the genetic elements within the *Pj.D6D* and *Nc.Fad3* expression cassettes of MON 87769, which was identical to those cassettes in plasmid PV-GMPQ1972. Finally, heritability and stability of the insert within MON 87769 were as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the DNA insert at a single chromosomal locus.

VI. CHACTERIZATION OF THE INTRODUCED PjΔ6D AND NcΔ15D PROTEINS

This section summarizes the safety assessment of the PjΔ6D and NcΔ15D proteins produced in MON 87769 based on the following:

1. Characterization of their identity and function,
2. Isolation from immature soybean seed for use in subsequent laboratory and regulatory safety evaluations,
3. Quatitation of expression levels in key soybean tissues,
4. An assessment of the potential allergenicity, and
5. An evaluation of potential toxicity and human and animal dietary risk assessment.

Results show that the PjΔ6D and NcΔ15D proteins share sequence similarity to several proteins that are ubiquitous in the human diet and are consumed directly in many common foods. These proteins are expressed at low levels in the seed and not detectable in other tissues analysed. Both proteins lack biologically-relevant amino acid sequence similarities to known protein allergens and toxins. Additionally, both proteins are rapidly digested in simulated gastric and intestinal fluids and do not exhibit any signs of toxicity when administered to mice via oral gavage. Ultimately, the safety assessment supports the conclusion that dietary exposure to the PjΔ6D and the NcΔ15D proteins derived from MON 87769 poses no meaningful risk to human or animal health.

These results, all of which have been submitted to FDA as part of the pre-market consultation, support a conclusion of safe dietary consumption for humans and animals. The following sections provide further details regarding the safety assessment of the PjΔ6D and NcΔ15D proteins produced in MON 87769.

VI.A. Identity and Function of the PjΔ6D and NcΔ15D Proteins

Fatty acid desaturases are enzymes that convert a single bond between two carbon atoms (C-C) to a double bond (C=C) in a fatty acyl chain (Stumpf, 1980). The resultant double bond often is referred to as an unsaturated bond, and the reactions catalyzed by these enzymes are known as desaturation reactions. The degree of unsaturation of a fatty acid chain is a major determinant of the fluidity of biological membranes that consist of a bilayer of phospholipids (Aguilar and Mendoza, 2006). All organisms synthesize unsaturated fatty acids *de novo* using a common metabolic pathway (Somerville and Browse, 2000; Voet and Voet, 1995). Hence, enzymes involved in this metabolic pathway, including fatty acid desaturases, are conserved across all kingdoms (Los and Murata, 1998).

The PjΔ6D and NcΔ15D proteins are members of a family of integral membrane fatty acid desaturases found in all eukaryotic organisms (plants, animals, fungi) and some prokaryotes, e.g. cyanobacteria (Hashimoto et al., 2008; Los and Murata, 1998). Fatty

acid desaturases are non-heme iron-containing enzymes that introduce a double bond between defined carbons of fatty acyl chains with strict regioselectivity and stereoselectivity (Shanklin and Cahoon, 1998). Solubilization of integral membrane desaturases requires detergents which are likely to disrupt the association with one or more required accessory proteins; therefore, the functional activity of integral membrane proteins usually is studied in the context of an intact membrane system (Reed et al., 2000; Sayanova et al., 2001). Due to the tremendous difficultly in the purification and assay of the activity of these integral membrane fatty desaturase proteins (Warude et al., 2006), the tertiary structure of the integral membrane desaturase proteins is not known.

VI.A.1. PjΔ6D Protein

The PjΔ6D protein expressed in MON 87769 is nearly identical to the native protein produced by *Primula juliae*. The protein consists of 446 amino acids with a calculated molecular mass of 50,985 Da and a predicted isoelectric point of 8.8. Analysis of the PjΔ6D amino acid sequence demonstrates three histidine motifs that are characteristic of integral membrane desaturases (Figure VI-1). The active site of integral membrane desaturases consists of three histidine motifs with a total of eight essential histidine residues (Shanklin et al., 1994). The protein is thought to be folded in such a way that the histidine boxes align to form the di-iron binding site at the catalytic center of the desaturases (Los and Murata, 1998). Analysis of PjΔ6D deduced amino acid sequence has shown that, similar to other Δ6 desaturases (Nakamura and Nara, 2004) PjΔ6D contains the amino-terminal cytochrome b5 domain carrying the heme-binding motifs (Figures VI-1 and VI-2). As in other Δ6 desaturases, the first His residue of the third His-box in PjΔ6D is substituted with glutamine (QXXHH instead of HXXHH) and, therefore, is called Q-box. The cytochrome b5 domain and glutamine residue are essential for the activity of Δ6 desaturases because mutations abolish the enzyme activity (Sayanova et al., 2001; Sayanova et al., 2000).

The PjΔ6D protein also contains multiple membrane-spanning regions that are characteristic of the integral membrane desaturase proteins (Figure VI-2) based on the patterns observed from hydropathy plots.

```
  1 MTKTIYITSS ELEKHNKPGD LWISIHGQVY DVSSWAALHP GGIAPLLALA GHDVTDAFLA
 61 YHPPSTSRLL PPFSTNLLLE KHSVSETSSD YRKLLDSFHK MGMFRARGHT AYATFVIMIL
121 MLVSSVTGVL CSENPWVHLV CGAAMGFAWI QCGWIGHDSG HYRIMTDRKW NRFAQILSSN
181 CLQGISIGWW KWNHNAHHIA CNSLEYDPDL QYIPLLVVSP KFFNSLTSRF YDKKLNFDGV
241 SRFLVQYQHW SFYPVMCVAR LNMLAQSFIL LFSRREVANR VQEILGLAVF WLWFPLLLSC
301 LPNWGERIMF LLASYSVTGI QHVQFSLNHF SSDVYVGPPV GNDWFKKQTA GTLNISCPAW
361 MDWFHGGLQF QVEHHLFPRM PRGQFRKISP FVRDLCKKHN LTYNIASFTK ANVLTLETLR
421 NTAIEARDLS NPIPKNMVWE AVKNVG
```

Figure VI-1. Deduced Amino Acid Sequence of PjΔ6D from PV-GMPQ1972
Three histidine-motifs are represented by black shaded blocks while the heme binding motif is shaded in light gray.

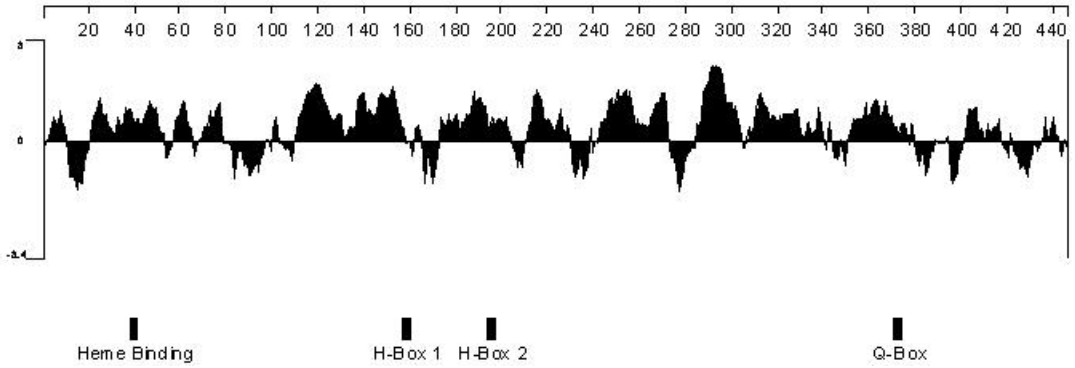


Figure VI-2. Hydropathy Plot of the Deduced Amino Acid Sequence of PjΔ6D
The deduced amino acid sequence was analyzed by the DNAStar-Protean software package. The positive half of the hydropathy plot represents regions that are hydrophobic. Assuming ~20 amino acids are required to span a membrane, there would be at least six transmembrane spanning domains predicted. The heme binding motif, the two histidine rich motifs, and the Q-box are shown below the hydropathy plot. The first ~80 residues comprise the cytb5 domain.

The functional activity of the PjΔ6D protein was confirmed *in vivo* after expression from the yeast expression vector in *S. cerevisiae*. There are only four fatty acids present in *S. cerevisiae* (palmitic acid [16:0], stearic acid [18:0], palmitoleic acid [16:1] and oleic acid [18:1]). *S. cerevisiae* cells containing the *Pj.D6D* expression vector were grown in the absence or presence of exogenous fatty acids. In the absence of the exogenous fatty acids, yeast cells were shown to produce two new fatty acids as, *cis*-6,*cis*-9-hexadecadienoic acid (16:2 c6,9) and *cis*-6,*cis*-9-octadecadienoic acid (18:2 c6,9), which are both the products of Δ6 desaturation of the endogenous monounsaturated fatty acids palmitoleic acid (16:1) and oleic acid (18:1). Cultures grown in the presence of exogenous oleic acid showed even higher levels of *cis*-6,*cis*-9-octadecadienoic acid (18:2 c6,9). Both GLA and SDA were produced when an equal mixture of LA and ALA was included in the medium.

The minimum components required for *in vitro* activity of an integral membrane desaturase would include an intact membrane system containing the desaturase and the

requisite electron transfer proteins. In the case of PjΔ6D, this would include a source of cytb5 and b5 reductase. Additionally, the membranes would need to be comprised of lipids that could serve as an appropriate substrate. Enzymatic activity specific to the PjΔ6D protein was observed in the crude homogenate obtained from very young (stage 0-1, green immature seed, up to 4 mm) fresh MON 87769 seed. The starting seed material was collected on the day of the assay, cotyledons removed, and a homogenate prepared by gently grinding the tissue at 4 °C with a mortar and pestle. When ^{14}C-ALA-CoA was incubated with the immature seed homogenate of MON 87769, ^{14}C-SDA was detected in the assay mixture. The formation of SDA is a unique reaction attributed to the presence of the PjΔ6D protein in MON 87769.

The results obtained from the yeast expression system and crude SDA immature seed extracts demonstrate the Δ6 desaturase function of the PjΔ6D protein encoded by the *Pj.D6D* gene.

VI.A.2. NcΔ15D Protein

The NcΔ15D protein is identical to the native protein produced by *Neurospora crassa*, with the exception of a single amino acid change from threonine to alanine at the first amino acid after the start codon that was introduced to facilitate the insertion of the gene into the plant transformation vector. The MON 87769-expressed NcΔ15D protein consists of 429 amino acids, has a calculated molecular mass of 49,195 Da, and a predicted isoelectric point of 7.1. Analysis of the NcΔ15D amino acid sequence demonstrates the presence of three histidine motifs that are characteristic of integral membrane desaturases (Figure VI-3). The active site of integral membrane desaturases is thought to be comprised of three histidine-rich motifs with a total of nine essential histidine residues (Shanklin et al., 1994). Similar to PjΔ6D, the NcΔ15D proteins shows multiple membrane-spanning regions (Figure VI-4) based on the patterns observed from hydropathy plots.

```
1   MAVTTRSHKA AAATEPEVVS TGVDAVSAAA PSSSSSSSSQ KSAEPIEYPD IKTIRDAIPD
61  HCFRPRVWIS MAYFIRDFAM AFGLGYLAWQ YIPLIASTPL RYGAWALYGY LQGLVCTGIW
121 ILAHECGHGA FSRHTWFNNV MGWIGHSFLL VPYFSWKFSH HRHHRFTGHM EKDMAFVPAT
181 EADRNQRKLA NLYMDKETAE MFEDVPIVQL VKLIAHQLAG WQMYLLFNVS AGKGSKQWET
241 GKGGMGWLRV SHFEPSSAVF RNSEAIYIAL SDLGLMIMGY ILYQAAQVVG WQMVGLLYFQ
301 QYFWVHHWLV AITYLHHTHE EVHHFDADSW TFVKGALATV DRDFGFIGKH LFHNIIDHHV
361 VHHLFPRIPF YYAEEATNSI RPMLGPLYHR DDRSFMGQLW YNFTHCKWVV PDPQVPGALI
421 WAHTVQSTQ
```

Figure VI-3. Deduced Amino Acid Sequence of NcΔ15D from PV GMPQ1972
Three histidine-motifs are represented by shaded blocks.

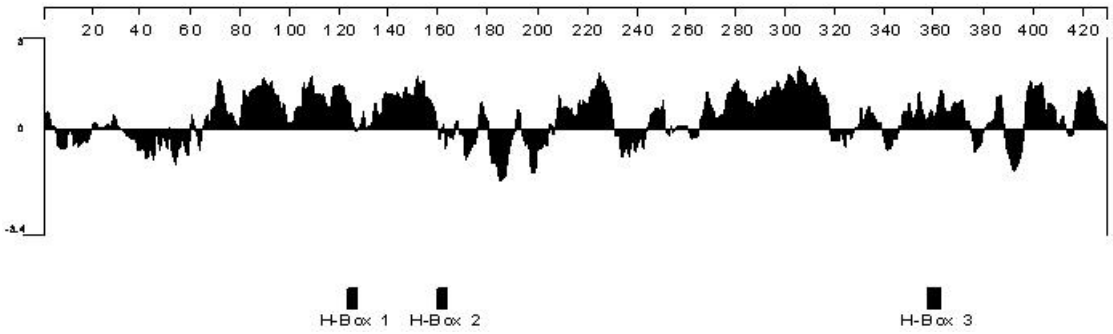


Figure VI-4. Hydropathy Plot of the Deduced Amino Acid Sequence of NcΔ15D
The deduced amino acid sequence was analyzed in the DNAStar-Protean software package. The positive half of the hydropathy plot represents regions that are hydrophobic. Assuming ~20 amino acids are required to span a membrane, there are at least six transmembrane spanning domains predicted. The locations of the three histidine-rich motifs are shown below the hydropathy plot.

The functional activity and specificity of the NcΔ15D protein was confirmed *in vivo* via the expression of the yeast expression vector containing *Nc.Fad3* (NcΔ15D-encoding gene) in *S. cerevisiae* as described for PjΔ6D protein in Section VI.A.1. *S. cerevisiae* was grown in the absence or presence of various 18 and 20 carbon fatty acids. In the absence of the exogenous fatty acids, the yeast cells produced ALA from oleic acid. The Δ15D protein is the only known enzyme capable of catalyzing this reaction. Results also demonstrated that other 18 (e.g., LA and GLA) or 20 carbon fatty acids (e.g., di-homo-γ-linolenic acid and arachidonic acid tested) were substrates for NcΔ15D and all of the exogenous fatty acid substrates were desaturated three carbons from the methyl terminus (omega-3 desaturation).

A demonstration of the functional activity of the NcΔ15D protein in the crude homogenate from immature MON 87769 soybean seed was not successful because the 18:3 fatty acid isomers (ALA and GLA) from the assay were not distinguishable. It should be noted that soybean seed contains an endogenous Δ15 desaturase; therefore, this Δ15 desaturation reaction occurs in the conventional control soybean seed. The fatty acid composition of yeast containing the *Nc.Fad3* vector and MON 87769 demonstrate the omega-3 desaturation function of the NcΔ15D protein encoded by *Nc.Fad3* gene.

VI.B. Characterization of the PjΔ6D and NcΔ15D Proteins Produced in MON 87769

The safety assessment of crops improved through biotechnology includes characterization of the introduced protein(s), confirmation of their functional and physicochemical properties, and confirmation of the safety of the proteins produced from the inserted genes. A common approach used for the safety assessment of the proteins introduced into crop plants by biotechnology is to use heterologously expressed proteins

as surrogates for the plant-expressed proteins. In such cases, the physicochemical and functional properties of the plant-produced and heterologously produced proteins must be established to justify the use of the surrogate protein for safety testing. In the case of the two integral membrane desaturase proteins, PjΔ6D and NcΔ15D, safety assessment studies were conducted with proteins isolated directly from MON 87769 immature seed. Therefore, no equivalence study was required.

Expression of desaturase proteins is higher during seed development, therefore, the level of PjΔ6D and NcΔ15D is higher in immature seeds than mature seeds (Tables VI-1 and VI-2). Purification of integral membrane desaturases is a challenging task that requires removal of membranes and replacement of the lipids surrounding the protein's hydrophobic membrane-spanning regions with an appropriate detergent that will keep the protein in solution (Wiener, 2004). To purify the integral membrane PjΔ6D and NcΔ15D proteins from immature seeds as the source material for safety testing, the membranes were isolated and a panel of detergents was tested for ability to release these proteins from the seed membranes while maintaining their solubility. As a result, the zwitterionic detergent Fos-choline 12 was selected. Fos-choline 12 is a member of a relatively new class of detergents that are phospholipid analogs. These lipid-like detergents have a phosphocholine head group, but in contrast to phospholipids, possess simple hydrophobic tails. Fos-choline 12 has been rapidly adopted for use in NMR studies of membrane proteins due to its structure-stabilizing properties (Choowongkomon et al., 2005; Farès et al., 2006; Oxenoid and Chou, 2005). Furthermore, Fos-choline 12 was an essential tool in allowing for the refolding of the integral membrane protein diacylglycerol kinase (Gorzelle et al., 1999) and had less deleterious effects than other tested detergents on functional activities of some integral membrane proteins (Li et al., 2001; Narayanan et al., 2007). After the PjΔ6D and NcΔ15D proteins were solubilized from the membranes, multiple chromatographic steps were applied to further purify these proteins. Standard precautions to retain the integrity of the protein during purification were undertaken, including conducting all chromatographic steps in a cold room, inclusion of protease inhibitors at critical steps, and the addition of reducing agents and glycerol to buffers. This approach allowed the isolation of these desaturases in soluble form that enabled the characterization of their physico-chemical properties. Appendix C describes the purification and characterization of PjΔ6D and NcΔ15D proteins.

Although the activity of integral membrane desaturases can be demonstrated in crude extracts when the appropriate substrates are supplied, as with other desaturases (Reed et al., 2000), it has not been possible to assay PjΔ6D and NcΔ15D enzymatic activities following their solubilization from the membranes. This most likely is due to the desaturases' requirement for the requisite electron transfer proteins co-localized in the membrane.

To characterize the purified PjΔ6D and NcΔ15D proteins produced in MON 87769, a number of analyses were performed. The analyses employed for the characterization of MON 87769-produced PjΔ6D and NcΔ15D proteins included:

1. N-terminal sequence analysis,

2. Matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry to generate a tryptic peptide map,
3. Immunoblot analysis to establish protein identity through immunoreactivity with PjΔ6D and NcΔ15D-specific antibodies,
4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to assess the apparent molecular weight of the protein, and
5. Glycosylation analysis to evaluate potential post-translational modification of the protein.

Results show that the identity of the MON 87769-produced PjΔ6D and NcΔ15D proteins was confirmed by: 1) their recognition by anti-PjΔ6D and anti-NcΔ15D antibodies, respectively; 2) the identification of the first 15 amino acids of the expected N-terminus sequence of each protein; 3) the identification of tryptic peptide masses of each protein that yielded >40% overall coverage of the expected protein sequence; 4) confirmation of the apparent molecular weights estimated to be 46 kDa; and, 5) confirmation that the proteins are non-glycosylated. Taken together, these data confirm the identity of the PjΔ6D and NcΔ15D proteins isolated from MON 87769. The PjΔ6D and NcΔ15D proteins purified from MON 87769 immature seed were used in the protein safety assessment, which is described in section VI E.

VI.C. Expression of PjΔ6D and NcΔ15D Proteins in MON 87769

PjΔ6D and NcΔ15D are integral membrane proteins, and their detection in plant tissue extracts is a difficult task using a standard ELISA format. When plant tissue is extracted in neutral buffers that do not contain detergent, cell membranes tend to form vesicles of different orientation depending on the charge and hydrophobicity of the surface (Rosenberg, 1996). Hydrophilic portions of the integral membrane proteins are often trapped inside membrane vesicles and not available for interaction with antibody. Due to their membrane localization, the PjΔ6D and NcΔ15D proteins require detergent-containing buffers in order to be effectively solubilized from the membranes and to decrease the potential for aggregation. Because detergent in the extraction buffer interferes with the protein's ability to interact with antibody, extracts containing solubilized PjΔ6D and NcΔ15D proteins are not well suited for analysis in liquid phase assays such as an ELISA, but are compatible with a solid phase assay such as western blot. For western blot (or immunoblot), protein extracts are separated on SDS-PAGE performed in the presence of a denaturing detergent. The subsequent electrotransfer of proteins from the gel to a blotting membrane yields an immunoblot that can be probed in buffer conditions that facilitate specific protein-antibody interactions. For PjΔ6D and NcΔ15D protein detection in MON 87769, optimized tissue extraction conditions were combined with standardized electrophoretic, blotting, and immunodetection methodologies to utilize PjΔ6D and NcΔ15D peptide antibodies specific to a fragment (peptide) of soluble portion of the respective proteins. This method allowed for the reproducible and accurate estimation of the PjΔ6D and NcΔ15D protein levels in plant tissue samples. Densitometric analysis of the PjΔ6D- and NcΔ15D-specific immunoblots yielded the reported quantitative values of PjΔ6D and NcΔ15D proteins by interpolation from standard curves prepared using purified PjΔ6D and NcΔ15D protein standards,

respectively. Limit of quantitation (LOQ) for PjΔ6D and NcΔ15D in each plant tissue type was determined from the corresponding immunoblot and was defined as the lowest amount of the standard that could be reliably determined. Limit of detection (LOD) was defined as the lowest amount of PjΔ6D and NcΔ15D protein standards visually observed on X-ray films. The levels of the PjΔ6D and NcΔ15D proteins in various tissues of MON 87769 that are relevant to the risk assessment were estimated by densitometric analysis of X-ray films exposed to immunoblots probed with PjΔ6D- and NcΔ15D-specific antibodies and visualized using chemiluminescent detection reagents (refer to Appendix D).

VI.C.1. Protein Expression Analysis of Tissues from the 2006 U.S. Field Trials

Tissue samples for analysis were collected from five field locations in the U.S. during the 2006 soybean growing season. The trial locations were in the states of Iowa (two sites), Illinois, Michigan, and Ohio, which represent major soybean-growing regions of the U.S. and provide a range of environmental conditions that would be encountered in the commercial production of soybean. At each site, three replicated plots of MON 87769 and conventional soybean control (A3525) were grown using a randomized complete block field design. Over-season leaf (OSL), forage, root, mature seed, and immature seed were collected from each replicated plot from all field sites. A description of the tissues collected is provided below.

Twenty sets of the youngest expanded trifoliate leaves were randomly collected from plants of each plot at each site. The twenty leaves were combined to form the leaf sample for each plot. OSL samples were collected as follows:

Over-season leaf (OSL)	*Development stage*	*Days after planting (DAP)*
OSL-1	V3-V4	25-37
OSL-2	V6-V8	38-46
OSL-3	V10-V12	46-59
OSL-4	V14-V16	56-73

The above ground portion of six plants was collected from each plot at each site at the R6 stage and combined to form the forage sample. Roots remaining from four to six plants after the collection of the forage samples from each plot were combined to form the root sample. Forage and root samples were collected as follows:

Tissue	*Development stage*	*Days after planting (DAP)*
Forage	R6	81-108
Root	R6	81-108

Immature seed was collected from each plot, with a sufficient number of plants being sampled to yield ~15-56 g of tissue. Mature seed was harvested from all plants of each plot yielding 3.0 to 8.1 lb of mature seed per plot. Immature and mature seed were harvested as follows:

Tissue	*Development stage*	*Days after planting (DAP)*
Immature seed	R5 to early R6	76-96
Mature seed	R8 (95% mature pod color)	120-153

All tissue samples, except mature seed, were stored and shipped on dry ice for processing and analysis. Mature seed was stored and shipped at room temperature. All tissue samples were stored in a -80 ºC freezer upon receipt. Tissue samples were extracted and analyzed using an immunoblot according to applicable standard operating procedures (SOP). Appendix D describes the materials and methods used for the protein expression analysis.

The levels of PjΔ6D and NcΔ15D proteins in OSL-1 through OSL-4, forage, root, immature and mature harvested seed were determined using immunoblot and the results are summarized in Tables VI-1 and VI-2. Protein levels for all tissue types were calculated on a microgram (μg) per gram (g) fresh weight (FW) basis. For tissues displaying measurable quantities of PjΔ6D and NcΔ6D proteins, moisture content also was measured and protein levels were also expressed on a microgram (μg) per gram (g) dry weight of tissue (DW) basis.

The expression of the PjΔ6D and NcΔ15D proteins is driven by a 7Sα' and 7Sα seed specific promoter, respectively. As expected, both PjΔ6D and NcΔ15D proteins were undetectable across all field sites in OSL-1, OSL-2, OSL-3, OSL-4, and root tissues of MON 87769 due to the lack of a measurable signal specific to each protein at or above the tissue-specific LOD of the immmunoblot (Tables VI-1 and VI-2). Both proteins were detected in immature seed, mature seed, and at low levels in forage because this tissue usually contains a small amount of immature seed. The mean PjΔ6D protein levels across all sites for immature seed, mature seed and forage were 100, 1.8, and 16 μg/g DW, respectively (Table VI-1). The mean NcΔ15D protein levels across all sites for immature seed, mature harvested seed and forage were 200, 10, and 14 μg/g DW, respectively (Table VI-2). The PjΔ6D and NcΔ15D proteins were not detected in the conventional soybean control, A3525.

Table VI-1. Summary of PjΔ6D Protein Levels in Tissues Collected from MON 87769 Produced in the 2006 U.S. Growing Season

Tissue Type	PjΔ6D Mean (µg/g FW) (SD)[1]	Range[2] (µg/g FW)	PjΔ6D Mean (µg/g DW) (SD)[3]	Range (µg/g DW)	LOD (µg/g FW)
OSL-1	< LOD	n.a.	n.a.	n.a.	0.2
OSL- 2	< LOD	n.a.	n.a.	n.a.	0.2
OSL -3	< LOD	n.a.	n.a.	n.a.	0.1
OSL- 4	< LOD	n.a.	n.a.	n.a.	0.1
Root	< LOD	n.a.	n.a.	n.a.	0.1
Forage	4.3 (2.4)	1.0-7.4	16 (9.5)	3.6-28	0.1
Immature seed	27 (15)	5.6-45	100 (63)	19-210	0.2
Mature[4] seed	1.7 (0.86)	0.45-3.0	1.8 (0.95)	0.50-3.2	0.4

n.a – not applicable; LOD – Limit of Detection.

[1] Protein quantities are expressed as micrograms (µg) of protein per gram (g) of tissue on a fresh weight (FW) basis. When applicable, an arithmetic mean and a standard deviation (SD) were calculated for each tissue type across all sites (n=15 for all tissues).

[2] When applicable, minimum and maximum values were determined for each tissue type across all sites.

[3] When applicable, protein quantities are expressed as "µg/g" of tissue on a dry weight (DW) basis. The dry weight values were calculated by dividing the fresh weight values by the dry weight conversion factors obtained from moisture analysis data.

[4] For the PjΔ6D levels in mature seed, the low end of range was below the LOQ of the western blot assay, but above the LOD of 0.4 µg/g FW that was determined by serially diluting protein standard to the lowest amount that produced a visible band in the western blot. The reported values were based on the protein signal observed on the western blot and extrapolation of the standard curve.

Limit of Quantitation (LOQ) for the PjΔ6 desaturase western blot are as follows:

Limit of Quantitation (µg/g FW)[1]	
Tissue Type	**PjΔ6D Western Blot**
OSL-1, OSL-2, OSL-3 and OSL-4	1.0
Root	1.0
Forage	1.0
Immature Seed	4.0
Mature Seed	2.0

[1] For each tissue type, the Limit of Quantitation (LOQ) was calculated based on the lowest amount of protein standard visualized and recorded from the western blot in the study.

Table VI-2. Summary of NcΔ15D Protein Levels in Tissues Collected from MON 87769 Produced in the 2006 U.S. Growing Season

Tissue Type	NcΔ15D Mean (µg/g FW) (SD)[1]	Range[2] (µg/g FW)	NcΔ15D Mean (µg/g DW) (SD)[3]	Range (µg/g DW)	LOD (µg/g FW)
OSL-1	< LOD	n.a.	n.a.	n.a.	0.5
OSL-2	< LOD	n.a.	n.a.	n.a.	1.0
OSL-3	< LOD	n.a.	n.a.	n.a.	0.5
OSL-4	< LOD	n.a.	n.a.	n.a.	1.0
Root	< LOD	n.a.	n.a.	n.a.	0.5
Forage	3.7 (1.7)	1.3-7.9	14 (6.8)	4.6-30	1.0
Immature seed	55 (21)	20-85	200 (89)	66-330	4.0
Mature seed	9.5 (5.9)	4.3-23	10 (6.5)	4.8-25	2.0

n.a – not applicable; LOD – Limit of Detection.

[1] Protein quantities are expressed as micrograms (µg) of protein per gram (g) of tissue on a fresh weight (FW) basis. When applicable, an arithmetic mean and a standard deviation (SD) were calculated for each tissue type across all sites (n=15 for all tissues).

[2] When applicable, minimum and maximum values were determined for each tissue type across all sites.

[3] When applicable, protein quantities are expressed as "µg/g" of tissue on a dry weight (DW) basis. The dry weight values were calculated by dividing the fresh weight values by the dry weight conversion factors obtained from moisture analysis data.

Limit of Quantitation (LOQ) for the NcΔ15 desaturase western blot are as follows:

Limit of Quantitation (µg/g FW)[1]

Tissue Type	NcΔ15D Western Blot
OSL-1, OSL-2, OSL-3 and OSL-4	2.0
Root	2.0
Forage	1.0
Immature Seed	10.0
Mature Seed	2.0

[1] For each tissue type, the Limit of Quantitation (LOQ) was calculated based on the lowest amount of protein standard visualized and recorded from the western blot in the study.

VI.D. Assessment of the Potential for Allergenicity of the PjΔ6D and NcΔ15D Proteins Produced in MON 87769

The NcΔ15D and PjΔ6D proteins have been assessed for their potential allergenicity according to the recommendations of the Codex Alimentarius Commission (Codex, 2003). Both proteins are from nonallergenic sources, lack structural similarity to known allergens, are digested in simulated gastric and intestinal fluids, and constitute a small portion of the total protein present in MON 87769 seed.

The PjΔ6D protein is from *Primula juliae*, an organism that is not known to be allergenic. Bioinformatics analyses demonstrated that the PjΔ6D protein does not share structurally or immunologically relevant amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes. Digestive fate experiments conducted with the PjΔ6D protein demonstrated that the full-length protein is rapidly digested in SGF, a characteristic shared among many proteins with a history of safe consumption. The transiently stable protein fragments in the SGF assay were quickly degraded during a short exposure to SIF. Rapid digestion of the full-length PjΔ6D protein in SGF and SIF, together with rapid degradation of the transiently stable fragment from the SGF assay by SIF, indicates that it is highly unlikely that the PjΔ6D protein and its fragment will reach absorptive cells of the intestinal mucosa. Finally, the PjΔ6D protein represents no more than 0.00043% of the total protein in MON 87769 mature seed.

The NcΔ15D protein is derived from *Neurospora crassa*, a fungus that is ubiquitous in the environment, is not allergenic, and found in the digestive tracts of vertebrate species, including humans. Bioinformatics analyses demonstrated that the NcΔ15D protein does not share structurally or immunologically relevant amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes. Digestive fate experiments conducted with the NcΔ15D protein demonstrated that the full-length protein is rapidly digested in SGF, a characteristic shared among many proteins with a history of safe consumption. The transiently stable protein fragments from the SGF assay of NcΔ15D were very quickly degraded during short exposure to SIF. Rapid digestion of the full-length NcΔ15D protein in SGF and SIF, together with rapid degradation of the transiently stable fragment from the SGF assay by SIF, indicates that it is highly unlikely that the NcΔ15D protein and its fragment will reach absorptive cells of the intestinal mucosa. Finally, the NcΔ15D protein represents no more than 0.00239% of the total protein in MON 87769 mature seed.

Taken together, these data lead to the conclusion that the PjΔ6D and NcΔ15D proteins do not pose a significant allergenic risk.

VI.E. Assessment of the Potential for Toxicity of the PjΔ6D and NcΔ15D Proteins

The PjΔ6D and NcΔ15D proteins have been assessed for their potential toxicity. Homologues of NcΔ15D and PjΔ6D are present in all kingdoms, from prokaryotes to

humans. Many organisms that either are consumed as food or used to produce food constituents contain homologous desaturases, supporting the general familiarity of these proteins in human and animal diets. The proteins lack structural similarity to known toxins or biologically active proteins known to have adverse effects to mammals. The PjΔ6D and NcΔ15D proteins are present at a very low level in the harvested seed of MON 87769 and, therefore, constitute a negligible portion of the total protein present in food and feed derived from MON 87769. The PjΔ6D and NcΔ15D proteins were readily digestible in simulated gastric and intestinal fluids.

Acute toxicology studies were conducted with PjΔ6D and NcΔ15D purified from MON 87769 immature seed. The PjΔ6D and NcΔ15D did not cause any adverse effects on mice at a NOAEL of 4.7 mg/kg and 37.3 mg/kg BW, respectively. Human margin of safety was calculated by comparing the NOAEL with acute dietary consumption of soybean. The margin of safety was estimated to be 29,000 for PjΔ6D and 47,000 for NcΔ15D. Estimated dietary intake of the PjΔ6D and NcΔ15D proteins by animals was at extremely low levels compared to the consumption of total proteins present in animal feed. These data indicate that the PjΔ6D and NcΔ15D proteins are safe for human and animal consumption.

VII. COMPOSITIONAL AND NUTRITIONAL ASSESSMENT OF MON 87769

Compositional analyses were conducted to assess whether the nutrients and antinutrient levels in harvested seed and forage derived from MON 87769 are comparable to those in the conventional soybean control, A3525. In addition, 10 commercial conventional soybean varieties were included in the analysis as references to establish a range of natural variability for each analyte, where the range of variability is defined by a 99% tolerance interval for that particular analyte. Compositional analysis included the significant nutrients, antinutrients, and key secondary metabolites, consistent with OECD guidelines (OECD, 2001). As expected, MON 87769 contains SDA and GLA. Due to the known spontaneous isomerization of polyunsaturated fatty acids, small amounts of the trans-isomers of SDA and ALA that are formed during seed extraction and processing are also expected in MON 87769 analysis. Therefore, MON 87769 harvested seed was also analyzed for SDA, GLA, trans-SDA and trans-ALA. Results of the comparisons indicate that except for the intended fatty acid change, MON 87769 is compositionally and nutritionally equivalent to conventional soybean that is currently in commerce and has a history of safe consumption by humans and animals.

Seed and forage of MON 87769 and the conventional soybean control were harvested from soybean grown in three replicated plots at each of five sites across the U.S., during the 2006 growing season. Samples were collected from MON 87769 and the conventional soybean control from all three plots; samples from the three different commercial reference varieties grown at each site were collected from a single plot. Forage was collected at the R6 plant growth stage and soybean seed was collected at physiological maturity. The seed and forage collected from MON 87769, the conventional control, and the reference varieties were analyzed for compositional components. Appendix E describes the materials and methods used for the compositional analysis.

In all, 75 different analytical components were measured, 68 in seed and seven in forage. Of the measured components, 26 in seed had more than 50% of the observations below the assay limit of quantitation (LOQ) and were excluded from the statistical analysis. Thus, statistical analyses were conducted for 49 components (42 in seed and seven in forage). The overall data set was examined for evidence of biologically relevant changes using a mixed model of variance. Six sets of statistical analyses were conducted, five based on the data from each of the replicated field sites (individual-site) and the sixth analysis based on data from a combination of all five field sites (combined-site). The compositional data set was examined for evidence of statistically significant differences between MON 87769 and the conventional soybean control. Statistically significant differences were determined at the 5% level of significance ($p<0.05$) using established statistical methods.

A 99% tolerance interval was calculated for each compositional component, except for the fatty acid components that are intentionally changed. This interval is expected to contain, with 95% confidence, 99% of the values obtained from the population of commercial reference varieties. It is important to establish the 99% tolerance interval from representative conventional soybean varieties for each of the analytes, because such

data illustrate the compositional variability that occurs naturally in commercially grown varieties. By comparison to the 99% tolerance interval, any significant difference ($p<0.05$) between MON 87769 and the conventional control can be assessed for biological relevance in the context of the natural variability in soybean. This comparative evaluation also considers natural ranges in soybean component levels published in the literature or in the International Life Sciences Institute Crop Composition Database (ILSI-CCD, 2008).[2]

VII.A. Intended Changes to Fatty Acid Levels in MON 87769 Soybean Seed

MON 87769 was developed to contain SDA at levels of approximately 20 to 30% of total fatty acids. As expected, composition analysis showed that the levels of SDA in MON 87769 harvested seed ranged from 16.83 to 33.92% of total fatty acids, with a mean of 26.13% (Table VII-1). Associated with the expected levels of SDA in MON 87769 is the production of GLA from the Δ6 desaturation of linoleic acid (LA) by PjΔ6D (Figure VII-1). The GLA levels in MON 87769 ranged from 6.07 to 8.03% of total fatty acids, with a mean of 7.09%. Lower levels of two other fatty acids, trans-SDA (mean = 0.18%, range = 0.058 - 0.26% of total fatty acids) and trans-ALA (mean = 0.44%, range = 0.38 - 0.48% of total fatty acids) also were observed (Table VII-1). The formation of trans-ALA and trans-SDA is due to the known spontaneous trans-isomerization of unsaturated fatty acids during seed extraction and processing. Trans isomerization of unsaturated fatty acids are known to increase with increasing degree of unsaturation (Chardigny et al., 1996). As SDA and ALA represent a significant proportion of total fatty acids in MON 87769 (approximately 35 - 40% in total), trans-ALA and trans-SDA are expected to be seen in the fatty acid analysis of MON 87769.

Since SDA, GLA, and the two trans-isomer analytes were not detected in the conventional soybean control seed, statistical comparisons between MON 87769 and the conventional soybean control were not possible for these analytes. The mean values, standard errors, and the range of values for the introduced fatty acids observed in MON 87769 are presented as % total fatty acids and % dry weight in Table VII-1.

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[2] ILSI-CCD. 2008. International Life Sciences Institute Crop Composition Database. Version 3.0 http://www.cropcomposition.org. Search criteria soybean seed, all locations, all years, all proximates, amino acids, fatty acids, bio-actives, fiber, dry weight other than moisture [Accessed June 3, 2008].

Table VII-1. Combined-Site Summary of SDA, GLA, trans-SDA and trans-ALA Levels in MON 87769 Seed

Analytical Component (Units)[1]	MON 87769 Mean (S.E.)	MON 87769 (Range)
Combined-Site Seed Fatty Acid (% Total FA)		
18:4 Stearidonic (SDA)	26.13 (1.64)	[16.83 - 33.92]
18:3 γ-Linolenic (GLA)	7.09 (0.19)	[6.07 – 8.03]
18:4 6c,9c,12c,15t (trans-SDA)	0.18 (0.019)	[0.058 - 0.26]
18:3 9c,12c,15t (trans-ALA)	0.44 (0.0091)	[0.38 – 0.48]
Combined-Site Seed Fatty Acid (% DW)		
18:4 Stearidonic (SDA)	3.94 (0.15)	[2.77 - 4.91]
18:3 γ-Linolenic (GLA)	1.09 (0.023)	[0.93 - 1.22]
18:4 6c,9c,12c,15t (trans-SDA)	0.027 (0.0023)	[0.011 - 0.036]
18:3 9c,12c,15t (trans-ALA)	0.068 (0.0018)	[0.055 - 0.081]

[1]DW = dry weight; S.E. = standard error.

VII.B. Compositional Comparisons of Soybean Seed and Forage from MON 87769 and the Conventional Control

Of the 42 comparisons made in the combined-site analysis between harvested seed from MON 87769 and the conventional soybean control, a total of 28 statistically significant differences, representing six fatty acids and 22 non-fatty acid analytes, were observed. In forage, no significant differences were observed for any analyte for the seven comparisons made between MON 87769 and the conventional soybean control in the combined-site analysis. A summary of the significant differences ($p<0.05$) from the combined-site statistical evaluation of MON 87769 versus conventional soybean control can be found in Table VII-2. Of the 28 detected differences in seed, 27 (except LA) were small in magnitude and/or their mean component values were all within the 99% tolerance interval for the population of conventional references grown at the same time and location as MON 87769 and also within the range of values found in the published literature and ILSI-CCD values. Therefore, these differences were not considered to be biologically meaningful from a food/feed safety and/or nutritional perspective. The LA levels in seed were lower for MON 87769 in the combined-site analysis and outside of

the 99% tolerance interval for the population of conventional references. These results were expected, because LA is the starting material from which SDA and GLA are produced and, therefore, its levels were expected to be significantly different in MON 87769 seed compared to conventional soybean seed.

Further assessment of the statistically significant differences observed between MON 87769 and the conventional soybean control are discussed in the sections below. The analytes that are significantly different between MON 87769 and the conventional soybean control in the combined-site analysis are presented in Table VII-2. The statistical summaries of the combined-site analysis data, individual-site analysis data, and reported literature and ILSI-CCD ranges for the analytical components present in the harvested seed are included in Appendix E.

VII.B.1. Fatty Acid Levels in Soybean Seed

Statistical comparisons of fatty acids between MON 87769 and the conventional soybean control seed could be made only for the eight fatty acids present in both MON 87769 and the conventional soybean control. Out of the eight fatty acids compared, significant differences ($p<0.05$) were observed for six fatty acids in the combined-site and in more than one individual-site analysis (Table VII-2). In MON 87769 seed, oleic acid, LA, and ALA were found to be significantly different from the conventional soybean control in the combined-site and in the five individual-site analyses. Since oleic acid, LA, and ALA are directly involved in the pathway to SDA (Figure VII-1), their concentrations are interdependent with that of other fatty acids and, therefore, were expected to be different in MON 87769 seed. Arachidic acid was found to be significantly different from the control in the combined-site analysis and in four of the five individual-site analyses. Palmitic acid and behenic acid values were significantly different in the combined-site analysis and in two individual-site analyses. Given the intended shift in the fatty acid metabolism toward an increase in SDA content in MON 87769, differences in fatty acid levels were expected. Except for LA, the differences in fatty acid levels were relatively small in absolute magnitude (<4 wt% of total fatty acids) and/or their mean values and ranges in MON 87769 seed were within the 99% tolerance interval for the population of the conventional reference varieties. Therefore the differences in fatty acid levels with the exception of the intended change are not considered to be biologically meaningful from a food/feed safety and/or nutritional perspective. The levels of LA in MON 87769 were significantly lower than the control in the combined-site as well as in the five individual-site analyses. Since LA is the starting material from which SDA and GLA are produced (Figure VII-1), LA levels were expected to be significantly lower across sites.

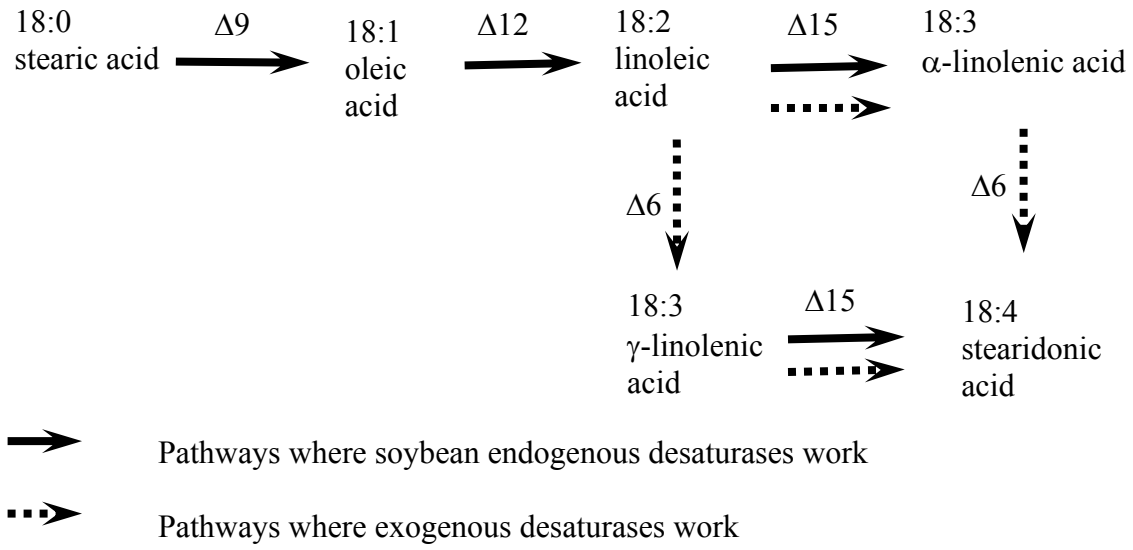


Figure VII-1. Fatty acid biosynthesis in plants and the introduced changes to produce MON 87769

VII.B.2. Nutrient Levels Other than Fatty Acids in Soybean Seed

Soybean seed also was analyzed for the following 26 nutrients: proximate (5), ADF, NDF, amino acids (18), and vitamin E. Nineteen nutrient analytes in the seed were statistically different ($p<0.05$) between MON 87769 and the conventional control in the combined-site analysis. For five of these 19 nutrient analytes (proline, arginine, cystine, glycine, and phenylalanine), statistically significant differences were observed in the combined-site analysis and in more than one individual-site analyses (Table VII-2). Proline values in MON 87769 were found to be significantly different from the conventional soybean control in the combined-site analysis and in three of the five individual-site analyses. The magnitude of the differences in proline between MON 87769 and the conventional control from the combined-site and individual-site analyses were very small, ranging from 2.94 to 5.77%. Statistically significant differences for arginine, cystine, glycine, and phenylalanine were observed in the combined-site and in two individual-site analyses. The magnitude of the differences between MON 87769 and conventional soybean control for arginine, cystine, glycine, and phenylalanine also were very small (≤ 10%). The mean and range values for proline, arginine, cystine, glycine, and phenylalanine in MON 87769 seed were within the 99% tolerance interval for the population of the conventional reference varieties.

Eight of the 19 nutrient analytes that were significantly different between MON 87769 and conventional control (aspartic acid, glutamic acid, histidine, isoleucine, leucine, lysine, valine and protein) were observed in the combined-site analysis and in one individual-site analysis. The magnitude of these differences were very small (<10%).

For the remaining six of 19 nutrient analytes, significant differences only were found in the combined-site analysis (alanine, methionine, serine, threonine, tyrosine and carbohydrates). The magnitude of these differences also were very small (<10%). The mean and range of values for all 19 nutrient analytes that were significantly different between MON 87769 and conventional control in the combined-site analysis and in the individual-site analyses were within the 99% tolerance interval for the population of conventional references and within the range of values found in the published literature and/or the ILSI-CCD (Tables VII-2 and E-14). Therefore, these differences were not considered to be biologically meaningful from a food/feed safety and/or nutritional perspective.

VII.B.3. Naturally Occurring Antinutrient Levels in Soybean Seed

Soybean seed contains several well-described antinutritional factors according to OECD (2001), which include: trypsin inhibitors, lectins, isoflavones (genistein, daidzein and glycitein), stachyose, raffinose, and phytic acid. The analytes that are significantly different between MON 87769 and the conventional soybean control are presented in Table VII-2.

Trypsin inhibitors are heat-labile antinutrients that interfere with the digestion of proteins and result in decreased animal growth (Liener, 1994). Lectins are also heat labile, and lectins can inhibit growth and cause death in animals if raw soybean is consumed (Liener, 1994). Both trypsin inhibitors and lectins are inactivated during processing of soybean protein products or soybean meal, and if processed appropriately, the final edible soybean fractions contain minimal levels of these antinutrients. No statistical differences were observed for all comparisons of trypsin inhibitors and lectin levels between MON 87769 and conventional control.

There are three basic categories of isoflavones in soybean seed; namely, daidzein, genistein, and glycitein. Although they have been reported to possess biochemical activities, including estrogenic, anti-estrogenic, and hypocholesterolemic effects, it is not universally accepted that the isoflavones are antinutrients as they have also been reported to have beneficial anti-carcinogenic effects (OECD, 2001). Genistein and daidzein levels in MON 87769 were found to be significantly different ($p>0.05$) from the conventional soybean control in the combined-site analysis and in four of the five individual-site analyses. The mean and range of values for these two isoflavones for MON 87769 in the combined-site analysis and in each of four individual-site analyses were lower than the values observed in the conventional soybean control (Table VII-2). Glycitein levels in MON 87669 were found to be significantly lower from the conventional soybean control in the combined-site and in one individual-site analysis. It is well documented that the soybean isoflavone levels are greatly influenced by many factors, ranging from environmental conditions, variety, and agronomic practices (Messina, 2001; Nelson et al., 2001; OECD, 2001). Furthermore, the significant differences are not biologically meaningful since the mean levels of genistein and daidzein in MON 87769 are well within the 99% tolerance intervals established from conventional soybean varieties, and within the literature and ILSI-CCD (Tables VII-2 and E-14). Therefore, these differences

do not raise any nutritional, antinutritional or other biological or toxicological concerns and are not considered biologically relevant.

Stachyose and raffinose are low molecular weight carbohydrates present in soybean seed that are considered to be antinutrients due to the gas production and resulting flatulence caused by consumption. There were no significant differences ($p>0.05$) observed for stachyose and raffinose between MON 87769 and the conventional soybean control (Table VII-2).

Phytic acid is present in soybean seed, where it chelates mineral nutrients, including calcium, magnesium, potassium, iron, and zinc, rendering them biologically unavailable to monogastric animals consuming the seed (Liener, 2000). Unlike trypsin inhibitors, phytic acid is not heat labile, and remains stable through most soybean processing steps. No significant differences ($p>0.05$) in phytic acid levels were observed when MON 87769 was compared to the conventional soybean control (Table VII-2).

Based on the data and information presented above, it is reasonable to conclude that MON 87769 is compositionally equivalent to conventional soybean with regard to the antinutrients in soybean seed.

VII.B.4. Proximate and Fiber Levels in Soybean Forage

Combined-site analysis of forage showed no significant differences ($p>0.05$) between MON 87769 and the conventional soybean control. In one of the five individual-site analyses, total fat and ADF were significantly different ($p<0.05$) between MON 87769 and the conventional control forage. All mean and range of values obtained from MON 87769 in the five individual-site analyses were within the calculated 99% tolerance interval for the population of conventional references grown at the same time and locations, therefore, the two differences found in one individual-site analysis were not considered to be biologically meaningful from a food/feed safety and/or nutritional perspective.

VII.B.5. Compositional Equivalence of MON 87769 to Conventional Soybean

The compositional data presented in Section VII.B establishes that MON 87769 seed is compositionally equivalent to conventional soybean seed, with the exception of the intended change in fatty acid composition. As expected, MON 87769 contains SDA and GLA. Due to the known spontaneous isomerization of polyunsaturated fatty acids during the seed extraction and processing, small amounts of the trans-isomers of SDA and ALA are also seen in MON 87769 compostion analysis. In addition, the forage from MON 87769 is compositionally equivalent to conventional soybean forage.

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A3525)	Significance (p-Value)		
Statistical Differences Observed in Combined-Site Analysis						
Seed Amino Acid (% DW)						
Alanine	1.78	1.74	2.51	0.001	[1.76 - 1.84]	[1.45, 2.02]
Arginine	3.23	2.95	9.35	<0.001	[3.00 - 3.61]	[2.13, 3.62]
Aspartic Acid	4.54	4.36	4.04	0.007	[4.41 - 4.73]	[3.45, 5.29]
Cystine	0.62	0.6	3.23	<0.001	[0.56 - 0.65]	[0.49, 0.68]
Glutamic Acid	7.63	7.29	4.7	<0.001	[7.42 - 7.90]	[5.51, 9.04]
Glycine	1.79	1.73	3.6	0.003	[1.76 - 1.87]	[1.39, 2.05]
Histidine	1.09	1.05	3.42	<0.001	[1.06 - 1.14]	[0.86, 1.27]
Isoleucine	1.87	1.78	4.95	<0.001	[1.75 - 1.97]	[1.34, 2.28]
Leucine	3.19	3.09	3.28	<0.001	[3.13 - 3.32]	[2.45, 3.76]
Lysine	2.67	2.6	2.69	<0.001	[2.61 - 2.75]	[2.13, 3.06]
Methionine	0.6	0.58	2.99	0.038	[0.54 - 0.62]	[0.48, 0.66]
Phenylalanine	2.14	2.06	3.63	0.002	[2.08 - 2.24]	[1.61, 2.55]
Proline	2.09	1.99	5.13	<0.001	[2.03 - 2.19]	[1.53, 2.45]
Serine	2.2	2.14	2.55	0.043	[2.08 - 2.25]	[1.75, 2.51]

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties (Continued)

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A3525)	Significance (p-Value)		
Seed Amino Acid (% DW)						
Threonine	1.6	1.57	1.9	0.035	[1.54 - 1.65]	[1.30, 1.82]
Tyrosine	1.4	1.34	4.43	0.013	[1.27 - 1.50]	[1.03, 1.67]
Valine	1.98	1.88	5.08	<0.001	[1.84 - 2.08]	[1.42, 2.41]
Seed Fatty Acid (% Total FA)						
16:0 Palmitic	12.06	11.77	2.5	<0.001	[11.53 - 12.54]	[7.28, 14.20]
18:1 Oleic	15.18	19.19	-20.92	0.001	[12.66 - 18.80]	[12.56, 27.98]
18:2 Linoleic	22.78	54.93	-58.53	<0.001	[16.46 - 30.81]	[50.46, 59.96]
18:3 Linolenic	11.18	9.2	21.51	0.016	[10.20 - 11.80]	[3.72, 13.46]
20:0 Arachidic	0.34	0.31	9.88	<0.001	[0.31 - 0.37]	[0.20, 0.45]
22:0 Behenic	0.29	0.32	-8.3	0.023	[0.26 - 0.31]	[0.22, 0.49]
Seed Proximate (%DW)						
Carbohydrates	36.45	38.68	-5.78	<0.001	[33.23 - 39.93]	[26.76, 45.99]
Protein	41.92	39.75	5.47	<0.001	[40.92 - 43.36]	[33.37, 46.00]
Seed Isoflavone (µg/g DW)						
Daidzein	1187.81	1807.36	-34.28	0.006	[957.23 - 1838.91]	[0, 2594.50]
Genistein	733.64	1136.52	-35.45	0.007	[576.70 - 1118.40]	[254.31, 1976.30]

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties (Continued)

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control) Mean Difference (% of A3525)	Mean Difference (Test minus Control) Significance (p-Value)	Test Range	Commercial Tolerance Interval[2]
Glycitein (µg/g DW)	82.73	102.18	-19.04	0.004	[65.37 - 106.72]	[0, 243.40]
Statistical Differences Observed in More than One Individual Site						
Seed Amino Acid (% DW)						
Arginine Site IA-2	3.42	3.11	10.03	0.003	[3.36 - 3.50]	[2.13, 3.62]
Arginine Site OH	3.11	2.86	8.67	0.012	[3.07 - 3.15]	
Cystine Site IA-2	0.62	0.6	4.05	0.005	[0.62 - 0.63]	[0.49, 0.68]
Cystine Site IL	0.63	0.61	3.21	0.01	[0.62 - 0.64]	
Glycine Site IA-1	1.8	1.72	4.38	0.046	[1.79 - 1.80]	[1.39, 2.05]
Glycine Site OH	1.76	1.71	2.87	0.024	[1.76 - 1.77]	
Phenylalanine Site IA-1	2.13	2.03	5.18	0.024	[2.08 - 2.16]	[1.61, 2.55]
Phenylalanine Site OH	2.12	2.05	3.69	0.015	[2.12 - 2.13]	
Proline Site IA-1	2.12	2	5.77	0.039	[2.11 - 2.13]	[1.53, 2.45]
Proline Site IA-2	2.12	2.06	2.94	0.014	[2.09 - 2.15]	
Proline Site OH	2.05	1.95	4.98	0.011	[2.03 - 2.06]	
Seed Fatty Acid (% Total FA)	12.31	12	2.51	0.024	[12.24 - 12.39]	
16:0 Palmitic Site IL	12.31	12	2,51	0.024	[12.24 – 12.39]	[7.28 – 14.20]
16:0 Palmitic Site MI	12.11	11.79	2.66	0.019	[12.03 - 12.19]	
18:1 Oleic Site IA-1	13.42	18.45	-27.29	0.001	[13.14 - 13.80]	[12.56, 27.98]

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties (Continued)

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control) Mean Difference (% of A3525)	Mean Difference (Test minus Control) Significance (p-Value)	Test Range	Commercial Tolerance Interval[2]
18:1 Oleic Site IA-2	13.56	18.53	-26.84	0.003	[12.93 - 14.36]	
18:1 Oleic Site IL	17.89	20.89	-14.35	0.01	[17.52 - 18.18]	
18:1 Oleic Site MI	12.92	17.44	-25.95	<0.001	[12.66 - 13.16]	
18:1 Oleic Site OH	18.1	20.65	-12.31	0.046	[16.73 - 18.80]	
18:2 Linoleic Site IA-1	18.46	54.9	-66.39	<0.001	[18.24 - 18.68]	[50.46, 59.96]
18:2 Linoleic Site IA-2	21.19	55.33	-61.7	<0.001	[20.36 - 22.78]	
18:2 Linoleic Site IL	30.48	54.33	-43.9	<0.001	[30.26 - 30.81]	
18:2 Linoleic Site MI	18.4	55.6	-66.92	<0.001	[16.46 - 19.58]	
18:2 Linoleic Site OH	25.37	54.5	-53.45	<0.001	[25.06 - 25.75]	
18:3 Linolenic Site IA-1	11.11	9.85	12.85	<0.001	[11.08 - 11.13]	[3.72, 13.46]
18:3 Linolenic Site IA-2	11.14	9.96	11.84	0.018	[11.10 - 11.18]	
18:3 Linolenic Site IL	10.27	7.59	35.25	0.002	[10.20 - 10.38]	
18:3 Linolenic Site MI	11.76	10.59	10.99	0.001	[11.72 - 11.80]	
18:3 Linolenic Site OH	11.63	8.02	45.05	0.001	[11.41 - 11.75]	
20:0 Arachidic Site IA-1	0.33	0.3	10.66	0.005	[0.32 - 0.34]	[0.20, 0.45]
20:0 Arachidic Site IL	0.36	0.34	8.18	0.025	[0.36 - 0.37]	

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties (Continued)

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control) Mean Difference (% of A3525)	Mean Difference (Test minus Control) Significance (p-Value)	Test Range	Commercial Tolerance Interval[2]
20:0 Arachidic Site MI	0.32	0.29	10.39	0.029	[0.31 - 0.32]	
20:0 Arachidic Site OH	0.36	0.33	8.57	0.011	[0.35 - 0.36]	
22:0 Behenic Site IA-1	0.29	0.31	-8.9	0.018	[0.26 - 0.31]	[0.22, 0.49]
22:0 Behenic Site IL	0.31	0.33	-6.71	0.046	[0.30 - 0.31]	
Seed Isoflavone (µg/g DW)						
Daidzein Site IA-1	995.39	1550.96	-35.82	0.001	[978.37 - 1026.28]	[0, 2594.50]
Daidzein Site IA-2	1076.04	1583	-32.03	0.002	[999.02 - 1130.31]	
Daidzein Site MI	1662.22	2750.13	-39.56	0.016	[1389.19 - 1838.91]	
Daidzein Site OH	1125.54	1668.07	-32.52	0.002	[1094.38 - 1183.11]	
Genistein Site IA-1	594.53	973.04	-38.9	<0.001	[584.75 - 612.91]	[254.31, 1976.30]
Genistein Site IA-2	656.8	1044.68	-37.13	0.001	[612.27 - 687.55]	
Genistein Site MI	1000.9	1683.74	-40.55	0.01	[841.05 - 1118.40]	
Genistein Site OH	760.07	1143.19	-33.51	0.003	[750.00 - 773.91]	
Seed Amino Acid (% DW)						
Aspartic Acid Site OH	4.43	4.27	3.72	0.021	[4.41 - 4.47]	[3.45, 5.29]
Glutamic Acid Site OH	7.51	7.17	4.67	0.02	[7.42 - 7.59]	[5.51, 9.04]
Histidine Site OH	1.07	1.03	3.2	0.003	[1.06 - 1.07]	[0.86, 1.27]

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties (Continued)

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control) Mean Difference (% of A3525)	Mean Difference (Test minus Control) Significance (p-Value)	Test Range	Commercial Tolerance Interval[2]
Isoleucine Site OH	1.87	1.75	6.53	0.025	[1.84 - 1.90]	[1.34, 2.28]
Leucine Site OH	3.16	3.05	3.42	0.016	[3.14 - 3.18]	[2.45, 3.76]
Lysine Site OH	2.62	2.56	2.46	0.02	[2.61 - 2.63]	[2.13, 3.06]
Valine Site OH	1.96	1.84	6.62	0.027	[1.94 - 2.01]	[1.42, 2.41]
Seed Fiber (% DW)						
Acid Detergent Fiber Site IL	16.16	17.76	-9	0.005	[15.91 - 16.61]	[10.36, 22.77]
Neutral Detergent Fiber Site IL	17.06	17.87	-4.52	0.043	[16.72 - 17.25]	[10.91, 22.59]
Seed Proximate (% DW)						
Ash Site OH	5.83	5.71	2.12	0.022	[5.78 - 5.93]	[5.16, 6.64]
Protein Site OH	41.37	39.54	4.63	0.008	[40.92 - 41.70]	[33.37, 46.00]
Seed Antinutrient (% DW, unless noted)						
Lectin (H.U./mg DW) Site MI	1.49	3.44	-56.79	0.019	[0.55 - 2.14]	[0, 16.00]
Phytic Acid Site IA-1	1.28	1.17	9.49	0.041	[1.22 - 1.34]	[0.51, 1.59]
Seed Isoflavone (µg/g DW)						
Glycitein Site IL	84.86	120.49	-29.57	0.044	[83.23 - 87.74]	[0, 243.40]

Table VII-2. Summary of the Soybean Seed and Forage Analyte Differences (p<0.05) in the Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) and Commercial Reference Varieties (Continued)

Component (Units)[1]	MON 87769 Mean	A3525 Mean	Mean Difference (Test minus Control) Mean Difference (% of A3525)	Mean Difference (Test minus Control) Significance (p-Value)	Test Range	Commercial Tolerance Interval[2]
Forage - Statistical Differences Observed in One Site						
Forage Fiber (% DW)						
Acid Detergent Fiber Site OH	32.29	28.31	14.09	0.009	[30.74 - 33.95]	[19.24, 38.36]
Forage Proximate (% DW)						
Total Fat Site IA-2	4.87	6.27	-22.39	0.037	[4.40 - 5.28]	[1.46, 9.88]

[1]DW = dry weight; FA = fatty acid.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

VII.C. Compositional Comparisons of Processed Fractions from Soybean Seed of MON 87769 and the Conventional Control

For preparing soybean processed fractions, seed samples were collected from a field trial conducted with MON 87769, the conventional soybean control, and eight conventional varieties at two field sites (Monmouth, IL and Carlyle, IL) in the U.S. during the 2006 growing season. The seed samples were processed into defatted toasted soybean meal (DT soybean meal); refined, bleached, and deodorized soybean oil (RBD oil); protein isolate; and crude lecithin fractions. The processed fractions were analyzed according to the principles outlined in the OECD consensus document for soybean composition (OECD, 2001). The DT soybean meal was analyzed for proximates (moisture, protein, fat, and ash), ADF, NDF, amino acids, trypsin inhibitors and phytic acid. The RBD oil was analyzed for fatty acids and vitamin E (α-tocopherol). The protein isolate fraction was analyzed for amino acids and moisture. The crude lecithin fraction was analyzed for phosphatides (α-phosphatidic acid, α-phosphatidyl-choline, α-phosphatidyl-ethanolamine, and α-phosphatidylinositiol). The methods used for composition analysis of the processed fractions are summarized in Appendix E, Section E.6. The statistical analysis compared MON 87769 and the conventional control across the two sites (combined-site). Statistical summary of the composition of each processed fraction and summary of the significant differences observed between the processed fractions prepared from the seed of MON 87769 and the conventional control are included in Appendix E, Section E.6.

As expected, apart from the intended fatty acid changes, the composition of the soybean processed fractions from MON 87769 is equivalent to the composition of conventional soybean control (OECD, 2001). Thus, the processed fractions from MON 87769 are concluded to be as safe and nutritious as the processed fractions prepared from conventional soybean from a food/feed perspective.

Collectively, these compositional data from processed fractions support the conclusion that MON 87769, with the exception of the expected changes in fatty acid composition, does not have biologically meaningful differences from conventional soybean from a food/feed safety and/or nutritional perspective.

VII.D. Safety and Nutritional Assessment of the Intended Changes in MON 87769

Beside the components typically present in soybean, MON 87769 is expected to contain SDA, GLA, and small amounts of trans-ALA and trans-SDA resulting from the intended change. As expected, the intended change also decreased the levels of LA in MON 87769. The safety of SDA soybean oil is supported by the results of a published 90-day/one-generation reproductive rat toxicity study in which a no observable adverse effect level (NOAEL) of 1 g SDA/kg body weight/day (4 g SDA soybean oil/kg body weight/day) was determined (Hammond et al., 2008). In addition to this, many published studies on SDA and GLA from other sources corroborate the safety of SDA soybean oil. A discussion of the safety and nutritional impact resulting from the intended fatty acid change in MON 87769 is included in Appendix F.

VII.E. Safety and Nutrition Assessment Conclusion

In conclusion, having demonstrated: (a) the compositional equivalence of MON 87769 seed (except for the intended fatty acid change) and forage to seed and forage from conventional soybean already on the market, (b) the safety of the expected fatty acids resulting from the intended change, (c) the history of safe use of the introduced proteins, and (d) familiarity of the host organism from which the genes are derived, MON 87769 is as safe and nutritious as conventional soybean for food and feed use with the added nutritional improvement of SDA, an omega-3 fatty acid.

VIII. PHENOTYPIC, AGRONOMIC, AND ENVIRONMENTAL INTERACTIONS ASSESSMENT

This section provides an evaluation of the phenotypic and agronomic characteristics, and the environmental interactions of MON 87769 compared to the control, A3525. The A3525 control is a conventional soybean variety that has a genetic background similar to MON 87769 but does not contain the *Pj.D6D* or *Nc.Fad3* genes.

These data support a determination that MON 87769 is no more likely to pose a plant pest risk or to have a significant environmental impact compared to conventional soybean. The conclusions are based on the results of the multiple evaluations reported herein.

Phenotypic and agronomic characteristics of MON 87769 were evaluated in a comparative manner to assess plant pest potential (OECD, 1993). These assessments included 14 plant growth and development characteristics, five seed germination parameters, two pollen characteristics, six plant-symbiont interaction characteristics, and observations for plant-insect and plant-disease interactions and plant responses to abiotic stressors. Results from the phenotypic and agronomic assessments indicate that MON 87769 neither possesses characteristics that would increase plant pest potential, nor would it have a significant environmental impact compared to conventional soybean. Data on environmental interactions also indicate that MON 87769 does not confer any increased susceptibility or tolerance to specific diseases, insects, or abiotic stressors.

VIII.A. Characteristics Measured for Assessment

In the phenotypic, agronomic, and environmental interactions assessment of MON 87769, data were collected to evaluate specific aspects of altered plant pest potential based on requirements of USDA-APHIS set forth at 7 CFR § 340.6. The MON 87769 plant characterization encompasses six general data categories: 1) germination, dormancy, and emergence; 2) vegetative growth; 3) reproductive growth (including pollen characteristics); 4) seed retention on the plant and lodging; 5) plant-symbiont associations; and 6) plant interactions with insect, disease, and abiotic stressors. An overview of the characteristics assessed is presented in Table VIII-1.

The phenotypic, agronomic, and environmental interactions data were evaluated on the basis of familiarity (OECD, 1993) and were comprised of a combination of field, greenhouse, and laboratory studies conducted by scientists who are familiar with the production and evaluation of soybean. In each of these assessments, MON 87769 was compared to an appropriate conventional control, A3525, which had a genetic background similar to MON 87769 but did not possess the genes introduced in MON 87769 (*Pj.D6D* and *Nc.Fad3)*, the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change. In addition, multiple commercial soybean varieties (see Appendix G and Tables G-1 and G-2) were included to provide a range of values that are representative of commercial soybean varieties for each measured phenotypic, agronomic, and environmental interaction characteristic.

Data collected from the commercial reference varieties reflect a range of selection and breeding for desirable characteristics and therefore can provide context for interpreting experimental results.

Table VIII-1. Phenotypic, Agronomic and Environmental Interaction Characteristics Evaluated in U.S. Field Trials

Characteristics	Characteristics measured	Evaluation timing[1]	Evaluation description (measurement endpoints)
Plant phenotypic and agronomic characteristics	Dormancy, germination	5, 8, and 13 days after planting	Percent normally germinated, abnormally germinated, viable hard (dormant), dead, and viable firm-swollen seed
	Seedling vigor	V2–V4	Rated on a 1-9 scale, where 1-3 = excellent, 4-6 = average, and 7-9 = poor vigor
	Early stand count	V2–V4	Number of emerged plants per plot, standardized to 20 ft rows
	Growth stage assessment[1]	Every 2-3 weeks; V2–R8	Average soybean plant growth stage per plot
	Days to 50% flowering	Flowering; R1–R2	Calendar day number when approx. 50% of the plants in each plot were flowering
	Pollen viability	Flowering; R1–R2	Viable and nonviable pollen based on pollen grain staining characteristics
	Pollen morphology	Flowering; R1–R2	Diameter of viable pollen grains
	Flower color	Flowering; R2	Color of flowers: purple, white, or mixed
	Plant pubescence	Maturity; R8	Pubescence on plants in each plot categorized as hairy, hairless, or mixed
	Plant height	Maturity; R8	Distance from the soil surface to the uppermost node on the main stem of five representative plants per plot
	Lodging	Maturity; R8	Rated on 0-9 scale, where 0 = completely erect and 9 = completely flat or lodged
	Pod shattering	Maturity; R8	Rated on 0-9 scale, where 0 = no shattering and 9 = completely shattered
	Final stand count	Maturity; R8	Number of plants per plot, standardized to 20 ft rows
	Seed moisture	Harvest	Percent moisture content of harvested seed
	100 seed weight (g)	Harvest	Weight of 100 harvested seed
	Test weight (lb/bu)	Harvest	Weight of a bushel of harvested seed
	Yield (bu/ac)	Harvest	Bushels of harvested seed produced per acre, adjusted to 13% moisture
Plant environmental interactions	Plant response to abiotic stressors, disease damage, and arthropod damage	Variable, from planting to harvest	Each plot rated using a continuous 0-9 or 0-5 rating scale of increasing symptomology
	Arthropod abundance	R1 – R2 R3 – R5 R6 – R8	Number of specific pest and beneficial arthropods collected per plot using beat sheet sampling method
	Plant × symbiont relationship	4 and/or 6 weeks after emergence in greenhouse	Nodule number and dry weight, root and shoot dry weight, shoot total nitrogen

[1] Soybean plant growth stages were determined using descriptions and guidelines outlined in Soybean Growth and Development (Iowa State University, 2004).

VIII.B. Interpretation of Phenotypic and Environmental Interaction Data

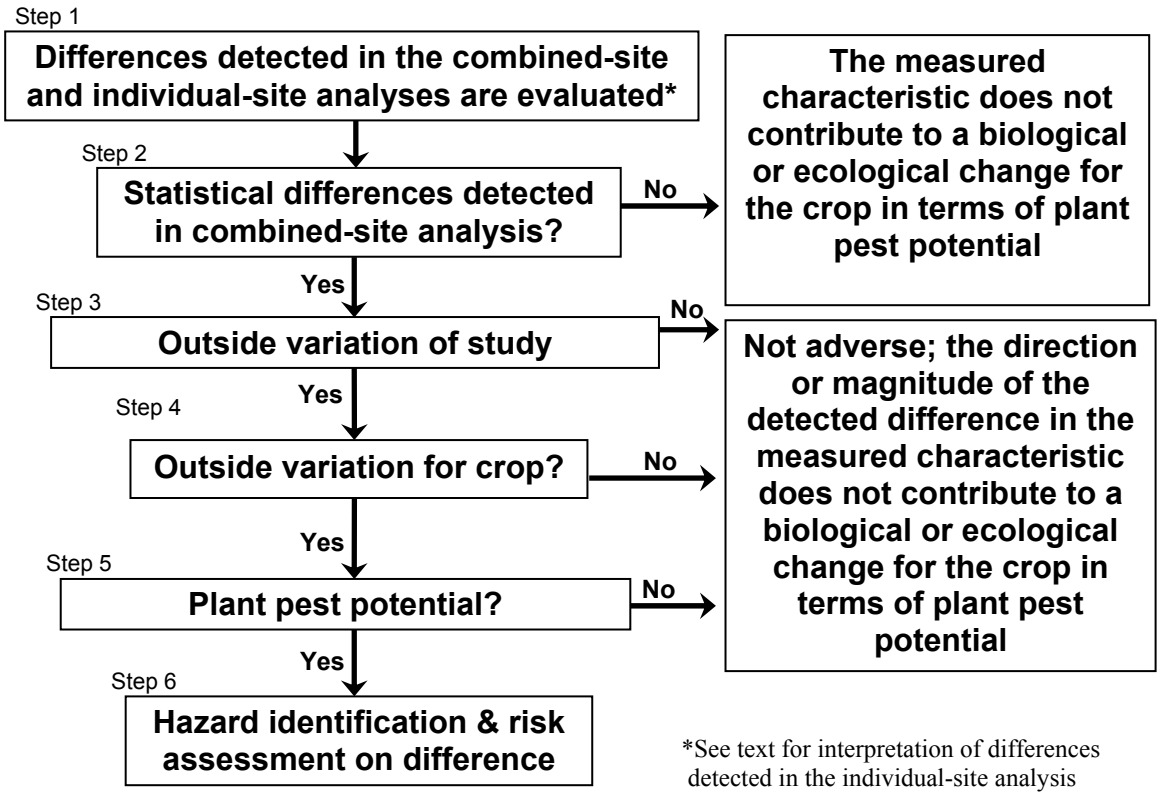
Plant pest risk assessments for biotechnology-derived crops are, by OECD (1993) standards, comparative assessments. Familiarity provides a basis from which the potential environmental impact of a biotechnology-derived plant can be evaluated. The concept of familiarity is based on the fact that the biotechnology-derived plant is developed from a well-characterized conventional plant variety. 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 were the basis for selecting appropriate endpoints and estimating the range of responses that would be considered typical for soybean. Thus, assessment of phenotypic and agronomic characteristics and environmental interactions was essential to compare the biotechnology-derived plant to the conventional counterpart. An overview of the characteristics assessed is presented in Table VIII-1. A subset of the data relating to well understood weediness criteria (e.g., dormancy, pre-harvest seed loss characteristics, and lodging) was used to assess whether there is an increased weediness potential, an element of APHIS's plant pest determination. Based on all of the data collected, an assessment was made whether the biotechnology-derived plant is likely to pose an increased plant pest risk compared to the conventional counterpart.

During the processes of data collection, summarization, and analysis, experienced scientists familiar with each experimental design and evaluation criteria were involved in all steps. This level of oversight ensured that the evaluation system was functioning appropriately, measurements were taken properly, and data were consistent with expectations based on experience with the crop. In addition, the overall dataset was evaluated for evidence of biologically relevant changes, and for possible evidence of an unexpected plant response. Data then were submitted to statistical analysis.

VIII.C. Interpretation of Detected Differences Criteria

Comparative plant characterization data between a biotechnology-derived crop and the control are interpreted in the context of contributions to increased plant pest 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 potential of the biotechnology-derived crop compared to the conventional crop. Characteristics for which differences are detected are considered in the step-wise method (Figure VIII-1 or a similar method). All detected differences for a characteristic are considered in the context of whether the difference would increase the plant pest potential of the biotechnology-derived crop. Ultimately, a weight of evidence approach considering all characteristics and studies was used for the overall risk assessment of differences and their significance. In detail, Figure VIII-1 illustrates the stepwise assessment process employed:



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

Figure VIII-1. Schematic Diagram of Data Interpretation Methods

- **Steps 1 & 2. Evaluate Detected Statistical Differences.** Combined-site and individual-site statistical analyses are conducted and evaluated on each measured characteristic. Differences detected in the individual-site analysis must be observed in the combined-site analysis to be considered further for plant pest potential. Any difference detected in the combined-site analysis is further assessed.
- **Step 3. Evaluate Differences Relative to Reference Range.** If a difference is detected in the combined-site analysis across multiple environments, then the test substance mean value is assessed relative to the reference substances.
- **Step 4. Evaluate Differences in the Context of the Crop.** If the test substance mean is outside the variation of the reference substances (e.g., reference range), the test substance mean is considered in the context of known values common for the crop.
- **Step 5. Plant Pest Potential.** If the test substance mean is outside the range of values common for the crop, the detected difference is then assessed for whether or not it is adverse in terms of plant pest 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 potential of the crop itself, the impact of differences detected in other measured characteristics, and potential for

and effects of trait transfer to feral populations of the crop or a sexually compatible species.

VIII.D. Phenotypic, Agronomic and Environmental Interactions Characteristics

As a significant part of the evaluation of MON 87769, plant phenotypic and agronomic characteristics including seed dormancy and germination, phenotypic, agronomic and environmental interactions, pollen characteristics, and symbiont interactions were evaluated. The phenotypic, agronomic, and environmental interaction evaluations are based on replicated laboratory, greenhouse, and/or multi-site field trials and experiments. In evaluating the phenotypic and agronomic characteristics of MON 87769, data were collected that address specific plant pest risks, as defined by APHIS.

VIII.D.1. Seed Dormancy and Germination Characteristic

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 a survival mechanism that prevents germination when conditions for survival are poor and is an important characteristic that is often associated with plants that are considered as weeds (Anderson, 1996; Lingenfelter and Hartwig, 2003). Thus, information on germination and dormancy is relevant to a weediness assessment. However, it is important to note that it is not uncommon to observe low levels of hard seed in soybean (Mullin and Xu, 2001; Bradford and Nonogaki, 2007). Standardized germination assays are available and routinely used to measure the germination characteristics of soybean seed. The Association of Official Seed Analysts, an internationally recognized seed testing organization, recommends a temperature range of 20/30 °C as optimal for germination of soybean (AOSA, 2007) (Table VIII-2).

Comparative assessments of seed dormancy and germination characteristics were conducted on MON 87769 and A3525, where A3525 served as a comparable control as it has a genetic background similar to MON 87769 but does not contain the *Pj.D6D* or *Nc.Fad3* gene. In addition, nine commercial soybean varieties were included as references to provide a range of values for each assessed characteristics that is representative of commercial soybean. The seed lots for MON 87769, the conventional soybean control and reference varieties were produced during 2006 in Iowa (IA), Illinois (IL), and Indiana (IN), which represent environmentally relevant conditions for soybean production for this product. In addition to the AOSA recommended temperature range of 20/30 °C, seed were tested at five other temperature regimes of 10, 20, 30, 10/20, and 10/30 °C to assess seed germination characteristics. For the alternating temperature regimes of 10/20, 10/30, or 20/30 °C, the lower temperature was maintained for 16 hours and the higher temperature for eight hours. The temperature regimes and types of observation are listed in Table VIII-2. The details of the materials, experimental methods, and data from all individual production sites are presented in Appendix H.

A total of 25 comparisons were made between MON 87769 and the conventional control for seed germination characteristics across the three seed production sites (Table VIII-3). No statistically or biologically significant differences were detected between MON 87769

and the conventional control for percent normal germinated, abnormal germinated, viable hard, dead, or viable firm-swollen seed in the AOSA temperature regime (20/30 °C) or for percent germinated, viable hard, dead, or viable firm-swollen seed in the additional temperature regimes (10, 20, 30, 10/20, 10/30 °C). In some cases, it was not possible to conduct an analysis of variance because there was no variability present in the data. For these comparisons, the values for MON 87769 and the control were all the same, indicating no biological differences.

One viable hard seed was observed at each of the 10 °C and 10/20 °C temperature regimes for MON 87769 produced from the IL site. In soybean, it is not uncommon to observe low levels of hard seed (Mullin and Xu, 2001; Potts et al., 1978). Thus, the observance of a total of two hard seeds out of 7,188 seeds of MON 87769 evaluated in this study was not unexpected.

No statistically significant differences in percent normal germinated and abnormal germinated seeds were detected between MON 87769 and control. However, the germination means of both MON 87769 and control were outside the range of reference varieties included in the study for the AOSA temperature regime (20/30 ºC). The lack of significant differences in germination between MON 87769 and conventional soybean control demonstrate that the observed values of MON 87769 are within the range of values expected of soybean. Thus, the data are indicative of no increased plant pest potential of MON 87769.

The biological characteristics evaluated in this study were used to characterize MON 87769 in the context of a plant pest risk assessment. The results demonstrate that the assessed dormancy and germination characteristics of MON 87769 were not different from those of the conventional control. The observed dormancy and germination values of MON 87769 were within the range expected for soybean. The results of this study, in particular the lack of change in hard seed, are indicative of no increased plant pest or weediness potential of MON 87769 relative to conventional soybean.

Table VIII-2. Seed Dormancy and Germination Characteristics Evaluated

Evaluation Timing[1]	Temperature Regimes and Seed Characteristics Evaluated	
	AOSA[2]	Additional Temperatures
	20/30 °C	10 °C, 20 °C, 30 °C, 10/20 °C and 10/30 °C
Day 5	Normal germinated Dead	Germinated Dead
Day 8	Normal germinated Abnormal germinated Dead Hard viable and non-viable[3] Firm swollen viable and non-viable[3]	Germinated Dead
Day 13	No data collected	Germinated Dead Hard viable and non-viable[3] Firm swollen viable and non-viable[3]

[1] Seed in the 20/30 °C temperature regime were evaluated in Days 5 and 8 (according to AOSA guidelines), while seed in the additional temperature regimes were evaluated on Days 5, 8, and 13.
[2] Association of Official Seed Analysts (AOSA, 2007).
[3] Hard and firm swollen were confirmed to be viable or non-viable by Tetrazolium (Tz) test (AOSA, 2000).

Table VIII-3. Germination Characteristics of MON 87769 and A3525

Temperature Regime	Germination Category[1]	Mean %[2]		Reference Range[3]	
		MON 87769	Control	Min.	Max.
10 °C	Germinated	99.8	100.0	99.8	100.0
	Viable Hard	0.1	0.0	0.0	0.0
	Dead	0.1	0.0	0.0	0.3
	Viable Firm-swollen	0.0†	0.0	0.0	0.0
20 °C	Germinated	100.0†	100.0	99.8	100.0
	Viable Hard	0.0†	0.0	0.0	0.0
	Dead	0.0†	0.0	0.0	0.3
	Viable Firm-swollen	0.0†	0.0	0.0	0.0
30 °C	Germinated	99.7	99.6	99.3	100.0
	Viable Hard	0.0†	0.0	0.0	0.0
	Dead	0.3	0.4	0.0	0.8
	Viable Firm-swollen	0.0†	0.0	0.0	0.0
10/20 °C	Germinated	99.8	99.8	99.5	100.0
	Viable Hard	0.1	0.0	0.0	0.0
	Dead	0.2	0.2	0.0	0.5
	Viable Firm-swollen	0.0†	0.0	0.0	0.0
10/30 °C	Germinated	100.0†	100.0	99.5	100.0
	Viable Hard	0.0†	0.0	0.0	0.0
	Dead	0.0†	0.0	0.0	0.5
	Viable Firm-swollen	0.0†	0.0	0.0	0.0
20/30 °C	Normal Germinated	89.1	92.1	93.8	98.5
(AOSA)	Abnormal Germinated	10.7	7.7	1.4	6.0
	Viable Hard	0.0†	0.0	0.0	0.0
	Dead	0.3	0.2	0.0	0.5
	Viable Firm-swollen	0.0	0.1	0.0	0.0

Note: The data in this table are the combined-site results for the seeds from the three production sites. No statistically significant differences were detected ($p>0.05$) between MON 87769 and the conventional control (A3525). The germination characteristic means for seeds from each individual production site are included in Appendix H.

† No statistical comparisons were made due to lack of variability in the data.

[1] Germinated seed in the AOSA temperature regime (20/30 °C) were categorized as either normal germinated or abnormal germinated seed.

[2] The total percentage of all germination characteristics of MON 87769 or the control in some temperature regimes is greater than 100% due to numerical rounding of the means.

[3] Minimum and maximum mean values from among nine commercial reference soybean varieties.

VIII.D.2. Field Phenotypic, Agronomic Characteristics and Environmental Interactions

Plant growth, development, and yield characteristics were assessed under field conditions as part of the plant characterization assessment of MON 87769. These data were developed to provide APHIS with a detailed description of any unintended phenotypic differences in MON 87769 relative to the conventional soybean control, A3525, and commercial soybean. Environmental interactions also were assessed as an indirect indicator of phenotypic changes to MON 87769 relative to the same comparators described above. The purpose of these field evaluations was to assess whether the introduced genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change altered the phenotypic and agronomic characteristics or the plant-arthropod, plant-disease, or plant-abiotic stressor interactions of MON 87769 compared to the control. Certain growth, reproduction, and pre-harvest seed loss characteristics (such as lodging and pod shattering) can be used in the assessment of whether MON 87769 has enhanced plant pest potential.

Data were collected at 21 field locations over two consecutive years (17 locations in 2006 and four in 2007) to thoroughly evaluate phenotypic, agronomic, and environmental interaction characteristics. These 21 locations provided a diverse range of environmental and agronomic conditions representative of commercial soybean production areas in the U.S. (Table VIII-4). The experiment at each field site was established in a randomized complete block design with three replications. Fourteen phenotypic and agronomic characteristics were evaluated: early stand count, seedling vigor, plant growth stage, days to 50% flowering, flower color, plant pubescence, plant height, lodging, pod shattering, final stand count, seed moisture, 100 seed weight, test weight, and yield. In addition, observational data were collected on plant response to abiotic stressors, disease damage, and arthropod damage. These data were collected four times during the growing season at all 21 sites over the two years to assess the susceptibility and tolerance of MON 87769 to specific abiotic stressors, diseases, or arthropod pests compared to that of the conventional soybean control. Additionally, data were collected on the abundance of specific pest and beneficial arthropods in the plots to assess the interactions between MON 87769 and these arthropods. The categories and timing of phenotypic characteristic and environmental interaction evaluations are included in Table VIII-1. The methods and detailed results of these individual site data comparisons are presented and discussed in Appendix G, while the combined-site analyses are summarized below. The results of this assessment showed the introduced trait did not unexpectedly alter the phenotypic or agronomic characteristics of MON 87769 compared to conventional soybean and are indicative of no increased weediness of MON 87769. The lack of biologically meaningful differences in plant response to abiotic stressors, disease damage, arthropod damage, and the abundance of various pest and beneficial arthropods further indicate that the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change are unlikely to increase plant pest potential.

VIII.D.2.1. Field Phenotypic and Agronomic Characteristics

A total of 14 phenotypic and agronomic characteristics were evaluated. In the combined-site analysis of the data from the 2006 and 2007 field trials, no statistically significant differences were detected between MON 87769 and the control for any of the assessed phenotypic characteristics (Tables VIII-5 and VIII-6). All plants of MON 87769 and the control had purple flowers and hairy pubescence at each site as expected (Appendix G, Tables G-3 and G-5). Furthermore, no differences in plant growth stage were observed between MON 87769 and the control for any of the 126 and 27 observations conducted among the sites in 2006 and 2007, respectively (Appendix G).

The phenotypic and agronomic characteristics evaluated in this study were used to provide a detailed description of any differences between MON 87769 compared to the nontransformed control (A3525). A subset of these characteristics were useful to assess the weediness potential of MON 87769 compared to the conventional soybean control. The results from the 2006 and 2007 field trials demonstrate that the assessed phenotypic and agronomic characteristics of MON 87769 were not altered compared to the conventional control as a result of the introduction of the genes (Pj.*D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change. The observed values of phenotypic and agronomic characteristics of MON 87769 were within the range expected for soybean. These results are indicative of no increased plant pest or weediness potential of MON 87769 compared to conventional soybean.

Table VIII-4. Field Phenotypic Evaluation Sites for MON 87769 during 2006 and 2007

Site Code	County, State	USDA-APHIS Notification Number
2006 Field Sites		
IA1	Jefferson, IA	06-045-22n
IA2	Benton, IA	06-045-22n
IA3	Jefferson, IA	06-074-05n
IA4	Guthrie, IA	06-074-05n
IL1	Stark, IL	06-045-22n
IL2	Warren, IL	06-045-22n
IL3	Clinton, IL	06-074-05n
IN1	Parke, IN	06-045-22n
IN2	Boone, IN	06-045-22n
KS	Pawnee, KS	06-045-22n
MI	Ottawa, MI	06-074-05n
MO1	Macon, MO	06-045-22n
MO2	Lincoln, MO	06-045-22n
NE	York, NE	06-045-22n
NE2	York, NE	06-074-05n
OH	Fayette, OH	06-074-05n
WI	Walworth, WI	06-074-05n
2007 Field Sites		
IL	Stark, IL	07-052-104n
IN	Boone, IN	07-052-104n
MI	Ottawa, MI	07-052-104n
WI	Walworth, WI	07-052-104n

Table VIII-5. Plant Growth and Development Data across 17 Locations during 2006

	MON 87769	Control	Reference Range[1]	
Phenotypic Characteristic (units)	**Mean**	**Mean**	**Minimum**	**Maximum**
Early stand count (#/plot)	217.0	219.7	150.5	298.6
Seedling vigor (1-9 scale)	3.2	3.1	2.3	5.0
Days to 50% flowering[2]	195.4	195.6	184.7	206.5
Flower color[3]	Purple	Purple	Purple	Purple
Plant pubescence[3]	Hairy	Hairy	Hairy	Hairy
Plant height (in)	36.2	36.1	30.4	43.9
Lodging (0-9 scale)	1.1	1.3	0.0	4.3
Pod shattering (0-9 scale)	0.3	0.3	0.0	1.5
Final stand count (#/plot)	199.6	203.4	154.3	271.3
Seed moisture (%)	12.2	12.2	11.0	16.0
100 seed weight (g)	15.1	15.4	14.0	18.0
Test weight (lb/bu)[4]	56.5	56.1	49.7	57.7
Yield (bu/ac)[4]	52.7	55.7	28.8	66.9

Note: No statistically significant differences were detected ($p>0.05$) between MON 87769 and the conventional control (A3525).
[1] Reference range = Minimum and maximum mean values among 18 commercial reference soybean varieties.
[2] Calendar day number when approximately 50% of the plants in each plot were flowering.
[3] Flower color and plant pubescence data were categorical and not statistically analyzed (see Appendix G, Table G-3).
[4] Test weight and yield data from the IA4 site were excluded from the statistical analysis because an inaccurate measuring device was used to collect the data.

Table VIII-6. Plant Growth and Development Data across Four Locations during 2007

	MON 87769	Control	Reference Range[1]	
Phenotypic Characteristic (units)	**Mean**	**Mean**	**Minimum**	**Maximum**
Early stand count (#/plot)	357.8	354.2	180.0	492.7
Seedling vigor (1-9 scale)	2.8	2.5	1.0	6.0
Days to 50% flowering[2]	205.4	205.3	201.5	211.7
Flower color[3]	Purple	Purple	Purple	Purple
Plant pubescence[3]	Hairy	Hairy	Hairy	Hairy
Plant height (in)	38.2	39.3	30.6	49.4
Lodging (0-9 scale)	1.7	1.8	0.0	3.7
Pod shattering (0-9 scale)	0.2	0.0	0.0	0.3
Final stand count (#/plot)	331.6	339.6	158.7	455.7
Seed moisture (%)	11.8	11.7	10.6	12.2
100 seed weight (g)[4]	17.3	17.0	13.9	20.0
Test weight (lb/bu)	54.2	54.5	51.2	57.0
Yield (bu/ac)	46.4	50.4	39.1	53.5

Note: No statistically significant differences were detected ($p>0.05$) between MON 87769 and the conventional control (A3525).
[1] Reference range = Minimum and maximum mean values among 12 commercial reference soybean varieties.
[2] Calendar day number when approximately 50% of the plants in each plot were flowering.
[3] Flower color and plant pubescence data were categorical and not statistically analyzed (see Appendix G, Table G-5).
[4] 100 seed weight data from the WI site were excluded from the statistical analysis because the balance used to weigh the samples did not have sufficient precision.

VIII.D.2.2. Environmental Interaction Analyses

Evaluations of environmental interactions were conducted as part of the plant characterization for MON 87769. In the 2006 and 2007 U.S. field trials conducted for evaluation of phenotypic and agronomic characteristics of MON 87769, data were collected on plant response to abiotic stressors (e.g., drought, nutrient deficiency, wind, etc.), disease damage, and arthropod damage. These data were used to assess the susceptibility and tolerance of MON 87769 to specific abiotic stressors, diseases, or arthropod pests compared to that of the conventional soybean control. Additionally, data were collected on the abundance of specific pest and beneficial arthropods in the plots to assess the interactions between MON 87769 and these arthropods (Appendix I; Tables I-1 through I-10). These data are used as part of the environmental risk assessment to evaluate plant pest potential and impact on NTOs for MON 87769 compared to the conventional soybean control. In addition, multiple commercial soybean varieties were included in the analysis to establish a range of natural variability for each characteristic. The lack of biologically meaningful differences in plant response to abiotic stressors, disease damage, arthropod damage, or the abundance of various pest and beneficial arthropod indicate that the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change are unlikely to be biologically meaningful in terms of increased plant pest potential.

Plant response to abiotic stressors, disease damage, and arthropod damage were assessed qualitatively four times during the growing season at 17 sites in 2006 and four sites in 2007. The observed abiotic stressors, diseases, and arthropod pests were "natural" (i.e., no artificial infestation or interference was used) and, therefore, often varied between observations at a site and between sites. For each abiotic stress response, disease damage, and arthropod damage observation at a site, the reported values represent the range of ratings observed across the three replicatons at each site. MON 87769 and the control were considered qualitatively different in response to a stressor on a particular observation date if the range of ratings across all three replications of MON 87769 and the conventional soybean control did not overlap (e.g., "none" rating vs. "slight-moderate" rating). The ratings observed among the commercial reference soybean varieties provide qualitative assessment data common to the crop for each stressor assessed. Arthropod abundance data were collected quantitatively from plots three times during the growing season at four sites each in 2006 and 2007 to assess the interactions between MON 87769 and these arthropods.

A total of 703 comparative observations of plant response to abiotic stressors, disease damage, and arthropod damage and 260 comparative observations of the abundance of various pest and beneficial arthropods were conducted in field trials over two consecutive years to assess environmental interactions of MON 87769 compared to the conventional control. No biologically meaningful differences were observed across sites over two U.S. soybean growing seasons between MON 87769 and the conventional control in their susceptibility or tolerance to specific abiotic stressors, diseases, or arthropod pests or in their interactions with specific pest and beneficial arthropods, as indicated by the

arthropod abundance data. Thus, the results from the field trials demonstrate that the assessed interactions between MON 87769 and abiotic stressors, diseases, and arthropods were not altered as a result of the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change, compared to the conventional control and were within the range expected for soybean. These results are indicative of no increased plant pest potential or adverse ecological interactions of MON 87769 relative to conventional soybean.

VIII.D.3. Pollen Characteristics

In determining the potential for a biotechnology-derived plant to increase weedy or invasive characteristics in other plants, APHIS considers the potential for gene flow and introgression of the biotechnology-derived trait into other plant varieties or wild relatives. Information on pollen morphology and viability are pertinent to this assessment, and therefore, were assessed for MON 87769. In addition, morphological characterization of pollen produced by MON 87769 and the conventional soybean control are relevant to APHIS's plant pest risk assessment because they add to the detailed description of the phenotype of MON 87769 compared to conventional soybean.

The purpose of this evaluation was to assess the morphology and viability of pollen collected from MON 87769 compared to a conventional soybean control. Pollen was collected from MON 87769, the control (A3525), and four commercial reference soybean varieties grown under similar agronomic conditions in a field trial in Missouri. The trial was arranged in a randomized complete block design with three replications. A minimum of 20 flowers were collected from each plot. Pollen was extracted, combined among flowers collected from the same plot, and stained with Alexander's stain (Alexander, 1980). Pollen viability was evaluated for each sample and pollen grain diameter was measured for ten representative viable pollen grains per replication. General morphology of the pollen was observed for each of the three replications of MON 87769, the control, and the reference soybean varieties (see Appendix J).

No statistically significant differences were detected ($p>0.05$) between MON 87769 and the control for percent viable pollen or pollen grain diameter (Table VIII-7). Furthermore, no visual differences in general pollen morphology were observed between MON 87769 and the control. These results demonstrate that the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change did not alter the overall morphology or viability of MON 87769 pollen compared to the conventional soybean control. The absence of statistically significant differences between the pollen collected from MON 87769 compared to the conventional soybean control for the assessed characteristics demonstrate that the observed values of MON 87769 were within the range of values expected for soybean. Thus, these data further support no change in plant pest potential for MON 87769 compared to the nontransformed control and other soybean varieties.

Table VIII-7. Pollen Grain Diameter and Viability Analyses

Pollen Characteristic	Mean		Reference Range[1]	
	MON 87769	Control	Minimum	Maximum
Viability (%)	98.2	98.7	94.3	98.7
Diameter (µm)	23.4	24.3	23.4	23.8

Note: No statistically significant differences were detected (p>0.05) between MON 87769 and the conventional control (A3525).
[1] Reference range is the minimum and maximum mean value observed among the four commercial reference soybean varieties.

VIII.D.4. Symbiont Interactions

Members of the bacterial family *Rhizobiaceae* and *Bradyrhizobiaceae* form a highly complex and specific symbiotic relationship with leguminous plants, including soybean (Gage, 2004). The *Rhizobium*-legume symbiosis results in the formation of root nodules, providing an environment in which differentiated bacteria called bacteroids are capable of reducing or "fixing" atmospheric nitrogen. The product of nitrogen fixation, ammonia, can then be utilized by the plant. In soybean, atmospheric nitrogen is fixed into organic nitrogen through a symbiotic association with the bacterium *Bradyrhizobium japonicum*. As a result of this relationship, no nitrogen inputs are needed for agricultural production of soybean.

As part of the plant pest risk assessment, APHIS considers the impact of the biotechnology-derived crop to agricultural or cultivation practices (7 CFR § 340.6). Changes in the symbiotic relationship with *Rhizobiaceae* and *Bradyrhizobiaceae* could directly impact cultivation practices (e.g., the need to add additional nitrogen to soybean production). Thus, the purpose of this evaluation was to assess whether the *B. japonicum*-soybean symbiosis of MON 87769 had been altered as a result of the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change, compared to the conventional soybean control.

The relative effectiveness of the symbiotic association between a leguminous plant and its rhizobial symbiont can be assessed by various methods. Assessment of nodule number and mass along with plant growth and nitrogen status are commonly used to assess differences in the symbiotic association 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 (Israel et al., 1986; Appunu et al., 2006).

MON 87769, a conventional soybean control (A3525), and reference soybean varieties were produced in a greenhouse from seeds planted in pots containing nitrogen-deficient

potting medium. Seeds were inoculated with a solution of *B. japonicum*. The pots were arranged in a randomized strip-plot or randomized complete block design with eight replicates. At four and/or six weeks after emergence, plants were excised at the surface of the potting medium, then shoot and root plus nodule material were removed from the pots. Nodules were separated from roots prior to enumeration and determination of dry weight. Detailed information on materials and methods used for symbiont evaluation is presented in Appendix K.

An initial study was conducted to assess the symbiotic interactions between *B. japonicum* and MON 87769; however, results of the study were variable and did not provide conclusive evidence to assess the objectives of the study. Therefore, a subsequent study was conducted using a refined methodology in which a negative isoline control was included to determine whether differences detected between MON 87769 and the conventional control in the initial study were associated with the trait or the plant transformation process. Additionally, reference soybean varieties representing more diverse soybean germplasm were used to determine whether the measured endpoint values of MON 87769 are within the range of responses familiar to what is expected for soybean. The results from both studies were considered in assessing the impact from the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and intended fatty acid change on the symbiotic relationship between *B. japonicum* and MON 87769, plant pest potential, and cultivation practices relative to nitrogen inputs. The results from both studies are summarized below and in Appendix K.

In the initial study, four out of the ten measurement endpoints assessed among the four and six week sampling times were significantly different between MON 87769 and the control, although the detected differences were not consistent among the two sampling times (Table VIII-8). Soybean varieties are known to be heterogeneous for characteristics associated with symbiotic nitrogen fixation (Sinclair et al., 1991; Pazdernick et al., 1996; Graham et al., 2004). Since MON 87769 was derived from a single seed of the conventional control variety A3525, it was not possible to conclude whether the detected differences between MON 87769 and the control were associated with the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change or were due to inherent variability within the A3525 variety. In addition, the mean values for both MON 87769 and the control were greater than the range of the reference varieties for five of the ten measurement endpoints among the two sampling times. This likely was due to varietal differences, since both MON 87769 and the conventional control have the same genetic background, and indicates that the reference varieties used in the experiment may not have sufficiently represented the inherent variability in soybean for characteristics associated with symbiotic nitrogen fixation. Therefore, an additional assessment was conducted using refined methodology to further investigate the symbiotic interactions between *B. japonicum* and MON 87769 and to determine whether the measured endpoint values of MON 87769 are within the range of responses familiar to what is expected for soybean.

In the subsequent study, the negative isoline of MON 87769, derived from progeny of the regenerated R0 plant, was included as a control, in addition to the conventional soybean control (variety A3525), to determine if differences between MON 87769 and the conventional control were due to the introduction of the trait or the plant transformation process. Additionally, six conventional reference soybean varieties representing more diverse soybean germplasm from maturity groups II to V were included in the evaluation to determine whether the measured endpoint values of MON 87769 are within the range of responses familiar to what is expected for soybean. The evaluation was conducted using the same methods and measurement endpoints as in the first study with the exception of the following refinements. The data were collected at a single time point of six-weeks after emergence when the soybean plants were at approximately the R4 (full pod) growth stage and not at four weeks after emergence. Since nodule number and size and N-fixation increase over time until the R5-R6 stage (Iowa State University, 2004; Lersten and Carlson, 2004), it was determined that the six-week time point was more appropriate for assessing symbiotic interactions between soybean and *B. japonicum.* Consequently, pots were arranged in the greenhouse in a randomized complete block design with eight replications instead of the randomized strip-plot design used in the initial study. In addition, smaller pots and a single *B. japonicum* inoculation, instead of two inoculations (as was performed in the initial study), were used to further reduce variability within the test system. The concentration of inoculum was reduced from approximately 1×10^8 cells to approximately 1×10^7 cells of *B. japonicum* due to the use of smaller pots and to reduce the potential for pseudo-nodulation. MON 87769 was compared to the conventional control as well as to the negative isoline for nodule number, nodule dry weight, shoot dry weight, root dry weight, and shoot nitrogen. A summary of the results is provided below, while the details of the materials and methods are provided in Appendix K.

No statistically significant differences were detected between MON 87769 and either the conventional or negative isoline control for any measured parameter, including nodule number, shoot nitrogen (percent and total nitrogen), and biomass (dry weight) of nodules, shoot material, or root material (Table VIII-9). Apart from shoot total nitrogen (% dry weight), each measurement endpoint evaluated for MON 87769 was within the range of the reference varieties. The mean shoot total nitrogen of MON 87769 (3.77%) was only slightly outside the reference range (2.85 – 3.76%). The percent differences between MON 87769 and the negative isoline were less than or equivalent to the percent differences between MON 87769 and the conventional control for five of the six measurement endpoints (Table VIII-10).

The results from the refined assessment support the hypothesis that differences between MON 87769 and the conventional control observed in the first study were not attributed to the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and intended fatty acid change or plant transformation process. The results also demonstrate that the measured endpoint values of MON 87769 are within the range expected for soybean. Considering all of the data, the evidence supports a conclusion that the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and intended fatty acid change does not alter the symbiotic relationship between *B. japonicum*

and MON 87769 compared to that of conventional soybean. Thus, there is no expected impact to cultivation practices relative to nitrogen inputs and no increased plant pest potential of MON 87769 compared to conventional soybean.

Table VIII-8. Symbiont Interaction Assessment of MON 87769 and the Conventional Control at 4- and 6-Weeks after Emergence in an Initial Assessment

Measurement Endpoint		Mean			Reference Range[1]	
	Sampling Time	MON 87769	Control		Minimum	Maximum
Nodule Number (per plant)	4 week	77.63	68.29		44.25	75.88
	6 week	180.13*	140.50		60.00	128.25
Nodule Dry Wt (g/plant)	4 week	0.13*	0.10		0.07	0.10
	6 week	0.30	0.25		0.13	0.22
Root Dry Wt (g/plant)	4 week	0.89	0.82		0.81	0.88
	6 week	1.94	1.71		1.35	1.67
Shoot Dry Wt (g/plant)	4 week	1.81	1.49		1.33	1.53
	6 week	4.57*	3.50		2.64	3.71
Shoot Total Nitrogen (% dry wt)	4 week	2.83	2.74		2.34	2.46
	6 week	2.54*	2.80		2.11	2.21

* Indicates a statistically significant difference ($p<0.05$) between MON 87769 and the conventional control (A3525).

[1] Reference range is the minimum and maximum mean value observed among the three commercial reference soybean varieties.

Table VIII-9. Symbiont Interaction Assessment of MON 87769, the Conventional Control, and the Negative Isoline at 6-Weeks after Emergence in a Refined Assessment

Measurement Endpoint	Mean				Reference Range[1]	
	MON 87769	A3525	Negative Isoline		Minimum	Maximum
Nodule Number (per plant)	208	185	206		83	265
Nodule Dry Wt (g/plant)	0.56	0.49	0.53		0.40	0.80
Root Dry Wt (g/plant)	1.53	1.63	1.57		1.08	2.58
Shoot Dry Wt (g/plant)	6.21	6.29	6.36		5.05	11.25
Shoot Total Nitrogen (% dry wt)	3.77	3.66	3.74		2.85	3.76
Shoot Total Nitrogen (g)	0.23	0.23	0.23		0.19	0.33

Note: No significant differences were detected ($p>0.05$) between MON 87769 and the conventional control (A3525) or the negative isoline for any measured endpoint.

[1] Reference range is the minimum and maximum mean value observed among the six conventional reference soybean varieties.

Table VIII-10. Percent Difference between MON 87769 and the Conventional Control or Negative Isoline Control

	MON 87769	
Measurement Endpoint	**Difference from Conventional Control (%)[1]**	**Difference from Negative Isoline (%)**
Nodule Number (per plant)	11.3	1.0
Nodule Dry Wt (g/plant)	12.5	5.4
Root Dry Wt (g/plant)	-6.5	-2.6
Shoot Dry Wt (g/plant)	-1.3	-2.4
Shoot Total Nitrogen (% dry wt)	2.9	0.8
Shoot Total Nitrogen (g/plant)	0	0

[1]Conventional control = variety A3525.

VIII.E. Overall Conclusions for Phenotypic, Agronomic, and Environmental Interactions Evaluation

An extensive and robust set of information and data were used to assess whether the introduction of the genes (*Pj.D6D and Nc.Fad3*) genes, the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change altered the plant pest potential of MON 87769 compared to the conventional soybean control, A3525. Phenotypic and agronomic characteristics of MON 87769 were evaluated and compared to those of the conventional soybean control. These assessments included 14 plant growth and development characteristics; five seed dormancy and germination parameters under six different temperature regimes; two pollen characteristics; more than 960 observations for plant interactions with arthropods, diseases, and abiotic stressors under field conditions; six plant-symbiont interaction characteristics; and compositional evaluation (Section VII) of 75 different components (seven in forage, and 68 in soybean seed).

Results from the phenotypic and agronomic assessments demonstrate that MON 87769 does not possess characteristics that would increase plant pest potential compared to conventional soybean. Data on environmental interactions indicate that MON 87769 does not confer any biologically meaningful increased susceptibility or tolerance to specific diseases, arthopods, or abiotic stressors, or changes in agronomic and phenotypic

characteristics. Taken together, these data support the conclusion that MON 87769 is not likely to pose increased plant pest risk compared to conventional soybean.

IX. U.S. AGRONOMIC PRACTICES

IX.A. Introduction

This section provides a summary of the key agronomic practices in the U.S. for producing soybean and is provided to serve as a baseline for comparative purposes in an analysis of the impacts to current agronomic practices due to cultivation of MON 87769. Discussions include soybean production, growth and development, general management practices, management of weeds, insects and diseases, soybean rotational crops, and volunteer soybean management. Compared to traits such as herbicide tolerance or resistance to insects that directly benefit the growers, the production of SDA through cultivation of MON 87769 is neutral with respect to agronomic benefits. Information presented in the previous section demonstrated that MON 87769 is no more susceptible to diseases or pests and is phenotypically comparable to conventional soybean. Thus, there are no impacts to the inputs needed to obtain a crop from MON 87769, and as such, there are no specific impacts to most of the agronomic practices employed for production of soybean. MON 87769 will be produced under an identity preservation system (IDP) requiring specific management to preserve the value of the SDA soybean oil. Therefore, emphasis is placed on the anticipated impacts on agronomic practices used for production of specialty type soybean upon deregulation of MON 87769.

Soybean is planted in over 30 states, demonstrating its wide adaptation to soils and climate. The soil, moisture, and temperature requirements for producing soybean are generally similar to those for corn and thus the two crops share a similar cultivation area. Proper seedbed preparation, appropriate variety selection, proper planting dates and plant population, and good integrated pest management practices are important to optimizing the yield potential and economic returns of soybean.

MON 87769 is expected to bring added value for growers, soybean handlers, crushers and food processors. An identity preservation system will be used for production, post-harvest handling and processing to preserve the enhanced value of MON 87769. As such, production practices, post-harvest handling of soybean, and processing will fall under a separate production system and distribution channel termed for this specialty soybean. MON 87769 is expected to utilize existing agricultural practices employed for production and identity preservation of specialty soybean.

Annual and perennial weeds are perceived to be the greatest pest problem in soybean production. Economic thresholds for controlling weeds in soybean require some form of weed management practice on all soybean acreage. Approximately 98% of the soybean acreage receives an herbicide application. Soybean insects and diseases are generally considered less problematic, although infestations do occasionally reach economic thresholds requiring treatment.

Volunteer soybean is not considered a significant concern in rotational crops, primarily because of climatic conditions and adequate control from tillage practices. Additionally,

mechanical and chemical control methods are available to manage the occasional volunteer soybean plant. Due to the lack of weediness potential, introduction of MON 87769 in the soybean production system would have a negligible impact on managing soybean volunteer plants in rotational crops such as corn, cotton, and rice because control measures are available for volunteer soybean when they arise. Pre-plant tillage is the first management tool for control of emerging volunteer soybean in the spring. If volunteer soybean emerges after planting, shallow cultivation will control most of the plants and effectively reduce competition with the crop. Several postemergence herbicides also are available to control volunteer soybean (conventional or glyphosate-tolerant soybean) in each of the major rotational crops.

As shown in Sections VII and VIII, with the exception of the intended fatty acid change (presence of SDA and GLA in MON 87769 seed), no phenotypic, compositional, or environmental differences between MON 87769 and conventional soybean have been observed. Therefore, it is not anticipated that commercialization of MON 87769 in the U.S. would have a notable impact on current soybean cultivation practices, including the management of weeds, diseases, and insects.

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

IX.B.1. Soybean Production

Soybean first entered North America in the 18th century (Hoeft et al., 2000). Sometime during the 1930s, soybean started to be processed industrially in the U.S. for edible oil and protein meal. Currently, the U.S. produces approximately 32% of the global soybean supply (ASA, 2008). In 2007, the U.S. exported 1 billion bushels (27.9 million metric tons) of soybean, which accounted for 37 percent of the world's soybean exports (ASA, 2008). In total, the U.S. exported $12.9 billion USD worth of soybean and soybean products globally in 2007 (ASA, 2008). China is the largest export market for U.S. soybean with purchases totalling $4.1 billion. Japan is the second largest export market with sales of $1.1 billion in the same year. Other significant markets include the European Union and Mexico.

The production of soybean is highly dependent upon soil and climatic conditions. In the U.S., the soil and climatic requirements for growing soybean are very similar to corn. The soils and climate in the eastern half of the U.S. provide sufficient water supplies under normal climatic conditions to produce a soybean crop. The general water requirement for a high-yielding soybean crop is approximately 20 inches of water during the growing season (Hoeft et al., 2000). Soil texture and structure are key components determining water availability in soils, where medium-textured soils hold more available water, allowing soybean roots to penetrate deeper in medium-textured soils than in clay soils. Irrigation is used on approximately 9% of the acreage to supplement the water supply during dry periods in the western and southern soybean growing regions (ASA, 2008).

Most of the soybean acreage is grown as a full-season crop. Approximately 8% of the soybean acres are planted in a double-crop system following winter wheat south of 35º

North latitude (Boerma and Specht, 2004). However, this percentage can vary significantly from year to year. The decision to plant double-crop soybean is influenced by both agronomic and economic factors. Agronomic factors include harvest date of the wheat crop, which determines the double-crop soybean planting date, and available soil moisture. Economic factors include expected soybean price and anticipated economic return (Boerma and Specht, 2004).

The vast majority of soybean grown in the U.S. is grown for animal feed and is usually fed as soybean meal. However, approximately 12% of soybean grown is specialty soybean produced for a specific market or use.[3] Examples of specialty soybean include high protein, tofu, high oil, high oleic, non-biotechnology-derived (also referred to as non-genetically modified), and organic soybean.[4] The uses of these soybean varieties include human consumption, food processing or specialty products. The soybean varieties used in the specialty market are typically specified by buyers and endusers of soybean for production, and a premium relative to commodity soybean is paid for delivering a product that meets purity and quality standards (Pritchett et al., 2002; Elberhi, 2007; Sundstrom et al, 2002; Lee and Herbek, 2004; Muth et al 2003; and Smyth and Phillips 2002) for the soybean variety. Product differentiation and market segmentation in the specialty soybean industry includes mechanisms to keep track of the soybean (traceability), methods for identity preservation (IDP), including closed-loop systems, and quality assurance processes (e.g., ISO9001-2000 certification), as well as contracts between growers and buyers that specify delivery agreements. MON 87769 is an alternate source of omega-3 fatty acid and will be considered a specialty soybean product and marketed in a manner similar to other high-value specialty crops.

The U.S. soybean acreage in the past ten years has varied from approximately 64.7 to 75.7 million acres, with the lowest acreage recorded in 2007 and the highest in 2008 (Table IX-1). Average soybean yields have varied from 33.9 to 43.3 bushels per acre over this same time period. Soybean production ranged from 2.45 to 3.19 billion bushels over the past ten years, with 2006 being the largest production year on record. According to data from USDA-NASS (2009), soybean was planted on approximately 75.7 million acres in the U.S. in 2008, producing 2.96 billion bushels of soybean (Table IX-1). Soybean acreage and production in 2007 was down significantly from 2006, mainly due to a large increase in corn acreage. The value of soybean reached $27.4 billion in the U.S. in 2008 (USDA-NASS, 2009). In comparison, corn and wheat values in 2008 were $47.37 and $16.57 billion, respectively (USDA-NASS, 2009).

For purposes of this agronomic practices discussion, soybean production is divided into three major soybean growing regions accounting for 99.1% of the 2008 U.S. soybean acreage: Midwest/Great Plains region (IL, IN, IA, KS, KY, MI, MN, MO, NE, ND, OH, SD, and WI), Southeast region (AL, AR, GA, LA, MS, NC, SC, and TN) and the Eastern Coastal region (DE, MD, NJ, NY, PA, and VA) (Table IX-2). The vast majority of soybean was grown in the Midwest region, representing 82.1% of the total U.S. acreage.

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[3] http://usb.adayana.com:8080/usb/jsp/login.jsp
[4] http://usb.adayana.com:8080/usb/jsp/login.jsp

The Southeast and Eastern Coastal regions represented 14.3% and 2.7% of the acreage, respectively. Among the three regions, the Midwest region produced the highest average yield at 38.6 bushels per acre in 2008, and average state yields in this region ranged from 28.0 to 47.0 bushels per acre. The average yield in the Southeast region was 34.4 bushels per acre, with states within this region averaging from 30.0 to 40.0 bushels per acre. The average yield in the Eastern Coastal region was 34.1 bushels per acre, with individual state averages ranging from 27.5 to 46 bushels per acre. It is anticipated that MON 87769 will be grown mainly in the Midwest/Great Plains region, where MON 87769 is anticipated to be grown on less than 3.5 million acres (~5% of the total U.S. soybean acreage). MON 87769, as a speciality soybean product is not expected to impact overall soybean production acreage in the U.S., and the impact on total soybean acreage in the Midwest/Great Plains region is anticipated to be negligible as well because MON 87769 plantings are expected to replace existing soybean acreage. Additionally, the total U.S. soybean acreage has fluctuated over the past ten years (64.7 to 75.7 million acres) and the MON 87769 acreage is anticipated to be well within this historical fluctuation.

Managing input costs is a major component to the economics of producing a soybean crop. Key decisions on input costs include choosing what soybean varieties to plant, amounts of fertilizer to apply, and what herbicide program to use. The average operating cost for producing soybean in the U.S. in 2006 was $93.41 per acre, according to statistics compiled by the USDA-Economic Research Service (USDA-ERS, 2006). The value of the production less operating cost was reported to be $161.43 per acre. A summary of potential production costs and returns are presented in Table IX-3.

Table IX-1. Soybean Production in the U.S., 1999 – 2008

Year	Acres Planted (×1000)	Acres Harvested (×1000)	Average Yield (bushels/acre)	Total Production (×1000 bushels)	Value (billions $)
2008	75,718	74,641	39.6	2,959,174	27.40
2007	64,741	64,146	41.7	2,677,117	26.97
2006	75,522	74,602	42.7	3,188,247	20.42
2005	72,142	71,361	43.3	3,086,432	16.93
2004	75,208	73,958	42.2	3,123,686	17.89
2003	73,404	72,476	33.9	2,453,665	18.01
2002	73,963	72,497	38.0	2,756,147	15.25
2001	74,075	72,975	39.6	2,890,682	12.61
2000	74,266	72,408	38.1	2,757,810	12.47
1999	73,730	72,446	36.6	2,653,758	12.21

Source: USDA-NASS, 2009.

Table IX-2. U.S. Soybean Production by Region and State in 2008

Region/State	Acres Planted[1] (thousands)	Acres Harvested[1] (thousands)	Average Yield[1] (bushels/acre)	Total Production[1] (×1000 bushels)	Value[1] (billions $)
Midwest Region					
Illinois	9,200	9,100	47.0	427,700	4.00
Indiana	5,450	5,430	45.0	244,350	2.27
Iowa	9,750	9,670	46.0	444,820	4.29
Kansas	3,300	3,250	37.0	120,250	1.03
Kentucky	1,390	1,380	34.0	46,920	0.42
Michigan	1,900	1,890	37.0	69,930	0.64
Minnesota	7,050	6,950	38.0	264,100	2.54
Missouri	5,200	5,030	38.0	191,140	1.72
Nebraska	4,900	4,860	46.5	225,990	2.12
North Dakota	3,800	3,760	28.0	105,280	0.96
Ohio	4,500	4,480	36.0	161,280	1.55
South Dakota	4,100	4,060	34.0	138,040	1.25
Wisconsin	1,610	1,590	35.0	55,650	0.51
Region Totals	**62,150**	**61,450**	**38.6**	**2,495,450**	**23.30**
Southeast Region					
Alabama	360	350	35.0	12,250	0.12
Arkansas	3,300	3,250	38.0	123,500	1.09
Georgia	430	415	30.0	12,450	0.11
Louisiana	1,050	950	33.0	31,350	0.29
Mississippi	2,000	1,960	40.0	78,400	0.69
North Carolina	1,690	1,670	33.0	55,110	0.47
South Carolina	540	530	32.0	16,960	0.15
Tennessee	1,490	1,460	34.0	49,640	0.43
Region Totals	**10,860**	**10,585**	**34.4**	**379,660**	**3.35**
Eastern Coastal Region					
Delaware	195	193	27.5	5,308	0.05
Maryland	495	485	30.0	14,550	0.13
New Jersey	92	90	29.0	2,610	0.02
New York	230	226	46.0	10,396	0.09
Pennsylvania	435	430	40.0	17,200	0.15
Virginia	580	570	32.0	18,240	0.16
Region Totals	**2027**	**1994**	**34.1**	**68,304**	**0.60**

[1] Source: USDA-NASS, 2009.

Table IX-3. U.S. Soybean Production Costs and Returns in 2006

Production Cost or Return Category	Itemized Costs	Return per Planted Acre ($ USD)
Total Gross Value of Production		**254.84**
Operating Costs:	Seed	32.30
	Fertilizer	13.05
	Chemicals	14.46
	Custom operations	6.01
	Fuel, lube and electricity	13.51
	Repairs	11.80
	Purchased irrigation water	0.11
	Interest on operating capital	2.17
Total, operating costs		**93.41**
	Hired labor	1.78
Allocated overhead:	Opportunity cost of unpaid grower's labor	15.20
	Capital recovery of machinery and equipment	60.38
	Opportunity cost of land (rental rate)	86.17
	Taxes and insurance	7.93
	General farm overhead	13.22
Total, allocated overhead		**184.68**
Total cost listed		**278.09**
Value of production less total cost listed		**(23.25)**
Value of production less operating costs		**161.43**

Supporting Information: Yield = 46 bushels/acre, Price = $5.54/bushel, Enterprise size = 268 planted acres, Irrigated = 9%, Dry land = 91%.
Source: USDA-ERS. 2006. Soybean production costs and returns per planted crop acre, by region, excluding government payments for 2006. United States Department of Agriculture-Economic Research Service. (http://www.ers.usda.gov/data/costsandreturns/Soy_all.xls) [Accessed June 26, 2009].

IX.B.2 Specialty Soybean

Commodity and specialty soybean are the two primary production and distribution systems for soybean produced in the U.S. The majority of soybean commonly is grown and marketed through commodity markets for the oil and protein content. Commodity soybean is not consumed directly, but is crushed for meal that is predominantly used for animal feed and as a minor protein source for food. The oil produced during the crushing of the soybean is used for cooking or food ingredients. The goal of the commodity chain is to supply a homogenous product to the enduser. The grower producing soybean for this chain has a choice from many different varieties for production; however, harvested soybean is viewed to be the same for all commodity soybean varieties. At harvest, the grower either delivers soybean to a handler or stores them on farm for later delivery. The first handler is not interested in differentiating the commodity soybean for later use. Commodity soybean handlers typically have large volume storage capacity. Similarly, commodity soybean processors crush large volumes of soybean to produce homogeneous oil and meal products. The commodity system is designed to maximize efficiency at a low profit margin resulting in commingling of different sources of soybean that do not affect the price received for the final product. This production system has been in place in the U.S. since the production of soybean began in earnest in the 1960s (Sonka et al., 2004).

In recent years, there has been an increased demand by consumers and food processers for soybean that has specific physical or chemical characteristics that are required by certain customers to meet specific food or feed needs. As a result a separate specialty soybean channel has developed. This production system and distribution channel is focused on value-added traits that involve much smaller volumes than commodity soybean (Sonka et al., 2004). Specialty soybean varieties are produced on approximately 14% of the U.S. soybean acreage,[5] and according to the Midwest Shippers Association (MSA, 2009) this acreage could grow to over 25% of the crop acreage in certain states within the next decade. This supply chain for specialty soybean typically consists of a specialty firm that contracts production of a specific variety and sets standards for quality of the harvested soybean (Lee and Herbek, 2004). In return, growers receive a premium over the price paid for commodity soybean. Growers may store harvested soybean on farm or deliver the product directly to a processor or to special containers for international shipment. The goal of this identity preserved system is to minimize handling so that value is maintained. The cost incurred from an identity preservation system is offset by the higher value received for the final product.

According to the American Soybean Association (2009), specialty soybean can be grouped into ten broad categories: Non-biotechnology-derived, certified seed, organic food-grade, low saturated fat, clear hilum, tofu, natto, high sucrose, high oleic, low

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[5] http://usb.adayana.com:8080/usb/jsp/login.jsp: Percent U.S. soybean acreage estimate based on U.S. Domestic Consumption by Segment – 2008/09. D. Ludwig, personal communication, 2009.

linolenic, and high protein. The categories refer to soybean with characteristics such as altered seed composition (e.g., low saturated fat, high sucrose, high oleic, low linolenic, and high protein), varieties of soybean with unique physical characteristics suited to their specific uses (e.g., clear hilum for direct human consumption), or refer to a production process (e.g., organic, certified seed). The categories are not meant to be exclusive; for instance, soybean used to produce natto or tofu may employ organic production processes, and soybean from all of the categories is often derived from varieties produced according to certified seed production practices. Tofu and soymilk produced from the tofu soybean category represent a large segment of the specialty soybean market and are produced from unique soybean varieties that have clear hilum and large seed size (Lee and Herbek, 2004). Tofu varieties also must be high in protein (40% or higher) and low in oil concentration compared to commodity soybean. Clear hilum and other characteristic are required for soybean used in the production of other soybean food products consumed directly by humans, such as natto, soybean sprouts, edamame (vegetable soybean), and soy nuts (Lee and Herbek, 2004; UK Cooperative Extension Service, 2009). Organic food-grade and non-biotechnology-derived soybean are identity protected and produced not to contain biotechnology-derived traits. Organic soybean have additional production restrictions requiring the soybean to be produced using no synthetic fertilizers or pesticides. In recent years, public and private soybean breeders have developed soybean varieties with improved nutritional characteristics (e.g., high sucrose, high oleic, low linolenic, and high protein). The characteristics or compositional modifications in this group include varieties with fatty acid profiles that reduce the need for hydrogenation of the resulting soybean oil, increased sugar concentration, increased protein concentration, decreased concentration of saturated and polyunsaturated fats, and lipoxygenase free soybean, which reduces the production of some of the flavors that are objectionable to some consumers (Lee and Herbek, 2004).

The majority of specialty soybean varieties are offered in Group II and early Group III varieties which are adapted to the upper Midwest/Great Plains region (Lee and Herbek, 2004). Maturity Groups II and III soybean varieties are grown on approximately 42 to 45 million acres, occupying the largest percentage of soybean acreage (see Section IX.C). The varieties were developed for this area primarily due to proximity to processing facilities as well as international routes of shipment and to take advantage of efficiencies in soybean breeding programs. With a few exceptions, the agronomic or management practices for growing specialty soybean from planting to harvest are similar to commodity soybean (Lee and Herbek, 2004). Since certain varieties are utilized in the production of each specialty soybean, the variety selection is dictated by the specialty soybean buyer or processor. Nonbiotechnology-derived and organic soybean by definition must be produced utilizing only conventional soybean varieties. As with commodity soybean, weed control is extremely important for specialty soybean to maintain a high-yield potential and because certain weeds, such as nightshade (*Solanum nigrum*), can stain harvested soybean, which is particularly undesirable in food-grade soybean (TCM, 2009). Since organic soybean must be grown without synthetic fertilizers or pesticides for three or more years prior to the current crop of soybean, fertilization and pest management is more difficult (Lee and Herbek, 2004). Weed control in organic soybean relies on a combination of crop rotation, tillage, in-crop cultivation, and hand-weeding. Insect and disease control is managed primarily through

crop rotation. Certain organic pesticides are permitted in the production of certified organic soybean.

All equipment and storage facilities for specialty soybean must be clean of seed from other soybean varieties or plants, dirt, pathogens, and other foreign material. Some soybean contracts may require a special inspection of the handling and storage facilities. The specialty soybean for soybean foods may require special harvesting equipment since some of this soybean is harvested before full maturity (e.g., edemame or vegetable soybean).

IX.B.3. Identity Preservation

Identity Preservation (IDP) refers to a system of production, handling, and marketing practices that maintains the integrity and purity of agricultural commodities (Sundstrom et al., 2002). Commodity grains, on the other hand, are marketed in mass according to USDA grading standards. Specialty crops require some form of segregation or full-scale identity preservation to keep these grains separate from conventional commodity grains (Elberhi, 2007). This market segmentation within the grain channeling system is driven by the need to preserve market value or ensure a specified purity of the product. With certain specialty crops, IDP is required to prevent accidental or unintended commingling (e.g., nonbiotechnology, organic) or to segregate products that are approved only for certain uses (industrial use only).

IDP grain production has been in existence for a long time. Agricultural producers have over many decades developed practices that allowed for differentiation between food vs. feed grain, or grain vs. seed production, or organic vs. nonorganic (Massey, 2002). Seed certification programs such as those used by the Association of Official Seed Certifying Agencies often are cited as the model of IDP systems. These programs date back to the 1920s and 1930s when the certification process was implemented to verify the genetic purity of seed made available to growers (see Section IX.B.4). Standards were established to ensure production of seed from known pedigree with high purity and quality. Similarly, commodity grain traders, marketing organizations, and food processors have established purity and quality tolerances for specific end-product uses. The need for segregation and IDP production systems has increased with the development of specialty crops or crops with special output traits, such as high oil corn, high oleic sunflower, and low-linolenic soybean (Sundstrom et al., 2002).

The production of IDP grains requires special processes in order for growers to meet buyers' criteria for variety identification, composition, and quality. Buyers of IDP soybean typically contract for the production of seed-variety specific soybean and work directly with seed suppliers, growers, independent certification agencies, intermediate processors, and freight companies to deliver the preferred product within specified tolerances.[6] Contract specifications are written to ensure the delivery of the desired

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[6] United Soybean Board, International Buyer's Guide at http://www.ussoyexports.org/ussoy/buyersguide/Chap6.pdf

attribute-specific product and that predetermined management practices are used (Massey, 2002). Lack of compliance with a product specification can lead either to a price discount or rejection of a shipment by the buyer. Depending on the end use, contracts can be extensive, defining many of the production, harvest and storage activities, or less stringent dealing more with pricing, quality specifications and only the most critical production practices. Premiums are paid to growers for the additional risk and management only when the grain meets the contract specifications. Premium prices provide the impetus to maintain the specialty grain's purity and identity separate from commodity grains (Massey, 2002). Premiums are affected by various factors, including the proximity of suppliers to buyers and the cost and availability of substitutes (Sundstrom et al., 2002). For many trait-specific crops, price premiums rise or fall, depending on the supply conditions for the generic commodity.

Growers of IDP grains must have good managerial ability and implement certain management practices to produce and deliver grain possessing the desired physical and chemical characteristics. The production and marketing of trait-specific grains involves additional financial risks (Elberhi, 2007). The growers' managerial ability can affect both yield performance and proficiency in meeting the contract specifications. From a soybean buyer's perspective, contracts help the buyer meet the demand for specific product qualities, improve cost efficiencies of product processing, and reduce transaction costs.

While specific IDP production practices vary depending on the characteristics of the product to be delivered, general elements are implemented to ensure that the enduser or processor receives the grain or end-product with the intended identity and desired quality. As mentioned above, many IDP systems were developed using the principles similar to those used in seed certification. IDP production begins with a system of standards, records, and auditing that are put in place throughout the entire crop production, harvesting, handling, and marketing process (Sundstrom et al., 2002). Some key considerations in the establishment of an IDP system include: 1) planted seed identity and tolerances, 2) appropriate field isolation, 3) inspection and clean-out of equipment and facilities, 4) end-product sampling and testing, and 5) record maintenance and identity labeling (Sundstrom et al., 2002). Each of these components are described in greater detail below:

Planted Seed Identity and Tolerances

The purity and identity of starting seed should be tested and confirmed. The purity of the seed stock should equal or exceed the purity standards of the desired final product. Single or multiple quality tolerances may be established in specialized IDP programs based on market-driven standards.

Field Isolation

Crops must be isolated either spatially or temporally from pollen sources that could impact the quality or purity of the harvested seed. The amount of isolation depends on flower characteristic, sexual compatibility with neighboring crops, pollen quantity and

viability, and mode of pollen dissemination. A self-pollinating crop, such as soybean, requires relatively small isolation distances to effectively preclude cross-pollination.

Equipment and Facilities

Equipment used in production should be cleaned and inspected before and after use in an IDP crop. Storage facilities and transporting vehicles are cleaned and inspected to assure that segregation is maintained and no physical contamination occurs.

Sampling and Testing

In some cases, the IDP grain is sampled and tested at various stages to confirm the product identification, purity, and quality. Special consideration should be given to sampling and testing techniques that ensure reliable results.

Record Maintenance and Identity Labeling

Records typically are maintained on field designations, harvest amounts, storage bin locations, and product transfers. IDP products must be identified, segregated, and labeled in the market chain.

As with current IDP systems used for specialty soybean to preserve market value or ensure purity of the product, MON 87769 will employ an IDP system based on established practices. The IDP practices will be implemented to ensure that the enduser or processor receives the soybean with the identity, fatty acid composition of the oil, and desired quality for this value-added specialty soybean. This system will also serve to direct this speciality soybean to its intended use and away from the commodity processing stream.

IX.B.4. Soybean Seed Production

Standardized seed production practices are responsible for maintaining high quality seed stocks, an essential basis for U.S. agriculture. By the early 20th century, agronomists learned how to develop specific plant varieties with desirable traits. In the U.S., state agricultural experiment stations developed many varieties which were distributed to growers for use. Growers saved seed and later sold to neighbors; however, the desirable traits of the varieties often were lost through random genetic changes and contamination with other crop and weed seed (Sundstrom et al., 2002). The value of seed quality (including genetic purity, vigor, absence of weed seed, and seed-borne diseases and inert materials such as dirt) was quickly identified as a major factor in crop yields. States developed seed laws and certification agencies to ensure that purchasers who received certified seed could be assured that the seed met established seed quality standards (Bradford, 2006). The federal government passed the U.S. Federal Seed Act of 1939 to recognize seed certification and official certifying agencies. Regulations first adopted in 1969 under the Federal Seed Act recognize land history, field isolation, and varietal purity standards for foundation, registered, and certified seed. Under international agreements such as the Organization for Economic Co-Operation and Development scheme, the U.S. and other countries mutually recognize minimum seed quality standards

(Bradford, 2006). The Association of Official Seed Certifying Agencies represents state and private seed certification in the U.S., and includes international member countries in North and South America, as well as in Australia and New Zealand.

Soybean seed is separated into four seed classes: 1) breeder, 2) foundation, 3) registered, and 4) certified (AOSCA, 2009). Breeder seed is seed directly controlled by the originating or sponsoring plant breeding organization or firm. Foundation seed is first generation seed increased from breeder seed and is handled to maintain specific varietal purity and identity. Registered seed is the progeny of foundation seed that is handled to maintain satisfactory variety purity and identity. Certified seed is the progeny of breeder, foundation or registered seed and is two generations from foundation seed. All soybean seed sold may not be officially certified; however, commercial soybean seed sold and planted for normal soybean production predominately is produced to meet or exceed certified seed standards. This section of the petition will provide a broad overview of the practices utilized in producing certified seed.

Soybean seed breeders and producers have put in place practical measures to assure the quality and genetic purity of soybean seed varieties for commercial planting. The need for such systems arose from the recognition that the quality of improved soybean varieties quickly deteriorated in the absence of monitoring for quality and genetic purity (CAST, 2007). Seed certification programs were initiated in the early 1900s in the U.S. to preserve the genetic identity and variety purity of seed. There are special land requirements, seed stock eligibility requirements, field inspections and seed labeling standards for seed certification. Seed certification services are available through various state agencies affiliated with the Association of Official Seed Certifying Agencies. Large seed producers implement their own seed quality assurance programs. However, large seed producers often will utilize the services of state certifying agencies as a third-party source to perform certain field inspections and audits.

The U.S soybean production for all purposes according to USDA-NASS statistics has varied from approximately 64.1 to 74.6 million acres in the past ten years, with the lowest acreage recorded in 2007 and the highest in 2008 (USDA-NASS, 2009; Table IX-1). This range of soybean acreage would require 105 to 125 million units (50 lbs/unit) of soybean seed. This seed volume includes allowances for seed losses due to weather, poor yields, and quality issues. Additional allowances are included for distribution excess, seed returns, replants and potential increases in soybean acreage. Assuming an average soybean yield of 45 bushels, or 54 units (50 lbs/unit) per acre, 1.9 to 2.3 million acres would be required to produce this volume of commercial certified soybean seed each year.

Soybean seed is produced throughout most of the U.S. soybean growing regions. Soybean varieties are developed and adapted to certain geographical zones and are separated into ten maturity groups – Group 00 to Group VIII (see Section IX.C). Seed production for these maturity groups is grown in the respective geographical zone for each maturity group. However, the production areas generally are on the northern edge of the respective zone to minimize incidences of disease.

Soybean seed is produced by companies which produce and sell seed, such as Monsanto Company, Pioneer Hi-Bred International, Syngenta Seeds, Kruger Seed Co., Becks Hybrids, and tollers, which are companies that produce but do not sell certified seed, such as Remington Seeds LLC and Precision Soya. Seed companies and tollers in turn contract acreage with growers to produce the required amount of soybean seed. Production or processing plants at these seed companies identify top soybean growers to produce the seed and also monitor and inspect seed fields throughout the growing season. The production plants also clean, condition, and bag the harvested soybean seed as well as monitor and inspect all the processes at the plant. Production plants typically produce between 100,000 units to 2,000,000 units of soybean seed. Production plants will produce the various soybean varieties in different climates or environments to spread production risks.

The entire seed production process at the majority of private seed companies and tollers is International Organization for Standardization (ISO) certified and, therefore, includes internal and external audits (ISO, 2009). The ISO standards ensure desirable characteristics of seeds and services such as quality, safety, reliability, and efficiency. ISO standards represent an international consensus on good management practices with the aim of ensuring that the organization can consistently deliver excellent product or services. The standards not only must meet the customer's requirements and applicable seed regulatory requirements, but must aim to enhance customer satisfaction and achieve continual improvement of its performance in pursuit of these objectives.

The field operations and management practices for producing soybean seed are similar to normal soybean production. However, special attention is needed in certain areas to produce seed with high quality, high germination rates, and high genetic purity (Helsel and Minor, 1993). General guidelines specific for seed production are discussed below. The seed production field should not have been planted with soybean the previous crop in order to avoid volunteer soybean plants (even though the risk of soybean volunteer plants is negligible) and to ensure genetic purity.

Very early planting should be avoided because the seed produced from early planting often results in poorer quality seed (Helsel and Minor, 1993). Every effort must be made to eliminate weeds in a seed field through the use of herbicides and cultivation to prevent weed seed in the harvested soybean seed. Fields are scouted frequently for insect pests and insecticides are applied when insect pest infestations reach economic threshold levels. Foliar-applied fungicides should be considered when disease infestations are predicted in the area. Harvest should occur as soon as the mature soybean seed reaches 13% moisture content. Harvesting soybean seed with less than 13% moisture can cause damage to the seed coat and result in split soybean seed that can affect germination and viability. Harvesting equipment must be adjusted to minimize or avoid seed damage. Harvesting equipment must be cleaned before entering the seed fields to minimize genetic contamination. Certain handling equipment such as auger elevators, should be avoided because they can increase seed damage.

Field inspections are vital to ensure the soybean seed meets seed certification requirements, ISO certification standards, regulatory standards, and trait licensing

agreement standards. Field inspections are conducted on seed production fields throughout the soybean growing season to evaluate variety purity, ensure soybean plants are developing properly, and fields are maintained free of weeds, insects, and diseases. The fields also are mapped to ensure the seed field has the minimum federal isolation requirement of five feet (AOSCA, 2009). Some states and seed producers have a stricter isolation requirement of 10 feet.

Production plant personnel make every effort to avoid mechanical damage to the harvested seed during the screening, cleaning, and bagging processes. Specific methods are used to assure the genetic purity and identity of the seed is maintained throughout the handling and storage operation. Bin inspections and sample collections are conducted at storage locations at the plant to examine the physical characteristics of the soybean seed and ensure proper bin cleanout. Seed is inspected for appearance, disease, discoloration, seed coat, mechanical damage, inert matter, and weed seed. Warm and cold germination tests are conducted on all seed lots to verify acceptable germination rates. Many seed companies will also conduct tetrazolium staining tests to assess seed viability.

Commercially certified soybean seed must meet state and federal seed standards and labeling requirements. AOSCA standards for certified soybean seed are as follows: 98% pure seed (minimum), 2% inert matter (maximum), 0.05% weed seed (maximum, not to exceed 10 per lb.), 0.60% total of other crop seeds (maximum), 0.5% other varieties (maximum, includes off-colored beans and off-type seeds), 0.10% other crop seeds (maximum, not to exceed three per lb.), and 80% germination and hard seed (minimum) (AOSCA, 2009). State seed certification standards vary slightly from state to state and can be more restrictive than the seed standards of AOSCA.

MON 87769 seed will be produced in the same manner as commercially certified soybean seed, such that it will meet all state and federal seed standards and labeling requirements.

IX.C. Production Management Considerations

Pre-Season

Crop rotation, tillage system, row spacing, planting equipment, seed or variety selection(s), and soil fertility are areas that require production decisions well in advance of planting the soybean crop. Many of the decisions in this area are made immediately after harvest of the previous crop or sooner. There are many benefits to crop rotation, with the majority of the soybean acreage planted in a two-year corn-soybean rotation (see Section IX.G). Crop rotation is generally a long-term decision, but the rotation sequence can be modified to take advantage of a particular economic or market opportunity. The decision to plant soybean in a conservation tillage or no-till system may require special equipment and will be made long before planting. In addition, this decision usually will be a long-term commitment, provided the system is successful. A decision to change row spacing is a similar long-term commitment that generally requires new equipment.

The benefits of conservation tillage or no-till systems are well documented and include reduced soil erosion, reduced fuel and labor costs, and conserving soil moisture. In 2004, approximately 29.3 million acres (38.6%) of soybean were planted in a no-till system (CTIC, 2004). Slow soybean emergence and growth plus lower yields have been some of the concerns associated with adoption of conservation tillage systems in soybean, especially no-till. Research in Wisconsin and Minnesota shows that soil temperatures can be four to five degrees colder in no-till than conventional tillage systems, which can slow emergence but have little effect on soybean yield (Pedersen, 2008a). Improved planters for establishment of good soybean populations and planting Roundup Ready soybean to effectively control weeds in no-till fields have made no-till a viable production system for soybean. Researchers still recommend some spring tillage on fine-textured and poorly drained soils for proper seedbed preparation (Pedersen, 2008a).

Most field crops, including soybean, respond very well to fertilizer when planted in soils with low fertility levels. Soybean requires 16 essential elements for growth and development. Deficiencies in any of these elements can reduce yields (Hoeft et al., 2000). The primary or major essential nutrients are nitrogen, phosphorus and potassium. The soybean plant is a member of the legume family, like alfalfa and clover, and fixes a significant portion of its own nitrogen through the symbiotic relationship with the nitrogen-fixing Bradyrhizobia bacteria (*Bradyrhizobium japonicum*) that live in the nodules on its roots. Bradyrhizobia are unicellar, microscopic bacteria that invade the soybean plant through its root hairs (Hoeft et al., 2000). The plant responds to this invasion by forming nodules which contain colonies of bacteria. After being established on the soybean root, bacteria in the nodule take gaseous nitrogen from the atmosphere and fix it in forms easily used by the soybean plant. Since the bacteria are not native to U.S. soils, inoculation of the soybean seed is recommended when soybean has not been grown in a field for three to five years. Nitrogen fertilizer applications at planting generally do not improve yield and decreases nodulation while increasing the plant's dependency on the soil for nitrogen (Pedersen, 2008a). Therefore, nitrogen fertilizer seldom is applied prior to planting a soybean crop.

Soil tests are the only reliable way to determine the pH, phosphorus, and potassium levels in the soil. Liming and fertilizer requirements are subsequently determined based on soil test results. Ideal soil test results for corn are also ideal for soybean (Scott and Aldrich, 1970). In corn-soybean rotations in the Midwest, phosphorus and potassium fertilizers generally are applied prior to a corn crop in accordance with soil test recommendations but seldom are applied prior to a soybean crop. However, for soybean plants which require large amounts of phosphorus and potassium, fertilizer often is needed in some of the southern growing areas due to differences in crop rotations and soil types.

Although not common, deficiencies can occur in secondary nutrients (calcium, magnesium, and sulfur) or micronutrients (boron, chloride, copper, iron, manganese, molybdenum, and zinc). The availability of soil nutrients is dependent on soil acidity or pH level. Soybean is adversely affected when the pH is below approximately 5.8 (Hoeft et al., 2000). Since soybean is grown in rotation with corn and other crops, soil pH should be maintained at about 6.0 to 6.5 on acidic soils through the addition of limestone.

Soybean varieties are developed and adapted to certain geographical zones and are separated into ten maturity groups – Group 00 to Group VIII (Hammond et al., 1991; Zhang et al., 2004). Groups 00 and 0 are the earliest maturity groups and are adapted best to the area north of latitude 46º north. Succeeding groups are adapted further south with Groups I and II within latitudes 41º and 46º north, and Group III within latitudes 38º and 41º North. Group 00 through Group IV soybean varieties are planted in the Midwest and Eastern Coastal regions. Groups II, III and IV account for approximately 75%, 24%, 36%, and 16%, respectively of the soybean planted in the U.S., with Group III having the largest acreage (T. Schlueter, personal communication, 2008). Groups IV through VIII are planted in the southern states with Groups V, VI and VII representing 7%, 2%, and 2% of the planted soybean, respectively (T. Schlueter, personal communication, 2008).

Soybean variety selection is crucial for high yield and quality, and is the foundation of an effective management plan (Pedersen, 2008a). Soybean characteristics to consider in selecting a variety include maturity, yield potential, disease and pest resistance, iron deficiency tolerance (chlorosis), lodging score, height, and specific soybean quality traits, such as protein and oil content. If a field has a history of a particular disease or pest, planting soybean varieties that have resistance or tolerance to these pests and diseases can be an effective and economical method of control.

Row spacing is important to maximize soybean yield. Research in the Midwest over the past 20 years consistently shows that row spacing of less than 20 inches is preferred for soybean regardless of tillage system, rotation sequence or planting date (Pedersen, 2008a). In the Southern states, the advantage from narrow rows is less consistent and less beneficial. In 2000, approximately 40% of soybean was planted in row spacing of 10 inches or less, 27% in 10.1 to 28.5 inches, and 33% in rows wider than 28.5 (Hoeft et al., 2000).

Planting and Early Season

An understanding of the growth stages of soybean is also important for the proper timing of certain management practices, such as herbicide and insecticide applications. In addition, the impact of certain weather conditions, insect pests, and diseases on soybean yield is dependent on growth stage. The system of soybean growth stages divides plant development into vegetative (V) and reproductive (R) stages (Pedersen, 2008a). The vegetative stages begin with VE, which designates emergence. V stages continue and are numbered according to how many fully developed trifoliate leaves are present (i.e., V1, V2, etc.). The reproductive (R) stages begin at flowering (R1) and include pod development and plant maturation. Full maturity is designated as R8.

Adequate soil moisture and warm temperatures facilitate rapid seed germination and emergence. The ideal soil temperature for soybean germination and emergence is 77 ºF (Pedersen, 2008a). However, waiting for soils to reach this soil temperature will delay planting beyond the optimum planting date that will maximize yield. Soybean can germinate at a soil temperature of 50 ºF when planted at a depth of two inches. However, emergence is slow and can take up to three weeks in Northern climates. Because of fluctuations in soil temperature in early spring, soil temperature should not be the only criteria for optimum planting time. Planting into a good seedbed is the most important

consideration. Planting into soil that is too wet will reduce emergence and plant population, and can lead to reduced yield.

Planting date has the greatest impact on yield according to research conducted in the Northern states (Hoeft et al., 2000). Highest yields generally are obtained when planting is in early to mid May. Yields begin to drop off quite rapidly when planting is delayed until late May. For example, the optimum planting dates for soybean in Iowa are the last week of April in the southern two-thirds of the state and the first week of May in the northern one-third of the state (Pedersen, 2008a). In the Southern U.S., planting adapted varieties before late April results in shorter plants and, in many cases, lower yields than when the same varieties are planted in May or early June. Planting after early June generally decreases plant height and yield due to water shortages in July and August.

Variations in plant spacing through row spacing and plant population have a significant effect on canopy development and soybean yield. Soybean has the ability to produce good yield over a wide range of plant populations. Most soybean varieties have the ability to branch and adjust the number of pods on branches to compensate for large differences in seeding rate. Maximum yields generally require planting rates that result in about 2.5 to five plants per square foot (Hoeft et al., 2000). Therefore, a full stand of soybean is approximately eight to 10 plants per foot of row at harvest for 40-inch rows, six to eight plants per foot of row in 30-inch rows, four to six plants in 20-inch rows, and two to three plants in 10-inch rows. This translates to 109,000 to 218,000 plants per acre at harvest. Higher populations are recommended in narrow rows for maximum yields because plants are more uniformly spaced in narrow rows. Seeding rates are generally 10 to 25% higher than the desired harvest population, especially in no-till, to account for the losses in germination, emergence, and seedling diseases. The accuracy of the planting equipment also can impact the decision on seeding rate. Soybean seed traditionally has been sold by weight. Therefore, the farmer must know the number of seeds per pound for the particular soybean varieties being planted for accurate seeding rates.

Treating soybean seed with a fungicide (e.g., pyraclostrobin, metalaxyl, mefenoxam) to prevent damping-off diseases may be beneficial when planting in cold, wet soils, using reduced till and no-till planting systems, and when planting seed with a low germination rate (<80%) or low seed vigor.

Annual and perennial weeds are considered to be the greatest pest problem in soybean production (Aref and Pike, 1998). In order to maximize yields, weeds must be controlled during the early growth stages of soybean because weeds compete with soybean for water, nutrients, and light. A combination of tillage and herbicides are utilized to control weeds throughout the growing season.

Mid to Late Season

Ideal daytime temperatures for soybean growth are between 75 ºF and 85 ºF (Hoeft et al., 2000). Warmer temperatures result in larger plants and earlier flowering. Sustained temperatures below 75 ºF will delay the beginning of flowering significantly. Seed set also is affected by temperature. Seed set is generally good when pollination follows

night temperatures around 70 ºF. Soybean varieties differ in their response and tolerance to temperatures.

Soybean is photoperiod sensitive, which means that it transitions from vegetative to flowering stage in direct response to length of daylight (Scott and Aldrich, 1970). Most soybean varieties begin flowering soon after the day length begins to shorten. Flowering of southern varieties is initiated by a shorter day than varieties adapted to the north. The extent of vegetative growth occurring after the initiation of flowering depends not only on environmental factors but also on the growth habit. Soybean varieties are described as either indeterminate or determinate in their growth habit (Scott and Aldrich, 1970). Indeterminate varieties increase their height by two to four times after flowering begins. These are grown in the Northern and Central U.S. Determinate varieties increase their height very little after flowering and generally are grown in the Southern U.S. Indeterminate and determinate varieties also differ in flowering characteristics. Indeterminate plants generally bloom first at the fourth or fifth node and progress upward. Flowering on determinate plants begins at the eight or tenth node and progresses both downward and upward.

The first appearance of flowers signals the beginning of the reproductive stage, namely the R1 stage (Hoeft et al., 2000). The reproductive period consists of flowering, pod set, and seed formation. Climatic conditions such as temperature and moisture supply during the flowering period will affect the number of flowers. The soybean plant does not form a pod for each flower. It is common for the soybean plant to have 75% of the flowers fail to develop a pod (Scott and Aldrich, 1970). This characteristic makes soybean less susceptible than corn to short periods of adverse weather during flowering. Under normal conditions, pod set occurs over about a three-week period. Good soil moisture is most critical during the pod-filling stages to prevent pod abortion and to ensure high yields (Hoeft et al., 2000). Another critical period is during the seed-filling stages to assure high rates of photosynthesis. High humidity and temperatures during seed development and maturity can result in poor seed quality since these conditions promote the development of reproductive-stage diseases.

Harvest Season

When dry matter accumulation ends, the plant is considered to be physiologically mature. The seed moisture content is approximately 55 to 60% at this stage (Hoeft et al., 2000). At this stage, namely R7, at least one normal pod on the plant reaches the mature pod color. Under warm and dry weather conditions, seed moisture content will drop to 13 to 14% in 10 to 14 days from physiological maturity (Hoeft et al., 2000). Soybean can be harvested when the moisture content drops below 15%. However, soybean should be at 13% moisture to be stored without artificial drying (Scott and Aldrich, 1970). Moisture content below 12% may increase seed cracking and seed coat damage.

Pre-harvest losses are influenced by variety, weather, and timeliness of harvest (Scott and Aldrich, 1970). Timely harvest when the moisture content is 13 to 14% also will minimize losses. Proper operation and adjustment of the combine is essential to minimizing harvest losses in the field.

IX.D. Weed Management

Annual weeds are typically the greatest pest problem in soybean production, followed by perennial weeds (Aref and Pike, 1998). Soybean insects and diseases are generally 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. Weeds can harbor insects and diseases, and can also interfere with harvest, causing extra wear on harvest equipment (Pedersen, 2008a). The primary factors affecting soybean yield loss from weed competition are the weed species, weed density, and the duration of the competition. When weeds are left to compete with soybean for the entire growing season, yield losses can exceed 75% (Dalley et al., 2001). Generally, the competition increases with increasing weed density. The time period that weeds compete with the soybean crop influences the level of yield loss. In general, the later the weeds emerge, the less impact the weeds will have on yield. Soybean plants withstand early-season weed competition longer than corn, and the canopy closes earlier in soybean than corn. In addition, canopy closure is much sooner when soybean is drilled or planted in narrow rows.

Crop rotations and environment have a significant impact on the adaptation and occurrence of weeds in soybean. Foxtail spp. (foxtail species group), pigweed, velvetleaf, lambs quarters, and cocklebur are common weeds in Midwest corn and soybean fields. However, growers consider giant ragweed (*Ambrosia artemisiifolia*), lambsquarters (*Chenopodium album*), Canada thistle (*Cirsium arvense)*, cocklebur (*Xanthium strumarium*), and velvetleaf (*Abutilon theophrasti*) to be the top five most problematic weeds in corn and soybean because of the difficulty to control these weeds (Nice and Johnson, 2005). The most frequently reported common weeds in the Southeast region are morning glory (*Ipomoea spp.*), prickly sida (*Sida spinosa*), johnsongrass (*Sorghum halepense*), sicklepod (*Cassia obtusifolia*), and broadleaf signalgrass (*Brachiaria platyphylla*) (Webster et al., 2005).

Cultural and mechanical weed control practices are important components of an effective weed management program (Baumann et al., 2008). Crop rotation, narrow row spacing, and planting date are a few of the crop management practices that are implemented to provide the crop with a competitive edge over weeds. Although the primary purpose of tillage is for seedbed preparation, tillage still is used to supplement weed control with selective herbicides in soybean production. Approximately 98 percent of the soybean acreage received an herbicide application in 2006, indicating the importance of excellent weed control in maximizing soybean yield (USDA-NASS, 2007b). Herbicide-tolerant soybean was introduced to provide growers with additional options to improve crop safety and/or improve weed control. The Roundup Ready soybean system, i.e., planting Roundup Ready soybean and applying glyphosate in crop has become the standard weed control program in U.S. soybean production. Currently, Roundup Ready soybean is planted on 91 percent of the soybean acreage (USDA-NASS, 2007a). Consequently, glyphosate is the most widely used herbicide in soybean, being applied on 96 percent of the soybean acreage in 2006 (USDA-NASS, 2007b).

IX.E. Management of Insects

Although insects are typically 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 the yield of soybean (Aref and Pike, 1998). Understanding the impact of insects on soybean growth is essential for proper management (Way, 1994). It is important to understand the way that insects injure soybean as well as how the soybean plant responds to insect injury. Insect injury can impact yield, plant maturity, and seed quality. Injury is defined as a stimulus producing an abnormal change in plant physiological processes. Injury may produce stress which is a departure from optimal physiological conditions (Way, 1994). The ultimate impact of injury is damage, a measurable reduction in plant growth development or reproduction. Insect injury in soybean seldom reaches levels to cause an economic loss as indicated by the low percentage (16%) of soybean acreage that receives an insecticide treatment.

Characterizing soybean responses to insect injury is essential in establishing economic injury levels (Way, 1994). Most often, soybean insects 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 their damage. The leaf-feeding insects comprise the biggest group of insects, but not necessarily the most damaging insects. Recent research on defoliation has determined that a major effect of injury is to reduce light interception by the soybean canopy which in turn can have a significant effect on yield (Way, 1994). Soybean has an extraordinary capacity to withstand considerable defoliation early in the season without significant yield loss, whereas, defoliation during the flowering and pod filling stages poses a greater threat to yield because the soybean plant has less time to compensate for injury compared to other growth stages. Research indicates that the soybean plant can sustain a 35% leaf loss prior to the pre-bloom period without lowering yield (NDSU, 2002). However, from pod-set to maturity, the plant can tolerate only a 20% defoliation level before yield is impacted.

IX.F. 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; and Funderburk et al., 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., 2000). 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; and Funderburk et al., 1999).

One or more diseases generally can be found in fields wherever soybean is grown (Bowers and Russin, 1999; and Funderburk et al., 1999). However, a pathogen may be very destructive one season and difficult or impossible to find the next season. The extent and severity of soybean diseases depend on the degree of compatibility between the host and the pathogen and the influence of the environment.

According to field surveys conducted in 15 soybean producing states during 1996 to 1998, soybean cyst nematode (*Heterodera gylcines*) caused the greatest soybean yield losses (Wrather et al., 2000). Phytophthora root and stem rot (*Phytophthora sojae*), brown stem rot (*Phialophora gregata*), sclerotinia stem rot (*Sclerotinia sclerotiorum*), and seedling diseases followed in economical importance. As expected, yield losses varied by region. Sclerotinia stem rot caused yield losses in several northern states, but not in other states. *Rhizoctonia* foliar blight losses were greatest in Arkansas, Louisiana, and Texas where humidity and temperature conditions are suitable for disease development.

Selecting resistant varieties is the primary tool growers have for disease control (Bowers and Russin, 1999; and Funderburk et al., 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; and Funderburk et al., 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 inoculums. One exception is *Rhizontonia*, a soil-inhabitant pathogen that grows on a wide variety of crops and can survive sufficiently in the soil to make crop rotation as a means of controlling this pest impractical. Row spacing, plant population, and planting date can also be changed to manage soybean diseases.

Soybean cyst nematode (SCN) is one of the most damaging pathogens of soybean throughout the soybean growing regions of the U.S. (Pedersen, 2008b). Losses have been estimated to be at about $1.5 billion in the U.S. (Pedersen, 2008a). SCN can cause yield losses up to 50%. This pest in 2004 caused an estimated loss of 50 million bushels in Iowa (Pedersen, 2008b). Soybean cyst nematodes feed on the roots causing severely stunted and yellow plants. The simplest, least expensive method to reduce populations of this pest is to rotate soybean with a non-host crop, such as corn, small grains, or sorghum. Planting resistant varieties is regarded as the best and most effective management practice to prevent losses from this pest. Several public and private soybean varieties offer sources of resistance to certain races of nematode. Alternating varieties with different sources of resistance may be beneficial.

High-quality seed is essential for controlling seedling diseases. The most important seedling diseases in soybean are *Phytophthora, Pythium, Rhizoctonia, and Fusarium* (Pedersen, 2008a). Many soybean varieties have race-specific resistance to *Phytophthora.* Treating soybean seed with a fungicide (e.g., pyraclostrobin, metalaxyl, mefenoxam) is effective against damping-off disease (seedling blight) caused by common soil fungi, such as *Phytophthora* and *Pythium*. Fungicide seed treatments are recommended where there is a history of these seedling diseases.

Asian soybean rust is a foliar fungal disease that typically infests soybean during reproductive stages of development and can cause defoliation and reduce yields significantly in areas such as Brazil (Dorrance et al., 2007). Soybean rust is caused by

the fungus *Phakopsora pachyrhizi*. This disease in the U.S. was first detected in Louisiana in 2004. Foliar application of fungicides is the standard disease management practice to limit yield losses due to soybean rust at this time.

Foliar fungicide applications can effectively reduce the incidence of many diseases (Bowers and Russin, 1999; and Funderburk et al., 1999). However, the economic return from a fungicide application may be limited to select production programs, e.g., primarily to high-yield environments or when producing soybean seed. According to USDA-NASS statistics, fungicides were applied on approximately 4% of the soybean acreage in 2006 (2007b).

IX.G. Crop Rotation Practices in Soybean

The well-established farming practice of crop rotation is still a key management tool for growers. The purpose of growing soybean in rotation with other crops is 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; Boerma and Specht, 2004). According to USDA Economic Research Service crop residue management studies, 95% of the soybean-planted acreage has been in some form of a crop rotation system since 1991 (USDA-ERS, 2006a). Corn- and wheat-planted acreage has been rotated at a slightly lower level of 75% and 70%, respectively. Although the benefits of crop rotation can be substantial, the farmer must make cropping decisions by evaluating both the agronomic and economic returns on various cropping systems. Crop rotations also afford growers the opportunity to diversify farm production in order to minimize market risks.

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). Corn and soybean occupy more than 80% of the farmland in many of the Midwestern states, and the two-year cropping sequence of a soybean-corn rotation is used most extensively in this region. However, a soybean crop sometimes is grown after soybean and then rotated to corn in a three-year rotation sequence (soybean-soybean-corn) in the Midwest. Compared to corn, soybean shows a greater response to being grown after a number of years without soybean. The yields of both corn and soybean are approximately 10% higher when grown in rotation than when either crop is grown continuously (Hoeft et al., 2000).

A combination of conservation tillage practices and crop rotation has been shown to be very effective in improving soil physical properties. Long-term studies in the Midwest indicate that a corn-soybean rotation improves yield potential of no-till systems compared to continuous corn production (Al-Kaisi, 2001). The reduction in yield of continuous corn production in no-till systems is attributed to low soil temperature during seed germination, which is especially evident on poorly drained soils under no-till practices.

Unique to the southern portion of the Midwest region and the mid-south states, soybean is grown in a double-cropping system. Double-cropping refers to the practice of growing

two crops in one year. This practice can improve income and reduce soil and water losses by having the soil covered with a plant canopy most of the year (Hoeft et al., 2000). In the Midwest, winter wheat is harvested in late June or July, and then soybean is planted into the wheat stubble in a no-till system to conserve moisture. Due to the uncertainty of double-cropping yields, farmers sometimes do not plant if soils are too dry at the time of wheat harvest. Soybean is typically grown in a corn-wheat-soybean rotation sequence when grown in a double-cropping system. In the northern soybean growing areas, wheat often will follow soybean in the rotation.

IX.H. Soybean Volunteer Management

Volunteer soybean is defined as a plant that has germinated and emerged unintentionally in a subsequent crop. Soybean seeds can remain in a field after soybean harvest 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 the combine is improperly adjusted for efficient harvesting. Volunteer soybean in rotational crops is typically not a concern in the Midwest region because the soybean seed is typically not viable after the winter period (Carpenter et al., 2002; OECD, 2000). In southern soybean growing areas of the U.S. where the winter temperatures are milder, it is possible for soybean seed to remain viable over the winter and germinate the following spring.

Volunteer soybean is normally not a concern in rotational crops such as corn, cotton, rice, and wheat that are the significant rotational crops following soybean due to control measures that are available for volunteer soybean when they arise (Carpenter et al., 2002; OECD, 2000). Preplant tillage is the first management tool for control of emerging volunteer soybean in the spring. If volunteer soybean should emerge after planting, shallow cultivation will control most of the plants and effectively reduce competition with the crop. Several postemergence herbicides also are available to control volunteer soybean (conventional or glyphosate-tolerant soybean) in each of the major rotational crops. Table IX-4 provides control ratings on volunteer glyphosate tolerant soybean for several herbicides used in the major rotational crops.

To provide control of volunteer soybean in corn, postemergence applications of AAtrex (atrazine), Clarity (dicamba), Distinct (diflufenzopyr + dicamba), Hornet (flumetsulam + clopyralid) and Widematch (clopyralid + fluroxypyr) provide excellent control (Zollinger, 2005). In wheat, Bronate Advanced (bromoxynil), Clarity (dicamba) and Widematch postemergence provide excellent control of volunteer soybean (Zollinger, 2005).

Volunteer soybean in cotton is normally not a concern. However, hurricanes or other extreme weather conditions can damage a soybean crop preceding cotton production in the Mid-south states, where the unharvested soybean seed can produce volunteer plants. Preplant applications of paraquat or herbicide mixtures containing paraquat will effectively control volunteer glyphosate-tolerant soybean (Montgomery et al., 2002; Murdock et al., 2002). Recent research in North Carolina indicates Envoke (trifloxysulfuron) will provide excellent postemergence control of soybean with traits for

glyphosate and sulfonylurea herbicide tolerance in Roundup Ready cotton (York et al., 2005).

Volunteer soybean in rice is rarely a concern due to the combination of preplant tillage, flooding practices, and herbicides utilized in producing rice. If volunteer plants should emerge in rice, the postemergence applications of Grasp (penoxsulam), Permit (halosulfuron) and Regiment (bispyribac) typically used for weed control in rice will effectively alleviate competition from volunteer soybean (Dillon et al., 2006).

Table IX-4. Ratings for Control of Volunteer Glyphosate-Tolerant Soybean in Labeled Rotational Crops[1]

Product	Rate (Product/Acre)	Soybean V2 – V3	Soybean V4- V6
Corn[2]			
AAtrex	0.38 qts	E	P
	0.50 qts	E	F
Clarity	4 fl oz	E	E
	5 fl oz	E	E
Distinct	1 oz	E	G
	2 oz	E	E
Hornet	1 oz	E	F
	2 oz	E	F-G
Widematch	0.25 pt	E	G
Wheat[2]			
Bronate Advanced	0.8 pt	E	E
Clarity	4 fl oz	E	E
	5 fl oz	E	E
Widematch	0.25 pt	E	G
Cotton[3]			
Envoke	0.1 oz	E	E
Rice[4]			
Grasp	2 oz	E	NA
Permit	1 oz	E	NA
Regiment	0.4 oz	E	E

NA denotes "not applicable."

[1] Weed control ratings: E = Excellent (90 to 99% control), G = Good (80 to 90% control), F = Fair (65 to 80 control), and P = Poor (40 to 65% control).

[2] Zollinger, 2005.

[3] York et al., 2005.

[4] Dillon et al., 2006.

IX.I. Stewardship of MON 87769

Monsanto Company is firmly committed to its legal, ethical, and moral obligation to ensure 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[SM] Program (http://www.excellencethroughstewardship.org/). These policies and practices include rigorous field compliance and quality management systems and verification through auditing.

As with all of our products, Monsanto is committed to the rigorous product stewardship of MON 87769. In keeping with past practice, Monsanto will seek regulatory approval for MON 87769 in all key soybean import countries with a functioning regulatory system to assure global compliance and support the flow of international trade. Monsanto continues to monitor other countries that are key importers of soybean from the U.S., for the development of formal biotechnology approval processes. If new functioning regulatory processes are developed, Monsanto will make appropriate and timely regulatory submissions.

Once appropriate approvals are received, as with other value-added specialty soybean products, MON 87769 will be grown using an appropriate IDP system based on established practices as described in Section IX.B.3. IDP practices are implemented for value-added specialty soybean to capture the enhanced value of the product and ensure that the enduser or processor receives the soybean with the identity, fatty acid composition of the oil, and desired quality.

Prior to obtaining all key global regulatory approvals, Monsanto will conduct seed testing, variety development and production, and oil manufacturing, testing and commercial activities leading up to the commercial introduction of MON 87769. Monsanto will work in a closed loop system under contract with partners to plant, harvest and process MON 87769 to produce the SDA soybean oil

A closed loop system will utilize appropriate processes for containment, documentation and traceability of seed production, planting, harvest, processing and use of the product. Grain production processes will include mass balance and accounting of all planting, harvested seed, secured storage facilities, labeling of all soybean, training of personnel, identification and audit of field production sites and acreage, spatial isolation of fields, equipment clean out procedures and documentation. Soybean processing will include segregation, control and traceability of handling, processing, packaging, and shipping of products and co-products.

Before implementing a closed loop system, Monsanto will dialogue with the appropriate value chain stakeholders in the countries of production and product use to gain feedback on and confirm the robustness and validity of the closed loop production system. Monsanto will not employ the closed loop system without adequate assurance that the system will be effective in containing, preventing the escape of, and controlling the disposition of MON 87769, so it is not comingled with commodity soybean. As part of this process, Monsanto will continue to provide regular updates on MON 87769 to key members of the soybean industry grain trade, processing industry and food industry throughout the regulatory and product development process.

IX.J. Impact of the Introduction of MON 87769 on Agricultural Practices

Introduction of MON 87769 is expected to have no impact on current cultivation and management practices for soybean. MON 87769 has been shown to be no different from conventional soybean in its agronomic, phenotypic, ecological, and compositional characteristics (refer to Sections VII, VIII, IX), except for the intended change in fatty

acid composition (presence of SDA and GLA in MON 87769 seed). MON 87769 is a nutritionally improved specialty soybean product that is expected to bring added value to consumers. The added value of MON 87769 is expected to bring higher returns to growers compared to commodity soybean.

X. PLANT PEST ASSESSMENT

This section provides a brief review and assessment of the plant pest potential of MON 87769 and its impact on agronomic practices. USDA-AHPIS has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. APHIS 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 no longer should 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.

The definition of "plant pest" in the Plant Protection Act (PPA) includes living organisms that could directly or indirectly injure, damage, or cause disease in any plant or plant product [7 U.S.C. § 7702(14)]. Information in this petition related to plant pest risk characteristics include disease and pest susceptibilities, expression and characteristics of the gene products (PjΔ6D and NcΔ15D proteins), impacts to NTOs, changes to plant metabolism, weediness of the regulated article, impacts on agronomic practices, any impacts on the weediness of any other plant with which it can interbreed, and the transfer of genetic information to organisms with which it cannot interbreed.

The regulatory end-point under the PPA for biotechnology-derived crop products is not zero risk, but rather a determination that deregulation of the regulated article is not likely to pose a plant pest risk. The plant pest risk assessment of MON 87769 is based primarily on eight lines of evidence: (1) modern soybean has inherently low plant pest potential, (2) insertion of a single functional copy of the inserted expression cassette, (3) characterization of the PjΔ6D and NcΔ15D proteins expressed in MON 87769 and the altered soybean oil profile, (4) mode of action and safety of the PjΔ6D and NcΔ15D proteins and the altered soybean oil profile, (5) compositional equivalence of MON 87769 seed as compared to conventional soybean, with the exception of intended fatty acid change, (6) phenotypic and agronomic characteristics demonstrating no increased plant pest potential, (7) negligible risk to NTOs and threatened or endangered species, and (8) no greater likelihood to impact agronomic practices, including land use, cultivation practices, or the management of weeds, diseases, and insects than conventional soybean.

As part of the plant pest risk assessment, the genetic construct inserted into MON 87769 was evaluated to determine if those sequences cause plant disease. Morphological characteristics of MON 87769 were analyzed to determine if it will be any more weedy or invasive relative to soybean varieties currently on the market. Agronomic practices associated with MON 87769 were considered relative to potential changes that could lead to increased plant pest potential. The potential for gene flow and introgression of the genetic construct into other plant varieties or wild relatives also was evaluated to determine the potential of increased weedy or invasive characteristics in sexually compatible plants. Finally, the propensity of MON 87769 to become a greater reservoir

of plant pests (insects or pathogens) compared to conventional soybean and the potential for horizontal gene transfer were evaluated. Using this risk assessment process, the data and analysis presented in this petition lead to a conclusion that MON 87769 is unlikely to be a plant pest and, therefore, should no longer be subject to regulation under 7 CFR § 340.

The assessment of the impact of MON 87769 on threatened and endangered species and other NTOs concludes that risk to these organisms from the use of MON 87769 is negligible. This risk assessment took into consideration several components, including similarity of PjΔ6D and NcΔ15D proteins to other desaturases present in plants, the functional activity of the PjΔ6D and NcΔ15D proteins, and the expression level of the PjΔ6D and NcΔ15D proteins in MON 87769. The evaluation of weediness and gene flow potential concluded that MON 87769 is no more likely to become a weed than conventional soybean, and MON 87769 is expected to be similar to conventional soybean regarding the potential for and impact from gene flow. Due to the lack of sexually compatible relatives in the U.S., pollen-mediated gene flow is expected to occur only within cultivated soybean. Given the reproductive biology of soybean, pollen-mediated gene flow is expected to be negligible within cultivated soybean. The probability for horizontal gene flow is exceedingly small. Even if it were to occur, the consequences would be negligible because the genes introduced into MON 87769 (*Pj.D6D* and *Nc.Fad3*) are of plant and fungal origin and the proteins produced from the introduced genes (PjΔ6D and NcΔ15D) and the intended fatty acid change have no meaningful toxicity to humans and other NTOs under the conditions of use.

An assessment of current soybean agronomic practices in the U.S. indicates that the introduction of MON 87769 will not impact current U.S. soybean cultivation practices and the management of weeds, diseases, and insects (see Section IX). The impact of MON 87769 on agronomic practices associated with production of soybean is addressed in Sections IX and XI.

APHIS has recently proposed to amend 40 CFR § 340 to include elements derived from the noxious weed authority provided under the Plant Protection Act. Because the data show that MON 87769 has no potential to cause injury or damage to any protected crops, livestock or interests of agriculture, MON 87769 also would not be considered a "noxious weed" as defined by the Plant Protection Act.

X.A. Plant Pest Assessment of MON 87769

X.A.1. Characteristics of the Genetic Insert

MON 87769 was developed through *Agrobacterium*-mediated transformation of meristematic soybean tissue using the binary transformation plasmid PV-GMPQ1972 (Section IV: Figure IV-1and Table IV-1). Molecular analyses indicate that MON 87769 contains one copy of the insert at a single integration locus. No additional elements from the transformation vector were detected in the genome of MON 87769, including T-DNA II or backbone sequence from plasmid PV-GMPQ1972. Additionally, the data confirm the organization and sequence of the insert, and demonstrate the stability of the

insert over several generations. On the basis of these data, it is concluded that only the expected PjΔ6D and NcΔ15D proteins are produced from the inserted DNA. Molecular analysis confirmed that MON 87769 does not contain elements that promote plant disease (refer to Table IV-1).

X.A.2. Mode of Action and Safety of PjΔ6D and NcΔ15D Proteins

The PjΔ6D and NcΔ15D proteins expressed in MON 87769 are members of a family of integral membrane fatty acid desaturases found in all eukaryotic organisms (plants, animals, and fungi) and some prokaryotes, e.g. cyanobacteria (Hashimoto et al., 2008; Los and Murata, 1998). The PjΔ6D and NcΔ15D proteins expressed in MON 87769 are nearly identical to the native proteins produced by *Primula juliae* (primrose) and *Neurospora crassa*, respectively.

The general mode of action of the PjΔ6D and NcΔ15D proteins are well understood (see Section IV.A). PjΔ6D catalyzes the desaturation of ALA (18:3) to form SDA (18:4) in the seed of MON 87769 and also converts LA (18:2) to GLA (18:3). NcΔ15D catalyzes the desaturation of LA to ALA and, therefore, reduces the substrate pool for GLA production and increases the substrate pool for SDA production (Figure VII-1).

The safety assessment of the PjΔ6D and NcΔ15D proteins included extensive protein characterization that demonstrated the lack of similarity to known allergens and toxins and a long history of safe consumption of similar proteins. Expression of the two proteins in leaf and root tissue was below the limit of detection of 0.1 – 0.2 μg/g of tissue on a fresh weight (FW) basis for PjΔ6D and 0.5 – 1.0 μg/g on a FW basis for NcΔ15D. The mean PjΔ6D protein levels in immature seed, mature seed, and forage were 100, 1.8 and 16 μg/g DW, respectively. The mean NcΔ15D protein levels in immature seed, mature seed, and forage were 200, 10 and 14 μg/g DW, respectively. The forage samples, consisting of the above ground portion of plants with developing pods, were harvested at the R6 growth stage and included immature soybean seed. Thus, it was expected that the PjΔ6D and NcΔ15D proteins would be detected at low levels in the forage samples due to the presence of immature seed. Data from the safety assessment also confirmed that the full-length PjΔ6D and NcΔ15D proteins are readily digestible in simulated gastric and intestinal fluids and lack acute oral toxicity in mice (see Sections VI.D and VI.E.). Collectively, these data establish the safety of the PjΔ6D and NcΔ15D proteins. From these data, it can be concluded with reasonable certainty that the PjΔ6D and NcΔ15D proteins have no meaningful toxic potential to exposed organisms in the environment. Furthermore, these integral membrane proteins are structurally and functionally related to other desaturases that are widely distributed in plants and animals and are highly labile *ex vivo* (see Section VI). Therefore, it is expected that these proteins would neither persist in the environment, nor have any adverse effects on soil-dwelling organisms when post-harvest soybean biomass or detritus decomposes in soil.

X.A.3. Environmental Safety of Fatty Acids Produced in MON 87769

Data presented in Section VII summarize the composition of forage and the seed from MON 87769, the conventional control and ten commercial soybean varieties. Compositional analyses compared a total of 75 different analytical components, seven in forage and 68 in seed, between MON 87769 and a conventional soybean control with genetics comparable to MON 87769, but lacking the introduced trait. Data presented in Section VII indicate that there are no meaningful differences in compositional or nutritional quality of MON 87769 compared to conventional soybean, except for the intended seed fatty acid change (presence of SDA and GLA). Compositional data were statistically analyzed and while there were some statistical differences between MON 87769 and the conventional control, it is concluded that the statistical differences represent the natural variability for these analytes in soybean such that they were not regarded as biologically meaningful. Seed and forage analytical component values also were comparable to published scientific literature and the ILSI-CCD, further supporting the conclusion that seed and forage from MON 87769 are compositionally equivalent to those of conventional soybean, except for the intended seed fatty acid compositional changes. Thus, except for the intended fatty acid change, the composition of MON 87769 is not different from conventional soybean.

X.A.3.1. Biological Role and Metabolism of Polyunsaturated Fatty Acids

Polyunsaturated fatty acids are biologically significant compounds that play a role in most organisms in metabolic energy storage, as components of phospholipids, which are essential for cellular membrane formation and function, and as precursors to the biosynthesis of eicosanoids, which affect numerous biochemical reactions and physiological processes (MacDonald and Sprecher, 1991; Watkins, 1991and 1995). In insects, PUFAs also serve as precursors in the biosynthesis of waxes, sex pheromones, and as components of defensive secretions (Stanley-Samuelson et al., 1988). Most animals, including mammals, birds, and most insects, have a dietary requirement for PUFAs, particularly for LA and ALA. These fatty acids often are referred to as "essential fatty acids" (EFA) because of their biological significance, and since they cannot be synthesized *de novo*, they must be obtained from the diet. The 18-carbon (C18) PUFAs, including GLA and SDA, and C20 PUFAs, are derived from LA and ALA by a sequence of desaturation and elongation processes. One significant difference among insect species, as it relates to fatty acid biosynthesis, is their ability or inability to biosynthesize LA. Most insects have a dietary requirement for LA (Canavoso et al., 2001). However, at least 15 species are known to biosynthesize LA from acetate and, therefore, do not require dietary supplied LA (Stanley-Samuelson et al., 1988).

Linoleic acid is an essential fatty acid in vertebrates. It is the first fatty acid of the *n*-6 series of PUFAs which includes GLA. Deficiencies of LA in poultry have been reported to include retarded growth, increased water consumption, reduced disease resistance, enlarged livers, and reduced testes and delayed development of secondary sexual characteristics in males. Decreased egg size and weight and changes in yolk fatty acids result from LA-deficient laying hens (Watkins, 1991).

ALA is the first fatty acid of the *n*-3 series of PUFAs which includes SDA. ALA is considered an essential fatty acid; however, an absolute requirement of ALA in mammals, birds, and insects has not been empirically determined (Canavoso et al., 2001; Spector, 1999; Watkins, 1991). Nonetheless, ALA is necessary for normal brain and retina development in mammals and birds (Watkins, 1991) and metamorphosis in Lepidoptera and Hymenoptera (Canavoso et al., 2001).

In most mammals, birds, and insects, the metabolic fate of EFA is similar when they are either *de novo* biosynthesized by the organism or derived from a dietary source. Primary routes of metabolism include β-oxidization in the mitochondria to generate ATP, incorporation into cellular phospholipids, or further desaturation and elongation to form long chain (LC) PUFAs (Watkins, 1991; Stanley-Samuelson, 1988). The Δ6, Δ5, and Δ4 desaturases along with the elongases, mostly found in animals, are primarily responsible for the conversion of C18 to C20 PUFAs (Pan et al., 1994; Watkins, 1991; Stanley-Samuelson, 1988). Generally, the desaturation/elongation pathways of vertebrates, including mammals and birds, apply to most insect species in that ALA and LA are the precursors of the *n*-3 and *n*-6 PUFAs and are the immediate precursors of SDA and GLA, respectively. SDA is a normal metabolic intermediate in the conversion of ALA to eicosapentaenoic acid (EPA) by the actions of a Δ6 desaturase, an elongase, and a Δ5 desaturase (Cook, 1991). GLA is an intermediate in the conversion of LA to dihomo-γ-linolenic acid (DGLA) and arachidonic acid following the same pathway. C20 PUFAs, particularly EPA, DGLA, and AA, play important metabolic roles in most animals as precursors in the biosynthesis of physiologically active eicosanoids, which include the prostaglandins (PG), thromboxanes, leukotrienes, and lipoxins (Beare-Rogers et al., 2001).

Thus, PUFAs are important compounds in most animals. They are oxidized in energy production, incorporated in phospholipids and are essential for cellular membrane formation and function, desaturated/elongated, and oxygenated into physiologically active eicosanoids. SDA and GLA are PUFAs that are present in MON 87769 seed and are also consumed from other sources in the environment including plants, marine algae, fish/fish oil (see Section X.A.3.2). Therefore, these PUFAs in MON 87769 seed are expected to be absorbed, transported, and metabolized in animals in the same manner as they are from other sources. Their lack of toxicity is further supported by ecological interaction assessments conducted under field conditions (see Section VIII.D.2.2.).

X.A.3.2. Environmental Exposure to Fatty Acids in MON 87769

With the exception of SDA and GLA, the fatty acids present in MON 87769 are the same as those present in conventional soybean. Thus, the analysis here focuses on the potential impact from exposure of SDA and GLA. Environmental exposure to SDA and GLA from MON 87769 is expected to be limited primarily to soybean pests and animals that feed on seeds or potentially through trophic interactions. However, these fatty acids are not new and are already present in the environment. SDA, GLA, and other polyunsaturated fatty acids, such as ALA and LA, exist in many sources in the environment without known adverse ecological effects. SDA and GLA are produced in many plant seed oils. More than 230 species from among 17 plant families are

documented to produce SDA in seed oil at concentrations greater than 1% of their total fatty acid composition.[7] Twenty of those species from among three families (*Boraginaceae*, *Elaengaceae*, and *Primulaceae*) are reported to produce greater than 16% SDA, which is comparable to the levels of SDA produced in MON 87769. More than 360 species from 23 plant families are documented to produce GLA in seed oil at concentrations greater than 1% of their total fatty acid composition.[8] Over 230 of those species from 16 families are reported to produce greater than 6% GLA (approximately 6 – 8% GLA is produced in the MON 87769 seed). The seed oil from many of the commonly recognized plant-based sources of these two fatty acids are known to contain 0.3-22.5% SDA, 0.7-24.4% GLA, and varying concentrations of other related PUFAs, such as ALA and LA (Table X-1). The GLA content in many of these species exceeds that found in MON 87769. GLA is also present in oats, barley, and human breast milk (Horrobin, 1992). Flax seed (*Linum usitatissimum*) is consumed as a source of dietary fiber and is used to produce linseed oil. The oil from flaxseed is not a significant source of SDA or GLA but is a rich source (approximately 55% in total fatty acid composition) of ALA, an omega-3 fatty acid (Dubois et al., 2007).

Many fish species, particularly marine fish, contain SDA, which the fish obtain in their diet by consuming plankton, algae, and other fish (Table X-2). Fish meal has been used as an animal feed ingredient for over a century. The meal is fed to poultry, pigs, ruminants, farmed fish, crustaceans, fur-producing animals, laboratory animals, and household pets (Bimbo and Crowther, 1992; FAO, 1986; Thomson, 1990). In addition, fish meal also has been used as fertilizer for crop production, although its use in fertilizers has diminished as increasing amounts of the meal are diverted for use in animal feed (FAO, 1986). Fish oils that contain SDA include cod liver (0.94% w/w), herring (2.31% w/w), menhaden (2.74% w/w), salmon (2.80% w/w), and sardine (3.03% w/w) (USDA-ARS National Nutrient Database for Standard Reference).[9] Fish oil is used for human consumption and in animal feed and industrial applications, including soaps, lubricating greases, and waterproofing agents (Bimbo and Crowther, 1992; FAO, 1986). Oil from some fish species has been approved by the FDA as Generally Recognized as Safe (GRAS) and is used in many dietary supplements, along with plant-based sources of SDA, such as black currant. GLA may be found in some fish oils but at concentrations generally less than 0.3% (w/w).

A number of species of marine microalgae and macroalgae also contain SDA. Liu and Lin (2001) report that marine microalgae widely used as aquaculture feed contain SDA, including *Isochrysis* spp. (15.1-24.9% of total fatty acids) and *Pavlova* spp. (5.9-6.8% of total fatty acids). Edible macroalgae species *Ulva pertusa* and *Undaria pinnatifida* contain 16.3 and 26.3% SDA, respectively (Ishihara et al., 2000).

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[7] Federal Research Centre for Nutrition and Food – Institute for Lipid Research. 2008. Seed Oil Fatty Acids Online Database. http://sofa.bfel.de/ [Accessed on 8 December 2008].
[8] Federal Research Centre for Nutrition and Food – Institute for Lipid Research. 2008. Seed Oil Fatty Acids Online Database. http://sofa.bfel.de/ [Accessed on 8 December 2008].
[9] http://www.ars.usda.gov/nutrientdata [Accessed on 8 December 2008].

Additionally, even though conventional soybean seed is not a source of SDA and GLA exposure to the environment, conventional soybean is a well known source of fatty acids used in food and feed. Fatty acids such as ALA and LA are structurally similar to SDA and GLA. These closely related fatty acids have an established history of exposure in soybean without known adverse ecological effects.

Thus, the fatty acids in MON 87769 seed have an established history of exposure through other routes in the environment and have no known plant pest characteristics as defined in 7 U.S.C. § 7701-7772.

X.A.3.3. Environmental Fate of Fatty Acids Present in MON 87769

As natural components of the plant and animal world, SDA, GLA, and other PUFAs are not expected to accumulate, persist or to be detrimental to the environment. A major route for decomposition of PUFAs in the environment is enzymatic oxidation. The enzyme lipoxygenase, which is present in soybean, beans, peas and other plants, is a major transformation agent for PUFAs (Gardner, 1995; Feussner and Wasternack, 2002). PUFAs can also undergo non-enzymatic oxidative changes such as peroxidation, photo-oxidation, thermal oxidation, oxidative fission, hydrolysis and other reactions that convert the fatty acids into breakdown products (DeMan, 2000). The decomposition of PUFAs is primarily a result of their reactivity towards oxygen. The more unsaturated fatty acids are more reactive, especially the *n*-3 and *n*-6 fatty acids, as shown by the following oxygen reactivity index: stearic (18:0) = 1; oleic (18:1) = 100; linoleic (18:2) = 1200; and linolenic (18:3) = 2500 (DeMan, 2000). It is expected that SDA (18:4) and GLA (18:3) are at least as reactive, and likely more reactive as their immediate precursors (ALA and LA, respectively).

Based on the oxygen reactivity of *n*-3 and *n*-6 fatty acids, as well as the presence of lipoxygenase in soybean, it is very likely that any SDA, GLA, or other PUFAs from MON 87769 seed that reaches soil, water, or other environmental compartments will undergo rapid decomposition via oxidative and enzymatic processes and will not persist in or be detrimental to the environment or have any adverse effects on soil-dwelling organisms.

Table X-1. Plants that Contain Stearidonic Acid (SDA), Gamma-Linolenic Acid (GLA), and Other Polyunsaturated Fatty Acids (PUFAs)

Taxa[1]	Fatty acid composition of seed oil (%)				Source of Information
	C18:2*n*-6 (Linoleic)	C18:3*n*-6 (γ-Linolenic)	C18:3*n*-3 (α-Linolenic)	C18:4*n*-3 (Stearidonic)	
Aleuritia farinosa L.	29.9	1.8	29.2	17.5	Sayanova et al., 1999
Aleuritia scotica W.J. Hooker	26.9	2.2	29.0	22.5	Sayanova et al., 1999
Borago officinalis L. (starflower)	35.0	24.4	0.9	0.3	Velasco and Goffman, 1999
Dodecatheon meadia L. (pride of Ohio)	27.0	4.5	19.9	11.9	Sayanova et al., 1999
Dodecatheon tetrandrum L. (sierra shooting star)	26.8	2.5	27.9	12.2	Sayanova et al., 1999
Echium italicum L. (Italian viper's bugloss)	10.6	7.4	43.0	14.6	Özcan, 2008
Echium plantagineum L. (salvation jane)	13.8-15.2	9.2-11.3	33.3-36.7	12.9-13.4	Guil-Guerrero et al., 2000; 2006; Kleiman, 1964; Özcan, 2008
Echium vulgare L. (common viper's bugloss)	16.9	12.2	36.1	12.9	Özcan, 2008
Lappula squarrosa (Retz.) Dumort. (European stickseed)	13.6	7.3	27.6	17.1	Velasco and Goffman, 1999
Lithospermum arvense L. (field gromwell)	10.6	5.2	41.5	17.4	Velasco and Goffman, 1999
Lithospermum officinale L. (European stoneseed)	17.2	12.3	29.1	13.3	Velasco and Goffman, 1999
Primula juliae Kusn. (primrose)	22.1	0.7	35.5	11.5	Sayanova et al., 1999
Ribes nigrum (black currant)	48.5	15-19	13.5	3.5	Traitler et al., 1984
Ribes uva-crispa L. (European Gooseberry)	40.0	10-12	n.r.	n.r.	Traitler et al., 1984

[1] Common names provided in parentheses. n.r. = not reported.

Table X-2. Fish that Contain Stearidonic Acid (SDA)

Fish	% SDA[1]	Fish	% SDA	Fish	% SDA
Anchovy	0.055	Mullet	0.082	Shark	0.031
Bluefish	0.167	Ocean perch, Atlantic	0.024	Sheepshead	0.027
Bass, freshwater	0.039	Perch, mixed species	0.008	Smelt	0.025
Carp	0.058	Pollock, Atlantic	0.005	Snapper	0.009
Catfish, farmed	0.037	Rockfish, Pacific	0.016	Spot	0.16
Catfish, wild	0.013	Roe	0.012	Sturgeon	0.128
Cisco	0.029	Roughy, orange	0.001	Sucker, white	0.032
Cod, Atlantic	0.001	Sablefish	0.114	Tilapia	0.003
Croaker	0.011	Salmon, Atlantic, farmed	0.121	Trout, mixed species	0.064
Dolphinfish	0.005	Salmon, Atlantic, wild	0.083	Trout, rainbow, farmed	0.05
Flatfish	0.016	Salmon, chinook	0.188	Trout, rainbow, wild	0.066
Haddock	0.003	Salmon, chum	0.068	Tuna, fresh, bluefin	0.039
Halibut, Atlantic/Pacific	0.039	Salmon, coho, farmed	0.112	Tuna, fresh, skipjack	0.004
Halibut, greenland	0.132	Salmon, coho, wild	0.119	Tuna, fresh, yellowfin	0.005
Herring, Atlantic	0.214	Salmon, pink	0.09	Whitefish	0.05
Herring, Pacific	0.248	Salmon, sockeye	0.102	Whiting	0.041
Mackerel, king	0.278	Seabass	0.046	Wolffish	0.061
Mackerel, Atlantic	0.09	Seatrout	0.008		
Mackerel, Pacific	0.125	Shad, American	0.328		

Source: USDA-ARS National Nutrient Database for Standard Reference (http://www.ars.usda.gov/nutrientdata). [Accessed on 8 December 2008]
[1] Concentration of stearidonic acid (SDA) in raw fish expressed as the % of total mass.

X.A.4. Impact on NTOs

An evaluation of the potential risks to (NTOs) is an important component of an overall environmental risk assessment of a biotechnology-derived crop. An assessment of MON 87769 for potential risks to NTOs was conducted based on the finding that the presence of the inserted genes, the expression of the PjΔ6D and NcΔ15D proteins, and the production of the expected fatty acids (SDA and GLA) in MON 87769 seed were meaningful differences compared to conventional soybean. The nature of MON 87769 as a source of SDA and GLA with no pesticidal activity is such that all exposed organisms are considered to be NTOs. The assessment considered pertinent product characterization information, information from the protein safety assessments, history of environmental exposure to SDA and GLA (the additional fatty acids present in MON 87769 seed), and results from the ecological interaction assessment. Based on the weight of evidence of all the information reported herein, there is no indication that MON 87769 would have an adverse impact on NTOs.

X.A.4.1 Impact Due to introduced Genes and Proteins

Molecular analyses indicate that MON 87769 contains one copy of the insert at a single integration locus. No additional elements from the transformation vector were detected in the genome of MON 87769. Additionally, the data confirm the organization and sequence of the insert and demonstrate the stability of the insert over several generations. On the basis of these data, it is concluded that only the expected PjΔ6D and NcΔ15D proteins are produced from the inserted DNA (Section X.A.1).

From the protein safety assessment data (Sections VI and X.A.2), it can be concluded with reasonable certainty that the PjΔ6D and NcΔ15D proteins have no meaningful toxic potential to exposed organisms in the environment. Furthermore, these integral membrane proteins are structurally and functionally related to other desaturases that are widely distributed in plants and animals and are highly labile *ex vivo* (see Section VI). Therefore, it is expected that these proteins would neither persist in the environment, nor have any adverse effects on NTOs including soil-dwelling organisms when post-harvest soybean biomass or detritus decomposes in soil.

X.A.4.2. Potential Adverse Effects of MON 87769 Fatty Acids on NTOs

With the exception of the intended changes in fatty acid composition (presence of SDA and GLA), MON 87769 seed is compositionally equivalent to conventional soybean (see Section VII). While SDA and GLA are not typically produced in soybean, they are already present in the environment (Section X.A.3). Importantly, the history of exposure across a wide taxonomic range provides evidence that these fatty acids when present at the levels seen in MON 87769 are no more toxic than other fatty acids present in soybean to fish, birds, arthropods, or other animals. Their lack of toxicity is further supported by the known biological role and metabolism of polyunsaturated fatty acids, the ecological interaction assessments of MON 87769 conducted under field conditions, and published literature (Hammond et al., 2008; Harris et al., 2008).

X.A.4.3. Interactions with NTOs under Field Conditions

In an extensive environmental interaction assessment of MON 87769, more than 700 comparative observations for plant interactions with arthropods and diseases under field conditions over two consecutive years, including over 500 observations of plant damage caused by various arthropods and diseases, and 260 observations of the abundance of various pest and beneficial arthropods were conducted (see Section VIII.D.2.2.). Data support the conclusion that MON 87769 does not have increased susceptibility or tolerance to the evaluated arthropods or diseases or altered interactions with the evaluated pest and beneficial arthropods, as indicated by the abundance data, when compared to conventional soybean. In particular, there were no meaningful differences observed between MON 87769 and the control for damage from or abundance of soybean pests that directly feed on soybean seed and, thus, would be exposed to the PjΔ6D and NcΔ15D proteins and the additional fatty acids present in MON 87769 immature and mature seed. Thus, the results of the environmental interactions assessment further corroborate the hypothesis that the introduced genes (*Pj.D6D and Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change have no direct adverse or toxic effects on soybean pests or other NTOs.

X.B. Weediness Potential of MON 87769

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., 1979; Muenscher, 1980), nor is it identified as a noxious weed species on the list distributed by the federal government (7 CFR § 360). Soybean does not possess any of the attributes commonly associated with weeds (Baker, 1965), such as long persistence of seed in the soil, the ability to disperse, invade, and become a dominant species in new or diverse landscapes, or the ability to compete well with native vegetation. Due to a pronounced absence of dormancy, a trait that has been removed through commercial breeding, soybean seed can germinate quickly under adequate temperature and moisture and can potentially grow as volunteer plants. However, volunteer plants likely would be killed by frost in the soybean growing regions during autumn or winter of the year they were produced. If they 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). In addition, since the wild populations of *Glycine* species are not known to exist in the U.S., the potential does not exist for MON 87769 to outcross to wild or weedy relatives and alter their weediness potential.

In comparative studies between MON 87769 and a conventional soybean control, phenotypic, agronomic and environmental interaction data were evaluated (Section VIII) for changes that would impact the plant pest potential and, in particular, plant weediness potential. Results of these evaluations indicate that there is no fundamental difference between MON 87769 and the conventional control for traits associated with weediness. Collectively, these findings support the conclusion that MON 87769 has no increased weediness potential compared to conventional soybean. Data on environmental

interactions also indicate that MON 87769 does not confer any biologically meaningful increased susceptibility or tolerance to specific disease, arthropod, or abiotic stressors.

X.C. Potential for Pollen-mediated Gene Flow

Gene flow and gene introgression are processes whereby one or more genes successfully integrate into the genome of a recipient plant. 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. Since gene introgression is a natural biological process, it does not constitute an environmental risk in and of itself (Southerland and Poppy, 2005). 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 transgenes and their subsequent transfer to recipient plants will result in increased plant pest potential.

X.C.1. Vertical Gene Flow

X.C.1.1. Hybridization with Cultivated Soybean *Glycine max*

Although soybean is a largely self-pollinated species, low levels of natural cross-pollination can occur (Caviness, 1966; OECD, 2000). In studies with cultivated soybean where conditions have been optimized to ensure close proximity and flowering synchrony, natural cross-pollination has been found to be generally very low. Most outcrossing occurred with surrounding plants and cross-pollination frequencies vary, depending on growing season and genotype. Insect activity does increase the outcrossing rate, but soybean generally is not a preferred plant for pollinators (Erickson, 1975 and 1984).

Numerous studies on soybean cross-pollination have been conducted, and the published results (with and without supplemental pollinators) are summarized in Table X-3. Under natural conditions, cross pollination among adjacent plants in a row or among plants in adjacent rows ranged from 0.3 to 3.62% (Beard and Knowles, 1971). In experiments where supplemental pollinators (primarily 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: 0.02% at 8.2 m (Caviness, 1966), 0.05% at 5.4 m (Ray et al., 2003), and 0% at 6.5 m (Abud et al., 2003).

The potential for cross-pollination 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 so long as the distance is adequate to prevent mechanical mixing (USDA-APHIS, 2006).

In the event when cross-pollination may occur, MON 87769 and its progeny are not expected to have a significant environmental impact because evaluations have shown that

the introduced genes (*Pj.D6D* and *Nc.Fad*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D) and the intended fatty acid change are not likely to enhance weediness or plant pest potential. Therefore, the environmental consequence of pollen-mediated gene flow from MON 87769 to other soybean plants is considered negligible.

Table X-3. Summary of Published Literature on Soybean Cross-Pollination

Distance from Pollen Source	% Cross-Pollination	Comments	Reference
0.3 m	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 m	0.07 to 0.18%	Adjacent rows. Experiment conducted over two years. Several male and female parental varieties.	Garber and Odland, 1926
0.1 m	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 m	0.2 to 1%	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 m 2.7 – 4.6 m 6.4 – 8.2 m 10 – 15.5 m	0.03 to 0.44 % 0.007 to 0.04% 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 honeybee.	Abrams et al., 1978
0.1 – 0.6 m	0.5 to 1.03% (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 m	0.09 to 1.63%	Adjacent rows. Experiment conducted over two years. Several male and female parental varieties.	Ahrent and Caviness, 1994
0.5 m 1.0 m 6.5 m	0.44 to 0.45% 0.04 to 1.4% 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 m 5.4 m	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 m	1.8%	Interspaced plants within a row. Experiment conducted in a single year. Single male and female parental varieties.	Ray et al., 2003

X.C.1.2. Hybridization with the Wild Annual Species within Subgenus *Soja*

The subgenus *Soja* includes the cultivated soybean *G. max* and the wild annual species *G. soja.* *G. soja* is found in China, Taiwan, Japan, Korea, and Russia and can hybridize naturally with the cultivated soybean, *G. max* (Hymowitz, 2004). Hybridization between female *G. soja* and male *G. max* was less successful than hybridization in the opposing 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 F1 hybrids of *G. max* and *G. soja* carry similar genomes and are fertile (Singh and Hymowitz, 1989). Abe et al. (1999) notes that "natural hybrids between *G. max* and *G. soja* are rare ... and hybrid swarms involving both species have never been reported." Many barriers exist to natural hybridization 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 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.

As described earlier, the subgenus *Soja* also contains an unofficial species, *G. gracilis* (Hymowitz, 2004). *G. gracilis* is known only from Northeast China, and is considered 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). Interspecific fertile hybrids formed by intentional crosses between *G. max* and *G. soja* and between *G. max* and *G. gracilis* have been easily obtained (Dorokhov et al., 2004). Although hybridization between *G. max* and members of the subgenus *G. soja* can take place, *G. soja* is not found in North or South America, and it is highly unlikely that gene transfer will occur.

X.C.1.3. Hybridization with Wild Perennial Species of Subgenus *Glycine*

The wild perennial species of the *Glycine* subgenus occur in Australia, West Central and South Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan. Therefore, the only opportunities for inter-subgeneric hybridization would occur in areas where those species are endemic (Hymowitz et al., 1992; Hymowitz and Singh, 1992). Nonetheless, the likelihood of interspecific hybridization between *G. max* and the wild perennial *Glycine* species is extremely low because they are genomically dissimilar (see Table II-2) and pod abortion is common. From time to time, immature seeds of the crosses could be germinated aseptically *in vitro*, but the resulting F1 hybrids are slow-growing, morphologically weak, and completely sterile. Their sterility is due to poor chromosome pairing. Furthermore, species distantly related usually produce nonviable F1 seeds that either have premature death of the germinating seedlings or suffer from seedling and vegetative lethality (Kollipara et al., 1993; Singh and Hymowitz, 1989). In North and South America, it is not possible for gene transfer between cultivated soybean and wild perennial species of *Glycine* subgenera, because these wild species do not exist in these regions.

X.C.2. Transfer of Genetic information to Species with which Soybean Cannot Interbreed (Horizontal Gene Flow)

Monsanto is not aware of any reports regarding the unaided transfer of genetic material from soybean species to other sexually incompatible species. The probability for horizontal gene flow to occur is judged to be exceedingly small. Even if it were to occur, the consequences would be negligible since the genes introduced into MON 87769 are of plant and fungal origin and the PjΔ6D and NcΔ15D proteins as well as the two fatty acids not previously produced in soybean (SDA and GLA) have no meaningful toxicity to animals, including humans, and other NTOs under the conditions of use.

X.D. Summary of Plant Pest Assessments

Plant pests are defined in the Plant Protection Act as any living organisms that can directly or indirectly injure, cause damage to, or cause disease to any plant or plant product [7 U.S.C. § 7702(14)]. Characterization data presented in Sections III through X of this petition confirm that except for the introduced genes (*Pj.D6D and Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and the intended fatty acid change, MON 87769 is not different from conventional soybean in its phenotype, environmental interactions, or susceptibility to pest or disease. A plant pest assessment was conducted to assess the potential impact of the introduced genes (*Pj.D6D and Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and intended fatty acid change (SDA and GLA) on NTOs and the weediness potential of MON 87769. Based on the results of this assessment, it is concluded that the potential risk of MON 87769 to cause adverse effects on NTOs and endangered species is negligible. MON 87769 is no more likely to become a weed than conventional soybean. The environmental risk associated with pollen-mediated gene flow from MON 87769 to other sexually compatible plants is considered negligible because the potential for gene flow is low. Evaluations have shown that the introduction of the genes (*Pj.D6D* and *Nc.Fad3*), the proteins produced from the introduced genes (PjΔ6D and NcΔ15D), and intended fatty acid change is not likely to enhance weediness or plant pest potential. There are no changes expected in agronomic practices for MON 87769 and the production systems currently used with specialty soybean. Thus, compared to conventional soybean, there are no increased plant pest characteristics associated with MON 87769.

Based on the data and information presented in this petition, it is concluded that MON 87769 is not likely to be a plant pest. Therefore, Monsanto Company requests a determination from APHIS that MON 87769 and any progeny derived from crosses between MON 87769 and conventional soybean or deregulated biotechnology-derived soybean be granted nonregulated status under 7 CFR Part 340.

XI. POTENTIAL FOR ENVIRONMENTAL CONSEQUENCES

XI.A. Background

USDA-AHPIS has responsibility, under the Plant Protection Act (7 U.S.C. § 7701-7772), to prevent the introduction and dissemination of plant pests into the U.S. Additionally, APHIS must comply with the National Environmental Policy Act (NEPA) when making the decision whether to grant nonregulated status to MON 87769. MON 87769 has been the subject of numerous field trials conducted in the U.S. under APHIS notifications and permits since 2003. Information has been developed from these field trials, other tests, and the public literature to specifically assess whether the alteration in the soybean oil profile through the introduction of two desaturase genes, *Primula juliae* Δ6 desaturase (*Pj.D6D*) and *Neurospora crassa* Δ15 desaturase (*Nc.Fad3*) that encode for the PjΔ6D and NcΔ15D proteins, the presence of stearidonic acid (SDA) and/or the plant transformation process altered MON 87769 in any way that would impart plant pest characteristics or cause significant environmental impacts, including cumulative impacts. The purpose of this section is to provide relevant, trait-specific information regarding the potential for reasonably foreseeable, significant environmental impacts.

An analysis of the potential impact of deregulation of MON 87769 on current soybean agronomic production systems, and related activities such as soybean processing, food and feed uses as well as marketing of soybean and soybean products is presented in this section. Factors evaluated as part of the assessment include potential impacts to:

- land use patterns, non-agricultural lands, farming practices, commodity and specialty soybean production,
- marketability of soybean seed for planting and seed for specialty and commodity markets, and
- public health, impacts to non-target organisms, and threatened or endangered species and biodiversity.

The analysis conducted considers current conditions, the potential to impact these conditions, and potential cumulative impacts. In most cases, there are no impacts to current conditions (e.g., no differences between deregulation versus continuing to regulate). Where differences were noted, these differences are described and their significance evaluated.

XI.B. Purpose and Need

Monsanto Company (Monsanto) is submitting to APHIS this petition for the determination of nonregulated status for MON 87769 plants genetically modified to produce stearidonic acid (SDA), which is not found in conventional soybean but which is found in other plants, fish and marine micro and macroalgae (see Table X-1). MON 87769 is a sustainable alternate source of an omega-3 fatty acid to help meet the

needed intake of long chain omega-3 fatty acids. The American Heart Association (AHA) has recommended a dietary intake of 1 gram/day of omega-3 fatty acid for heart patients and the incorporation of two servings of fatty fish per week for healthy individuals (or 500 mg/day of omega-3 fatty acid in the diet). Due in large part to food preferences, the current average intake of omega-3 fatty acids in the diet is well below these recommendations (Kris-Etherton et al., 2002, 2003). MON 87769 is intended to be a value-added specialty soybean crop commercialized as an alternate source of omega-3 fatty acid, the oil of which will replace non-hydrogenated soybean oil and/or be added to select food products such as margarine, mayonnaise, shortenings, salad dressings and ready-to-eat foods.

Common sources of omega-3s include ALA found in canola, soybean and other vegetables oils, flax and walnuts, fatty fish and algae. Studies have shown that the conversion of ALA to the more desirable long chain PUFAs, EPA and DHA, found in fatty fish and algae, is inefficient in humans. As a result, studies have shown that it can take 14-20 grams of ALA to convert to 1 gram EPA (James et al., 2003). SDA is an eighteen carbon fatty acid with four double bonds (18:4n-3) that is naturally found in fish and fish/algal oil products and some plants. SDA is the intermediate in the biochemical pathway between ALA and EPA and does not require the action of delta-6 desaturase for conversion to EPA. As a result, conversion of SDA to EPA is much more efficient (see Appendix F).

The development of MON 87769 involved the introduction of two desaturase genes, *Primula juliae* Δ6 desaturase (*Pj.D6D*) and *Neurospora crassa* Δ15 desaturase (*Nc.Fad3*) that encode for the PjΔ6D and NcΔ15D proteins. The genes and subsequent enzymes responsible for production of SDA in MON 87769 are derived from plant and algal sources and are not from fish.

Soybean plants lack the Δ6 desaturase gene, which is a minimal requirement for the production of SDA. However, Δ6 desaturase also converts LA to GLA. The addition of a Δ15 desaturase with temporal expression similar to the Δ6 desaturase increases the flux of ALA to SDA and lowers the substrate pool for GLA production (see Section VI.A). To produce SDA in soybean, the conventional soybean variety A3525 was transformed with a vector that contained the *Nc.Fad3* and the *Pj.D6D* genes driven by promoters that are known to be spatially and temporally active only in the developing soybean seed. The oil from MON 87769 contains 20% to 30% SDA (weight % of total fatty acids).

Oil derived from MON 87769 (referred to as SDA soybean oil) is expected to help food manufacturers develop foods that can assist consumers in meeting the American Heart Association's (AHA) goals for dietary intake of omega-3 long chain fatty acids and to improved heart health. SDA has been utilized in several human clinical studies. For example, James et al. (2003) found that by incorporating SDA in the diet at 1500 mg/day, EPA levels in red blood cells increased significantly and conversion was several-fold more efficient for SDA than for ALA. Harris (2007) found that SDA enriched the red blood cells and cardiac lipids with EPA in dogs. Likewise, Harris et al. (2008) found in a human clinical study using SDA soybean oil over a 16 week period, that there was a significant increase in red blood cell EPA levels whereas none was observed for participants using only conventional soybean oil containing ALA.

Fish oil and plant oils rich in omega-3 fatty acids are also currently used in certain feed applications especially in aquaculture and poultry feeds. SDA soybean oil may eventually be used in aquaculture and feed applications, as an alternative to fish oil and other omega-3 rich feed components.

XI.C. Soybean Production

The following section describes the setting for the proposed deregulation and provides the context for evaluating the intensity of the impact due to APHIS granting deregulated status to MON 87769. The proposed deregulation would be relevant to the production of an intensively cultivated row crop – soybean. Soybean is grown as a commercial crop in over 35 countries. In the United States, it is generally grown on greater than 70 million acres in at least 27 states with over a million acres grown in each of the following states: IA, IL, MN, IN, MO, NE, OH, SD, AR, ND, KS, MI, MS, WI, NC, KY, and TN (USDA-NASS, 2006a-b). On the basis of predicted demand for food and feed applications with omega-3 fatty acids, MON 87769 is expected to remain a specialty soybean product (Section IX.B.2) that is projected to be grown on less than 5% of the approximately 70 million acres of soybean grown annually in the U.S.

Soybean is the dominant oilseed in the U.S. and in global markets. Commodity soybean is grown on the majority of U.S. soybean acreage for its meal which is largely used for animal feed and for oil that is widely used in commercial processed foods (Sonka et al., 2004). Approximately 12% of soybean grown is specialty soybean produced for a specific market or use[10]. Examples of specialty soybean products include non-biotechnology-derived, certified seed, organic food-grade, low saturated fat, clear hilum, tofu, natto, high sucrose, high oleic, low linolenic and high protein (ASA, 2009). These soybean varieties are specified by buyers and end-users of soybean for production, and premiums are paid for delivering a product that meets purity and quality standards of that soybean variety. The specialty, value-added, product may be the whole soybean or, in the case of MON 87769, a processed fraction such as the oil. Product differentiation and market segmentation in the specialty soybean industry includes mechanisms to keep track of the soybean (traceability), methods for identity preservation (IDP), including closed-loop systems, and quality assurance processes (e.g., ISO9001-2000 certification), as well as contracts between growers and buyers that specify delivery agreements within product specifications (see Section IX.B.3).

This deregulation is being sought in an environment that has rapidly adopted biotechnology-derived soybean varieties (James, 2008). Thirteen different biotechnology-derived soybean crop products have been deregulated by USDA since 1994. Biotechnology-derived herbicide-tolerant soybean varieties were grown on approximately 69 million of the 75 million acres of soybean grown in the U.S. in 2008 (USDA-NASS, 2008). Thus, soybean breeders, seed manufacturers, and soybean producers have developed practices and systems that allow for the concurrent breeding

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[10] http://usb.adayana.com:8080/usb/jsp/login.jsp. Percent U.S. Soybean acreage estimate based on U.S. Domestic Consumption by Segment – 2008/09. D. Ludwig, personal communication, 2009.

and manufacture of biotechnology-derived and specialty soybean and are capable of breeding, manufacturing seed, and producing harvested seed to meet the needs of various markets. MON 87769 is the first SDA-producing soybean crop product for which deregulated status is being requested. However, APHIS is currently reviewing a high oleic acid soybean product (Event DP-305423-1) and has granted nonregulated status to Laurate canola (Events pCGN3828-212/86 18 & 23) with modified oil characteristics, and there are other modified oil soybean products produced by conventional breeding on the market.

In recent years, there has been an increased demand by consumers and food processers for soybean and other oilseed crops that have specific physical or chemical characteristics to meet specific food or feed needs. Traditional plant breeding has been used to alter the fatty acid profiles of oil producing plants such as soybean. For example, soybean varieties with lower levels (1-3%) of the polyunsaturated linolenic fatty acid have been commercially introduced (Fehr, 2007) and programs to develop high oleic soybean through conventional breeding have resulted in soybean varieties with an oleic content of >70% (Alt et al., 2005). MON 87769 has been developed to meet a demand for a land-based, sustainable source of omega-3 fatty acid for food and possibly feed use. As a replacement for omega-3 rich oils or omega-3 fatty acid containing products, use of SDA soybean oil would be limited to incorporation into food applications where it is: 1) functionally suitable (discussed below); 2) provides acceptable sensory properties (reduced off-flavors relative to longer chain omega-3 fatty acids) to foods and feeds; and 3) gives an appropriate stability profile and shelf life during food and feed production and storage. As expected, the SDA soybean oil from MON 87769 contains high levels of SDA (approximately 20-30 weight % of total fatty acids), a polyunsaturated fatty acid. Vegetable oils containing high levels of polyunsaturated fatty acids, such as oil from MON 87769, are known to undergo rapid oxidation during frying applications and will impart undesirable taste and odor to fried foods. Therefore, SDA soybean oil will not be suitable for all food applications and may not be suitable as a substitute for saturated fats in food recipes. However, SDA soybean oil can partially replace unhydrogenated soybean oil or other oils in many food categories. The current anticipated food applications for SDA soybean oil are in baked goods and baking mixes, breakfast cereals and grains, cheeses, dairy product analogs, fats and oils, fish products, frozen dairy desserts and mixes, grain products and pastas, gravies and sauces, meat products, milk products, nuts and nut products, poultry products, processed fruit juices, processed vegetable products, puddings and fillings, snack foods, soft candy, and soups and soup mixes (GRAS Notice No. GRN 000283).

Hydrogenation of vegetable oils makes the oil more stable relative to unhydrogenated oil and thus prolongs the shelf life of products containing these oils. Through the process of hydrogenation, the chemical structure of the fatty acids are changed. In the case of SDA soybean oil, hydrogenation would convert SDA (18:4) to other fatty acids present in soybean oil (e.g. 18:3, 18:2, and 18:1) and thereby eliminate the benefits of the long chain PUFA produced by MON 87769. Thus, as is the case with fish oils, and for the same reasons, MON 87769 oil will not be hydrogenated.

Because oil produced from MON 87769 is not suitable for all applications that currently use soybean oil, and because MON 87769 oil will be more valuable than commodity

soybean oil, it is Monsanto's intent that MON 87769 will be marketed and produced in a manner similar to other value-added, specialty soybean products (e.g., low linolenic, high protein, and high oleic). These value-added products are typically produced using IDP systems that include contracts between all handlers of soybean (seed suppliers, growers, handlers, and processors), traceability, product tracking, process verification and separate channeling to minimize commingling. Monsanto has introduced the value-added low linolenic Vistive® soybean, developed via conventional breeding, to the marketplace and will utilize similar management practices in order to identity preserve the SDA soybean oil produced from MON 87769.

XI.D. Potential Environmental Impacts

The soybean production environment and MON 87769 are described in detail throughout this petition. The discussion of impacts below is focused on specific areas of the production environment and related activities including land use patterns, farming practices, commercial activities such as seed production and marketing of soybean and impacts to the quality of agricultural products.

XI.D.1. Impacts on Land Use

Soybean is grown as a commercial crop on over 75 million acres in at least 27 states in the U.S. (USDA-NASS, 2009). Soybean acreage in the past five years has been relatively stable varying from 64.7 to 75.7 million acres with a 10-year average of 73.3 million acres (Table IX-1). Fluctuations in soybean acreage are due to environmental, agronomic and economic factors[11], as well as government programs such as the crop reserve program (CRP) or ethanol mandates imposed by the U.S. government. Soybean fields are typically highly managed agricultural areas that can be expected to be dedicated to crop production for many years and cultivation of MON 87769 is not expected to differ from typical soybean cultivation practices (see Section IX). The modified oil trait in MON 87769 provides growers with an option to produce a value added soybean and potentially greater profitability in their farming operation. MON 87769 will likely be used in common rotations on land previously used for agricultural purposes.

Deregulation of MON 87769 is not expected to cause any significant impact to land devoted to agricultural uses. The cumulative land area in the U.S. planted to principal crops, which include corn, sorghum, oats, barley, winter wheat, rye, durum, spring wheat, rice, soybean, peanuts, sunflower, cotton, dry edible beans, potatoes, canola, proso millet, and sugar beets, has remained relatively constant over the past 25 years. From 1983 to 1995, the average yearly acreage of principal crops was 328 million. This average is statistically unchanged at 326 million acres since the introduction of biotechnology-derived crops in 1996[12]. In fact, the total U.S. cropland acres have been relatively constant since the 1940s (Vesterby and Krupa, 1997). Therefore, there is no indication that

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[11] 2008 NASS crop acreage report [http://www.nass.usda.gov/Newsroom/2008/06_30_2008.asp] accessed 6/2009

[12] Calculated from datasets at http://usda.mannlib.cornell.edu/usda/current/htrcp/ by comparing the total acres from 1983 to 1995 and from 1996 to 2007.

the introduction and widespread adoption of biotechnology-derived crops, or specialty soybean varieties, has resulted in a significant change to the total U.S. cropland acres. As with these other products, introduction of MON 87769 is not expected to have a significant impact on total U.S. cropland acres.

MON 87769 will likely be introduced into the Midwest/Great Plains region. This region is responsible for production of over 75% of the soybean grown in the U.S. (Table IX-2). On the basis of projections for use in food and possibly feed, it is expected that MON 87769 will remain a low acre "specialty" soybean produced on less than 5% of the U.S. soybean acreage. Granting nonregulated status to MON 87769 is not expected to have a significant impact to land use for the following reasons. MON 87769 will not alter the range of soybean cultivation as the new trait (SDA production) does not change the growth habits compared to conventional soybean (see Section VIII). MON 87769 will be grown on land already devoted to soybean production replacing existing commodity or other specialty soybean production. As discussed above, soybean acreage fluctuates annually based upon the dynamics of the marketplace and other environmental and agronomic factors. Thus, assuming that demand for commodity and other specialty soybean remains the same, if MON 87769 achieves maximum adoption (3.5 million acres), soybean acres could slightly increase. However, the increase in acreage is well within the range of normal acreage fluctuations observed for soybean. Based on these reasons, Monsanto does not consider that deregulation of MON 87769 will result in significant impacts to land use.

A decision to deregulate MON 87769 similarly is not expected to result in any cumulative impacts to land use. It is foreseeable that MON 87769 could and likely will be stacked with MON 89788 which is tolerant to glyphosate. This herbicide tolerance trait is not expected to change the demand for SDA oil and is not considered a factor impacting the acreage devoted to MON 87769 production. As discussed in Section IX.B.2, numerous specialty soybean varieties have been introduced over the past 10 years, including non-biotechnology-derived, certified seed, organic food-grade, low saturated fat, clear hilum, tofu, natto, high sucrose, high oleic, low linolenic and high protein (ASA, 2009). Adoption and continued use of these specialty soybean varieties has not correlated with an increase in the total acreage for soybean (Table IX-1).

XI.D.2. Potential for Non-crop and Non-agricultural Impacts

Soybean does not grow in the wild in the U.S. (Hymowitz and Singh, 1987; CFIA, 1996; OECD, 2000). Soybean does not grow and persist in unmanaged habitats and would not be expected to invade and/or persist in the natural environment, including streams, lakes, oceans or other aquatic environments. With the exception of production of the PjΔ6D and NcΔ15D proteins and the presence of SDA and GLA as components of the fatty acids in the seed, MON 87769 is similar to other commercial soybean varieties currently grown in the U.S. including both conventional and biotechnology-derived, and would be expected to similarly have no significant impact to non-agricultural lands or aquatic systems. MON 87769 could be produced on land where soybean may be grown. There are no changes in agricultural inputs needed to produce varieties containing MON 87769 relative to commodity or specialty soybean and specifically no altered pest or disease susceptibility. Hence, pesticide applications on MON 87769 that may result in drift

impacting non-crop or nonagricultural lands would be comparable to those used on conventional or currently available soybean varieties.

XI.D.3. Potential Impacts to Agricultural Practices

MON 87769 has been shown to be no different from conventional soybean in its agronomic and ecological characteristics (see Sections VII, VIII, IX and X), and has the same levels of resistance to insects and diseases as current commercial soybean. A summary of agronomic practices for soybean production is presented in Section IX of this petition. Agricultural practices for MON 87769 are expected to be similar to those used for production of commodity or specialty soybean. Specifically, MON 87769 requires no changes to current cultivation practices, including seeding, tillage, fertilizer and pesticide applications and crop rotation practices. Growers will utilize best practices associated with production and identity preservation (IDP) of harvested soybean similar to those currently used for other specialty soybean varieties (see Section IX.B.3).

Potential Impact to Soybean Commodity and Specialty Soybean Production

Deregulation of MON 87769 would allow for unconfined commercial planting of varieties containing MON 87769 and would provide an alternative commercial use of soybean oil. It would also provide growers with a higher value crop resulting in greater profit potential for U.S. soybean producers. Soybean growers and processors are accustomed to accommodating a diversity of specialty soybean varieties in their normal handling of commodity soybean. Currently specialty soybean varieties are produced on approximately 12% of the U.S. soybean acreage (see Section IX.B.2). Soybean growers have demonstrated an ability to provide customers with their soybean product of choice. As discussed in Section IX, MON 87769 will be produced using IDP practices consistent with those used for other specialty soybean varieties. IDP practices will mitigate potential significant impacts to commodity or other specialty soybean. MON 87769 will be segregated from commodity soybean by producers as part of their contract and to maintain the value of the crop. Because soybean is primarily a self-pollinated crop with minimal gene movement and due to the identity preservation production system, no significant impact to commodity or other specialty soybean production is anticipated, should APHIS grant nonregulated status to MON 87769.

No cumulative impacts to commodity or other specialty soybean production are expected from deregulation. Soybean producers have demonstrated their ongoing ability to produce multiple specialty soybean varieties in a diverse marketplace.

Potential Impact to Certified Seed Production

Certified seed production is a carefully managed process (see Section IX.B.4). MON 87769 is not expected to impact certified seed production practices or production of other certified conventional, specialty or organic soybean seed for the reasons described in this section. As discussed elsewhere, MON 87769 is expected to be a small acre specialty product grown on less than five percent of total soybean acres. The amount of acreage needed to supply seed for five percent of the total soybean acreage is

approximately 100,000 acres (see Section IX-B.4). Thus, MON 87769 would occupy a small percentage of the roughly 1.9 to 2.3 million annual soybean seed producing acres.

If MON 87769 is deregulated, seed production could occur within production systems already developed by seed producers for certified seed varieties (see Section X). MON 87769 has been thoroughly characterized and (with the exception of the modified fatty acid profile) is not phenotypically different. The implementation of management practices to avoid pollen from a biotechnology-derived crop in organic, specialty or conventional soybean seed or commodity seed production operations is directly impacted by the nature of soybean pollination (see Section IX.B.4 and Table X-3). Soybean is a highly self-pollinated species that exhibits very low levels of outcrossing. When soybean plants were grown in very close proximity to each other (15 cm), average cross-pollination rates were 1.8% (Ray et al., 2003). At greater distances, cross-pollinations rates were 0.41 and 0.03% at 0.9 and 5.4 m, respectively (Table X-3). Hence, certified soybean seed producers can and have effectively implemented practices (e.g., isolation distances during the growing season, equipment cleaning during harvest, and post-harvest separation of harvested seed) that allow them to maintain commercially acceptable levels of varietal purity.

No cumulative effects are anticipated to certified seed production from deregulation of MON 87769. The use of management practices that prevent trait movement and comingling of soybean varieties has resulted in production of soybean varieties with improved genetics over time. As a result of the implementation of these management practices, growers today can choose from numerous varieties of soybean including those used to produce commodity and specialty soybean varieties.

Potential Impacts to Organic Soybean Production

Organic soybean markets typically enjoy a market premium offsetting the additional production and record-keeping costs associated with this production method (see Section IX.B.2). Organic farming operations as described by the National Organic Program, which is administered by USDA's Agricultural Marketing Service, requires organic production operations to have distinct, defined boundaries and buffer zones to prevent unintended contact with prohibited substances or products of excluded methods from adjoining land that is not under an organic production management plan. Organic production operations must also develop and maintain an organic production system plan approved by an accredited certifying agent. This plan enables the production operation to achieve and document compliance with the National Organic Standards, including the prohibition of the use of excluded methods. Excluded methods include a variety of methods used to genetically engineer organisms or influence their growth and development by means that are not possible under natural conditions or processes. The use of biotechnology such as that used to produce MON 87769 is an excluded method under the National Organic Program [7 C.F.R. § 205.2].

Organic certification involves oversight by an accredited certifying agent of the materials and practices used to produce or handle an organic agricultural product. This oversight includes an annual review of the certified operation's organic system plan and on-site inspections of the certified operation and its records. Although the National Organic

Standards prohibit the use of excluded methods, they do not require testing of inputs or products for the presence of excluded methods. The presence of a detectable residue of a product of excluded methods alone does not necessarily constitute a violation of the National Organic Standards. The unintentional presence of the products of excluded methods will not affect the status of an organic product or operation when the operation has not used excluded methods and has taken reasonable steps to avoid contact with the products of excluded methods as detailed in an approved organic system plan. Organic certification certifies that organic production and handling processes have been followed, not that the product itself is "free" from any particular substance.

Production systems designed prior to the introduction of MON 87769 or even prior to the introduction of biotechnology-derived soybean have allowed for production of soybean to meet varied customer demands. In addition to the market segments that produce organic or conventional soybean, distinct identity-preserved specialty soybean with such traits as clear hilum or high protein have also been grown and successfully marketed for specific food uses in domestic and export markets for many years (Zhanglin et al., 2004). The choice to grow biotechnology-derived, organic or conventional soybean depends on many factors and the dynamics of the marketplace. The dynamics of the marketplace, choice between various varieties of soybean, and the existing production practices will not be impacted by the introduction of MON 87769.

Organic soybean producers utilize production practices designed to specifically avoid the presence of both soybean products using conventional herbicide or other pesticide treatments, as well as biotechnology-derived crops. These well established practices to avoid "excluded methods" will continue with the introduction of MON 87769 varieties. They include isolation zones, use of buffer rows surrounding the organic crop, adjusted planting dates and varietal selection (www.attra.ncat.org). The implementation of management practices to avoid pollen from a biotechnology-derived crop in organic or conventional soybean production operations is facilitated by the nature of soybean pollination. As noted previously in this petition, soybean is a highly self-pollinated species and exhibits very low level of outcrossing. Hence, organic or conventional soybean producers can and have effectively implemented practices (e.g., isolation during the growing season, equipment cleaning during harvest, and post-harvest separation of harvested seed) that allow them to avoid the presence of biotechnology-derived soybean and maintain organic or conventional production status.

Currently, biotechnology-derived herbicide tolerant soybean is planted on 92% of the soybean acreage (USDA-NASS, 2008) and the Roundup Ready soybean system – that is, planting Roundup Ready soybean and applying glyphosate in crop – has become the most widely used weed control program in U.S. soybean production. Despite the high adoption rates of Roundup Ready soybean by growers, organic and conventional soybean production remains an option for farmers who choose to produce using these production practices or varieties of soybean. The decision to grow organic, conventional, or biotechnology-derived soybean varieties is typically an economic one based on market dynamics. Organic soybean producers and those growing conventional soybean for sensitive non-biotechnology markets typically enjoy a market premium offsetting the additional production and record-keeping costs. While the widespread adoption of Roundup Ready soybean has reduced the number of conventional soybean varieties that

are available, conventional and organically produced soybean seed is currently available from numerous seed suppliers (Table XI-1). Additional information on organic seed sources is provided though the U.S. Agricultural Marketing Service (AMS) at: www.ams.usda.gov. Thus, growers have a choice in the soybean variety they plant, and this is not expected to change with the introduction of MON 87769.

Buyers recognize that where there are biotechnology-derived crop varieties on the market, as with soybean, a guarantee that a commodity crop is 100% "free" of biotechnology-derived material is not feasible based on the limitations of testing and sampling methodology (Born, 2005). While the presence of a biotechnology-derived product like MON 87769 is unlikely in instances where producers utilize production practices designed to avoid biotech presence, in some instances buyer allowances between 0.1 to 5% biotechnology-derived commodity soybean in organic soybean are specified. This also is consistent with the USDA National Organic Program allowing for detectable residues of excluded methods (including biotechnology-derived crop products) as long as the producer has taken steps to avoid those methods (www.ams.usda.gov/nop/Q&A.html). Similarly, international regulatory organizations have recognized that testing and sampling methodologies limit the ability to confirm that commodity or specialty soybean is 100% free of biotechnology-derived material. Thus, they have set allowable tolerances for this material in conventional products to support food labeling and traceability laws. These tolerances allow from 0.9% (European Union) up to 5% (Japan) of the food to be biotechnology-derived in products considered "conventional." Levels above the threshold may trigger special labeling. Thus, *de minimis* levels of approved biotechnology-derived soybean are allowable in certified organic or conventional soybean.

Table XI-1. Organic and Conventional Soybean Seed Sources

Organic Soybean Seed Sources*:	**Conventional Soybean Seed Sources**
Albert Lea Seed House	AgVenture Seeds (modified oil)
Blue River Hybrids	Campbell Seed (modified oil)
Golden Grains	Becks Hybrids (food grade)
Great Harvest Organics	Monsanto (Asgrow)
Greis Seed Farm	Schillinger Seed
Lancaster Ag Products	Pioneer
Lawler Farm Center	Soy Genetics
Prairie Gold Seeds	Stewart Seed (modified oil)
Superior Organic Grains, Ltd	Stine Seed
Walter Seed and Honey Co	Syngenta - multiple brands
	Terral Seed
	Various State Crop Improvement Organizations

* From: www.organicgrains.ncsu.edu

XI.D.4. Potential Impacts to Raw or Processed Agricultural Commodities

With this petition, extensive data have been presented relating to plant growth parameters, disease susceptibility, insect susceptibility, and forage and seed composition of MON 87769 compared to conventional soybean varieties. These data indicate that there are no biologically relevant differences between MON 87769 and conventional soybean, except for the deliberate change in the fatty acid composition of the oil derived from MON 87769. Monsanto has initiated a voluntary consultation with the FDA, and information has been provided to the agency supporting the food and feed safety of soybean, forage and SDA soybean oil derived from MON 87769 (BNF 00117, GRAS Notice No. GRN 000283).

From a grain quality standpoint (e.g., foreign material, damaged grain, etc; see www.gipsa.usda.gov for soybean grain standards), the soybean produced from MON 87769 varieties is expected to be of comparable quality to soybean commercially produced in the U.S. for commodity markets based on the data presented herein. The intended use of MON 87769 will be for processing for the production of SDA soybean oil within an IDP system, which is described in Section IX.B.3. MON 87769 will be grown under a contract with growers that will direct harvested soybean to its intended purpose. If for any reason a load of MON 87769 soybean is not suitable to be processed within the IDP system for the production of SDA soybean oil, the soybean will be directed to appropriate uses.

Soybean is typically processed into two major fractions, the oil and meal, and several minor fractions including lecithin and protein isolate. With the exception of the oil fraction, the other processed fractions produced by MON 87769 are compositionally equivalent to, and will be put to the same uses as commodity soybean processed fractions (see Section VII.C). The meal, and other non-oil processed fractions are intended to be distributed into the commodity stream and will be marketed and sold in the commodity market. Because of their substantial equivalence, no significant impact will occur to these commodity soybean processed fractions when APHIS grants deregulated status to MON 87769.

As described elsewhere in this petition, MON 87769 and SDA soybean oil produced from MON 87769 will be produced and processed utilizing an identity preservation (IDP) system for use in specific food applications. If for any reason, the oil from MON 87769 is not used for the intended food applications, it would be directed to an appropriate use.

The fatty acid profile of SDA soybean oil produced from MON 87769 would not be suitable for all of the food applications that currently use commodity soybean oil. Vegetable oils containing high levels of polyunsaturated fatty acids, such as SDA soybean oil, are known to undergo rapid oxidation during frying applications that impart undesirable taste and odor to the fried foods. For this reason, undiluted SDA soybean oil would not be suitable for use in frying applications.

It is not expected that the introduction of MON 87769 will cause a significant impact to processed soybean oils (e.g., commodity or other specialty) due to the IDP system under which it will be commercialized, handled and used, and because of the systems employed

by the soybean processing and use chain to specify the characteristics of oils, and to identify, segregate and test those oils for different uses.

Before implementing an IDP system, Monsanto will identify relevant stakeholders in the value chain and engage them in dialogue to describe the intended uses and benefits of SDA soybean oil, to discuss and understand the potential impacts on commodity oils and processed soybean products from the IDP production, handling and use of MON 87769 soybean and SDA soybean oil. Based on the dialogue and the resulting assessment, Monsanto will take the following steps, where applicable and appropriate:

1. refine plans for production, handling and processing of MON 87769 soybean and SDA soybean oil to address valid concerns raised by the stakeholders,
2. assess potential impacts on processed soybean products due to the presence of SDA in commodity oil, and collaborate with stakeholders to support research required for the evaluation of those impacts and the levels of MON 87769 soybean or of the SDA soybean oil at which they occur,
3. develop management, mitigation and response plans to address potential instances where the trait is present at levels, if any, that are not compatible with commodity soybean and processing streams, and
4. engage in industry outreach and education regarding the MON 87769 soybean/SDA soybean oil IDP system, including product handling and use, and the implementation of any necessary risk management, mitigation and response plans.

Based on Monsanto's assessment, it is unlikely that there will be significant impacts on raw or processed soybean commodities due to APHIS granting nonregulated status to MON 87769.

Based on this assessment, Monsanto does not anticipate any significant cumulative impacts on either raw or processed soybean commodities resulting from the deregulation of MON 87769. Soybean does not survive outside of cultivation and does not readily survive as a volunteer in agricultural fields. Hence, the only source of SDA soybean oil will be from MON 87769 branded varieties produced under contract. The use of well established breeding and seed production methods assures the purity of soybean varieties. These same methods will be used to produce soybean varieties that contain MON 87769. Similarly, these methods will be used to minimize the presence of MON 87769 in other soybean varieties. Food ingredient and food manufacturers routinely manage and handle a wide variety of specialty oils derived from oilseed crops (including other modified oils produced by other deregulated events) with no adverse or cumulative effects on their manufacturing processes or on the quality of their products. Similarly, food manufactures will integrate SDA soybean oil into their existing handling and manufacturing process, and are expected to have the equivalent experience with SDA soybean oil, resulting in no adverse or cumulative effects due to APHIS granting deregulated status to MON 87769.

XI.D.5. Potential Impacts on Commercial Use of Soybean

The decision to deregulate MON 87769 would allow for breeding of this product with conventional and biotechnology-derived soybean varieties and would make MON 87769 available to breeders, certified seed producers, and growers. Like other biotechnology-derived traits, it is expected that breeders and certified seed producers would use MON 87769 to supply seed for planned commercial markets in the U.S. Monsanto anticipates that commercial use of MON 87769 may include export of soybean and soybean products, and has described import approval submission plans elsewhere in the petition. Monsanto's stewardship program for this product is described in Section IX.I.

XI.D.6. Potential Impacts to Human Health and Safety

Most human interactions with soybean occur through agricultural production, industrial operations, or through consumption. Therefore, this health and safety discussion will focus on food and feed safety as well as safety of workers exposed to MON 87769. SDA soybean oil derived from MON 87769 is intended to be commercialized as an omega-3 oil that will replace non-hydrogenated soybean oil and/or is added to select food products such as margarine, mayonnaise, shortenings, salad dressings and ready-to-eat foods. SDA soybean oil may also be used as a food/feed ingredient as a replacement for fish oil, flax seed oil, vegetable oils or animal fats.

Under the Federal Food, Drug, and Cosmetic Act (FFDCA), it is the responsibility of food and feed manufacturers to ensure that the products they market are safe and properly labeled. Food and feed derived from MON 87769 must be in compliance with all applicable legal and regulatory requirements. Biotechnology-derived crops for food and feed use undergo a voluntary consultation process with the FDA prior to release onto the market. Although a voluntary process, Monsanto completes a consultation with the FDA prior to placing a new biotechnology derived crop product on the market. A list of completed consultations on biotechnology-derived crop products is available at the FDA website. As part of the consultation process for MON 87769, Monsanto provided product characterization and safety data to the FDA on March 23, 2009 (BNF 00117).

Monsanto has also completed an assessment of the safety of the oil produced by MON 87769 and a panel of independent experts has concluded that SDA soybean oil is generally recognized as safe (GRAS) for its intended use in foods. Following a critical evaluation of the scientific data generally available in the public domain that pertain to the safety of SDA soybean oil, a panel of experts, qualified by scientific training and experience to evaluate the safety of SDA soybean oil as a component of food, unanimously concluded that the proposed uses of SDA soybean oil are safe and suitable and are GRAS, based on scientific procedures. Therefore, MON 87769 is as safe and nutritious as conventional soybean for food and feed use with the added nutritional improvement of the SDA omega-3 fatty acid. The GRAS assessment has been submitted to the FDA for review (GRAS Notice No. GRN 000283).

Soybean produced by MON 87769 contain approximately 20% to 30% stearidonic acid (SDA) and 7% gamma linolenic acid (GLA) (see Appendix F). Oil produced by MON 87769 represents a sustainable alternate source of an omega-3 fatty acid to help

meet the needed dietary intake of long chain omega-3 fatty acids. The health benefits of omega-3 fatty acid consumption are widely recognized (James, 2003). SDA is naturally present in some plants, fish and marine algae and has a long standing history of safe consumption in humans (see Table X-1). The safety of SDA soybean oil is supported by the results of a 90-day/one generation reproductive toxicity rat study (Hammond et al., 2008). Furthermore, no adverse effects attributable to SDA soybean oil were reported in a published human study (Harris et al., 2008). Additional published studies on SDA and GLA from other sources corroborate the safety of SDA soybean oil. The safety of SDA soybean oil is further supported by the regular dietary consumption of fats and oils containing the other primary fatty acids present in SDA soybean oil, namely ALA, LA, and palmitic acid (see Appendix F).

As part of the consultation with the FDA regarding MON 87769, Monsanto has provided the agency with information on the identity, function, and characterization of the genes, as well the expression levels of the gene products. Monsanto also provided information on the potential allergenicity and toxicity of the expressed PjΔ6D and NcΔ15D proteins. The development of MON 87769 involves the introduction of two proteins (PjΔ6D and NcΔ15D) produced by genes from plant and fungal sources (see Section VI for details). These two proteins are not currently present in soybean and the safety of each was assessed for both food and feed applications. A summary of the safety assessment conducted on these two proteins is provided in Section VI of this petition. These assessments lead to the conclusion that there is no meaningful risk to animal or human health from dietary exposure to the PjΔ6D and NcΔ15D proteins produced in MON 87769.

Extensive compositional analyses of forage and seed were conducted on samples from replicated, multi-site field trials conducted in the U.S. to compare the composition of MON 87769 to a conventional soybean control and to commercially available soybean varieties (see Section VII for details). The compositional analyses of MON 87769 demonstrated it to be compositionally equivalent to conventional soybean, except for the modified fatty acid profile. Composition data support the conclusion that there are no biologically relevant changes in MON 87769 due to the genetic modification, outside of the modified fatty acid profile.

On the basis of the assessment of laboratory data and scientific literature, it is reasonable to conclude that MON 87769 is as safe and nutritious as conventional soybean for food and feed use with the added nutritional improvement of the SDA omega-3 fatty acid. Therefore, a decision to grant nonregulated status to MON 87769 would have no significant impact on human or animal health. Possible beneficial effects could occur to human heart health if MON 87769 is deregulated. The availability of products that contain SDA from SDA soybean oil may increase the intake of omega-3 fatty acids thereby providing a health benefit. These effects are not considered significant because there are other sources of SDA and other heart healthy omega-3 fatty acids available in existing foods such as from fish, other plants and marine micro and macroalgae. MON 87769 provides an additional sustainable source of omega-3 fatty acid from which food processors and manufacturers can choose.

Worker Safety

During agricultural production of soybean, growers may be exposed to pesticides during application to the crop. Downstream processing and manufacturing workers deal with hazardous equipment and chemicals during the processing of soybean into food and feed and also for industrial uses. As discussed in Section IX, MON 87769 does not change the agronomic practices, or use of chemicals such as pesticides, associated with soybean production. Thus, deregulation of MON 87769 will have no significant impact to agricultural workers. Similarly, there are no changes to industrial processing of MON 87769 relative to the processing of commercial soybean.

SDA soybean oil derived from MON 87769 is expected to substitute for other oils currently used in foods and possibly in feeds. The composition of SDA soybean oil contains fatty acids that are already used in the food and feed industries. Since workers are already accustomed to handling various oils for formulation into foods, there are no significant impacts identified to processors or food formulators either.

XI.D.7. Potential Impacts to Plant, Animal and Microbial Communities Including Threatened or Endangered Species and Biodiversity

In assessing the potential impact to plant and animal communities, the potential for gene movement and introgression from MON 87769 was evaluated because movement and establishment of the gene and trait to related species could have indirect impacts to plant and animal communities that extend beyond the original recipient organism. Monsanto considered two primary issues: 1) the potential for gene flow and introgression, and 2) the potential impact of introgression. The genus *Glycine* has approximately nine species, with *G. max* being placed in the subgenus *Soja* along with one other species, *G. soja*. *G. max* is sexually compatible with only *G. soja* and no other *Glycine* species. *G. max* is the only *Glycine* species located in the United States. Therefore, the probability of gene flow and introgression of MON 87769 into other species in the U.S. is essentially zero (Stewart et al., 2003); thus, the potential impact of introgression of MON 87769 to sexually compatible relatives on plant and animal communities is nonexistent if APHIS were to grant the petition for nonregulated status. Additional discussion of the potential environmental impact due to gene movement may be found in Sections XI.D.4 and XI.D.5.

Animals

Soybean production systems in agriculture are host to many animal species. Mammals and birds may seasonally use grain, and invertebrates can feed on the plant during the entire growing season. Agronomic practices used to produce MON87769 will be substantially the same as those used to produce many types of specialty or conventionally grown soybean, thus this discussion for MON 87769 on animals will focus on the potential effects of the two introduced proteins (PjΔ6D and NcΔ15D) and the modified fatty acids in MON 87769. Animals that feed primarily on soybean are seed-feeding insects and rodents found in agricultural fields. Rodents, such as mice or squirrels, may seasonally feed exclusively on soybean seeds. Thus, these animals may have a diet

containing significant amounts of soybean seeds. Deer may also browse in soybean fields on the forage and on seed left after harvest.

Information included in this petition on the characterization of MON 87769 and safety of MON 87769 for food and feed uses is relevant to an assessment of potential impacts to animals that may visit soybean fields containing MON 87769. As discussed previously, there is no meaningful risk to animal or human health from dietary exposure to PjΔ6D and NcΔ15D from MON 87769. There are no toxic properties associated with either the PjΔ6D or NcΔ15D protein or with the fatty acids in the seed produced by MON 87769. The composition of the seed produced by MON 87769 is (with the exception of the intended change in fatty acid profile) comparable to that of conventional soybean seed. Furthermore, forage produced by MON 87769 is compositionally equivalent to forage produced by conventional soybean (see Section VII). This information indicates that there would be no negative effects to animals that forage on MON 87769 or consume seed. Additionally, animals have been previously exposed to SDA in the environment, as SDA is found in some plants, fish, and marine algae (James, 2003). During field trials, no changes in insect feeding damage were observed (see Section VIII), further supporting the lack of potential impact to animals. On the basis of this analysis, deregulation of MON 87769 will not result in significant impacts on animals, including insects that live near or in soybean fields containing MON 87769.

Plants

Soybean production systems in agriculture are host to many plant species as well. The landscape surrounding a soybean field varies depending on the region. In certain areas, soybean fields may be bordered by other soybean, corn (or any other crop); fields may also be surrounded by wooded and/or pasture/grassland areas. Therefore, the types of vegetation around a soybean field depend on the area where the soybean is planted.

If MON 87769 is granted nonregulated status, agricultural practices that are used for the production of commodity soybean would be used for plant management during the cultivation of MON 87769. MON 87769 does not exhibit characteristics associated with weedy growth and will not compete with plants found outside of agricultural production. Weeds within fields of MON 87769 will be managed using mechanical, cultural, and chemical control measures, similar to current management practices for commodity and many types of specialty soybean. As MON 87769 has no toxic effects on animals (see discussion above), pollinators of other plants in or around fields cultivated with MON 87769 will not be impacted and, likewise, no indirect effects are expected to other plants that rely on pollinators. Thus, granting nonregulated status to MON 87769 will have no significant impact to plant communities.

Threatened and Endangered Species

No significant impact to threatened and endangered species is expected due to APHIS granting nonregulated status to MON 87769. As previously noted, consumption of the PjΔ6D and NcΔ15D proteins has shown no toxicity in laboratory testing with mice (see Section VI.E) and it has been demonstrated that there has been previous environmental exposure to SDA with no documented adverse effects (see Sections X.A.3.2 and X.A.3.3).

MON 87769 does not express any additional proteins, natural toxicants, etc. that are known to directly or indirectly affect a listed threatened or endangered species (TES) or species proposed for listing by the U.S. Fish and Wildlife Service. MON 87769 is not sexually compatible with any federally listed TES or a species proposed for listing. The only TES animal listed that occupies habitat that is likely to include soybean fields and that might feed on soybean is the federally Endangered Delmarva Peninsula Fox Squirrel (*Sciurus niger cinereus*), found in areas of the mid-Atlantic Eastern seaboard. It is known to utilize certain agricultural lands readily, but its diet includes acorns, nuts/seeds of hickory, beech, walnut, and loblolly pine; buds and flowers of trees, fungi, insects, fruit, and an occasional bird egg. Given all these factors and the lack of noted adverse effects of the introduced proteins or SDA soybean oil on mammalian species (mice, rats) and information presented in Section VIII.D.2.2 on environmental interactions of MON 87769 with other non-target organisms in the field, it is concluded that MON 87769 will not have an effect on the Delmarva Peninsula Fox Squirrel.

No significant impact to any threatened or endangered plant species is expected from the cultivation of MON 87769. *G. max* has no sexually-compatible relatives in the U.S. and does not survive outside of cultivation. Thus, there is no opportunity for MON 87769 to interbreed with any plant species or displace natural vegetation in the U.S.

Soil Microorganisms

On the basis of the biochemical and physiochemical characteristics of the PjΔ6D and NcΔ15D proteins, no accumulation of these proteins is expected in soils where MON 87769 will be grown (see Section VI.A). Information has also been presented in the petition demonstrating the established presence of SDA in the environment (see Section A.A.3.2). No adverse effects on soil microorganisms are associated with SDA nor do the characteristics of the modified fatty acid profile merit concern to soil microorganisms because SDA will be degraded in the soil (see Section X.A.3.3) like other PUFAs. Monsanto presented data in this petition (see Section VIII) demonstrating the lack of impact to symbiotic microbes associated with soybean plants. The *B. japonicum*-soybean symbiosis of MON 87769 was not altered as a result of the introduction of the PjΔ6D and NcΔ15D proteins compared to a conventional soybean control. On the basis of these observations and in conjunction with related phenotypic measurements for MON 87769, no impact on soil microorganisms and other soil arthropods is expected due to deregulation of MON 87769.

Biodiversity

Analysis of available information indicates that MON 87769 exhibits no traits that would cause increased weediness, that its unconfined cultivation should not lead to increased weediness of other sexually compatible relatives (of which there are none in the United States), and it is likely to have no effect on non-target organisms common to agricultural ecosystems or threatened or endangered species. Based on this analysis, it is concluded that deregulation of soybean varieties containing MON 87769 would have no significant impact on biodiversity.

No impacts to plant and animal communities or threatened and endangered species have been identified for MON 87769. No cumulative impacts to plant and animal communities or threatened and endangered species have been identified.

XI.E. Other Cumulative Effects

Conventional Breeding with Other Biotechnology-derived or Conventional Soybean Varieties

As previously mentioned, several biotechnology-derived soybean products have been deregulated or are under consideration for deregulation, and a list of the events deregulated by USDA-APHIS is presented in Table XI-2. MON 87769 may be bred with these deregulated biotechnology-derived soybean crop products as well as with conventional soybean, creating new improved varieties. APHIS has determined that none of the biotechnology-derived individual soybean products it has deregulated display increased plant pest characteristics and that any progeny derived from crosses of these soybean varieties with other conventional or biotechnology-derived soybean are unlikely to exhibit new plant pest properties.

An assessment of the stability of the genetic insert in MON 87769 was conducted, and data have been presented in this petition demonstrating that MON 87769 is stable in progeny. Having established that the genetic material is stable and that MON 87769 is inherited in a Mendelian fashion, and based on experience with MON 87769 in Monsanto's plant breeding program, it can be concluded that the modified oil phenotype present in MON 87769 is likewise stable. Conventional breeding has an established history of safe use, and use of MON 87769 in breeding programs is expected to behave similar to other conventional traits and biotechnology-derived traits. For example, in an assessment by McCann et al. (2005), it was shown that during three years of breeding into multiple varieties, the composition of glyphosate-tolerant soybean remained equivalent to that of conventional soybean. Given that there have been no plant pest characteristics associated with MON 87769, or with any of the previously deregulated events listed below, no significant impacts are expected to other soybean varieties through the use of MON 87769 in breeding programs and in combination with any of the previously deregulated soybean crop products.

All biotechnology-derived soybean products on the market today have satisfactorily completed the FDA consultation process expressly established to review the safety of whole foods derived from biotechnology-derived crops for human and animal consumption (see Table XI-2). No impacts to public health (e.g., food or feed safety) are expected due to combination of these events through conventional breeding because the deregulated events have a history of safe use and on the basis of knowledge of the type of modifications made to each of the deregulated events, the biochemical pathways are not likely to unexpectedly interact or result in the production of novel constituents. As mentioned above, breeding with other modified oil soybean products on the list below, while not currently envisioned, would predictably alter the fatty acid composition of MON 87769 (i.e., the combined trait will have an altered fatty acid profile that is a combination of the individual trait characteristics).

Breeders use standard testing and assessment procedures to further examine and confirm the equivalence of combined trait products to the single event products in terms of phenotype, agronomic characteristics, and the efficacy of the traits. This testing is a part of the process to develop any new soybean variety. The use of conventional breeding to produce combined trait or combined event products would identify off-types and non-performing germplasm during development of new varieties and they would be removed from further development.

Table XI-2. Deregulated Biotechnology-derived Soybean Products

Phenotype	ID Code(s)	Institution	Date Deregulated
High Oleic Acid	DP-3Ø5423-1	Pioneer	Submitted
Glyphosate Tolerant	MON 89788	Monsanto	February, 2007
Phosphinothricin Tolerant	GU262	AgrEvo	October, 1998
Phosphinothricin Tolerant	A5547-127	AgrEvo	May, 1998
Altered Oil Profile	G94-1, G94-19, G-168	DuPont	May, 1997
Phosphinothricin Tolerant	W62, W98, A2704-12, A2704-21, A5547-35	AgrEvo	August, 1996
Glyphosate tolerant	40-3-2	Monsanto	May, 1994

XI.F. Highly Uncertain, Unique or Unknown Risks

MON 87769 has been thoroughly characterized and data submitted in the petition demonstrate that it does not pose an increased plant pest risk compared to conventional soybean. APHIS has previously deregulated 13 biotechnology-derived soybean products. MON 87769 represents an additional soybean product with a modified fatty acid profile in the soybean oil. Introduction of previous biotechnology-derived crops have resulted in no unexpected effects on the quality of the human environment as defined under NEPA and have provided benefits to growers, consumers and the environment. In this respect, a decision to deregulate a new biotechnology-derived soybean product is not precedent setting nor are the effects to the quality of the human environment highly uncertain or unpredictable.

XI.G. Summary

The extensive body of information presented in Sections I through X of this petition demonstrates that MON 87769 does not present a plant pest risk, and has no significant impact on threatened or endangered species or biodiversity. Soybean produced from MON 87769 has been demonstrated to be safe and wholesome for food and feed purposes. MON 87769 will not significantly impact the commercial interests of soybean producers or those involved in the marketing and sale of soybean and soybean products. The amount of land devoted to soybean production or crop production in general has not changed with the introduction of biotechnology-derived soybean products. Similarly, no significant change in the use of agricultural land or amount of land devoted to farming would be expected to occur with the commercial introduction of MON 87769.

MON 87769 will utilize common cultivation practices typically employed for production of commodity soybean and many types of specialty soybean, and management practices typically used for specialty soybean (i.e., IDP production practices). Hence, agricultural practices for MON 87769 are not likely to change.

The introduction and adoption of specialty soybean varieties in the past have provided differentiated high value soybean and soybean products that benefited U.S. growers and the end users of these products. The opportunity for growers to produce a land-based omega-3 fatty acid is a benefit from an economic and human health perspective. For these reasons, the proposed action to grant nonregulated status to MON 87769 does not represent a significant impact to the environment.

XII. ADVERSE CONSEQUENCES OF INTRODUCTION

Monsanto knows of no study results or observations associated with MON 87769 that indicate there would be an adverse environmental consequence from the introduction of MON 87769. MON 87769 is an alternate source of omega-3 fatty acid. As demonstrated by field results and laboratory tests, the only phenotypic difference between MON 87769 and conventional soybean is the intended change in seed fatty acid composition and the presence of the two desaturase proteins (PjΔ6D and NcΔ15D).

The data and information presented in this petition demonstrate that MON 87769 is unlikely to pose an increased plant pest potential or to have an adverse environmental consequence 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 and protein analyses, which confirmed the insertion of a single functional copy of the *Pj.D6D* and *Nc.Fad3* expression cassette at a single locus within the soybean genome and that PjΔ6D and NcΔ15D proteins were detected only in seed at low levels. Extensive characterization of the plant phenotype, including compositional analysis of key nutrient and antinutrients also indicated that MON 87769, with the exception of intended modification, was unchanged compared to conventional soybean. Allergenicity assessment and history of safe use of the PjΔ6D and NcΔ15D proteins concluded that the PjΔ6D and NcΔ15D proteins are unlikely to be an allergen for humans. Toxicity tests, including an acute mouse oral gavage with proteins produced in MON 87769 immature seed, showed no signs of adverse effects at high doses. An endangered species risk assessment also concluded that MON 87769 is unlikely to have adverse effects on these organisms. Therefore, the risks for humans, animals, and other NTOs from MON 87769 are negligible under the conditions of use.

The introduction of MON 87769 will not impact cultivation practices and the management of weeds, diseases, and insects in soybean production systems. Successful adaptation of MON 87769 will provide growers with an opportunity to produce this value-added speciality soybean containing SDA, which is a sustainable source of omega-3 fatty acid.

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APPENDICES

Appendix A. USDA Notifications

Field trials of MON 87769 were conducted in the U.S. since 2001. The protocols for these trials include field performance, breeding and observation, agronomics, and generation of field materials and data necessary for this petition. In addition to the phenotypic assessment data provided for MON 87769, 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 2007-2008 seasons, are still in preparation. A list of trials conducted under USDA notification and the status of the final reports for these trials are provided in Table A-1.

Table A-1. USDA Notification Approved for MON 87769 and Status of Trials Conducted under These Notifications

USDA #	Effective Date	Release Site (State)	Trial Status
2003 Field Trials			
03-225-02n	9/12/2003	PR(2)	Submitted to USDA
03-325-02n	1/21/2004	HI(1)	Submitted to USDA
2004 Field Trials			
04-035-02n	3/12/2004	IA(3), IL(9), KS(3)	Submitted to USDA
04-035-09n	3/26/2004	IA(2), IL(2)	Submitted to USDA
04-090-02n	4/29/2004	IA(2), IL(3)	Submitted to USDA
04-174-01n	8/11/2004	PR(2)	Submitted to USDA
2005 Field Trials			
05-035-01n	3/10/2005	IA(2), IL(10)	Submitted to USDA
05-035-02n	3/10/2005	IL(5), IN(2), KS(8)	Submitted to USDA
05-035-03n	3/10/2005	IA(12)	Submitted to USDA
05-035-04n	3/10/2005	IA(2), IL(11), IN(2)	Submitted to USDA
05-046-02n	3/28/2005	IL(1)	Submitted to USDA
05-073-15n	5/11/2005	IA(2), IL(10), IN(2), WI(1)	Submitted to USDA
05-131-05n	6/15/2005	PR(2)	Submitted to USDA
05-196-01n	8/17/2005	HI(1)	Submitted to USDA
05-217-03n	9/28/2005	PR(3)	Submitted to USDA
05-245-01n	10/3/2005	PR(1)	Submitted to USDA
05-299-09n	1/4/2006	HI(3)	Submitted to USDA
2006 Field Trials			
06-026-05n	2/27/2006	PR(2)	Submitted to USDA
06-027-03n	4/5/2006	IA(7), IL(5), IN(2)	Submitted to USDA
06-027-05n	2/27/2006	IL(8), KS(5)	Submitted to USDA
06-037-06n	3/14/2006	IL(3)	Submitted to USDA
06-045-12n	5/18/2006	HI(5)	Submitted to USDA
06-045-19n	5/18/2006	PR(3)	Submitted to USDA

Table A-1 (continued). USDA Notification Approved for MON 87769 and Status of Trials Conducted under These Notifications

USDA #	Effective Date	Release Site (State)	Trial Status
06-045-21n	3/22/2006	IL(1)	Submitted to USDA
06-045-22n	4/26/2006	IA(2), IL(3), IN(3), KS(1), MI(1), MO(3), NE(1), WI(1)	Submitted to USDA
06-069-10n	4/24/2006	PR(4)	Submitted to USDA
06-074-05n	5/15/2006	AR(1), IA(2), IL(3), MI(1), NE(1), OH(1), WI(1)	Submitted to USDA
06-076-12n	5/5/2006	IA(1), IL(2), KS(1), KY(1), MN(1), NE(1), OH(1), SD(1)	Submitted to USDA
06-101-02n	5/30/2006	IA(1), IL(3)	Submitted to USDA
06-118-04n	5/9/2006	IA(2)	Submitted to USDA
06-137-116n	6/7/2006	IA(1)	Submitted to USDA
06-195-102n	9/11/2006	PR(3)	Submitted to USDA
06-271-101n	10/28/2006	IA(2), IL(1), IN(2), MO(3)	Submitted to USDA
06-319-101n	12/15/2006	PR(4)	Submitted to USDA
2007 Field Trials			
07-019-101n	2/18/2007	IA(1), IL(10), IN(3), MO(1)	Submitted to USDA
07-019-104n	3/17/2007	IA(7), KS(6)	Submitted to USDA
07-029-103n	3/18/2007	IA(1), MN(2), PR(4)	Submitted to USDA
07-031-115n	3/21/2007	IL(2)	Submitted to USDA
07-039-122n	4/5/2007	IA(1), IL(2), IN(1), KS(1), KY(1), MN(1), NE(1), SD(1)	Submitted to USDA
07-046-108n	4/1/2007	IL(2)	Submitted to USDA
07-052-104n	4/10/2007	IL(1), IN(2), MI(1), MN(1), WI(2)	Submitted to USDA
07-058-101n	4/4/2007	AR(1), IA(1), IL(2), IN(1), MI(1), NE(1), OH(1), PA(1), WI(2)	Submitted to USDA
07-094-108n	5/4/2007	IL(1)	Submitted to USDA
07-095-101n	5/5/2007	IA(1)	Submitted to USDA

Table A-1 (continued). USDA Notification Approved for MON 87769 and Status of Trials Conducted under These Notifications

USDA #	Effective Date	Release Site (State)	Trial Status
07-099-102n	5/10/2007	IA(1), IN(1), MN(3), NE(1)	Submitted to USDA
07-159-101n	7/8/2007	PR(1)	In progress
07-283-101n	12/3/2007	PR(2)	In progress
07-296-101n	12/3/2007	PR(1)	In progress
2008 Field Trials			
08-038-101n	3/25/2008	IA(5), KS(2)	In progress
08-039-108n	3/9/2008	IA(2), IL(6), IN(2)	In progress
08-045-102n	3/15/2008	IL(2)	In progress
08-059-106n	4/1/2008	IA(2), MN(4), ND(1), SD(1), WI(2)	In progress
08-065-102n	4/4/2008	MI(1), WI(1)	In progress
08-078-108n	4/17/2008	IL(1)	In progress
08-079-101n	4/17/2008	IA(3)	In progress
08-084-102n	4/24/2008	IA(1), NE(1)	In progress
08-084-107n	4/24/2008	IL(5)	In progress
08-086-103n	4/25/2008	IL(2), IN(1)	In progress
08-098-101n	5/7/2008	IA(1), IL(1), IN(1), NE(1)	In progress

Appendix B. Materials and Methods Used for Molecular Analyses of MON 87769

B.1. Materials

The DNA used in the molecular analyses was isolated from leaf tissue grown from MON 87769 seed (seed lot number GLP-0509-16624-S). Additional DNA was extracted from leaf tissue grown from various generations of MON 87769 seed and used in generational stability analyses. The control DNA was isolated from leaf tissue from conventional soybean with the same genetic background (seed lot number GLP-0509-16625-S). The reference substances included the PV-GMPQ1972 plasmid, probe templates generated from this plasmid, and the size estimation molecular weight standards. As a positive control on Southern blots, PV-GMPQ1972 plasmid DNA was digested with a combination of enzymes to produce the banding patterns that were most relevant to the assessment of the test substance digested with appropriate enzyme(s). Probe templates generated from this plasmid also served as positive hybridization controls. Digested plasmid DNA or probe templates were added to predigested conventional soybean genomic DNA. The molecular weight standards include the 1 kb DNA Extension Ladder (Invitrogen, Carlsbad, CA) and λ DNA/*Hin*d III fragments (Invitrogen) for size estimations on Southern blots. The 100 bp and 500 bp DNA ladders (Invitrogen) were used for size estimations for PCR analyses**.**

B.2. Characterization of the Materials

The test and control materials identity were verified by PCR analysis to confirm the presence or absence of MON 87769, except for the materials used in the generational stability analyses. In this analysis the identity of the materials was confirmed by the generation stability Southern blots themselves. The stability of the genomic DNA was confirmed in each Southern analysis by observation of the digested DNA sample on an ethidium bromide-stained agarose gel.

B.3. DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA samples from MON 87769 and conventional soybean control were isolated from leaf tissue that was processed into a fine powder using a mortar and pestle under liquid nitrogen. DNA was extracted from the processed seed using a hexadecyltrimethylammonium bromide (CTAB) based method as follows: 2-4 mL equivalent of processed leaf tissue was placed in a 13 mL Sarstedt tube or 50 ml centrifuge tube and ~10 ml of CTAB extraction buffer (1.5% CTAB, 75 mM Tris pH 8.0, 100 mM EDTA, 1.05 M NaCl, 0.75% PVP [40K]) and 5-10 µL of 10 mg/mL RNase was added. The samples were incubated at 65°C for 40-50 minutes and mixed halfway through the incubation. The samples were cooled to room temperature, and divided between two tubes. 20 mL of 24:1 chloroform: isoamyl alcohol was added and the samples were mixed for 2-3 minutes and centrifuged for 8-10 minutes at 10,300 x *g* and 20-25°C to separate the aqueous and organic phases. The upper aqueous phase was transferred to a clean 50 mL centrifuge tube and 20 mL of 100% ethanol was added. The samples were then inverted to mix the samples until the DNA precipitated. The samples

were then centrifuged at 20-25°C to pellet the DNA, and the supernatant was discarded. The DNA pellet was air dried for ≤2 hours, and resuspended in an appropriate volume (250 – 750 mL) of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA, pH 8.0). To facilitate resuspension of the DNA, additional TE buffer was added and/or the solution was heated up to 70°C for 1-4 hours. The DNA was stored in a 4°C refrigerator, a -20°C, or a -80°C freezer.

B.4. Quantification of Genomic DNA

Quantification of DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer with Roche molecular size marker IX as a DNA calibration standard.

B.5. Restriction Enzyme Digestion of Genomic DNA

Approximately 10 or 20 µg of genomic DNA extracted from the test and control substances were used for restriction enzyme digestions. When digesting genomic DNA with *Lgu* I (Fermentas, Hanover, MD) or the enzyme combination *Bsr*G I and *Psh*A I (New England BioLabs, Ipswich, MA), Tango Buffer (Fermentas) was used. When digesting genomic DNA with the enzyme combination *Lgu* I and *Bst*X I (New England BioLabs), NEbuffer 2 (New England BioLabs) was used. All digests were performed at 55 °C or 37 °C according to SOP BR-ME-0316-01 in a total volume of approximately 500 µl using approximately 50-100 units of the appropriate restriction enzyme(s).

B.6. DNA Probe Preparation for Southern Blot Analyses

Probe template DNA containing sequences of plasmid PV-GMPQ1972 was prepared by PCR amplification according to SOP BR-ME-0486-01. Approximately 18-25 ng of each probe template were radiolabeled with ^{32}P-deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Invitrogen). Probe locations relative to the genetic elements in plasmid PV-GMPQ1972 are depicted in Figures V-1 and V-2.

B.7. Southern Blot Analyses of Genomic DNA

Digested DNA was separated using 0.8% (w/v) agarose gel electrophoresis. Except for generational stability analyses, DNA samples were loaded on the gels for a long run and a short run in an effort to provide better resolution of larger DNA fragments while retaining smaller DNA fragments on the gel. After transferring the DNA to the membrane, Southern blots were hybridized at 65°C with the exception of the *E9* 3′ nontranslated /Left Border regions and the T-DNA II Southern blots which were hybridized at 60 °C. Multiple exposures of each blot were then generated using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer.

B.8. DNA Sequence Analyses of the Insert

Overlapping PCR products were generated that span the insert in MON 87769 as well as genomic DNA directly adjacent to the insert. The PCR analyses were conducted

according to SOP BR-ME-0486-01 using 60 ng of genomic DNA template or approximately 0.9 ng plasmid DNA in a 50 µl reaction volume containing a final concentration of 1.8 mM MgCl2, 0.2 µM of each primer, 0.2 mM each dNTP, and 1 unit of DNA polymerase mix. The specific DNA polymerase mix used to generate the products was Platinum Taq (Invitrogen, Product A) or Platinum High Fidelity Taq (Invitrogen, Products B, C, D, and E). The amplification of Products A, B and E was performed under the following cycling conditions: 97°C for one minute; 34 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 2 minutes; one cycle at 68°C for 5 minutes. The amplification of Product C was performed under the following cycling conditions: 97°C for 1 minute; 32 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 3 minutes and 20 seconds; one cycle at 68°C for 5 minutes. The amplification of Products D was performed under the following cycling conditions: 97°C for 1 minute; 36 cycles at 96°C for 20 seconds, 55°C for 20 seconds, and 68°C for 2 minutes and 30 seconds; one cycle at 68°C for 5 minutes.

Aliquots of each PCR reaction were separated on a 1.0 % (w/v) agarose gel according to SOP BR-ME-0315-02 and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. The PCR product was sequenced with the multiple primers used for PCR amplification. In addition, primers internal to the PCR primers were used to sequence other regions of the amplified product. All sequencing was performed by the Monsanto Genomics Sequencing Center using dye-terminator chemistry.

Appendix C. Characterization of PjΔ6D and NcΔ15D Proteins Produced in MON 87769 – Materials, Methods and the Results

C.1. Materials and Methods Used for the Characterization of PjΔ6D Protein

C.1.1. Protein Purification

The PjΔ6D protein was purified from immature MON 87769 seed prior to initiation of this characterization plan. The purification of PjΔ6D from immature MON 87769 seed was not performed under a GLP study or plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. All operations were carried out at 4 °C unless otherwise indicated.

A total of 11 kg of immature MON 87769 seed (Orion Lot No. 10002214) was used for the isolation of PjΔ6D. The seed was homogenized for membrane isolation at a scale of ~1000-2500 g seed per extraction (a total of six runs). The isolated membrane was solubilized with 2% Fos-choline 12 and separated on a 3,000-mL cation exchange chromatography column (a total of three runs) followed by immuno-affinity purification (a total of 30 runs). The MON 87769-produced PjΔ6D protein isolated from the different batches were combined as a final preparation. After preliminary purity and concentration determination, the protein preparation was submitted to Monsanto's Analytical Protein Standards (APS) program under Orion lot # 10001532.

Each run included the following series of purification steps:

<u>*Homogenization and membrane isolation*</u> - Approximately 400 g of immature MON 87769 seed was homogenized with a Viking blender (Waring Commercial, Torrington, CT) for 3 min in 4 L of ice cold homogenization buffer consisting of 100 mM Tris-HCl, pH 8.0, 350 mM NaCl, 5.0 mM DTT, 0.5 mM PMSF, 1 µM leupeptin, and 1.0 mM sodium benzamidine hydrochloride. After filtration of the homogenate through four layers of cheese cloth, the filtrate was centrifuged at 2,000 x *g* to remove cell debris. The supernatant was centrifuged at 37,000 x *g* for 1 hr. After the centrifugation, the supernatant was discarded and the pellet re-suspended in ice cold de-ionized water (DI H_2O) at a total protein concentration of approximately 40 mg/mL. The membrane suspension was mixed with an equal volume of 2X Carbonate Wash Buffer (200 mM sodium carbonate, pH 11.5, 2 mM $MgCl_2$, 0.5 mM PMSF, 1 µM leupeptin, and 1.0 mM benzamidine hydrochloride). Immediately after mixing, the mixture was centrifuged at 37,000 x *g* at 4 °C for 30 min. The supernatant was discarded and the membrane pellets were stored at -80 °C until use. Extraction was repeated until all the remaining seed was homogenized.

<u>*Solubilization and cation exchange chromatography*</u> – The frozen membrane pellets (~100 g fresh weight) were thawed and re-suspended in 5 L of the solubilization buffer [50 mM sodium acetate, pH 5.6, 100 mM NaCl, 1.0 mM DTT, 10% Glycerol (v/v), 0.5 mM PMSF, and 1 µM leupeptin]. Solid Fos-choline 12 was added to a final concentration of 2% and the membrane/detergent solution was incubated at 4 °C for 2 hr with stirring. The mixture was centrifuged at 37,000 x *g* at 4 °C for 30 min to remove the insoluble fractions. The supernatant was applied onto a SP-Sepharose column (bed

volume ~3.0 L, GE Healthcare, Piscataway, NJ) that had been previously equilibrated with SP-Buffer A consisting of 50 mM sodium acetate, pH 5.6, 10% glycerol (v/v), and 0.1% Fos-choline 12 (w/v). After washing the column with SP-Buffer A, the column was further washed with 0.1 M NaCl in SP-Buffer A. The bound proteins were then eluted stepwise with 0.5 M NaCl in SP-Buffer A. Fractions containing the protein of interest were pooled, and stored at -80 °C.

Immuno-affinity chromatography - Immuno-affinity resin used in the purification was prepared by immobilizing anti-PjΔ6D peptide IgG to agarose resin using an IgG-orientation kit from Pierce (Cat. No. 44990, Rockford, IL), according to the manufacturer's instructions. The anti-PjΔ6D antibody (Lot Numbers: 7580967 and 7580957) was used for immunoaffinity resin preparation (total vol. of resin ~28 mL). The immuno-affinity column was equilibrated with Immunoaffinity Binding and Washing (IABW) Buffer consisting of 1X PBS, pH7.2, 1mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). Before applying the samples to the immuno-affinity column, the pH of the SP-Sepharose-purified protein preparation was adjusted to pH 7.4 with 2.0 M potassium phosphate, pH 8.5. The flow through fraction was re-applied to the column and the re-application of the flow through was repeated three times. The column was washed extensively with the IA washing buffer and PjΔ6D was eluted with 0.1 M Glycine, pH 2.8 containing 1 mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). The pooled protein fraction (~140 mL) was neutralized to pH 5.5 with 2.0 M MES, pH 10, and stored at -80 °C.

Protein concentration - To concentrate the IA-purified protein preparation, about 250 – 500 mL of the eluted fraction was loaded onto a 2-mL SP-Sepharose column (GE Healthcare, Piscataway, NJ) which had been previously equilibrated with SP-Buffer A. The flow rate was typically 4.0 mL/min. The protein was eluted with 0.5 M NaCl in SP-Buffer A and the fractions containing PjΔ6D protein were pooled and stored at -80 °C. A total of eight batches were prepared.

Final purification by Hydroxyapatite chromatography – All eight batches of the concentrated PjΔ6D preparations were combined and applied onto a 5-mL hydroxyapatite column (Type I ceramic hydroxyapatite, BioRad, Hercules, CA). While the majority of the contaminant proteins bound to the column, the flow through fraction was collected and saved as the final PjΔ6D protein preparation. After preliminary purity and concentration determination, the final preparation was submitted to Monsanto APS program under the Orion lot # 10001532.

C.1.2. Determination of Protein Concentration

The concentration of the MON 87769-produced PjΔ6D protein sample was determined by amino acid analysis (AAA) using AccQ-Tag derivatization (Waters Corporation, Milford, MA), a pre-column derivatization method which allows for high sensitivity fluorescent detection of amino acids. In order to avoid the interference from buffer components during protein hydrolysis, protein samples were precipitated using ethanol. The sample preparation was as follows: in a hydrolysis tube (~300 µl), approximately 1 µg of a protein sample was mixed with 200 µL of chilled 95% ethanol. After incubation overnight at -20 ºC, each sample was centrifuged for 45 min at 2-4 °C at

12,000 rpm in a microcentrifuge to precipitate proteins. The supernatant was removed and discarded. Each sample was then washed twice sequentially with 100 µL of both chilled acetone and water. Replicates of MON 87769-produced PjΔ6D protein, a hydrolysis blank, four dilutions of a calibration standard (NIST), and a BSA control were analyzed. An internal calibrant, α-aminobutyric acid, was included in all non-blank samples. All samples were evaporated to dryness in hydrolysis tubes using a Speed-Vac concentrator. A 500 µL volume of hydrolysis solution (6 N HCl, 1% phenol) was added and the tubes were transferred to a vacuum chamber. Samples were hydrolyzed for 90 min at 150±2 °C under vacuum. After cooling, the vacuum was released and the contents of the tube were evaporated to dryness using a Speed-Vac concentrator. A 20 µL volume of reconstitution solution (20 mM HCl) was added and tubes were vortexed to resuspend the sample. A 60 µl volume of AccQ-Fluor Borate Buffer and a 20 µl volume of AccQ-Fluor reagent were added sequentially to each vial with vortexing after each addition. The samples were transferred individually to autosampler vials, capped, and heated at 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corp.) in conjunction with reverse-phase C-18 column for separation of AccQ-Tag derivatized amino acids. Chromatographic data were collected using Atlas software (Thermo Electron Corp.).

C.1.3. Protein Identity

Immunoblot Analysis

Immunoblot analysis was performed to confirm the identity of PjΔ6D protein purified from MON 87769. Based on the concentration and purity, aliquots of the purified PjΔ6D protein preparation were diluted in 50 mM sodium acetate, pH 5.6, 10% glycerol and 0.1% Fos-choline 12 to give final amounts of 4, 6, 8, 10 and 12 ng, respectively. Samples were mixed with 5X Loading Buffer (LB) (312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8 and loaded directly onto gels without boiling. Membrane proteins in (a portion) the presence of detergents tends to self-associate via hydrophobic contact surfaces, forming irreversible nonspecific aggregates (McGregor et al., 2003; Sagne et al. 1996). This results in a smeared appearance on SDS-PAGE. Heating of the samples causes an increase in this behavior. As a result, none of the protein of interest (POI) samples was heated before analysis using SDS-PAGE. The samples were then separated on SDS-PAGE using 4-20% precast polyacrylamide gels (Invitrogen, Carlsbad, CA) at a constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min. Pre-stained molecular weight markers (Precision Plus protein standard, BioRad, Hercules, CA) were used to confirm the electrotransfer of proteins and estimate the size of the immunoreactive bands.

Following electrotransfer, the membrane was blocked for 30 min with 4% (w/v) NFDM in PBST. The membrane was then probed with a 1:5000 dilution of goat anti-PjΔ6D antibody (APS Lot No. 7580957¸ Orion number 10000821) in PBST containing 2% (w/v) NFDM for 1 hr. The IgG was purified from crude antiserum using immobilized PjΔ6D protein and was used at dilution of 1:5000. Rabbit anti-goat IgG conjugated with horse

radish peroxidase (Pierce, Rockford, IL) was used as secondary antibody at a dilution of 1:10,000. Excess antibody was removed using five 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase-conjugated rabbit anti-goat IgG (Pierce, Rockford, IL) at a dilution of 1:10,000 in PBST containing 2% (w/v) NFDM for 60 min. Following five 10 min washes with PBST, immunoreactive bands were visualized using the ECL detection system (GE Healthcare, Piscataway, NJ) and exposed (30 and 60 sec) to Hyperfilm ECL (G. E. Healthcare, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan). The MW of the immunoreactive bands was estimated using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

C.1.4. N-Terminal Sequencing

Edman degradation was used to confirm the N-terminal identity of the MON 87769-produced PjΔ6D.

Protein Blot for N-Terminal Analysis

An aliquot of MON 87769-produced PjΔ6D protein was removed from storage and mixed with 5X LB to a final concentration of 0.42 µg/µl and loaded in five lanes at 10.5 µg per lane. Samples were loaded directly onto gels without boiling. SDS-PAGE was conducted using 4-20% precast polyacrylamide gels (Invitrogen, Carlsbad, CA). Electrophoresis was carried out at constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to a PVDF (Invitrogen, Carlsbad, CA) membrane in the electrotransfer buffer containing 10 mM CAPS, pH 11, and 10% methanol at a constant voltage of 26 V for 120 min. Pre-stained molecular weight markers (Precision Plus protein standard, BioRad, Hercules, CA) were loaded in parallel to confirm electrotransfer of protein to the membrane and estimate the size of the stained bands observed. The blot was stained with Coomassie Blue R-250 (BioRad, Hercules, CA) to visualize the protein of interest.

N-Terminal Sequencing

Following electroblotting and staining, the band corresponding to MON 87769-produced PjΔ6D protein was excised from the blot and N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller and Hood, 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 pmole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the PjΔ6D protein, to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

C.1.5. MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the MON 87769-produced PjΔ6D protein.

Protein Gel for Tryptic Mass Map Analysis

A 100 µL aliquot of MON 87769-produced PjΔ6D protein was removed from storage, mixed with 5X LB to a final concentration of 0.42 µg/µL and loaded in five lanes at 10.5 µg per lane. Samples were loaded directly onto gels without boiling. SDS-PAGE was conducted using 4-20% precast polyacrylamide gels (Invitrogen, Carlsbad, CA). Electrophoresis was carried out at constant voltage of 50 V for 25 min followed by 160 V for 70 min. The gel was stained with Coomassie blue dye R-250 (BioRad, Hercules, CA) to visualize the protein of interest. The band corresponding to the MON 87769-produced PjΔ6D protein was excised from five lanes of the gel, destained, reduced, and alkylated. Briefly, each gel band was destained for 30 min by incubation in 100 µL of destaining solution (40% methanol, 10% acetic acid in water) in a microfuge tube. This step was repeated four times with the final destaining step proceeding for 60 minutes. Following destaining, the gel band was incubated in 100 µL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. The protein was reduced in 100 µL of 10 mM DTT solution for 2 h at 37 °C. The protein was alkylated by the addition of 100 µL of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. Gel band was incubated in 200 µL of 25 mM ammonium bicarbonate buffer for 15-25 min at room temperature. This step was repeated two additional times, following which the gel band was dried using a Speed Vac concentrator (Ramsey, MN). A single gel band was rehydrated with 20 µL of 0.02 µg/µL trypsin in 25 mM ammonium bicarbonate, 10% acetonitrile, and was incubated for about 1 h at room temperature, after which excess liquid was removed and the sample was incubated overnight at 37 °C in 40 µL of 25 mM ammonium bicarbonate, 10% acetonitrile. The following day, the sample was sonicated for 5 min, and the supernatant was transferred to a new tube and dried using a Speed Vac concentrator (Extract 1). The gel band was re-suspended in 30 µL 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% octyl-β-D-glucopyranoside solution, and sonicated for 5-10 min. After transfer of the supernatant to a new tube, this step was repeated one time, and the combined supernatants were dried using a Speed Vac concentrator (Extract 2). Extracts 1 and 2 were each resuspended in 20 µL 0.1% trifluoroacetic acid and then dried using a Speed Vac concentrator. Extract 1 was resuspended in 5 µL of 50% acetonitrile, 0.1% trifluoroacetic acid, while Extract 2 was re-suspended in 10 µL of the same solution. Each extract was sonicated for 5 min.

C.1.6. MALDI-TOF Mass Analysis

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture (CalMix 2; Applied Biosystems). Extract 1 and Extract 2 samples (0.3 µL) were co-crystallized with 0.70 µL each of the following matrix solutions: dihydroxybenzoic acid (DHB), α-cyano-4-hydroxy cinnamic acid (α-cyano), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) at separate

locations on the analysis plate. The samples in DHB matrix were analyzed in the 300 to 7500 Da range using 200 shots at a laser intensity setting of 3001. The laser intensity setting is a unit-less MALDI-TOF instrument-specific value. The samples in α-cyano matrix were analyzed in the 300 to 7500 Da range using 200 shots at a laser intensity setting of 2601. The samples in sinapinic acid matrix were analyzed in the 850 to 7500 Da range using 200 shots at a laser intensity setting of 3200. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993), except above 5000 Da, where mass-averaged values were used. GPMAW32 software (Applied Biosystems) was used to generate a theoretical trypsin digest of the expected PjΔ6D protein sequence based upon the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 8000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 Da from the mass analyzed.

C.1.7. Molecular Weight and Purity Estimation – SDS-PAGE

Aliquots of the MON 87769-produced PjΔ6D protein were diluted with Milli-Q water and mixed with 5X LB to a final protein concentration of 0.2 µg/µL. The MON 87769-produced PjΔ6D protein was analyzed in duplicate at 2, 3, and 4 µg of total protein per lane. Molecular weight standards were heated, though the test samples were not, and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (BioRad, Hercules, CA) were loaded in parallel. Electrophoresis was performed at a constant voltage of 120 V for 60 min followed by 180 V for 30 minutes. Proteins were stained using Colloidal Coomassie Brilliant Blue stain (Sigma-Aldrich, St. Louis, MO).

Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The molecular weight markers were used to estimate the apparent molecular weight of the MON 87769-produced PjΔ6D protein. For the purity evaluation, all visible bands within each lane were quantified. The purity and estimated molecular weight of the MON 87769-produced PjΔ6D protein were reported as the average of the six values obtained by densitometric analysis.

C.1.8. Glycosylation Analysis

The GE Healthcare Glycosylation Detection Module (Piscataway, NJ) was used to detect carbohydrate covalently bound to the MON 87769-produced PjΔ6D proteins after it was resolved by SDS-PAGE and electrotransferred onto PVDF membrane. The kit utilizes the specific labeling of biotin to the bound carbohydrate moiety followed by probing with streptavidin-conjugated horse radish peroxidase (Strep-HRP) and detection by ECL. The biotin labeling consists of two steps: pretreatment of the glycoprotein on the membrane and covalent conjugation of biotin to the carbohydrate moiety of the glycoprotein. The pre-treatment oxidizes hydroxyl groups of the carbohydrate moieties in the glycoproteins

to aldehydes which then react with biotin, resulting in covalent conjugation of biotin. This biotin-labeling procedure is specific for the detection of conjugation with carbohydrates and sensitive due to the use of biotin's high affinity binding partner, avidin, in an enzyme linked assay.

Aliquots of 0.5 µg and 1.0 µg of the purity-corrected PjΔ6D protein in SP-buffer A were mixed with 5X LB and loaded onto a 4-20% precast polyacrylamide gel (Invitrogen, Carlsbad, CA). Along with the PjΔ6D samples, 25, 50 100 and 200 ng of the glycosylated control protein, transferrin, were loaded in parallel. The PjΔ6D samples were not heated, but the transferrin controls were boiled prior to the loading. Electrophoresis was carried out at constant voltage of 90 V for 30 min followed by 159 V for 60 min. Proteins in the gel were electrotransferred to a PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min. Pre-stained molecular weight markers (Precision Plus Dual Color protein standard, BioRad, Hercules, CA) were loaded in parallel to confirm electrotransfer of protein to the membrane and estimate the size of the stained bands observed. Transferrin (~76 – 81 kDa, Sigma-Aldrich, St. Louis, MO) was loaded in parallel as a positive control of glycosylated protein in series of dilution.

The labeling and detection of carbohydrate was carried out according to the manufacturer's instructions and all the reagents except PBS were provided by the kit. Following electrotransfer to 0.45 µm PVDF membrane, the blot was incubated first in 30 mL of PBS for 10 min, then in 10 mM $NaIO_4$ for 20 min in darkness. The membrane blot was then rinsed twice with 15 mL PBS followed by three washes for 10 min each. To label the carbohydrate moiety on the protein with biotin, the membrane was incubated with biotin-hydrazide for 45 min followed by two rinses and three 10 min PBS washes as described above. The membrane was further blocked for 60 minutes with 5% blocking reagent in PBS followed by two rinses and three 10 min PBS washes. Strep-HRP at 1:6000 dilution was overlaid onto the membrane and incubated at room temperature for 30 min. After two rinses and three 10 min PBS washes, the membrane was then developed with ECL detection reagents by mixing 2 mL of Reagent 1 and 2 mL of Reagent 2. After 1 min incubation, the excess detection solution was removed by blotting with paper towel and the blot was exposed to Hyperfilm ECL (G. E. Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). The blot images were captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

C.1.9. Storage Stability

The short-term stability of the MON 87769-produced PjΔ6D protein was evaluated by comparing the purity and molecular weight values before and after storage for 29 days in a 4 ºC refrigerator, and in -20 ºC, and -80 ºC freezers. At the end of storage, aliquots of the PjΔ6D protein samples were removed and subjected to SDS-PAGE followed by staining, and subsequent purity and MW estimation. The MON 87769-produced PjΔ6D protein was analyzed in duplicate at 2, 3, and 4 µg of total protein per lane. MW standards were heated, though the test samples were not, and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (BioRad, Hercules, CA) were loaded in parallel.

Electrophoresis was performed at a constant voltage of 120 V for 20 min followed by 180V for 60 minutes. Proteins were stained using colloidal Coomassie Brilliant Blue stain (Sigma-Aldrich, St. Louis, MO).

Analysis of each gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The molecular weight markers were used to estimate the apparent molecular weight of the MON 87769-produced PjΔ6D protein. The protein samples were considered to have undergone degradation if a >10% decrease in purity and/or molecular weight was observed relative to the value determined on Day 0.

C.2. Materials and Methods Used for the Characterization of NcΔ15D Protein

C.2.1. Protein Purification

The NcΔ15 protein was purified from immature MON 87769 seed prior to initiation of this characterization plan. The purification was not performed under a GLP study or plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. All operations were carried out at 4 °C unless otherwise indicated.

A total of 13.5 kg of immature MON 87769 seed was used for the isolation of NcΔ15D protein in two separate batches. Approximately 2.5 kg of seed (Seed Lot No. GLP-0509-16624-S) were homogenized in four batches (500-800 g seed per batch) for the isolation of membrane fractions. The membrane was then solubilized in 2% Fos-choline 12 and separated on a 500-mL cation exchange chromatography column followed by immuno-affinity purification. The solubilization and cation exchange chromatography were performed in three different runs. The remaining 11 kg of seed (Seed Lot No. G-828102A) was homogenized for membrane isolation at a larger scale with 1,000-2,500 g of seed per extraction (a total of six runs). The solubilized membranes were then separated on a 3,000-mL cation exchange chromatography column (a total of three runs) followed by immuno-affinity purification (a total of 30 runs). The NcΔ15D protein preparations by both small-scale and large-scale operations were combined. Although at different scales, conditions (e.g., buffer to sample ratio) used to purify NcΔ15D protein at both small and large scales were proportionally comparable and therefore practically identical. After preliminary purity and concentration determination of the purified NcΔ15D preparation, the final preparation was submitted to Monsanto APS program under Orion lot # 10001516.

Each run included the following series of purification steps:

<u>*Homogenization and membrane isolation*</u>

In a 5-L Viking Blender (Waring Commercial, Torrington, CT), approximately 400 g of immature MON 87769 seed was homogenized for 3 min in 4,000 mL of ice-cold homogenization buffer consisting of 100 mM Tris-HCl, pH 8.0, 350 mM NaCl, 5.0 mM DTT, 0.5 mM PMSF, 1 µM leupeptin, and 1.0 mM sodium benzamidine hydrochloride. The process was repeated to homogenize the required amount seed (typically 1,000 g to 2,000 g seed per extraction). After filtration of the homogenate through four layers of

cheese cloth, the filtrate was centrifuged at 2,000 x *g* to remove cell debris. The supernatant was then centrifuged at 37,000 x *g* for 1 hr. After the centrifugation, the supernatant was discarded and the pellet re-suspended in ice-cold DI H_2O at a membrane concentration of approximately 40 mg/mL (fw/v). The membrane suspension was then mixed with an equal volume of 2X Carbonate Wash Buffer (200 mM sodium carbonate, pH 11.5, 2 mM $MgCl_2$, 0.5 mM PMSF, 1 μM leupeptin, and 1.0 mM benzamidine hydrochloride). Immediately after mixing, the mixture was centrifuged at 37,000 x *g* for 30 min. The supernatant was discarded and the membrane pellets were stored at -80 °C until use.

Solubilization and cation exchange chromatography

The frozen membrane pellets (~100 g FW) were thawed and re-suspended in 5 liters of the solubilization buffer [50 mM sodium acetate, pH 5.6, 100 mM NaCl, 1.0 mM DTT, 10% Glycerol (v/v), 0.5 mM PMSF, and 1 μM leupeptin]. Solid Fos-choline 12 was added to a final concentration of 2% (w/v) and the membrane/detergent solution was incubated for 2 hr with stirring. The mixture was centrifuged at 37,000 x *g* for 30 min to remove the insoluble fractions. The solubilized supernatant was applied onto a SP-Sepharose column (bed volume ~3.0 L, GE Healthcare, Piscataway, NJ) which had been previously equilibrated with SP-Buffer A consisting of 50 mM sodium acetate, pH 5.6, 10% glycerol (v/v), and 0.1% Fos-choline 12 (w/v). After washing the column with SP-Buffer A, the column was further washed with 0.1 M NaCl in SP-Buffer A. The bound proteins were then eluted stepwise with 0.5 M NaCl in SP-Buffer A. Fractions containing protein were pooled, and stored at -80 °C.

Immuno-affinity chromatography

Immuno-affinity resin used in the purification was prepared by immobilizing anti-NcΔ15D IgG to agarose resin using an IgG-orientation kit (Pierce Cat. No. 44990, Rockford, IL) according to the manufacture's instructions. The anti-NcΔ15D IgG (Lot Numbers: 7580958, 7580959, and 7580965) were used for immuno-affinity resin preparation. The immuno-affinity column (bed vol. ~28 mL) was equilibrated with IAWB consisting of 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). Before applying the SP-purified protein preparation to the immuno-affinity column, the pH of the sample was adjusted to pH 7.4 with 2.0 M potassium phosphate, pH 8.5. The flow through fraction was re-applied to the column and the flow through fraction re-application was repeated three times. The column was washed extensively with the IAWB and the NcΔ15D protein was eluted with 0.1 M glycine, pH 2.8 containing 1mM $MgCl_2$, 10% glycerol (v/v) and 0.1% Fos-choline 12 (w/v). The pooled protein fraction (~140 mL) was neutralized to pH 5.5 with 2.0 M MES, pH 10, and stored at -80 °C.

Concentration of Protein solution

To concentrate the immuno-affinity-purified NcΔ15D preparations, about 250 – 500 mL of the eluted fractions were loaded onto a 2-mL SP-Sepharose column (GE Healthcare, Piscataway, NJ) which had been previously equilibrated with the SP-Buffer A. The flow rate was typically at 4.0 mL/min. The protein was eluted with 0.5 M NaCl in the SP-Buffer A. Fractions containing the NcΔ15D protein were pooled and stored at -80 °C.

C.2.2. Protein Concentration

The total protein concentration of the MON 87769-produced NcΔ15D protein preparation was determined by amino acid analysis (AAA) using AccQ-Tag derivatization (Waters Corporation, Milford, MA), which allows for high sensitivity fluorescent detection of amino acids. In order to avoid the interference from buffer components during protein hydrolysis, protein was precipitated using ethanol. The sample preparation was as follows: in a hydrolysis tube (~300 µl), approximately 1 µg of protein samples (~10 µL) were mixed with 200 µL of chilled 95% ethanol. After incubation overnight at -20 ºC, samples were centrifuged at 12,000 rpm in a microcentrifuge for 30 min at 2-8 °C. The supernatant was removed and discarded. Precipitates were then washed sequentially with 100 µL of both chilled acetone and water. Along with replicates of the test sample, a hydrolysis blank, four dilutions of a calibration standard (NIST), and a BSA control were also analyzed. An internal calibrant, α-aminobutyric acid, was included in all non-blank samples. All samples were evaporated to dryness in hydrolysis tubes using a Speed-Vac concentrator. 500 µL of hydrolysis solution (6N HCl/1% phenol) was added and the tubes were transferred to a vacuum chamber. Samples were hydrolyzed for 90 min at 150 ± 2 °C under vacuum. After cooling, the vacuum was released and the hydrolysates were evaporated to dryness using a Speed-Vac concentrator and reconstituted in 20 µL of 20 mM HCl by vortexing. 60 µL of AccQ-Fluor Borate Buffer and 20 µL of AccQ-Fluor reagent were added sequentially to each vial with vortexing after each addition. The samples were transferred individually to autosampler vials, capped, and heated to 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corp.) equipped with a reverse-phase C-18 column for separation of AccQ-Tag derivatized amino acids. Chromatographic data were collected using Atlas software (Thermo Electron Corp. Waltham, MA).

C.2.3. Protein Identity

Immunoblot Analysis

Immunoblot analysis was performed to confirm the identity of NcΔ15D protein purified from MON 87769. Based on the concentration and purity, aliquots of the purified NcΔ15D protein preparation were diluted in 50 mM sodium acetate, pH 5.6, 10% glycerol and 0.1% Fos-choline 12 to give a final purity-corrected protein preparation of 2, 4, 6, 8, and 10 ng/10 µL, respectively. The samples (10 µL) were then mixed with 2.5 µL of 5X Loading Buffer (312 mM Tris-HCl, pH 6.8, 25% 2-mercaptoethanol, 10% sodium dodecyl sulfate, 0.025% bromophenol blue, 50% glycerol) and separated on the SDS-PAGE at constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min in the electrotransfer buffer containing 12 mM Tris, pH 8.3, 96 mM glycine, and 20% methanol. Pre-stained MW markers were loaded in parallel to confirm the transfer of proteins and estimate the size of the immuno-reactive bands observed.

Following the electrotransfer, the membrane was blocked for 30 min with 4% (w/v) NFDM in PBST. The membrane was then probed with a 1:5,000 dilution of goat anti-

NcΔ15D antibody (Lot No. 7580958) in PBST containing 2% (w/v) NFDM for 1 hr. Excess antibody was removed by three 15 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase-conjugated rabbit anti-goat IgG (Pierce, Rockford, IL) at a dilution of 1:10,000 in PBST containing 2% (w/v) NFDM for 60 min. Following five 15 min washes with PBST, immunoreactive bands were visualized using the ECL Detection System (GE Healthcare, Piscataway, NJ) and exposed (30 and 60 sec) to Hyperfilm ECL (G. E. Healthcare, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan). The MW of the immunoreactive bands were estimated using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

C.2.4. N-Terminal Sequencing

Edman degradation was used to confirm the N-terminal identity of the MON 87769-produced NcΔ15D and to determine if the N-terminal methionine was present in the protein.

Protein Blot for N-Terminal Analysis

Five 20 µL aliquots of MON 87769-produced NcΔ15D protein preparation were removed from storage, mixed with 5 µL of 5X LB and loaded in five lanes (25µL/lane) onto a 4-20% gradient polyacrylamide (10-well) gel. Pre-stained MW markers (Precision Plus protein standard, BioRad, Hercules, CA) were loaded in parallel to confirm electrotransfer of proteins to the membrane and estimate the size of the stained bands observed. Electrophoresis was carried out at constant voltage of 50 V for 25 min followed by 160 V for 70 min. Proteins in the gel were electrotransferred to PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 26 V for 2.0 hr in the electrotransfer buffer containing 10 mM CAPS, pH 11 and 10% methanol. The blot was stained with Coomassie Blue R-250 (BioRad, Hercules, CA) to visualize the protein of interest.

N-Terminal Sequencing

Following electroblotting and staining, the band corresponding to the NcΔ15D protein was excised from the blot and N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller and Hood, 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas[99] software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein, 10 pmole β-lactoglobulin, (Applied Biosystems, Foster City, CA) was analyzed before and after the analysis to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

C.2.5. MALDI-TOF Tryptic Mass Map Coverage Analysis

Protein Gel for Tryptic Mass Map Analysis

Five 20 μL aliquots of MON 87769-produced NcΔ15D protein preparation were removed from storage, mixed with 5 μL of 5X LB and loaded in five lanes (10.25 μg/lane) onto a 4-20% gradient polyacrylamide (10-well) gel. Pre-stained MW markers (Precision Plus Protein Standard, BioRad, Hercules, CA) were loaded in parallel to estimate the size of the stained bands observed. Electrophoresis was carried out at constant voltage of 50 V for 25 min followed by 160 V for 70 min. Pre-stained MW markers were loaded to estimate the size of the protein bands. Following electrophoresis, the gel was stained with Coomassie blue dye R-250 (BioRad, Hercules, CA). The bands corresponding to the MON 87769-produced NcΔ15D protein were excised from five lanes of the gel, destained, reduced, and alkylated. Briefly, each gel band was destained for 30 min by incubation in 100 μL of destaining solution in a microfuge tube. This step was repeated three times with the final destaining step proceeding for 60 minutes. Following destaining, the gel bands were incubated in 100 μL per band of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. The protein was reduced in 100 μL of 10 mM dithiothreitol solution for 2 h at 37 °C. After removing the reducing solution, the protein in the gel was alkylated by incubating in 100 μL of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. The gel containing the protein band was incubated in 200 μL of 25 mM ammonium bicarbonate buffer for 15-45 min at room temperature. This step was repeated two additional times, following which, gel band was dried using a Savant Speed Vac concentrator (Ramsey, MN). Each gel band was rehydrated with 20 μL of 0.02 μg/μL trypsin in 25 mM ammonium bicarbonate and 10% acetonitrile, and was incubated for about 1 h at room temperature. Following the incubation, the excess solution was removed and the gel/trypsin reaction mixture was incubated overnight at 37 °C in 40 μL of 25 mM ammonium bicarbonate and 10% acetonitrile. The following day, the sample was sonicated for 5 min, and the supernatant transferred to a new tube and dried using Speed Vac concentrator (Extract 1). The gel band was resuspended in 30 μL of a solution consisting of 60% acetonitrile, 0.1% trifluoroacetic acid and 0.1% octyl-β-D-glucopyranoside, and sonicated for 5-10 min. After transfer of the supernatant to a new tube, this step was repeated one more time, and the combined supernatants were dried using Speed Vac concentrator (Extract 2). Extracts 1 and 2 were separately dissolved in 20 μL 0.1% trifluoroacetic acid and then dried using a Speed Vac concentrator. Finally, Extract 1 was dissolved in 5 μL of 50% acetonitrile/0.1% trifluoroacetic acid, while Extract 2 was dissolved in 10 μL of the same solution. To maximize the solubilization, each sample was sonicated for 5 min. The extracts were ready for loading onto the MALDI-TOF sample plate.

C.2.6. MALDI-TOF Mass Analysis

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture (CalMix 2; Applied Biosystems, Foster City, CA). The samples Extract 1 and Extract 2 (0.1-0.25 μL) were co-crystallized with 0.75 μL each of the following matrix solutions: dihydroxybenzoic acid (DHB), α-cyano-

4-hydroxy cinnamic acid (α-cyano), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) at separate locations on the analysis plate. The samples in DHB matrix were analyzed in the 550 to 6,000 Da range using 200 shots at a laser intensity setting of 3,001. The laser intensity setting is a unit-less MALDI-TOF instrument-specific value. The samples in α-cyano matrix were analyzed in the 500 to 6,000 Da range using 200 shots at a laser intensity setting of 2,601. The samples in sinapinic acid matrix were analyzed in the 900 to 8,000 Da range using 200 shots at a laser intensity setting of 3,100. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993), except above 3,000 Da, where mass-averaged values were used. GPMAW32 software (Applied Biosystems) was used to generate a theoretical trypsin digest of the deduced NcΔ15D amino acid sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1,000 Da range if there were two or more isotopically resolved peaks, and in the 1,000 to 8,000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 Da from the mass analyzed.

C.2.7. Molecular Weight and Purity Analysis by SDS-PAGE

Aliquots of the MON 87769-produced NcΔ15D protein were diluted with the solubilization buffer (50 mM sodium acetate, pH 5.6, 0.1% Fos-choline 12, and 10% glycerol) and mixed with 5X LB to a final protein concentration of 0.2 μg/μL. The NcΔ15D protein was analyzed in duplicate at 1, 2, and 3 μg of total protein per lane. MW standards were heated, though the test samples were not, and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). MW markers (Broad Range MW Marker, BioRad, Hercules, CA) were loaded in parallel. Electrophoresis was performed at a constant voltage of 120 V for 60 min followed by 180 V for 30 minutes. The gel was stained using Colloidal Brilliant Blue G stain (Sigma-Aldrich, St. Louis, MO).

Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The MW markers were used to estimate the apparent MW of the NcΔ15D protein. For the purity evaluation, all visible bands within each lane were quantified. The purity and estimated MW of the MON 87769-produced NcΔ15D protein were reported as the average of the six values obtained by densitometric analysis.

C.2.8. Glycosylation Analysis

Glycosylation Detection Module (Cat. No. RPN 2190, GE Healthcare, Piscataway, NJ) was used to detect carbohydrate covalently bound to MON 87769-produced NcΔ15D after the proteins were resolved by SDS-PAGE and electrotransferred onto PVDF membrane. The kit utilizes the specific labeling of biotin to the bound carbohydrate moiety followed by probing with streptavidin-conjugated horse radish peroxidase (Strep-HRP) and detection by ECL. The biotin labeling consists of two steps: pretreatment of the glycoprotein on the membrane and covalent conjugation of biotin to the carbohydrate

moiety of the glycoprotein. This biotin-labeling procedure is specific for the conjugation with carbohydrates and is sensitive due to the use of biotin's high affinity binding partner, avidin, in an enzyme linked assay. This method of detecting glycoprotein on membrane blots is widely used for the determination of glycoproteins.

Aliquots of 0.5 µg and 1.0 µg of NcΔ15D protein were mixed with 5X LB and loaded onto a 4-20% precast polyacrylamide gel (Invitrogen, Carlsbad, CA). Along with the NcΔ15D samples, 25, 50, 100, and 200 ng of the glycosylated control protein, transferrin, were loaded in parallel. The NcΔ15D samples were not heated, but the transferrin controls were boiled prior to the loading. Electrophoresis was carried out at constant voltage of 90 V for 30 min followed by 159 V for 60 min. Following electrophoresis, proteins in the gel were electrotransferred to 0.45 µM PVDF membrane (Invitrogen, Carlsbad, CA) at a constant voltage of 25 V for 90 min in the electrotransfer buffer containing 12 mM Tris, pH 8.3, 96 mM glycine, and 20% methanol. Pre-stained MW markers were loaded to verify electrotransfer of proteins and estimate the size of the bands on the blot.

The labeling and detection of carbohydrate was carried out according to the manufacturer's instruction and all the reagents except PBS were provided in the kit. Following electrotransfer to PVDF membrane, the blot was incubated first in 30 mL of PBS for 10 min, then in 10 mM $NaIO_4$ for 20 min in darkness. Following the treatment, the membrane was then rinsed twice with 15 mL PBS and washed three times with 15 mL PBS for 10 min each. The membrane was incubated with biotin-hydrazide for 60 min followed by two PBS rinses and three 10 min washes with 15 mL PBS as described above. The membrane was blocked for 60 min with 5% blocking reagent in PBS followed by two PBS rinses and three 10 min washes with 15 mL PBS. Strep-HRP at 1:6000 dilution was overlaid onto the membrane and incubated at room temperature for 30 min. After two PBS rinses and three 10 min washes with 15 mL PBS, the membrane was then developed with ECL detection reagents by mixing 2 mL of the Reagent 1 and 2 mL of Reagent 2. After 1 min incubation, the excess detection solution was removed by blotting with paper towel and the blot was exposed to Hyperfilm ECL (G. E. Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). The blot images were captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

C.2.9 Storage Stability

The short-term stability of the MON 87769-produced NcΔ15D protein was evaluated by comparing the purity and MW values before and after the storage for 29 days in a 4 ºC refrigerator and, in-20 ºC and -80 º C freezers. At the end of storage, aliquots of the NcΔ15D protein samples equivalent to 1, 2, and 3 µg of the purity-corrected NcΔ15D at Day 0 were removed and analysed by SDS-PAGE followed by staining, and subsequent purity and MW estimation. The MON 87769-produced NcΔ15D protein was analyzed in duplicate at 1, 2, and 3 µg of total protein per lane. MW standards were heated, though the test samples were not, and all samples were applied to a pre-cast 4-20% polyacrylamide gradient 10-well gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 120 V for 20 min followed by 180 V for 60 minutes.

The gels were stained using Colloidal Brilliant Blue G stain (Sigma-Aldrich, St. Louis, MO).

Analysis of each gel was performed using a Bio-Rad GS-800 densitometer with the supplied 'Quantity One' software (version 4.4.0, Hercules, CA). The molecular weight markers were used to estimate the apparent molecular weight of the MON 87769-produced NcΔ15D protein on the gel. The protein samples were considered to have undergone degradation if a >10% decrease in purity and/or molecular weight was observed relative to the value determined on Day 0.

C.3. Characterization of the MON 87769-Produced PjΔ6D Protein

To characterize the purified PjΔ6D protein produced in MON 87769, a number of analyses were performed as described in Section C.1. The analyses employed for the characterization of MON 87769-produced PjΔ6D protein included:

1. N-terminal sequence analysis.
2. MALDI-TOF to generate a tryptic peptide map.
3. Immunoblot analysis to establish protein identity through immunoreactivity with PjΔ6D–specific antibody.
4. SDS-PAGE to assess the apparent molecular weight of the protein.
5. Glycosylation analysis to evaluate potential post-translational modification of the protein.

C.3.1. N-terminal Sequence Analysis

N-terminal sequence analysis of the first 15 amino acids performed on the major protein band with a molecular weight of ~46 kDa observed on stained SDS-PAGE (Figure C-1) and in immunoblot analysis with PjΔ6D antibody (Figure C-2) resulted in the expected sequence for the PjΔ6D protein (Table C-1). The N-terminal methionine was not observed, indicating that it was removed during post-translational processing of the protein. This result is expected as removal of the N-terminal methionine, catalyzed by methionine aminopeptidase, is a common modification that occurs co-translationally before completion of the nascent protein chain in many organisms and its removal has no effect on protein structure or activity (Arfin and Bradshaw, 1988; Polevoda and Sherman, 2000; Schmidt et al., 1992) and is common in many organisms. There was a second band of approximate molecular weight 7 kDa which comprised 10.6% of the total protein present on the gel. The sequence analysis of this band indicated the presence of multiple sequences that are not related to PjΔ6D protein. The protein preparation was not highly purified; therefore the presence of additional peptide fragments is not unexpected. The N-terminal sequence information, therefore, confirms the identity of the PjΔ6D protein isolated from MON 87769 and that its N-terminus is intact.

C.3.2. MALDI-TOF Mass Spectrometry Analysis

The identity of the MON 87769-produced PjΔ6D protein was further confirmed by tryptic peptide mass mapping analysis using MALDI-TOF MS. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). Observed tryptic peptides were considered a match to the expected tryptic mass when differences in molecular weight of less than one Da were found between the observed and predicted fragment masses. Such matches were made without consideration for potential natural amino acid modifications such as glycosylation. The MON 87769-produced PjΔ6D protein sample was heat-denatured, chemically reduced, alkylated, digested with trypsin, guanidinated, and the mass of the tryptic peptides were measured.

There were 30 unique protein fragments identified that matched the expected masses of the PjΔ6D trypsin-digested peptides. The identified protein fragments were used to assemble a coverage map of the entire PjΔ6D protein (Figure C-3), resulting in ~42% (188 out of 446 amino acids) coverage of the total protein. This analysis confirmed the identity of the MON 87769-produced PjΔ6D protein.

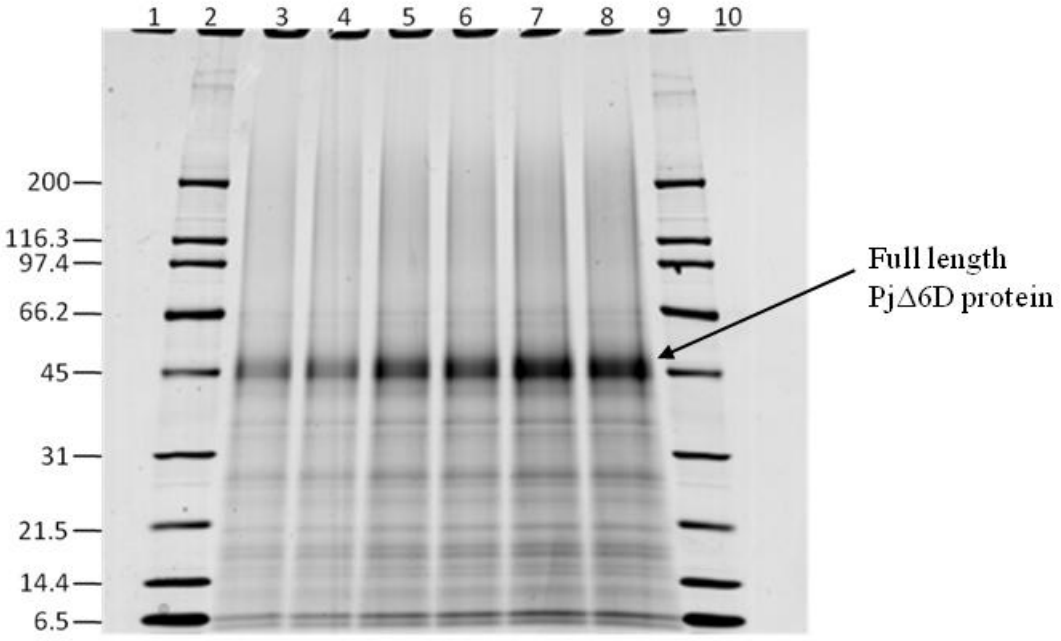

Figure C-1. SDS-PAGE of the MON 87769-Produced PjΔ6D Protein
Aliquots of the MON 87769-produced PjΔ6D protein were separated by SDS-PAGE, followed by Coomassie blue staining. Approximate molecular weights (kDa) are shown on the left and correspond to the marker loaded in lanes 2 and 9. Arrow indicates PjΔ6D protein.

Lane	Sample	Amount loaded (µg)
1	Blank	0
2	BioRad Broad Range Marker	4.5
3	MON 87769-produced PjΔ6D protein	2.0
4	MON 87769-produced PjΔ6D protein	2.0
5	MON 87769-produced PjΔ6D protein	3.0
6	MON 87769-produced PjΔ6D protein	3.0
7	MON 87769-produced PjΔ6D protein	4.0
8	MON 87769-produced PjΔ6D protein	4.0
9	BioRad Broad Range Marker	4.5
10	Blank	0

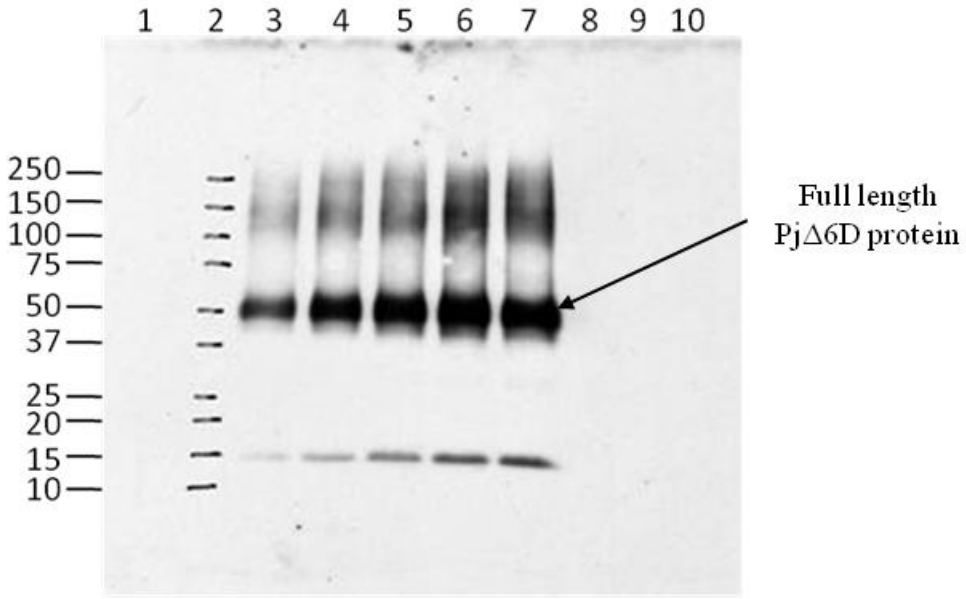

Figure C-2. Western Blot Analysis of MON 87769-Produced PjΔ6D Protein
Aliquots of the purified, MON 87769-produced PjΔ6D protein were separated by SDS-PAGE, and electro-transferred to a PVDF membrane. The membrane was probed with goat anti-PjΔ6D antibody and developed using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) of markers loaded in Lane 2 are shown on the left side of the blot.

Lane	Sample	Amount Loaded (ng)
1	Empty	-
2	Precision Plus All Blue protein Standards	7.5
3	MON 87769-produced PjΔ6D protein	4
4	MON 87769-produced PjΔ6D protein	6
5	MON 87769-produced PjΔ6D protein	8
6	MON 87769-produced PjΔ6D protein	10
7	MON 87769-produced PjΔ6D protein	12
8	Empty	-
9	Empty	-
10	Empty	-

Table C-1. N-terminal Amino Acid Sequence Analysis of the MON 87769-Produced PjΔ6D Protein

Amino acid residue # from the N-terminus	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Predicted PjΔ6D Sequence[1,2]	M	T	K	T	I	Y	I	T	S	S	E	L	E	K	H	N
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence[3]		T	K	T	I	Y	I	T	S	S	E	L	E	K	H	N

[1]The single letter IUPAC-IUB amino acid code is; E, glutamic acid; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; R, arginine; S, serine; T, threonine; Y, tyrosine.
[2]The predicted amino acid sequence of the PjΔ6D protein was deduced from the coding region of the full length *Pj.D6D* gene present in MON 87769 (Figure VI-1).
[3]The N-terminal methionine was removed by the action of methionine aminopeptidase during co-translation, and therefore, was not observed.

```
  1  MTKTIYITSS  ELEKHNKPGD  LWISIHGQVY  DVSSWAALHP  GGIAPLLALA
 51  GHDVTDAFLA  YHPPSTSRLL  PPFSTNLLLE  KHSVSETSSD  YRKLLDSFHK
101  MGMFRARGHT  AYATFVIMIL  MLVSSVTGVL  CSENPWVHLV  CGAAMGFAWI
151  QCGWIGHDSG  HYRIMTDRKW  NRFAQILSSN  CLQGISIGWW  KWNHNAHHIA
201  CNSLEYDPDL  QYIPLLVVSP  KFFNSLTSRF  YDKKLNFDGV  SRFLVQYQHW
251  SFYPVMCVAR  LNMLAQSFIL  LFSRREVANR  VQEILGLAVF  WLWFPLLLSC
301  LPNWGERIMF  LLASYSVTGI  QHVQFSLNHF  SSDVYVGPPV  GNDWFKKQTA
351  GTLNISCPAW  MDWFHGGLQF  QVEHHLFPRM  PRGQFRKISP  FVRDLCKKHN
401  LTYNIASFTK  ANVLTLETLR  NTAIEARDLS  NPIPKNMVWE  AVKNVG
```

Figure C-3. MALDI-TOF MS Coverage Map of the MON 87769-Produced PjΔ6D Protein

The amino acid sequence of the MON 87769 PjΔ6D protein was deduced from the coding region of the full-length *Pj.D6D* gene present in MON 87769 (see Figure VI-1). Shaded regions correspond to tryptic peptide masses that were identified from the protein sample using MALDI-TOF MS. In total, ~42% (188 of 446 total amino acids) of the expected protein sequence was identified.

C.3.3. PjΔ6D Protein Immuno-reactivity

A western blot analysis using goat anti-PjΔ6D antibody was conducted to further confirm the identity of the MON 87769-produced PjΔ6D protein. As demonstrated in Figure C-2, an immuno-reactive band was observed at the molecular weight of approximately 46 kDa. As expected, the immuno-reactive signal increased with increasing amount of protein loaded.

The immuno-reactive profiles shown in Figure C-2 reveal a slightly diffuse signal (streak) in the high molecular weight area between 100 kDa and 250 kDa. When membrane proteins are separated on SDS-PAGE, a portion tends to migrate as aggregates (McGregor et al., 2003; Von Jagow et al., 1994). While all possible precautions were taken during electrophoresis (i.e., the samples were not heated to prevent further aggregation), it is reasonable to assume that the diffuse signal observed in these blots consisted mainly of PjΔ6D protein as it is immuno-reactive with anti-PjΔ6D antibody. It is also possible that the diffuse signal observed in high molecular area originates from endogenous proteins cross-reacting with PjΔ6D antibody proteins. The PjΔ6D-specific antibody also recognized a band of approximately 15 kDa. This fragment is likely the result of proteolytic degradation of the PjΔ6D protein generated during the purification procedure or originates from endogenous cross-reacting proteins co-purified with the PjΔ6D protein.

Based on the above analysis, the identity of the MON 87769-produced PjΔ6D protein was confirmed.

C.3.4. PjΔ6D Protein Molecular Weight

The molecular weight of the MON 87769-produced PjΔ6D protein was determined using SDS-PAGE (Figure C-1). The MON 87769-produced PjΔ6D protein migrated with an apparent molecular weight of 46 kDa. The average purity of the full length PjΔ6D protein was estimated to be 47%.

C.3.5. PjΔ6D Protein Glycosylation Analysis

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988), while many prokaryotic organisms such as non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical synthetic capacity required for protein glycosylation. There are two forms of glycosylation. The first occurs on asparagine side chains, and is known as N-glycosylation. The other form is O-glycosylation, which is the addition of N-acetylglucosamine (N-GlcNAc) to the β-hydroxyl of either a serine or threonine residue. O-glycosylation sites are less well defined (Christlet and Veluraja, 2001) and may occur at any serine or threonine residue. PjΔ6D is an integral membrane protein of higher plant origin (*Primula juliae*) which contains two putative N-glycosylation sites Asn-Xxx-Ser/Thr (Marshall, 1972).

To test whether the MON 87769-produced PjΔ6D protein was glycosylated, the purified protein was analyzed for the presence of covalently bound carbohydrate moieties using a

Glycoprotein Detection Module (GE Healthcare, Piscataway, NJ) which detects N- and O-linked carbohydrates. A naturally glycosylated protein, transferrin, was utilized as a positive control for the assay. The transferrin protein and purified PjΔ6D protein isolated from MON 87769 were separated on SDS-PAGE, transferred to a PVDF membrane, and glycosylation analysis was performed to detect carbohydrate moieties on the proteins (Figure C-4). The positive control, transferrin, was detected at the expected molecular weights of ~75 kDa in a concentration-dependent manner (Figure C-4, Lanes 2-5). No detectable signal was observed at the expected molecular weight of approximately 46 kDa for the PjΔ6D protein (Figure C-4, lanes 7-8).

Three faint and slightly diffuse signals were detected by this analysis in the lane containing the highest protein concentration (Lane 8, Figure C-4) in the molecular weight range of 60-200 kDa. Taking into consideration that the purity of PjΔ6D protein is approximately 47% and that no signal was observed at the expected MW for PjΔ6D protein (~46 kDa), it was concluded that the faint signals observed on the blot are not derived from the PjΔ6D protein. Most likely, the observed faint bands originated from plant proteins that co-purified during the preparation of the PjΔ6D protein. Therefore, the above data demonstrate that the MON 87769-produced PjΔ6D is not glycosylated.

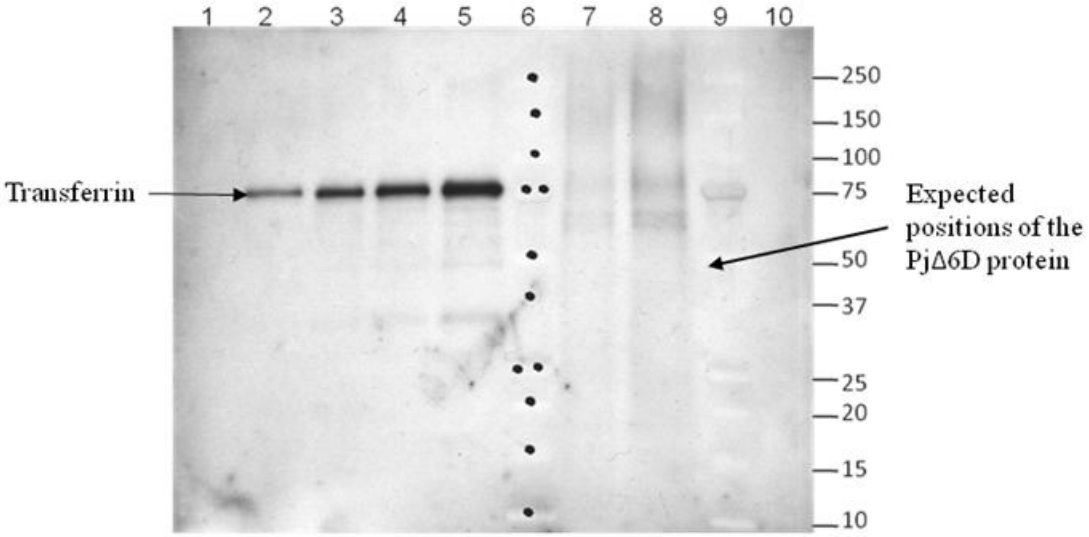

Figure C-4. Glycosylation Analysis of the MON 87769-Produced PjΔ6D Protein
Aliquots of transferrin (positive control) and MON 87769-produced PjΔ6D protein were separated by SDS-PAGE and transferred to a PVDF membrane. Carbohydrate moieties were labeled with biotin-hydrazide and detected using a Streptavidin-horseradish peroxidase (Streptavidin-HRP) antibody. Chemiluminescent activity of the HRP tag was detected using Hyperfilm ECL. Approximate molecular weights indicated in kDa correspond to protein standard markers (lane 6 and 9). The arrow indicates the expected position of PjΔ6D protein.

Lane	Sample	Amount Loaded (µg)
1	Blank	-
2	Transferrin	0.025
3	Transferrin	0.050
4	Transferrin	0.100
5	Transferrin	0.200
6	Precision Plus Dual Color MW marker	na
7	MON 87769-produced PjΔ6D	0.500
8	MON 87769-produced PjΔ6D	1.0
9	Precision Plus Dual Color MW marker	na
10	Empty	-

C.3.6. Conclusions on the Characterization of the MON87769-Produced PjΔ6D Protein

A panel of analytical techniques was used to characterize the purified MON 87769-produced PjΔ6D protein. The identity of the MON 87769-produced PjΔ6D protein was confirmed by its recognition with anti-PjΔ6D antibodies, identification of the first 15 amino acids of the expected N-terminus by amino acid sequencing, and identification of tryptic peptide masses that yielded ~42% overall coverage of the expected protein sequence. The apparent molecular weight of the MON 87769-produced PjΔ6D protein was estimated to be 46 kDa. The MON 87769-produced PjΔ6D protein was confirmed to be a non-glycosylated protein. Taken together, these data confirm the identity of the PjΔ6D protein isolated from MON 87769.

C.4. Characterization of the MON 87769-Produced NcΔ15D Protein

To characterize the purified NcΔ15D protein produced in MON 87769 a number of analyses were performed as described in Section C.2. The analyses employed for the characterization of MON 87769-produced NcΔ15D protein were the same as the analyses utilized to characterize PjΔ6D protein and included:

1. N-terminal sequence analysis.
2. MALDI-TOF mass spectrometry to generate a tryptic peptide map.
3. Immunoblot analysis to establish protein identity through immunoreactivity with NcΔ15D –specific antibody.
4. SDS-PAGE to assess the apparent molecular weight of the protein.
5. Glycosylation analysis to evaluate potential post-translational modification of the protein.

C.4.1. N-terminal Sequence Analysis

N-terminal sequence analysis performed on the major protein band with an apparent molecular weight of ~46 kDa observed on stained SDS-polyacrylamide gel (Figure C-5) resulted in the sequence expected for the NcΔ15D protein (Table C-2). The N-terminal methionine was not observed, indicating that it was removed during post-translational processing of the protein. This result is expected as removal of the N-terminal methionine, catalyzed by methionine aminopeptidase, is a common modification that occurs co-translationally before completion of the nascent protein chain and has no effect on protein structure or activity (Arfin and Bradshaw, 1988; Polevoda and Sherman, 2000; Schmidt et al., 1992) and is common in many organisms.

The N-terminal sequence information, therefore, confirms the identity of the NcΔ15D protein isolated from MON 87769 and that its N-terminus is intact.

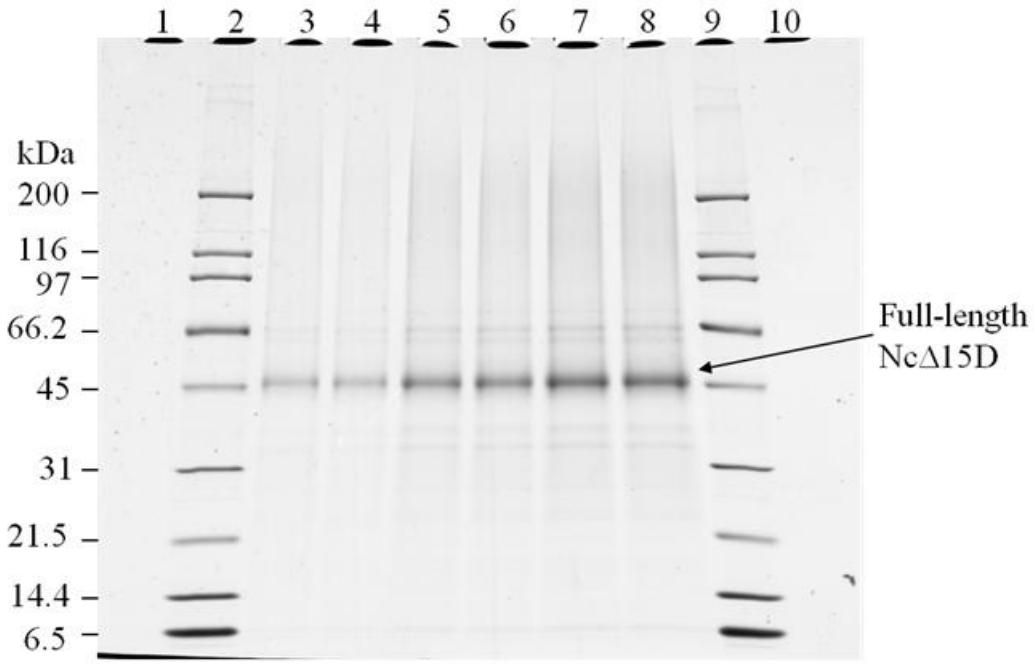

Figure C-5. SDS-PAGE of the MON 87769-Produced NcΔ15D Protein
Aliquots of the MON 87769-produced NcΔ15D protein were separated by SDS-PAGE, followed by Coomassie blue staining. Approximate molecular weights (kDa) are shown on the left and correspond to the marker loaded in lanes 2 and 9. Arrow indicates NcΔ15D protein.

Lane	Sample	Amount loaded (µg)
1	Empty	
2	BioRad Broad Range Marker	0.2
3	MON 87769-produced NcΔ15D protein	1
4	MON 87769-produced NcΔ15D protein	1
5	MON 87769-produced NcΔ15D protein	2
6	MON 87769-produced NcΔ15D protein	2
7	MON 87769-produced NcΔ15D protein	3
8	MON 87769-produced NcΔ15D protein	3
9	BioRad Broad Range Marker	0.2
10	Empty	

Table C-2. N-terminal Amino Acid Sequence Analysis of the MON 87769-Produced NcΔ15D Protein

Amino acid residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Predicted NcΔ15D Sequence[1,2]	→	M	A	V	T	T	R	S	H	K	A	A	A	A	T	E	P
			|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence[3]	→		A	V	T	T	R	S	H	K	A	A	A	A	T	E	P

[1]The single letter IUPAC-IUB amino acid code is; A, alanine; E, glutamate; H, histidine; M, methionine; P, proline; R, arginine; S, serine; T, threonine.
[2]The predicted amino acid sequence of the NcΔ15D protein was deduced from the coding region of the full length *Nc.Fad3* gene present in MON 87769 (see Figure VI-3).
[3]The N-terminal methionine was removed by the action of methionine aminopeptidase during co-translation, and therefore, was not observed.

C.4.2. MALDI-TOF Mass Spectrometry Analysis

The identity of the MON 87769-produced NcΔ15D protein was further confirmed by tryptic peptide mass mapping analysis using MALDI-TOF MS. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). Observed tryptic peptides were considered a match to the expected tryptic mass when differences in molecular weight of less than one Da were found between the observed and predicted fragment masses. Such matches were made without consideration for potential natural amino acid modifications such as glycosylation. The protein sample was heat-denatured, chemically reduced, alkylated, digested with trypsin, guanidinated, and the masses of the tryptic peptides were measured.

There were 15 unique protein fragments identified that matched the expected masses of the NcΔ15D trypsin-digested peptides. The identified masses were used to assemble a coverage map indicating the matched peptide sequences for the entire NcΔ15D protein (Figure C-6), resulting in ~45% (193 out of 429 amino acids) coverage of the total protein. This analysis confirmed the identity of the MON 87769-produced NcΔ15D protein.

```
  1 MAVTTRSHKA AAATEPEVVS TGVDAVSAAA PSSSSSSSSQ KSAEPIEYPD
 51 IKTIRDAIPD HCFRPRVWIS MAYFIRDFAM AFGLGYLAWQ YIPLIASTPL
101 RYGAWALYGY LQGLVCTGIW ILAHECGHGA FSRHTWFNNV MGWIGHSFLL
151 VPYFSWKFSH HRHHRFTGHM EKDMAFVPAT EADRNQRKLA NLYMDKETAE
201 MFEDVPIVQL VKLIAHQLAG WQMYLLFNVS AGKGSKQWET GKGGMGWLRV
251 SHFEPSSAVF RNSEAIYIAL SDLGLMIMGY ILYQAAQVVG WQMVGLLYFQ
301 QYFWVHHWLV AITYLHHTHE EVHHFDADSW TFVKGALATV DRDFGFIGKH
351 LFHNIIDHHV VHHLFPRIPF YYAEEATNSI RPMLGPLYHR DDRSFMGQLW
401 YNFTHCKWVV PDPQVPGALI WAHTVQSTQ
```

Figure C-6. MALDI-TOF MS Coverage Map of the MON 87769-Produced NcΔ15D Protein

The amino acid sequence of the MON 87769 NcΔ15D protein was deduced from the coding region of the full-length *Nc.Fad3* gene present in MON 87769 (see Figure VI-3). Shaded regions correspond to tryptic peptide masses that were identified from the protein sample using MALDI-TOF MS. In total, ~45% (193 of 429 total amino acids) of the expected protein sequence was identified.

C.4.3. NcΔ15D Protein Immuno-reactivity

A western blot analysis using goat anti-NcΔ15D antibody was conducted to further confirm the identity of the MON 87769-produced NcΔ15D protein. As demonstrated on Figure C-7, a predominant immuno-reactive band was observed at the expected molecular weight of approximately 46 kDa (Figure C-7, lanes 2-6). As expected, the immuno-reactive signal increased with increasing amount of protein loaded.

The immuno-reactive profiles shown in Figure C-7 reveal slightly diffuse immuno-staining in the high MW portion of the gel. When membrane proteins are separated on SDS-PAGE, a small portion tends to aggregate (McGregor et al., 2003; Von Jagow et al., 1994). While all possible precautions were taken during electrophoresis (i.e., the samples were not heated to prevent further aggregation), it is reasonable to assume that the diffuse signal observed in these blots consisted mainly of the NcΔ15D protein as it is immuno-reactive with anti-NcΔ15D antibody.

In addition, two minor bands with molecular weights between 25 kDa and 37 kDa were recognized by the antibody (Figure C-7, lanes 2-6). It is likely that these signals are the results of proteolytic degradation of the NcΔ15D protein that occurs during the purification procedure or originate from endogenous cross-reacting proteins co-purified with the NcΔ15D protein.

Based on the above analysis, the identity of the MON 87769-produced NcΔ15D protein was confirmed.

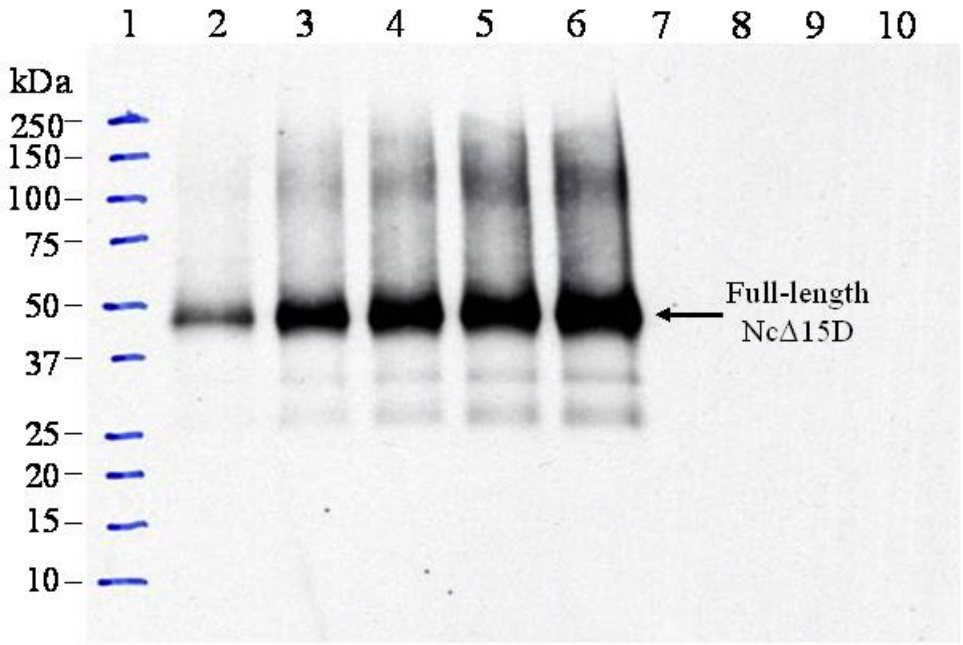

Figure C-7. Western Blot Analysis of MON 87769-Produced NcΔ15D Protein
Aliquots of the purified, MON 87769-produced NcΔ15D protein were separated by SDS-PAGE, and electro-transferred to a PVDF membrane. The membrane was probed with goat anti-NcΔ15D antibody and developed using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) of markers loaded in Lane 1 are shown on the left side of the blot.

Lane	Sample	Amount loaded (ng)
1	Precision Plus MWT Marker	198
2	MON 87769-produced NcΔ15D protein	2.0
3	MON 87769-produced NcΔ15D protein	4.0
4	MON 87769-produced NcΔ15D protein	6.0
5	MON 87769-produced NcΔ15D protein	8.0
6	MON 87769-produced NcΔ15D protein	10.0
7	Empty	
8	Empty	
9	Empty	
10	Empty	

C.4.4. NcΔ15D Protein Molecular Weight

The molecular weight of the MON 87769-produced NcΔ15D protein was determined using SDS-PAGE (Figure C-5). The MON 87769-produced NcΔ15D protein migrated with an apparent molecular weight of 46 kDa. The average purity of the full length NcΔ15D protein was estimated to be 74%.

C.4.5. NcΔ15D Protein Glycosylation Analysis

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988), while many prokaryotic organisms such as non-virulent *E. coli* strains used for cloning and expression purposes, lack the necessary biochemical synthetic capacity required for protein glycosylation. There are two forms of glycosylation. The first occurs on asparagine side chains, and is known as N-glycosylation. The other form is O-glycosylation, which is the addition of N-acetylglucosamine (N-GlcNAc) to the β-hydroxyl of either serine or threonine residue. O-glycosylation sites are less well defined (Christlet and Veluraja, 2001) and may occur at largely any serine or threonine residue. NcΔ15D is an integral membrane protein of microbial origin, which contains two putative N-glycosylation sites Asn-Xxx-Ser/Thr (Marshall, 1972).

To test whether the MON 87769-produced NcΔ15D protein was glycosylated, the purified protein was analyzed for the presence of covalently bound carbohydrate moieties using a Glycoprotein Detection Module (GE Healthcare, Piscataway, NJ) which detects N- and O-linked carbohydrates. A naturally glycosylated protein, transferrin, was utilized as a positive control for the assay. The transferrin protein and purified NcΔ15D protein isolated from MON 87769 were separated on SDS-PAGE, transferred to a PVDF membrane, and glycosylation analysis was performed to detect carbohydrate moieties on the proteins (Figure C-8). The positive control, transferrin, was detected at the expected molecular weights of ~75 kDa in a concentration-dependent manner (Figure C-8, lanes 2-5). No detectable signal was observed at the expected MW of approximately 46 kDa for the NcΔ15D protein (Figure C-8, lanes 7-8).

Two faint bands were detected at ~70 kDa in lanes 7-8 containing purified NcΔ15D protein (Figure C-8, lanes 7-8). An additional very weak signal was observed in the same lanes between 150 kDa and 250 kDa. Taking into consideration that the purity of NcΔ15D protein is only 74% and that no signal was observed at the expected MW for NcΔ15D protein ~46 kDa, it was concluded that the faint signals observed on the blot are not derived from the NcΔ15D protein. Most likely, the observed faint bands originate from plant proteins that co-purified during the preparation of the NcΔ15D protein. Therefore, the above data demonstrate that the MON 87769-produced NcΔ15D is not glycosylated.

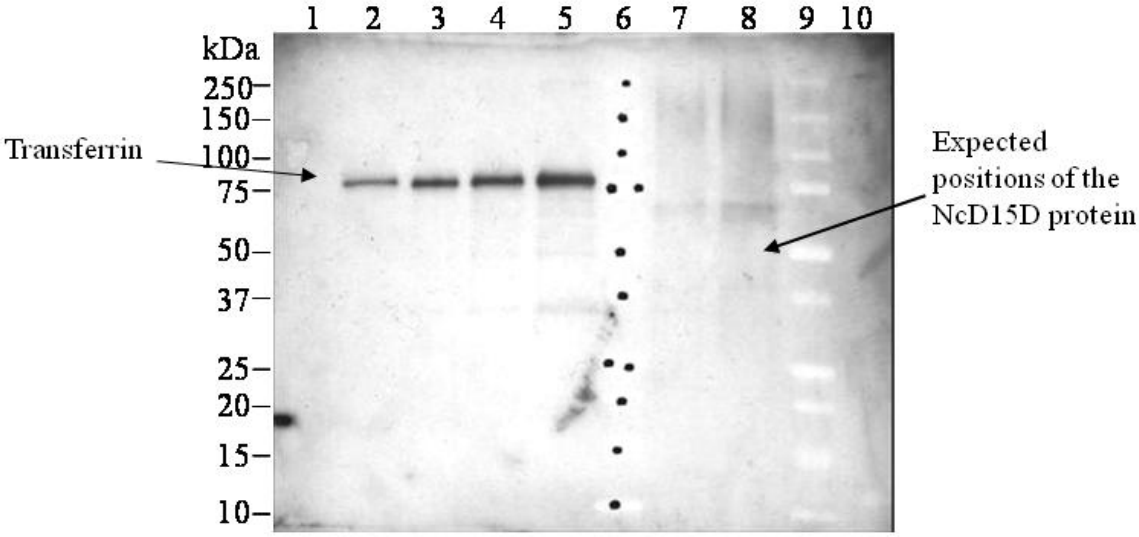

Figure C-8. Glycosylation Analysis of the MON 87769-Produced NcΔ15D Protein
Aliquots of transferrin (positive control) and MON 87769-produced NcΔ15D protein were separated by SDS-PAGE and transferred to a PVDF membrane. Carbohydrate moieties were labeled with biotin-hydrazide and detected using a Streptavidin-HRP antibody. Chemiluminescent activity of the HRP tag was detected using Hyperfilm ECL. Approximate molecular weights indicated in kDa correspond to protein standard markers (lane 6 and 9). The arrow indicates the expected position of NcΔ15D protein.

Lane	Sample	Amount loaded (μg)
1	Empty	
2	Transferrin	0.025
3	Transferrin	0.05
4	Transferrin	0.10
5	Transferrin	0.20
6	BioRad Precision Plus MWT Marker	0.20
7	MON 87769-produced NcΔ15D protein	0.50
8	MON 87769-produced NcΔ15D protein	1.00
9	BioRad Precision Plus MWT Marker	0.20
10	Empty	

C.4.6. Conclusions on the Characterization of the MON 87769-Produced NcΔ15D Protein

A panel of analytical techniques was used to characterize the purified MON 87769-produced NcΔ15D protein. The identity of the purified NcΔ15D protein was confirmed by its recognition with anti-NcΔ15D antibodies, identification of the first 15 amino acids of the expected N-terminus by amino acid sequencing, and identification of tryptic peptide masses that yielded 45% overall coverage of the expected protein sequence. The apparent molecular weight of the NcΔ15D protein was estimated to be 46 kDa. In addition, the MON 87769-produced NcΔ15D was confirmed to be a non-glycosylated protein. Taken together, these data confirm the identity of the NcΔ15D protein isolated from MON 87769.

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Appendix D. Materials and Methods Used for the Determination of Protein Expression in MON 87769 Tissue

D.1. Tissue Processing and Protein Extraction

All tissue samples produced at the field sites were shipped to the Monsanto Sample Processing facility. Processed tissue samples were stored in a -80 °C freezer until shipped on dry ice to Monsanto's analytical facility. All processed tissue samples were stored in a -80 °C freezer during the study.

The PjΔ6D and NcΔ15D proteins were extracted from soybean tissue by shaking tubes mounted in a Harbil mixer for two 3.5 minute cycles. Each extraction tube contained two ¼" diameter Chrome-steel beads, buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 2% Triton X-100, 1X protease inhibitors [Roche Diagnostics, Indianapolis, IN]) and a tissue-to-buffer ratio of 1:10 for root, leaf, and forage or 1:20 for immature and mature seed. Extracts were clarified by centrifugation (14,000 rpm x 15 minutes at 4 ^{o}C) and stored in a -80 °C freezer until western blot analyses.

D.2. PjΔ6D Antibodies

Goat polyclonal antibodies (lot # 7580971) were raised against a peptide fragment of the PjΔ6D protein (EARDLSNPIPKN), which is located close to the C terminus of the PjΔ6D protein. The antibodies were affinity purified by Invitrogen Life Sciences (Carlsbad, CA). The concentration of the purified IgG was determined to be 2.2 mg/mL by spectrophotometric methods. The purified antibody was stored in 25 mM sodium borate, 100 mM boric acid, 75 mM NaCl, 5 mM EDTA, pH 8.2 to 8.4 (Borate Buffered Saline).

D.3. NcΔ15D Antibodies

Goat polyclonal antibodies (lot # 7580965) were raised against a peptide fragment of the NcΔ15D protein (TEADRNQRKLANLYMDKET) located in the middle region of the NcΔ15D protein. The antibodies were affinity purified by Invitrogen Life Sciences. The concentration of the purified IgG was determined to be 2.6 mg/mL by spectrophotometric methods. The purified antibody was stored in Borate Buffered Saline.

D.4. PjΔ6D Western Blot Method

Extracts were analyzed by SDS-PAGE on a 4-20% Tris-HCl gradient gel. Prior to loading, the samples were diluted in 2X Laemmli Buffer (Bio-Rad, Hercules, CA). Sample extracts were loaded on the gels with the appropriate reference standards. The reference standards were prepared in tissue-specific nontransgenic extracts for each tissue type and diluted in 2X Laemmli buffer. The reference standards ranged from 20 ng to 1 ng. The nontransgenic extracts used for the reference standards for each tissue type were pooled from all sites. Samples were loaded in triplicate and at least two were used for densitometry and quantitation. Additionally, the Precision Plus molecular weight marker

(Bio-Rad) was loaded on the gel to demonstrate the transfer of protein to membrane and for the approximate molecular weight determination.

Electrophoresis was conducted according to the current version of SOP BR-ME-0388 at 200 V for approximately 5 minutes and at 120 V for approximately 80 minutes in 1X Tris-Glycine-SDS running buffer (Bio-Rad). Electrotransfer and western blot analysis were conducted according to the current versions of SOPs BR-ME-0924 and BR-ME-0392, respectively. Proteins separated by SDS-PAGE were electrophoretically transferred to 0.45 µm Criterion Nitrocellulose membrane (Bio-Rad) using 1X Tris-glycine transfer buffer (Bio-Rad) containing 20% methanol. After transfer, nonspecific sites on the membrane were blocked using 5% (w/v) NFDM (Bio-Rad, Hercules, CA) in 1X Phosphate-Buffered Saline with 0.05% (v/v) Tween-20 (1X PBST).

The membrane was probed for the presence of the PjΔ6D protein with a 1:2500 dilution of purified goat antibodies, anti-PjΔ6D peptide antibody (Lot 7580971) in 1X PBST with 1% (w/v) NFDM. Unbound antibodies were removed by rinsing the membrane briefly and then washing three times of 10 minutes each in 1X PBST. Bound antibodies were probed with a 1:5000 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL) in 1X PBST with 1% (w/v) NFDM. Unbound anti-goat IgG-HRP antibodies were removed by rinsing the membrane briefly and then washing four times of 10 minutes each in 1X PBST. The ECL substrate (Amersham, Piscataway, NJ) was added to the membrane according to the manufacturers' instructions. The membrane was exposed to Hyperfilm ECL (Amersham, Piscataway, NJ) to record an image of the immunoreactive bands.

D.5. NcΔ15D Western Blot Method

Extracts were analyzed by SDS-PAGE on a Novex 4-20% gradient gel (Invitrogen). Prior to loading, the samples were diluted in 2X Laemmli Buffer (Bio-Rad). Sample extracts were loaded on the gels with the appropriate reference standards. The reference standards were prepared in tissue-specific nontransgenic extracts for each tissue type and diluted in 2X Laemmli buffer. The reference standards ranged from 75 ng to 1 ng. The nontransgenic extracts used for the reference standards for each tissue type were pooled from all sites. Samples were loaded in triplicate and at least two were used for densitometry and quantitation. Additionally, the Precision Plus molecular weight marker (Bio-Rad) was loaded on the gel to demonstrate the transfer of protein to membrane and for the approximate molecular weight determination.

Electrophoresis was conducted according to the current version of SOP BR-ME-0388 at 120 V for approximately 120 minutes in 1X Novex Tris-Glycine SDS running buffer (Invitrogen, Carlsbad, CA). Electrotransfer and western blot analysis were conducted according to the current version of SOPs BR-ME-0924 and BR-ME-0392, respectively. Proteins separated by SDS-PAGE were electrophoretically transferred to Invitrolon PVDF membrane (Invitrogen) using 1X Novex Tris-Glycine transfer buffer (Invitrogen) containing 20% methanol. After transfer, nonspecific sites on the membrane were blocked using 5% (w/v) NFDM in 1X Tris-Buffered Saline with 0.1% (v/v) Tween-20 (1X TBST).

The membrane was probed for the presence of the NcΔ15D protein with a 1:2500 dilution of purified goat antibodies, anti-NcΔ15D peptide antibody (Lot 7580965) in 1X TBST with 5% (w/v) NFDM. Unbound antibodies were removed by rinsing the membrane briefly and then washing three times of 10 minutes each in 1X TBST. Bound antibodies were probed with a 1:5000 dilution of anti-goat IgG antibody conjugated to HRP (Pierce, Rockford, IL) in 1X TBST with 5% (w/v) NFDM. Unbound anti-goat IgG-HRP antibodies were removed by rinsing the membrane briefly and then washing four times of 10 minutes each in 1X TBST. The SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was added to the membrane according to the manufacturers' instructions. The membrane was exposed to Hyperfilm ECL (Amersham, Piscataway, NJ) to record an image of the immunoreactive bands.

D.6. Moisture Analysis

Forage and immature and mature seed were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) according to SOP AG-EQ-1023-01. A homogeneous tissue-specific site pool (TSSP) was prepared using MON 87769 and conventional soybean control samples of a given tissue type grown at a given site. These pools were prepared for all tissue types in this study. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$DWCF = 1 - [Mean\ \%\ TSSP\ Moisture / 100]$$

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:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ Fresh\ Weight)}{(DWCF)}$$

The protein levels that were reported to be less than or equal to the LOD or less than the LOQ on a fresh weight basis were not reported on a dry weight basis.

D.7. Data Analyses

Quantitation of the bands was performed according to the current version of BR-ME-0932. At least three standards were present on the film and used to generate the standard curve for sample quantitation. For the tissues that protein expression was not detected, only the standard curve was generated. For the tissue types where protein expression was detected, at least two sample replicates were used for densitometry and quantitation. The coefficient of variation (CV) met the pre-set criteria of ≤33%.

Appendix E. Materials and Methods Used for the Compositional Analysis of MON 87769 Soybean Seed, Forage and Processed Fractions from the Five Replicated Field Sites in the U.S. in 2006 Growing Season

E.1. Materials

MON 87769, a conventional soybean control (A3525) and conventional reference soybean varieties were grown at five U.S. locations in 2006. MON 87769 and the conventional soybean control were grown from seed lots GLP-0604-17267-S and GLP-0604-17278-S, respectively. The conventional soybean control has background genetics representative of MON 87769 but does not contain neither the *Nc.Fad3* nor the *Pj.D15D* coding sequence. In addition, 10 conventional soybean varieties produced alongside of MON 87769 were included for the generation of a 99% tolerance interval. The varieties, locations, and seed lot numbers are listed below:

Vendor/Variety	Starting Seed Lot No.	Field Site
Stine/ST3300	GLP-0605-17335-S	IA-1
Asgrow/A3244	GLP-0604-17273-S	IA-1
Asgrow/A2869	GLP-0604-17264-S	IA-1
Stine/ST2788	GLP-0605-17334-S	IA-2
Lewis 372	GLP-0604-17261-S	IA-2
Stine/ST3300	GLP-0605-17335-S	IA-2
Stine/ST3600	GLP-0605-17336-S	IL
P-93B82	GLP-0604-17260-S	IL
Lewis 392	GLP-0604-17262-S	IL
Asgrow/A2553	GLP-0604-17263-S	MI
Asgrow/A2804	REF-0506-16373-S	MI
Lewis 372	GLP-0604-17261-S	MI
Lewis 372	GLP-0604-17261-S	OH
Asgrow/A3244	GLP-0604-17273-S	OH
Stine/ST3300	GLP-0605-17335-S	OH

E.2. Characterization of the Materials

The identities of MON 87769, the conventional soybean control, and reference soybean varieties were verified prior to their use in the study by confirming the chain-of-custody documentation of the samples from the field cooperators. The seed samples from MON 87769, the conventional soybean control, and reference soybean varieties were

further characterized by an event-specific PCR analysis of the DNA extracted from the seed to confirm the presence or absence of MON 87769.

E.3. Field Production of the Samples

Soybean forage and seed of MON 87769 and conventional soybean control collected from each of three replicate plots and conventional reference soybean varieties collected from one replicate plot at each of five U.S. field sites were analyzed. Seed was planted in a randomized complete block design with three replicates per block of MON 87769, a conventional soybean control, and conventional reference soybean varieties. All the samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. The five U.S. sites were: Site IA-1, Richland, IA; Site IA-2, Bagley, IA; Site IL, Carlyle, IL; Site MI, Conklin, MI; and Site OH, New Holland, OH. Forage and seed samples were harvested from all plots and shipped on dry ice (forage) or ambient temperature (seed) to Monsanto Company, St. Louis, MO. A subsample for use in compositional analyses was prepared from each of the bulk forage and seed samples generated in the field. Each sub-sample was ground, stored in a -20 °C freezer located at Monsanto Company (St. Louis, MO), and then shipped overnight, on dry ice to Covance Laboratories Inc. (Madison, WI) for compositional analyses. The labels on the samples shipped to Covance Laboratories Inc. listed the composition study number, tissue type, material name, storage conditions, and a unique sample ID number.

E.4. Analytical Method Summaries and Reference Standards for Seed and Forage Analysis

E.4.1. Acid Detergent Fiber

The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash removed the fats and pigments. The lignocellulose fraction was collected on the frit and determined gravimetrically. The limit of quantitation for this study was 0.100%.

Reference

USDA. 1970. Forage and Fiber Analyses. Agriculture Handbook No. 379. United States Department of Agriculture, Washington, D.C.

E.4.2. Amino Acid Composition

Total aspartic acid (including asparagine)
Total threonine
Total serine
Total glutamic acid (including glutamine)
Total proline
Total glycine
Total alanine
Total valine
Total isoleucine
Total leucine

Total tyrosine
Total phenylalanine
Total histidine
Total lysine
Total arginine
Total tryptophan
Sulfur-containing amino acids: Total methionine
Total cystine (including cysteine)

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantitated using an automated amino acid analyzer. The limit of quantitation was 0.100 mg/g.

Reference Standards

- Beckman K18, 2.5 µmol/mL per constituent except cystine (1.25 µmol/mL), Lot Number S702114
- Sigma, L-Tryptophan, 100%, Lot Number 076K50075
- Fluka, L-Cysteic Acid Monohydrate, 100%, Lot Number 1157629
- Sigma, L-Methionine Sulfone, 100%, Lot Number 012H3349

Reference

AOAC. 2005. Method 982.30 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.

E.4.3. Ash

The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The limit of quantitation was 0.100%.

Reference

AOAC. 2005. Method 923 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.

E.4.4. Carbohydrates

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

$$\% \text{ carbohydrates} = 100\ \% - (\% \text{ protein} + \% \text{ fat} + \% \text{ moisture} + \% \text{ ash})$$

The limit of quantitation was 0.100%.

Reference

USDA. 1973. Energy value of foods. Agriculture Handbook No. 74. United States Department of Agriculture, Washington, D.C.

E.4.5. Fat by Acid Hydrolysis

The sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted with ether and hexane. The extract was evaporated on a steambath, re-dissolved in hexane and filtered through a sodium sulfate column. The hexane extract was then evaporated again on a steambath under nitrogen, dried, and weighed. The limit of quantitation was 0.100%.

Reference

AOAC. 2005. Methods 922.06 and 954.02 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.

E.4.6. Fat by Soxhlet Extraction

The sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed. The limit of quantitation was 0.100%.

Reference

AOAC. 2005. Method 960.39 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.

E.4.7. Fatty Acid Profile with Trans Fat by GC

The lipid was extracted, saponified with 0.5N methanolic sodium hydroxide, and methylated with 14% BF_3-methanol. The resulting methyl esters of the fatty acids were extracted with heptane. An internal standard was added prior to the lipid extraction. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The limit of quantitation was 0.0200%.

Reference Standards

- Nu Chek Prep GLC Reference Standard Hazelton No. 1, Lot Number AU22-P
- Nu Chek Prep GLC Reference Standard Hazelton No. 2, Lot Number M13-O
- Nu Chek Prep GLC Reference Standard Hazelton No. 3, Lot Number N28-Q
- Nu Chek Prep GLC Reference Standard Hazelton No. 4, Lot Number N28-Q
- Nu Chek Prep Methyl Gamma Linolenate, used as 100% Lot Number U-63M-N30-Q
- Sigma Methyl Tridecanoate, Lot Number 036K2636, used as 99%, Lot Numbers 035K1392 and 046K1065, used as 100%
- Nu Chek Prep Methyl Butyrate, used as 100%, Lot Number N-4M-A4-R
- Nu Chek Prep Methyl Hexanoate, used as 100%, Lot Number N-6M-S1-Q
- Nu Chek Prep Methyl Erucate, used as 100%, Lot Number U-79M-AU3-Q
- Nu Chek Prep Methyl Lignocerate, used as 100%, Lot Number N-24M-A5-R

- Nu Chek Prep Methyl Docosapentaenoate, used as 100%, Lot Number U-101M-MA27-R
- Nu Chek Prep Methyl Docosahexaenoate, used as 100%, Lot Number U-84M-D18-Q
- Nu Chek Prep Methyl Eicosapentaenoate, used as 100%, Lot Number U-99M-F13-R
- Cayman Chemicals Steariodonic Acid Methyl Ester, used as 100%, Lot Number 171721-172473
- Nu Chek Prep Methyl Elaidate, used as 100%, Lot Number U-47M-JA18-R
- Nu Chek Prep Methyl Linoelaidate, used as 100%, Lot Number U-60M-MA27-R

References

AOAC. 2005. Method 996.06 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.

AOCS. 1997. Method Ce 2c-66 and Ce 1C-89 in Official Methods and Recommended Practices of the AOCS, 5th ed. American Oil Chemists' Society, Champaign, IL.

AOAC. 2005. Method 983.23 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD

E.4.8. Isoflavones Analysis

The sample was extracted using a solution of hydrochloric acid and reagent alcohol heated on steam baths or hot plates. The extract was brought to volume, diluted, and centrifuged. An aliquot of the supernatant was 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 sample was analyzed on a high-performance liquid chromatography system with ultraviolet detection and was compared to an external standard curve of known standards for quantitation. The limit of quantitation for each component was 10.0 ppm.

Reference Standards
Indofine, Daidzein, 99%, Lot Number 020508146
Indofine, Genistein, ≥99%, (used as 100%), Lot Number 0604043
Indofine, Glycitein, 99%, Lot Number 0704034

References

Seo, A. and C.V. Morr. 1984. Improved high-performance liquid chromatographic analysis of phenolic acids and isoflavonoids from soybean protein products. Journal of Agricultural and Food Chemistry, 32(3):530-533, (1984).

Pettersson, H., and K.H. Kiessling. 1984. Liquid Chromatographic Determination of the Plant Estrogens Coumestrol and Isoflavones in Animal Feed. Association of Official Analytical Chemists Journal, 67(3):503-506.

E.4.9. Lectin

The sample was 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 sample and lectin control was 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. The limit of quantitation was 0.10 H.U./mg.

Reference Standard
Sigma-Aldrich, Red Blood Cells, Rabbit, Product #R1629, Lot Number 105K6042

References

Klurfeld, D. M. and Kritchevsky, D., "Isolation and Quantitation of Lectins from Vegetable Oils," *Lipids,* 22:667-668, (1987).

Klurfeld, D. M., Personal communication.

Liener, I. E., "The Photometric Determination of the Hemagglutinating Activity of Soyin and Crude Soybean Extracts," *Archives of Biochemistry and Biophysics,* 54:223-231, (1955).

E.4.10. Moisture

The sample was dried in a vacuum oven at approximately 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture. The limit of quantitation for this study was 0.100%.

Reference

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 926.08 and 925.09, AOAC INTERNATIONAL: Gaithersburg, Maryland, (2005).

E.4.11. Neutral Detergent Fiber, Enzyme Method

The sample was placed in a fritted vessel and washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. Hemicellulose, cellulose, and lignin fractions were collected on the frit and determined gravimetrically. The limit of quantitation was 0.100%.

References

Approved Methods of the American Association of Cereal Chemists, 9th Ed., Method 32.20, (1998).

USDA. 1970. Forage and Fiber Analyses. Agriculture Handbook No. 379. United States Department of Agriculture, Washington, D.C.

E.4.12. Phytic Acid

The sample was extracted using 0.5 M HCl with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The sample was analyzed on a polymer high-performance liquid chromatography column PRP-1, 5µm (150 x 4.1mm) with a refractive index detector. The limit of quantitation was 0.100%.

Reference Standard
Aldrich, Phytic Acid, Dodecasodium Salt Hydrate, 97%, Lot Number 035K0590

References

Lehrfeld, Jacob, "HPLC Separation and Quantitation of Phytic Acid and Some Inositol Phosphates in Foods: Problem and Solutions," *Journal of Agricultural and Food Chemistry*, 42:2726-2731, (1994).

Lehrfeld, Jacob, "High-Performance Liquid Chromatography Analysis of Phytic Acid on a pH-Stable, Macroporous Polymer Column," *Cereal Chemistry*, 66(6):510-515, (1989).

E.4.13. Protein

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25. The limit of quantitation was 0.100%.

References

Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., Methods 955.04 and 979.09, AOAC INTERNATIONAL, Gaithersburg, Maryland, (2005).

Bradstreet, R. B., *The Kjeldahl Method for Organic Nitrogen*, Academic Press: New York, New York, (1965).

Kalthoff, I. M., and Sandell, E. B., *Quantitative Inorganic Analysis*, MacMillan: New York, (1948).

E.4.14. Raffinose and Stachyose

The sample was extracted with deionized water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The quantitation limit was 0.0500%.

Reference Standards

- Sigma, Raffinose Pentahydrate, 99% (84% after correction for degree of hydration), Lot Number 035K1371
- Sigma, Stachyose, 98% (96.4% after correction for degree of hydration), Lot Number 065K3775

References

Brobst, K. M. 1972. Gas-Liquid Chromatography of Trimethylsilyl Derivatives in Methods in Carbohydrate Chemistry, Vol. 6. Academic Press, New York.

Mason, B.S., and H.T. Slover. 1971. A Gas Chromatographic Method for the Determination of Sugars in Foods. Journal of Agricultural and Food Chemistry, 19(3):551-554

E.4.15. Trypsin Inhibitor

The sample was ground and defatted with petroleum ether. A sample of matrix was extracted with 0.01N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride. The sample was allowed to react for 10 minutes at 37 °C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solution was centrifuged, then the 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. The limit of quantitation was 1.00 Trypsin Inhibitor Units (TIU)/mg.

Reference

AOCS. 1997. Method Ba 12-75 in Official Methods and Recommended Practices of the American Oil Chemists' Society. AOCS Press, Champaign, IL.

E.4.16. Vitamin E

The sample was saponified to break down any fat and release vitamin E. The saponified mixture was extracted with ethyl ether and then quantitated by high-performance liquid chromatography using a silica column. The limit of quantitation was 0.500 mg/100 g.

Reference Standard

USP, Alpha Tocopherol, 100%, Lot Number M

References

Cort, W.M., T.S. Vincente, E.H. Waysek, and B.D. Williams. 1983. Vitamin E content of feedstuffs determined by high-performance liquid chromatographic Fluorescence. Journal of Agricultural Food Chemistry, 31:1330-1333.

Speek, A.J., J. Schijver, and W.H.P. Schreurs. 1985. Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorometric quantitation. Journal of Food Science, 50(1):121-124.

McMurray, C.H., W.J. Blanchflower, and D.A. Rice. 1980. Influence of extraction techniques on determination of α-tocopherol in animal feedstuffs. Journal of the Association of Official Analytical Chemists, 63(6):1258-1261.

E.5. Data Processing

After compositional analyses were performed at Covance Laboratories Inc., data spreadsheets were forwarded to Monsanto Company. The data were reviewed, formatted, and sent to Certus International, Inc. for statistical analysis. A statistical sub-report was generated by Certus and sent to Monsanto Company.

In all, 75 different analytical components were measured. Of these evaluated components, 26 had more than 50% of observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from the statistical analysis. The following analytes 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, 18:1 total trans-octadecenoic acid, 18:2 isolinoleic acid, 18:2 total trans-linoleic acid, 18:3 9c,12c,15t trans-ALA, 18:3 gamma linolenic acid, 18:4 6c,9c,12c,15t trans-SDA, 18:4 stearidonic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, 20:5 eicosapentaenoic acid, 22:1 erucic acid, 22:5 docosapentaenoic acid, 22:6 docosehexaenoic acid, and 24:0 lignoceric acid. The LOQ for the fatty acid method was 0.0200% fresh weight.

For four of the seed fatty acids excluded from statistical analysis (GLA, trans-ALA, SDA, and trans-SDA), the majority of values were below the LOQ for the conventional soybean control and conventional reference soybean varieties, but were measured in MON 87769. Because of their presence in quantities higher than the LOQ in MON 87769, data for the above four fatty acids were excluded from the calculation of total fatty acids for use in the fatty acid composition data re-expression formula.

The following 21 observations for seed tissue samples were below the LOQ: 18:4 6c,9c,12c,15t trans-SDA (three values), 20:1 eicosenoic acid (17 values), and vitamin E (one value). To include a complete data set for these analytes in the statistical analysis, observations below the limit of quantitation were assigned a value equal to half the limit of quantitation (LOQ) for these 21 data points.

The data were assessed for potential outliers using a studentized PRESS residuals calculation. No outliers were identified in the data set.

Statistical Methodology

At the field sites, MON 87769, the conventional soybean control, and reference soybean varieties were grown in single plots randomly assigned within each of three replication blocks. The compositional components for MON 87769 and the conventional soybean control were statistically analyzed using a mixed-model analysis of variance. The data from the five replicated sites were analyzed separately and as a combined data set. Individual replicated site analyses used the model:

$$Y_{ij} = U + T_i + B_j + e_{ij} ,$$

where Y_{ij} = unique individual observation, U = overall mean, T_i = variety effect, B_j = random block effect, and e_{ij} = residual error.

Combined-site analyses used the 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 = variety effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by variety interaction effect, and e_{ijk} = residual error. For each of the forage and seed compositional components, MON 87769 was compared to the conventional soybean control.

A range of observed values from the reference soybean varieties was determined for each analytical component. Additionally, the reference soybean varieties' data were used to develop population tolerance intervals. 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, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional reference soybean varieties (George et al., 2004; Ridley et al., 2002). Each tolerance interval estimate was based upon one observation per unique reference variety. Since individual variety with multiple observations would first be summarized across replicates within each site and then summarized across sites to obtain a single value for inclusion in tolerance interval calculations, a single replicate from each unique reference variety was analyzed for inclusion in tolerance interval calculations. Because negative quantities are not possible, calculated negative lower tolerance bounds were set to zero. SAS programming was used to generate all summary statistics and perform all analyses (SAS Software Release 9.1, 2002-2003). Report tables present p-values from SAS as either <0.001 or the actual value truncated to three decimals.

Individual-Site Compositional Comparison of MON 87769 vs. the Conventional Soybean Control for Forage and Seed

Table E-1. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Forage from IA-1 Site for Fiber and Proximates

			Difference (Test minus Control)			
Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	**A3525 Mean (S.E.) [Range]**	**Mean (S.E.) [Range]**	**95% CI (Lower, Upper)**	**p-Value**	**Commercial (Range) [99% Tolerance Interval[2]]**
Fiber (% DW)						
Acid Detergent Fiber	32.11 (1.36) [31.12 - 32.90]	31.48 (1.36) [28.48 - 34.85]	0.63 (1.35) [-1.95 - 2.64]	-5.20, 6.46	0.688	(24.31 - 31.73) [19.24, 38.36]
Neutral Detergent Fiber	33.00 (2.18) [30.50 - 36.34]	29.47 (2.18) [25.48 - 34.20]	3.53 (0.83) [2.14 - 5.02]	-0.052, 7.11	0.051	(24.37 - 38.09) [19.01, 46.73]
Proximate (% DW, unless noted)						
Ash	6.26 (0.13) [6.06 - 6.46]	6.03 (0.13) [5.74 - 6.18]	0.23 (0.19) [-0.12 - 0.53]	-0.57, 1.03	0.342	(5.33 - 7.77) [3.73, 9.32]
Carbohydrates	64.75 (0.58) [64.27 - 65.07]	66.01 (0.58) [65.13 - 67.57]	-1.26 (0.82) [-3.30 - -0.055]	-4.79, 2.28	0.265	(64.94 - 72.18) [60.10, 76.68]
Moisture (% FW)	74.67 (0.57) [73.20 - 75.90]	73.07 (0.57) [72.80 - 73.30]	1.60 (0.80) [0.10 - 3.10]	-1.85, 5.05	0.183	(71.10 - 74.90) [67.41, 78.15]
Protein	23.17 (0.50) [22.13 - 23.82]	22.22 (0.50) [21.36 - 22.96]	0.95 (0.70) [-0.22 - 2.21]	-2.06, 3.97	0.306	(16.96 - 21.65) [13.69, 25.14]
Total Fat	5.82 (0.32) [5.22 - 6.34]	5.74 (0.32) [5.33 - 6.36]	0.075 (0.26) [-0.32 - 0.56]	-1.04, 1.19	0.800	(4.15 - 7.02) [1.46, 9.88]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-2. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-1 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.78 (0.015) [1.77 - 1.78]	1.72 (0.015) [1.68 - 1.75]	0.057 (0.021) [0.033 - 0.10]	-0.033, 0.15	0.112	(1.63 - 1.86) [1.45, 2.02]
Arginine	3.20 (0.056) [3.12 - 3.25]	2.94 (0.056) [2.81 - 3.04]	0.26 (0.079) [0.14 - 0.44]	-0.083, 0.60	0.082	(2.61 - 3.15) [2.13, 3.62]
Aspartic Acid	4.57 (0.055) [4.51 - 4.63]	4.39 (0.055) [4.27 - 4.51]	0.18 (0.065) [0.12 - 0.31]	-0.097, 0.46	0.106	(4.01 - 4.71) [3.45, 5.29]
Cystine	0.64 (0.0098) [0.63 - 0.65]	0.61 (0.0098) [0.60 - 0.64]	0.026 (0.0081) [0.010 - 0.036]	-0.0086, 0.061	0.083	(0.55 - 0.62) [0.49, 0.68]
Glutamic Acid	7.66 (0.079) [7.59 - 7.70]	7.29 (0.079) [7.10 - 7.46]	0.36 (0.11) [0.22 - 0.60]	-0.11, 0.84	0.081	(6.67 - 7.99) [5.51, 9.04]
Glycine	1.80 (0.012) [1.79 - 1.80]	1.72 (0.012) [1.69 - 1.75]	0.075 (0.017) [0.047 - 0.11]	0.0027, 0.15	0.046	(1.61 - 1.86) [1.39, 2.05]
Histidine	1.09 (0.010) [1.09 - 1.10]	1.05 (0.010) [1.03 - 1.08]	0.040 (0.014) [0.013 - 0.068]	-0.023, 0.10	0.111	(0.98 - 1.13) [0.86, 1.27]
Isoleucine	1.88 (0.026) [1.88 - 1.88]	1.79 (0.026) [1.72 - 1.84]	0.090 (0.036) [0.037 - 0.16]	-0.067, 0.25	0.132	(1.62 - 2.00) [1.34, 2.28]

Table E-2. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-1 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.19 (0.022) [3.19 - 3.20]	3.08 (0.022) [3.04 - 3.14]	0.11 (0.027) [0.061 - 0.15]	-0.00097, 0.23	0.050	(2.86 - 3.37) [2.45, 3.76]
Lysine	2.70 (0.020) [2.68 - 2.71]	2.62 (0.020) [2.59 - 2.67]	0.077 (0.024) [0.039 - 0.12]	-0.027, 0.18	0.085	(2.42 - 2.78) [2.13, 3.06]
Methionine	0.60 (0.0064) [0.60 - 0.61]	0.58 (0.0064) [0.57 - 0.59]	0.026 (0.0085) [0.0098 - 0.038]	-0.010, 0.063	0.089	(0.52 - 0.61) [0.48, 0.66]
Phenylalanine	2.13 (0.025) [2.08 - 2.16]	2.03 (0.025) [1.99 - 2.07]	0.10 (0.017) [0.086 - 0.14]	0.034, 0.18	0.024	(1.92 - 2.29) [1.61, 2.55]
Proline	2.12 (0.020) [2.11 - 2.13]	2.00 (0.020) [1.97 - 2.06]	0.12 (0.024) [0.070 - 0.15]	0.014, 0.22	0.039	(1.81 - 2.16) [1.53, 2.45]
Serine	2.21 (0.035) [2.16 - 2.24]	2.13 (0.035) [2.05 - 2.19]	0.073 (0.049) [-0.023 - 0.19]	-0.14, 0.28	0.278	(1.97 - 2.27) [1.75, 2.51]
Threonine	1.60 (0.026) [1.57 - 1.64]	1.57 (0.026) [1.51 - 1.61]	0.039 (0.029) [-0.0089 - 0.090]	-0.084, 0.16	0.304	(1.45 - 1.65) [1.30, 1.82]
Tryptophan	0.47 (0.011) [0.45 - 0.50]	0.45 (0.011) [0.44 - 0.46]	0.019 (0.016) [-0.0067 - 0.059]	-0.051, 0.088	0.363	(0.43 - 0.52) [0.35, 0.59]

Table E-2. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from Site IA for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.40 (0.056) [1.27 - 1.47]	1.34 (0.056) [1.26 - 1.41]	0.064 (0.079) [-0.072 - 0.21]	-0.28, 0.41	0.504	(1.21 - 1.49) [1.03, 1.67]
Valine	1.99 (0.027) [1.98 - 2.00]	1.89 (0.027) [1.82 - 1.94]	0.097 (0.038) [0.037 - 0.18]	-0.066, 0.26	0.124	(1.70 - 2.11) [1.42, 2.41]
Fatty Acid (% Total FA)						
16:0 Palmitic	12.39 (0.056) [12.27 - 12.54]	12.06 (0.056) [12.05 - 12.06]	0.34 (0.078) [0.21 - 0.48]	-0.0012, 0.67	0.050	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic	4.00 (0.027) [3.96 - 4.06]	4.04 (0.027) [4.01 - 4.08]	-0.043 (0.015) [-0.064 - -0.015]	-0.11, 0.019	0.096	(3.68 - 4.89) [2.87, 5.85]
18:1 Total 18:1	13.42 (0.14) [13.14 - 13.80]	18.45 (0.14) [18.35 - 18.52]	-5.04 (0.20) [-5.38 - -4.55]	-5.92, -4.15	0.001	(16.70 - 23.16) [12.56, 27.98]
18:2 9c,12c Linoleic	18.46 (0.11) [18.24 - 18.68]	54.90 (0.11) [54.80 - 55.07]	-36.45 (0.078) [-36.60 - -36.35]	-36.78, -36.11	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:3 Linolenic	11.11 (0.019) [11.08 - 11.13]	9.85 (0.019) [9.80 - 9.88]	1.27 (0.0091) [1.25 - 1.28]	1.23, 1.30	<0.001	(6.95 - 10.58) [3.72, 13.46]
20:0 Arachidic	0.33 (0.0038) [0.32 - 0.34]	0.30 (0.0038) [0.29 - 0.30]	0.032 (0.0024) [0.029 - 0.037]	0.021, 0.042	0.005	(0.27 - 0.36) [0.20, 0.45]

Table E-2. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from Site IA-1 for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
20:1 Eicosenoic	0.10 (0.017) [0.075 - 0.15]	0.086 (0.017) [0.082 - 0.089]	0.017 (0.023) [-0.013 - 0.063]	-0.084, 0.12	0.540	(0.071 - 0.19) [0, 0.31]
22:0 Behenic	0.29 (0.014) [0.26 - 0.31]	0.31 (0.014) [0.30 - 0.34]	-0.028 (0.0039) [-0.035 - -0.022]	-0.045, -0.011	0.018	(0.30 - 0.41) [0.22, 0.49]
Fiber (% DW)						
Acid Detergent Fiber	15.21 (0.50) [14.38 - 16.48]	16.19 (0.50) [15.70 - 16.70]	-0.98 (0.70) [-1.91 - 0.77]	-4.01, 2.05	0.298	(14.57 - 18.85) [10.36, 22.77]
Neutral Detergent Fiber	15.79 (0.80) [15.46 - 16.05]	15.80 (0.80) [14.43 - 18.01]	-0.0054 (1.13) [-2.55 - 1.44]	-4.87, 4.86	0.996	(15.03 - 18.92) [10.91, 22.59]
Proximate (% DW, unless noted)						
Ash	5.74 (0.079) [5.66 - 5.82]	5.72 (0.079) [5.60 - 5.92]	0.025 (0.11) [-0.17 - 0.21]	-0.46, 0.51	0.843	(5.59 - 6.20) [5.16, 6.64]
Carbohydrates	37.57 (0.89) [35.70 - 39.54]	40.99 (0.89) [39.93 - 41.94]	-3.41 (1.25) [-6.24 - -0.39]	-8.81, 1.98	0.112	(33.50 - 40.22) [26.76, 45.99]
Moisture (% FW)	7.55 (0.21) [7.14 - 8.01]	7.37 (0.21) [7.17 - 7.66]	0.18 (0.29) [-0.52 - 0.84]	-1.08, 1.44	0.607	(6.68 - 8.16) [5.23, 9.56]
Protein	41.93 (0.32) [41.35 - 42.27]	40.09 (0.32) [39.43 - 40.55]	1.84 (0.45) [1.07 - 2.75]	-0.080, 3.77	0.054	(37.52 - 42.37) [33.37, 46.00]

Table E-2. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-1Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Proximate						
Total Fat	14.75 (0.65) [13.35 - 16.31]	13.21 (0.65) [12.73 - 13.86]	1.54 (0.92) [-0.51 - 3.27]	-2.42, 5.50	0.235	(13.99 - 20.56) [11.04, 25.03]
Vitamin						
Vitamin E (mg/100g DW)	1.02 (0.11) [0.86 - 1.30]	1.10 (0.11) [0.97 - 1.19]	-0.082 (0.11) [-0.28 - 0.11]	-0.57, 0.40	0.543	(0.27 - 2.93) [0, 4.65]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg DW)	4.28 (1.49) [1.52 - 8.07]	2.31 (1.49) [0.75 - 3.23]	1.97 (1.59) [0.014 - 5.13]	-4.88, 8.82	0.341	(0.81 - 9.73) [0, 16.00]
Phytic Acid	1.28 (0.048) [1.22 - 1.34]	1.17 (0.048) [1.06 - 1.26]	0.11 (0.023) [0.079 - 0.16]	0.011, 0.21	0.041	(0.81 - 1.27) [0.51, 1.59]
Raffinose	0.35 (0.019) [0.32 - 0.40]	0.31 (0.019) [0.29 - 0.32]	0.039 (0.022) [0.0029 - 0.079]	-0.055, 0.13	0.215	(0.31 - 0.42) [0.19, 0.52]
Stachyose	3.05 (0.087) [2.93 - 3.27]	2.78 (0.087) [2.69 - 2.86]	0.27 (0.076) [0.16 - 0.41]	-0.055, 0.60	0.070	(2.23 - 3.29) [1.61, 4.05]
Trypsin Inhibitor (TIU/mg DW)	45.27 (5.77) [33.06 - 54.80]	35.70 (5.77) [25.67 - 41.69]	9.57 (8.16) [-6.68 - 29.14]	-25.56, 44.70	0.361	(24.29 - 46.29) [8.09, 57.27]
Isoflavone (µg/g DW)						
Daidzein	995.39 (13.83) [978.37 - 1026.28]	1550.96 (13.83) [1531.49 - 1572.77]	-555.58 (19.56) [-594.40 - -522.35]	-639.75, -471.40	0.001	(783.49 - 1691.97) [0, 2594.50]

Table E-2. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-1 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Isoflavone (µg/g DW)						
Genistein	594.53 (7.52) [584.75 - 612.91]	973.04 (7.52) [966.28 - 983.61]	-378.51 (4.09) [-384.49 - -370.70]	-396.09, -360.93	<0.001	(741.53 - 1580.48) [254.31, 1976.30]
Glycitein	87.40 (9.32) [73.40 - 106.72]	103.25 (9.32) [86.17 - 113.71]	-15.86 (6.20) [-27.80 - -6.99]	-42.55, 10.83	0.124	(74.87 - 189.64) [0, 243.40]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-3. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Forage from IA-2 Site for Fiber and Proximates

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	26.08 (0.67) [24.91 - 26.79]	25.76 (0.67) [24.39 - 26.92]	0.33 (0.23) [-0.13 - 0.59]	-0.66, 1.32	0.290	(24.31 - 31.73) [19.24, 38.36]
Neutral Detergent Fiber	30.83 (1.42) [28.06 - 34.06]	32.11 (1.42) [30.71 - 33.99]	-1.28 (2.00) [-5.94 - 2.44]	-9.90, 7.35	0.589	(24.37 - 38.09) [19.01, 46.73]
Proximate (% DW, unless noted)						
Ash	7.19 (0.13) [7.00 - 7.30]	7.45 (0.13) [7.25 - 7.75]	-0.26 (0.16) [-0.49 - 0.048]	-0.95, 0.43	0.244	(5.33 - 7.77) [3.73, 9.32]
Carbohydrates	64.72 (0.85) [62.88 - 65.63]	62.33 (0.85) [61.18 - 63.79]	2.39 (1.07) [0.88 - 4.45]	-2.19, 6.98	0.154	(64.94 - 72.18) [60.10, 76.68]
Moisture (% FW)	72.93 (0.94) [70.70 - 74.80]	74.03 (0.94) [72.90 - 74.70]	-1.10 (0.67) [-2.20 - 0.10]	-3.96, 1.76	0.240	(71.10 - 74.90) [67.41, 78.15]
Protein	23.23 (0.76) [22.18 - 24.53]	23.95 (0.76) [22.33 - 24.98]	-0.73 (0.64) [-2.01 - -0.017]	-3.50, 2.05	0.376	(16.96 - 21.65) [13.69, 25.14]
Total Fat	4.87 (0.20) [4.40 - 5.28]	6.27 (0.20) [6.13 - 6.49]	-1.40 (0.28) [-2.09 - -0.92]	-2.60, -0.20	0.037	(4.15 - 7.02) [1.46, 9.88]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-4. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-2 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.79 (0.018) [1.76 - 1.81]	1.78 (0.018) [1.74 - 1.81]	0.0095 (0.022) [-0.018 - 0.052]	-0.083, 0.10	0.703	(1.63 - 1.86) [1.45, 2.02]
Arginine	3.42 (0.037) [3.36 - 3.50]	3.11 (0.037) [3.05 - 3.15]	0.31 (0.020) [0.28 - 0.35]	0.23, 0.40	0.003	(2.61 - 3.15) [2.13, 3.62]
Aspartic Acid	4.56 (0.053) [4.51 - 4.61]	4.50 (0.053) [4.36 - 4.60]	0.060 (0.072) [-0.034 - 0.20]	-0.25, 0.37	0.490	(4.01 - 4.71) [3.45, 5.29]
Cystine	0.62 (0.0028) [0.62 - 0.63]	0.60 (0.0028) [0.59 - 0.61]	0.024 (0.0019) [0.021 - 0.027]	0.016, 0.032	0.005	(0.55 - 0.62) [0.49, 0.68]
Glutamic Acid	7.73 (0.088) [7.62 - 7.83]	7.56 (0.088) [7.36 - 7.71]	0.17 (0.11) [-0.0052 - 0.38]	-0.32, 0.66	0.283	(6.67 - 7.99) [5.51, 9.04]
Glycine	1.81 (0.018) [1.78 - 1.83]	1.78 (0.018) [1.74 - 1.81]	0.031 (0.022) [0.0039 - 0.074]	-0.062, 0.12	0.284	(1.61 - 1.86) [1.39, 2.05]
Histidine	1.10 (0.012) [1.08 - 1.11]	1.08 (0.012) [1.05 - 1.10]	0.015 (0.016) [-0.013 - 0.044]	-0.056, 0.086	0.455	(0.98 - 1.13) [0.86, 1.27]
Isoleucine	1.89 (0.027) [1.84 - 1.94]	1.81 (0.027) [1.78 - 1.86]	0.082 (0.038) [-0.018 - 0.16]	-0.081, 0.25	0.162	(1.62 - 2.00) [1.34, 2.28]

Table E-4. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-2 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.21 (0.028) [3.17 - 3.26]	3.16 (0.028) [3.10 - 3.19]	0.047 (0.032) [-0.015 - 0.094]	-0.092, 0.19	0.279	(2.86 - 3.37) [2.45, 3.76]
Lysine	2.70 (0.029) [2.65 - 2.73]	2.69 (0.029) [2.62 - 2.73]	0.014 (0.041) [-0.059 - 0.094]	-0.16, 0.19	0.760	(2.42 - 2.78) [2.13, 3.06]
Methionine	0.60 (0.0088) [0.59 - 0.61]	0.58 (0.0088) [0.56 - 0.60]	0.025 (0.0099) [0.0057 - 0.038]	-0.017, 0.067	0.126	(0.52 - 0.61) [0.48, 0.66]
Phenylalanine	2.14 (0.018) [2.13 - 2.15]	2.11 (0.018) [2.07 - 2.15]	0.032 (0.025) [-0.016 - 0.084]	-0.075, 0.14	0.330	(1.92 - 2.29) [1.61, 2.55]
Proline	2.12 (0.021) [2.09 - 2.15]	2.06 (0.021) [2.01 - 2.09]	0.061 (0.0073) [0.048 - 0.073]	0.029, 0.092	0.014	(1.81 - 2.16) [1.53, 2.45]
Serine	2.19 (0.028) [2.18 - 2.21]	2.22 (0.028) [2.17 - 2.29]	-0.030 (0.040) [-0.11 - 0.041]	-0.20, 0.14	0.529	(1.97 - 2.27) [1.75, 2.51]
Threonine	1.58 (0.015) [1.58 - 1.60]	1.60 (0.015) [1.56 - 1.62]	-0.013 (0.017) [-0.039 - 0.020]	-0.086, 0.061	0.542	(1.45 - 1.65) [1.30, 1.82]
Tryptophan	0.46 (0.0039) [0.46 - 0.46]	0.47 (0.0039) [0.46 - 0.48]	-0.0056 (0.0055) [-0.019 - 0.0016]	-0.029, 0.018	0.417	(0.43 - 0.52) [0.35, 0.59]

Table E-4. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-2 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.41 (0.022) [1.35 - 1.45]	1.32 (0.022) [1.30 - 1.34]	0.089 (0.031) [0.015 - 0.14]	-0.043, 0.22	0.100	(1.21 - 1.49) [1.03, 1.67]
Valine	2.01 (0.031) [1.95 - 2.07]	1.92 (0.031) [1.89 - 1.96]	0.097 (0.043) [-0.017 - 0.18]	-0.089, 0.28	0.154	(1.70 - 2.11) [1.42, 2.41]
Fatty Acid (% Total FA)						
16:0 Palmitic	11.77 (0.12) [11.53 - 11.90]	11.36 (0.12) [11.14 - 11.52]	0.41 (0.16) [0.0098 - 0.75]	-0.29, 1.12	0.128	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic	4.21 (0.058) [4.15 - 4.27]	4.11 (0.058) [3.96 - 4.21]	0.097 (0.056) [-0.0058 - 0.19]	-0.14, 0.34	0.224	(3.68 - 4.89) [2.87, 5.85]
18:1 Total 18:1	13.56 (0.31) [12.93 - 14.36]	18.53 (0.31) [18.29 - 18.72]	-4.97 (0.31) [-5.36 - -4.36]	-6.32, -3.63	0.003	(16.70 - 23.16) [12.56, 27.98]
18:2 9c,12c Linoleic	21.19 (0.57) [20.36 - 22.78]	55.33 (0.57) [55.21 - 55.58]	-34.14 (0.80) [-35.15 - -32.43]	-37.60, -30.68	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:3 Linolenic	11.14 (0.12) [11.10 - 11.18]	9.96 (0.12) [9.73 - 10.28]	1.18 (0.16) [0.82 - 1.45]	0.47, 1.89	0.018	(6.95 - 10.58) [3.72, 13.46]
20:0 Arachidic	0.34 (0.0072) [0.33 - 0.34]	0.30 (0.0072) [0.28 - 0.32]	0.036 (0.010) [0.017 - 0.058]	-0.0077, 0.080	0.071	(0.27 - 0.36) [0.20, 0.45]

Table E-4. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-2 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
20:1 Eicosenoic	0.13 (0.015) [0.086 - 0.15]	0.084 (0.015) [0.075 - 0.092]	0.043 (0.021) [-0.0061 - 0.072]	-0.048, 0.13	0.180	(0.071 - 0.19) [0, 0.31]
22:0 Behenic	0.27 (0.019) [0.27 - 0.27]	0.32 (0.019) [0.28 - 0.37]	-0.051 (0.027) [-0.11 - -0.019]	-0.17, 0.067	0.206	(0.30 - 0.41) [0.22, 0.49]
Fiber (% DW)						
Acid Detergent Fiber	17.23 (0.90) [16.45 - 17.62]	16.16 (0.90) [13.80 - 17.85]	1.07 (0.85) [-0.24 - 2.65]	-2.57, 4.71	0.333	(14.57 - 18.85) [10.36, 22.77]
Neutral Detergent Fiber	17.34 (0.97) [15.47 - 19.15]	17.64 (0.97) [16.73 - 19.37]	-0.31 (1.33) [-1.98 - 2.32]	-6.03, 5.42	0.839	(15.03 - 18.92) [10.91, 22.59]
Proximate (% DW, unless noted)						
Ash	5.52 (0.091) [5.46 - 5.63]	5.41 (0.091) [5.24 - 5.64]	0.11 (0.065) [-0.0074 - 0.22]	-0.17, 0.39	0.229	(5.59 - 6.20) [5.16, 6.64]
Carbohydrates	36.95 (0.79) [35.58 - 39.06]	40.25 (0.79) [39.89 - 40.93]	-3.30 (1.12) [-4.71 - -0.88]	-8.12, 1.52	0.098	(33.50 - 40.22) [26.76, 45.99]
Moisture (% FW)	8.09 (0.066) [7.99 - 8.21]	7.98 (0.066) [7.88 - 8.11]	0.11 (0.093) [-0.12 - 0.26]	-0.29, 0.51	0.348	(6.68 - 8.16) [5.23, 9.56]
Protein	42.80 (0.78) [42.50 - 43.36]	40.42 (0.78) [38.31 - 41.58]	2.37 (0.95) [0.96 - 4.19]	-1.73, 6.48	0.130	(37.52 - 42.37) [33.37, 46.00]

Table E-4. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-2 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Proximate						
Total Fat	14.73 (0.76) [12.95 - 15.65]	13.91 (0.76) [13.24 - 15.13]	0.81 (0.74) [-0.30 - 2.22]	-2.37, 4.00	0.386	(13.99 - 20.56) [11.04, 25.03]
Vitamin						
Vitamin E (mg/100g DW)	1.05 (0.11) [0.92 - 1.22]	1.18 (0.11) [0.94 - 1.37]	-0.13 (0.15) [-0.35 - 0.27]	-0.79, 0.53	0.489	(0.27 - 2.93) [0, 4.65]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg DW)	2.84 (0.65) [2.07 - 4.28]	1.69 (0.65) [0.71 - 2.71]	1.15 (0.36) [0.43 - 1.57]	-0.41, 2.70	0.086	(0.81 - 9.73) [0, 16.00]
Phytic Acid	0.97 (0.059) [0.86 - 1.11]	1.01 (0.059) [0.94 - 1.06]	-0.047 (0.057) [-0.13 - 0.064]	-0.29, 0.20	0.495	(0.81 - 1.27) [0.51, 1.59]
Raffinose	0.38 (0.024) [0.35 - 0.45]	0.34 (0.024) [0.31 - 0.37]	0.042 (0.034) [-0.019 - 0.099]	-0.10, 0.19	0.344	(0.31 - 0.42) [0.19, 0.52]
Stachyose	2.87 (0.19) [2.53 - 3.24]	2.74 (0.19) [2.43 - 3.00]	0.13 (0.26) [-0.46 - 0.44]	-0.99, 1.26	0.659	(2.23 - 3.29) [1.61, 4.05]
Trypsin Inhibitor (TIU/mg DW)	28.51 (1.63) [27.39 - 29.81]	29.19 (1.63) [24.81 - 31.50]	-0.69 (1.71) [-3.18 - 2.58]	-8.02, 6.65	0.726	(24.29 - 46.29) [8.09, 57.27]
Isoflavone (µg/g DW)						
Daidzein	1076.04 (29.87) [999.02 - 1130.31]	1583.00 (29.87) [1553.50 - 1599.74]	-506.96 (25.06) [-554.48 - -469.43]	-614.77, -399.15	0.002	(783.49 - 1691.97) [0, 2594.50]

Table E-4. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IA-2 Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Isoflavone (µg/g DW)						
Genistein	656.80 (21.40) [612.27 - 687.55]	1044.68 (21.40) [1023.36 - 1084.46]	-387.88 (16.63) [-411.09 - -355.65]	-459.42, -316.33	0.001	(741.53 - 1580.48) [254.31, 1976.30]
Glycitein	75.18 (6.78) [67.00 - 87.16]	76.19 (6.78) [65.51 - 90.33]	-1.01 (1.36) [-3.16 - 1.49]	-6.84, 4.82	0.533	(74.87 - 189.64) [0, 243.40]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-5. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Forage from IL Site for Fiber and Proximates

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	31.77 (1.82) [30.24 - 34.02]	31.12 (1.82) [27.30 - 35.23]	0.65 (1.56) [-1.21 - 3.76]	-6.06, 7.37	0.715	(24.31 - 31.73) [19.24, 38.36]
Neutral Detergent Fiber	36.52 (3.76) [29.02 - 42.44]	37.15 (3.76) [30.49 - 42.62]	-0.64 (4.89) [-9.33 - 7.60]	-21.69, 20.42	0.908	(24.37 - 38.09) [19.01, 46.73]
Proximate (% DW, unless noted)						
Ash	6.79 (0.18) [6.70 - 6.92]	6.72 (0.18) [6.44 - 7.20]	0.068 (0.18) [-0.28 - 0.31]	-0.70, 0.84	0.741	(5.33 - 7.77) [3.73, 9.32]
Carbohydrates	69.55 (0.72) [68.50 - 70.34]	69.94 (0.72) [68.21 - 70.84]	-0.39 (0.36) [-0.95 - 0.29]	-1.95, 1.17	0.396	(64.94 - 72.18) [60.10, 76.68]
Moisture (% FW)	71.63 (0.62) [70.60 - 72.90]	71.27 (0.62) [70.20 - 72.10]	0.37 (0.88) [-0.90 - 2.70]	-3.41, 4.14	0.716	(71.10 - 74.90) [67.41, 78.15]
Protein	18.60 (0.40) [17.76 - 19.23]	18.57 (0.40) [18.09 - 19.25]	0.028 (0.39) [-0.63 - 0.73]	-1.67, 1.72	0.949	(16.96 - 21.65) [13.69, 25.14]
Total Fat	5.06 (0.28) [4.61 - 5.35]	4.76 (0.28) [4.25 - 5.34]	0.29 (0.33) [-0.085 - 0.96]	-1.14, 1.73	0.470	(4.15 - 7.02) [1.46, 9.88]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-6. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IL Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.80 (0.020) [1.76 - 1.84]	1.74 (0.020) [1.71 - 1.76]	0.052 (0.024) [0.0060 - 0.084]	-0.049, 0.15	0.158	(1.63 - 1.86) [1.45, 2.02]
Arginine	3.07 (0.049) [3.00 - 3.14]	2.90 (0.049) [2.80 - 3.00]	0.17 (0.070) [0.0027 - 0.27]	-0.13, 0.47	0.129	(2.61 - 3.15) [2.13, 3.62]
Aspartic Acid	4.55 (0.063) [4.45 - 4.70]	4.35 (0.063) [4.26 - 4.42]	0.20 (0.085) [0.031 - 0.31]	-0.17, 0.56	0.146	(4.01 - 4.71) [3.45, 5.29]
Cystine	0.63 (0.0056) [0.62 - 0.64]	0.61 (0.0056) [0.60 - 0.62]	0.020 (0.0020) [0.016 - 0.022]	0.011, 0.028	0.010	(0.55 - 0.62) [0.49, 0.68]
Glutamic Acid	7.65 (0.11) [7.46 - 7.90]	7.26 (0.11) [7.08 - 7.36]	0.39 (0.14) [0.098 - 0.56]	-0.24, 1.01	0.116	(6.67 - 7.99) [5.51, 9.04]
Glycine	1.82 (0.019) [1.78 - 1.87]	1.75 (0.019) [1.72 - 1.76]	0.070 (0.023) [0.028 - 0.11]	-0.028, 0.17	0.091	(1.61 - 1.86) [1.39, 2.05]
Histidine	1.10 (0.015) [1.08 - 1.14]	1.06 (0.015) [1.04 - 1.07]	0.044 (0.020) [0.0068 - 0.075]	-0.042, 0.13	0.158	(0.98 - 1.13) [0.86, 1.27]
Isoleucine	1.91 (0.025) [1.87 - 1.97]	1.82 (0.025) [1.79 - 1.84]	0.087 (0.036) [0.022 - 0.18]	-0.066, 0.24	0.134	(1.62 - 2.00) [1.34, 2.28]

Table E-6. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IL Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.21 (0.048) [3.14 - 3.32]	3.10 (0.048) [3.03 - 3.14]	0.11 (0.059) [0.0023 - 0.20]	-0.14, 0.36	0.196	(2.86 - 3.37) [2.45, 3.76]
Lysine	2.68 (0.031) [2.63 - 2.75]	2.59 (0.031) [2.55 - 2.62]	0.085 (0.040) [0.014 - 0.15]	-0.087, 0.26	0.168	(2.42 - 2.78) [2.13, 3.06]
Methionine	0.61 (0.0070) [0.61 - 0.62]	0.59 (0.0070) [0.57 - 0.60]	0.028 (0.0068) [0.015 - 0.038]	-0.0014, 0.057	0.054	(0.52 - 0.61) [0.48, 0.66]
Phenylalanine	2.12 (0.026) [2.08 - 2.18]	2.07 (0.026) [2.03 - 2.10]	0.051 (0.025) [0.0051 - 0.092]	-0.058, 0.16	0.182	(1.92 - 2.29) [1.61, 2.55]
Proline	2.09 (0.030) [2.05 - 2.15]	1.99 (0.030) [1.95 - 2.04]	0.094 (0.043) [0.016 - 0.17]	-0.090, 0.28	0.159	(1.81 - 2.16) [1.53, 2.45]
Serine	2.20 (0.044) [2.14 - 2.25]	2.11 (0.044) [2.00 - 2.18]	0.094 (0.059) [0.0050 - 0.21]	-0.16, 0.35	0.253	(1.97 - 2.27) [1.75, 2.51]
Threonine	1.58 (0.028) [1.54 - 1.63]	1.56 (0.028) [1.50 - 1.60]	0.027 (0.040) [-0.058 - 0.087]	-0.14, 0.20	0.564	(1.45 - 1.65) [1.30, 1.82]
Tryptophan	0.50 (0.0096) [0.49 - 0.50]	0.48 (0.0096) [0.46 - 0.50]	0.013 (0.010) [-0.0014 - 0.032]	-0.030, 0.056	0.322	(0.43 - 0.52) [0.35, 0.59]

Table E-6. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IL Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.38 (0.032) [1.34 - 1.41]	1.37 (0.032) [1.33 - 1.44]	0.014 (0.042) [-0.058 - 0.087]	-0.17, 0.19	0.773	(1.21 - 1.49) [1.03, 1.67]
Valine	2.01 (0.029) [1.96 - 2.08]	1.92 (0.029) [1.88 - 1.95]	0.098 (0.040) [0.011 - 0.20]	-0.076, 0.27	0.136	(1.70 - 2.11) [1.42, 2.41]
Fatty Acid (% Total FA)						
16:0 Palmitic	12.31 (0.052) [12.24 - 12.39]	12.00 (0.052) [11.89 - 12.08]	0.30 (0.048) [0.21 - 0.35]	0.095, 0.51	0.024	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic	4.42 (0.027) [4.37 - 4.45]	4.33 (0.027) [4.28 - 4.37]	0.090 (0.036) [0.035 - 0.16]	-0.064, 0.24	0.128	(3.68 - 4.89) [2.87, 5.85]
18:1 Total 18:1	17.89 (0.22) [17.52 - 18.18]	20.89 (0.22) [20.42 - 21.17]	-3.00 (0.31) [-3.65 - -2.46]	-4.31, -1.68	0.010	(16.70 - 23.16) [12.56, 27.98]
18:2 9c,12c Linoleic	30.48 (0.20) [30.26 - 30.81]	54.33 (0.20) [54.05 - 54.77]	-23.86 (0.087) [-23.96 - -23.68]	-24.23, -23.48	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:3 Linolenic	10.27 (0.090) [10.20 - 10.38]	7.59 (0.090) [7.42 - 7.81]	2.68 (0.13) [2.42 - 2.82]	2.13, 3.22	0.002	(6.95 - 10.58) [3.72, 13.46]
20:0 Arachidic	0.36 (0.0038) [0.36 - 0.37]	0.34 (0.0038) [0.33 - 0.34]	0.027 (0.0045) [0.020 - 0.036]	0.0083, 0.047	0.025	(0.27 - 0.36) [0.20, 0.45]

Table E-6. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IL Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
20:1 Eicosenoic	0.20 (0.0013) [0.20 - 0.20]	0.19 (0.0013) [0.19 - 0.19]	0.0062 (0.0019) [0.0027 - 0.0098]	-0.0019, 0.014	0.081	(0.071 - 0.19) [0, 0.31]
22:0 Behenic	0.31 (0.0039) [0.30 - 0.31]	0.33 (0.0039) [0.32 - 0.34]	-0.022 (0.0049) [-0.029 - -0.013]	-0.043, -0.00089	0.046	(0.30 - 0.41) [0.22, 0.49]
Fiber (% DW)						
Acid Detergent Fiber	16.16 (0.20) [15.91 - 16.61]	17.76 (0.20) [17.47 - 18.03]	-1.60 (0.12) [-1.83 - -1.41]	-2.12, -1.08	0.005	(14.57 - 18.85) [10.36, 22.77]
Neutral Detergent Fiber	17.06 (0.12) [16.72 - 17.25]	17.87 (0.12) [17.78 - 17.92]	-0.81 (0.17) [-1.20 - -0.53]	-1.56, -0.060	0.043	(15.03 - 18.92) [10.91, 22.59]
Proximate (% DW, unless noted)						
Ash	6.06 (0.13) [5.90 - 6.17]	5.78 (0.13) [5.51 - 6.07]	0.28 (0.17) [0.099 - 0.62]	-0.44, 1.01	0.234	(5.59 - 6.20) [5.16, 6.64]
Carbohydrates	34.20 (0.77) [33.23 - 36.11]	36.20 (0.77) [35.30 - 37.13]	-2.00 (0.55) [-2.92 - -1.01]	-4.38, 0.37	0.068	(33.50 - 40.22) [26.76, 45.99]
Moisture (% FW)	6.98 (0.11) [6.71 - 7.24]	7.28 (0.11) [7.21 - 7.37]	-0.29 (0.16) [-0.66 - 0.030]	-0.98, 0.40	0.209	(6.68 - 8.16) [5.23, 9.56]
Protein	41.64 (0.45) [41.05 - 42.26]	39.58 (0.45) [38.97 - 40.65]	2.06 (0.63) [0.97 - 3.14]	-0.64, 4.76	0.081	(37.52 - 42.37) [33.37, 46.00]

Table E-6. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IL Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Proximate						
Total Fat	18.10 (0.45) [16.94 - 19.03]	18.44 (0.45) [18.14 - 18.64]	-0.34 (0.49) [-1.20 - 0.49]	-2.44, 1.75	0.554	(13.99 - 20.56) [11.04, 25.03]
Vitamin						
Vitamin E (mg/100g DW)	2.42 (0.070) [2.27 - 2.54]	2.10 (0.070) [2.01 - 2.21]	0.31 (0.099) [0.059 - 0.45]	-0.11, 0.74	0.087	(0.27 - 2.93) [0, 4.65]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg DW)	5.00 (1.67) [3.69 - 7.02]	7.07 (1.67) [4.88 - 11.32]	-2.07 (2.36) [-7.01 - 2.00]	-12.21, 8.07	0.472	(0.81 - 9.73) [0, 16.00]
Phytic Acid	1.07 (0.030) [1.02 - 1.10]	1.04 (0.030) [1.00 - 1.11]	0.023 (0.043) [-0.094 - 0.10]	-0.16, 0.21	0.641	(0.81 - 1.27) [0.51, 1.59]
Raffinose	0.43 (0.015) [0.40 - 0.44]	0.43 (0.015) [0.40 - 0.45]	0.00042 (0.021) [-0.049 - 0.044]	-0.091, 0.092	0.985	(0.31 - 0.42) [0.19, 0.52]
Stachyose	3.06 (0.088) [2.91 - 3.17]	3.07 (0.088) [2.88 - 3.21]	-0.0061 (0.12) [-0.30 - 0.29]	-0.54, 0.53	0.965	(2.23 - 3.29) [1.61, 4.05]
Trypsin Inhibitor (TIU/mg DW)	38.06 (1.03) [36.23 - 40.86]	35.27 (1.03) [34.82 - 35.95]	2.80 (1.46) [0.28 - 5.83]	-3.49, 9.09	0.195	(24.29 - 46.29) [8.09, 57.27]
Isoflavone (µg/g DW)						
Daidzein	1079.87 (87.82) [957.23 - 1193.55]	1484.63 (87.82) [1380.05 - 1691.99]	-404.76 (120.69) [-603.16 - -186.51]	-924.03, 114.51	0.078	(783.49 - 1691.97) [0, 2594.50]

Table E-6. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from IL Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Isoflavone (µg/g DW)						
Genistein	655.90 (47.11) [576.70 - 716.13]	837.93 (47.11) [770.81 - 940.83]	-182.04 (52.30) [-265.97 - -86.03]	-407.05, 42.97	0.073	(741.53 - 1580.48) [254.31, 1976.30]
Glycitein	84.86 (5.58) [83.23 - 87.74]	120.49 (5.58) [106.01 - 132.56]	-35.63 (7.78) [-49.33 - -22.40]	-69.10, -2.17	0.044	(74.87 - 189.64) [0, 243.40]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-7. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Forage from MI Site for Fiber and Proximates

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	33.02 (2.24) [27.92 - 36.39]	33.97 (2.24) [30.72 - 37.06]	-0.95 (3.17) [-9.14 - 5.67]	-14.61, 12.71	0.793	(24.31 - 31.73) [19.24, 38.36]
Neutral Detergent Fiber	36.16 (2.10) [31.87 - 40.66]	36.13 (2.10) [33.13 - 38.13]	0.029 (2.96) [-5.27 - 7.53]	-12.72, 12.78	0.993	(24.37 - 38.09) [19.01, 46.73]
Proximate (% DW, unless noted)						
Ash	5.99 (0.33) [5.29 - 6.36]	6.00 (0.33) [5.38 - 6.39]	-0.014 (0.077) [-0.091 - 0.14]	-0.35, 0.32	0.875	(5.33 - 7.77) [3.73, 9.32]
Carbohydrates	70.54 (1.12) [68.27 - 72.79]	70.07 (1.12) [68.44 - 71.51]	0.47 (1.26) [-2.00 - 2.12]	-4.94, 5.87	0.745	(64.94 - 72.18) [60.10, 76.68]
Moisture (% FW)	71.30 (0.93) [69.50 - 72.80]	71.37 (0.93) [70.10 - 73.10]	-0.067 (1.32) [-1.50 - 2.70]	-5.73, 5.60	0.964	(71.10 - 74.90) [67.41, 78.15]
Protein	18.80 (0.73) [17.05 - 20.44]	18.54 (0.73) [18.23 - 19.14]	0.26 (1.02) [-1.20 - 2.21]	-4.11, 4.63	0.822	(16.96 - 21.65) [13.69, 25.14]
Total Fat	4.67 (0.68) [3.80 - 5.99]	5.39 (0.68) [4.02 - 6.12]	-0.71 (0.54) [-1.80 - -0.13]	-3.05, 1.62	0.318	(4.15 - 7.02) [1.46, 9.88]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-8. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from MI Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.79 (0.021) [1.76 - 1.83]	1.72 (0.021) [1.69 - 1.76]	0.068 (0.030) [0.0015 - 0.11]	-0.062, 0.20	0.152	(1.63 - 1.86) [1.45, 2.02]
Arginine	3.32 (0.10) [3.16 - 3.61]	2.94 (0.10) [2.91 - 2.98]	0.39 (0.14) [0.19 - 0.68]	-0.23, 1.01	0.114	(2.61 - 3.15) [2.13, 3.62]
Aspartic Acid	4.57 (0.064) [4.48 - 4.73]	4.29 (0.064) [4.21 - 4.37]	0.28 (0.090) [0.11 - 0.43]	-0.10, 0.67	0.087	(4.01 - 4.71) [3.45, 5.29]
Cystine	0.58 (0.011) [0.56 - 0.60]	0.57 (0.011) [0.56 - 0.58]	0.012 (0.015) [-0.024 - 0.037]	-0.054, 0.077	0.528	(0.55 - 0.62) [0.49, 0.68]
Glutamic Acid	7.62 (0.11) [7.47 - 7.87]	7.16 (0.11) [7.03 - 7.32]	0.46 (0.15) [0.15 - 0.76]	-0.20, 1.12	0.094	(6.67 - 7.99) [5.51, 9.04]
Glycine	1.78 (0.019) [1.76 - 1.83]	1.70 (0.019) [1.67 - 1.72]	0.086 (0.027) [0.034 - 0.14]	-0.032, 0.20	0.088	(1.61 - 1.86) [1.39, 2.05]
Histidine	1.09 (0.017) [1.07 - 1.13]	1.04 (0.017) [1.02 - 1.07]	0.048 (0.024) [0.0020 - 0.090]	-0.053, 0.15	0.176	(0.98 - 1.13) [0.86, 1.27]
Isoleucine	1.82 (0.048) [1.75 - 1.94]	1.75 (0.048) [1.70 - 1.80]	0.068 (0.068) [-0.052 - 0.24]	-0.23, 0.36	0.422	(1.62 - 2.00) [1.34, 2.28]

Table E-8. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from MI Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.19 (0.047) [3.13 - 3.28]	3.06 (0.047) [3.01 - 3.15]	0.13 (0.066) [-0.019 - 0.26]	-0.15, 0.41	0.189	(2.86 - 3.37) [2.45, 3.76]
Lysine	2.67 (0.033) [2.62 - 2.75]	2.56 (0.033) [2.51 - 2.61]	0.11 (0.047) [0.013 - 0.19]	-0.092, 0.31	0.142	(2.42 - 2.78) [2.13, 3.06]
Methionine	0.56 (0.011) [0.54 - 0.59]	0.56 (0.011) [0.56 - 0.57]	0.00074 (0.016) [-0.030 - 0.033]	-0.068, 0.069	0.967	(0.52 - 0.61) [0.48, 0.66]
Phenylalanine	2.16 (0.032) [2.11 - 2.24]	2.05 (0.032) [2.02 - 2.10]	0.11 (0.046) [0.013 - 0.20]	-0.086, 0.31	0.135	(1.92 - 2.29) [1.61, 2.55]
Proline	2.09 (0.039) [2.03 - 2.19]	1.94 (0.039) [1.91 - 1.98]	0.14 (0.055) [0.056 - 0.25]	-0.091, 0.38	0.119	(1.81 - 2.16) [1.53, 2.45]
Serine	2.23 (0.022) [2.23 - 2.23]	2.12 (0.022) [2.06 - 2.17]	0.11 (0.030) [0.067 - 0.17]	-0.020, 0.23	0.067	(1.97 - 2.27) [1.75, 2.51]
Threonine	1.63 (0.023) [1.60 - 1.65]	1.55 (0.023) [1.49 - 1.58]	0.082 (0.032) [0.023 - 0.15]	-0.057, 0.22	0.125	(1.45 - 1.65) [1.30, 1.82]
Tryptophan	0.48 (0.014) [0.45 - 0.51]	0.46 (0.014) [0.44 - 0.47]	0.018 (0.011) [-0.00067 - 0.037]	-0.030, 0.065	0.253	(0.43 - 0.52) [0.35, 0.59]

Table E-8. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from MI Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.42 (0.049) [1.29 - 1.50]	1.35 (0.049) [1.32 - 1.40]	0.072 (0.048) [-0.020 - 0.14]	-0.14, 0.28	0.275	(1.21 - 1.49) [1.03, 1.67]
Valine	1.92 (0.054) [1.84 - 2.06]	1.86 (0.054) [1.79 - 1.91]	0.065 (0.076) [-0.063 - 0.26]	-0.26, 0.39	0.485	(1.70 - 2.11) [1.42, 2.41]
Fatty Acid (% Total FA)						
16:0 Palmitic	12.11 (0.057) [12.03 - 12.19]	11.79 (0.057) [11.70 - 11.92]	0.31 (0.044) [0.26 - 0.40]	0.12, 0.50	0.019	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic	3.87 (0.054) [3.73 - 3.98]	3.90 (0.054) [3.85 - 3.94]	-0.022 (0.070) [-0.16 - 0.059]	-0.32, 0.28	0.779	(3.68 - 4.89) [2.87, 5.85]
18:1 Total 18:1	12.92 (0.13) [12.66 - 13.16]	17.44 (0.13) [17.24 - 17.59]	-4.53 (0.097) [-4.66 - -4.34]	-4.94, -4.11	<0.001	(16.70 - 23.16) [12.56, 27.98]
18:2 9c,12c Linoleic	18.40 (0.71) [16.46 - 19.58]	55.60 (0.71) [55.36 - 56.04]	-37.21 (0.90) [-38.90 - -35.83]	-41.08, -33.33	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:3 Linolenic	11.76 (0.029) [11.72 - 11.80]	10.59 (0.029) [10.54 - 10.66]	1.16 (0.038) [1.09 - 1.22]	1.00, 1.33	0.001	(6.95 - 10.58) [3.72, 13.46]
20:0 Arachidic	0.32 (0.0037) [0.31 - 0.32]	0.29 (0.0037) [0.28 - 0.29]	0.030 (0.0052) [0.019 - 0.042]	0.0074, 0.052	0.029	(0.27 - 0.36) [0.20, 0.45]

Table E-8. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from MI Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
20:1 Eicosenoic	0.081 (0.014) [0.077 - 0.084]	0.096 (0.014) [0.069 - 0.14]	-0.015 (0.020) [-0.058 - 0.013]	-0.10, 0.072	0.535	(0.071 - 0.19) [0, 0.31]
22:0 Behenic	0.30 (0.0096) [0.27 - 0.31]	0.29 (0.0096) [0.29 - 0.30]	0.0060 (0.012) [-0.016 - 0.022]	-0.044, 0.056	0.656	(0.30 - 0.41) [0.22, 0.49]
Fiber (% DW)						
Acid Detergent Fiber	17.27 (0.32) [16.91 - 17.59]	16.77 (0.32) [15.98 - 17.35]	0.50 (0.21) [0.23 - 0.93]	-0.42, 1.43	0.143	(14.57 - 18.85) [10.36, 22.77]
Neutral Detergent Fiber	17.30 (0.55) [16.15 - 18.17]	17.34 (0.55) [16.42 - 18.11]	-0.038 (0.78) [-1.34 - 1.75]	-3.38, 3.30	0.965	(15.03 - 18.92) [10.91, 22.59]
Proximate (% DW, unless noted)						
Ash	5.46 (0.10) [5.23 - 5.66]	5.51 (0.10) [5.35 - 5.61]	-0.051 (0.15) [-0.34 - 0.31]	-0.68, 0.58	0.763	(5.59 - 6.20) [5.16, 6.64]
Carbohydrates	39.11 (1.09) [38.34 - 39.93]	39.75 (1.09) [37.70 - 42.60]	-0.63 (1.54) [-4.26 - 2.23]	-7.27, 6.00	0.720	(33.50 - 40.22) [26.76, 45.99]
Moisture (% FW)	7.15 (0.13) [7.01 - 7.31]	7.15 (0.13) [6.84 - 7.39]	0 (0.13) [-0.25 - 0.17]	-0.55, 0.55	1.000	(6.68 - 8.16) [5.23, 9.56]
Protein	41.86 (0.67) [41.08 - 43.29]	39.10 (0.67) [38.22 - 40.31]	2.76 (0.95) [0.90 - 5.07]	-1.33, 6.86	0.100	(37.52 - 42.37) [33.37, 46.00]

Table E-8. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from MI Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Proximate						
Total Fat	13.57 (0.77) [13.14 - 14.20]	15.65 (0.77) [13.61 - 16.96]	-2.08 (0.81) [-3.01 - -0.47]	-5.56, 1.40	0.123	(13.99 - 20.56) [11.04, 25.03]
Vitamin						
Vitamin E (mg/100g DW)	1.26 (0.087) [1.22 - 1.34]	0.86 (0.087) [0.70 - 1.09]	0.40 (0.12) [0.14 - 0.55]	-0.13, 0.93	0.083	(0.27 - 2.93) [0, 4.65]
Antinutrient						
Lectin (H.U./mg DW)	1.49 (0.63) [0.55 - 2.14]	3.44 (0.63) [1.95 - 4.39]	-1.95 (0.28) [-2.25 - -1.40]	-3.14, -0.77	0.019	(0.81 - 9.73) [0, 16.00]
Phytic Acid (% DW)	0.86 (0.063) [0.81 - 0.92]	0.88 (0.063) [0.75 - 1.03]	-0.017 (0.061) [-0.10 - 0.10]	-0.28, 0.25	0.811	(0.81 - 1.27) [0.51, 1.59]
Raffinose (% DW)	0.34 (0.0085) [0.32 - 0.36]	0.32 (0.0085) [0.32 - 0.33]	0.021 (0.012) [-0.0059 - 0.044]	-0.031, 0.073	0.225	(0.31 - 0.42) [0.19, 0.52]
Stachyose (% DW)	2.43 (0.066) [2.28 - 2.53]	2.52 (0.066) [2.45 - 2.63]	-0.093 (0.094) [-0.35 - 0.080]	-0.50, 0.31	0.425	(2.23 - 3.29) [1.61, 4.05]
Trypsin Inhibitor (TIU/mg DW)	25.92 (2.94) [24.30 - 27.94]	27.17 (2.94) [21.34 - 34.89]	-1.24 (4.16) [-10.58 - 6.60]	-19.15, 16.67	0.793	(24.29 - 46.29) [8.09, 57.27]
Isoflavone (µg/g DW)						
Daidzein	1662.22 (99.21) [1389.19 - 1838.91]	2750.13 (99.21) [2705.02 - 2775.08]	-1087.92 (140.30) [-1385.89 - -866.12]	-1691.59, -484.25	0.016	(783.49 - 1691.97) [0, 2594.50]

Table E-8. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from MI Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Isoflavone (µg/g DW)						
Genistein	1000.90 (59.25) [841.05 - 1118.40]	1683.74 (59.25) [1662.89 - 1706.74]	-682.83 (70.98) [-821.84 - -588.34]	-988.25, -377.41	0.010	(741.53 - 1580.48) [254.31, 1976.30]
Glycitein	83.59 (17.61) [65.37 - 101.09]	114.34 (17.61) [84.16 - 158.73]	-30.75 (24.91) [-93.36 - 0.95]	-137.92, 76.41	0.342	(74.87 - 189.64) [0, 243.40]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-9. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Forage from OH Site for Fiber and Proximates

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	32.29 (0.91) [30.74 - 33.95]	28.31 (0.91) [26.53 - 29.41]	3.99 (0.40) [3.21 - 4.54]	2.28, 5.70	0.009	(24.31 - 31.73) [19.24, 38.36]
Neutral Detergent Fiber	32.93 (1.50) [29.20 - 35.58]	31.22 (1.50) [29.79 - 32.90]	1.70 (1.80) [-1.78 - 4.22]	-6.04, 9.45	0.443	(24.37 - 38.09) [19.01, 46.73]
Proximate (% DW, unless noted)						
Ash	6.67 (0.24) [6.05 - 7.07]	6.59 (0.24) [6.44 - 6.88]	0.078 (0.25) [-0.40 - 0.44]	-0.99, 1.14	0.782	(5.33 - 7.77) [3.73, 9.32]
Carbohydrates	67.50 (0.78) [66.33 - 69.34]	66.87 (0.78) [65.73 - 67.76]	0.64 (0.90) [-0.91 - 2.22]	-3.26, 4.53	0.553	(64.94 - 72.18) [60.10, 76.68]
Moisture (% FW)	70.87 (0.56) [69.90 - 72.40]	71.27 (0.56) [71.00 - 71.60]	-0.40 (0.80) [-1.70 - 1.40]	-3.82, 3.02	0.664	(71.10 - 74.90) [67.41, 78.15]
Protein	19.77 (0.58) [18.74 - 20.87]	20.08 (0.58) [19.07 - 20.87]	-0.32 (0.81) [-1.58 - 1.80]	-3.82, 3.19	0.734	(16.96 - 21.65) [13.69, 25.14]
Total Fat	6.06 (0.34) [5.40 - 6.90]	6.46 (0.34) [6.13 - 6.72]	-0.40 (0.48) [-1.33 - 0.37]	-2.45, 1.65	0.490	(4.15 - 7.02) [1.46, 9.88]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-10. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from OH Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.77 (0.0068) [1.76 - 1.78]	1.74 (0.0068) [1.72 - 1.75]	0.031 (0.0096) [0.0084 - 0.043]	-0.010, 0.073	0.083	(1.63 - 1.86) [1.45, 2.02]
Arginine	3.11 (0.020) [3.07 - 3.15]	2.86 (0.020) [2.82 - 2.89]	0.25 (0.028) [0.21 - 0.32]	0.13, 0.37	0.012	(2.61 - 3.15) [2.13, 3.62]
Aspartic Acid	4.43 (0.017) [4.41 - 4.47]	4.27 (0.017) [4.25 - 4.29]	0.16 (0.024) [0.12 - 0.22]	0.056, 0.26	0.021	(4.01 - 4.71) [3.45, 5.29]
Cystine	0.60 (0.0030) [0.60 - 0.60]	0.59 (0.0030) [0.58 - 0.59]	0.015 (0.0043) [0.0058 - 0.021]	-0.0037, 0.033	0.075	(0.55 - 0.62) [0.49, 0.68]
Glutamic Acid	7.51 (0.034) [7.42 - 7.59]	7.17 (0.034) [7.16 - 7.19]	0.33 (0.048) [0.23 - 0.43]	0.13, 0.54	0.020	(6.67 - 7.99) [5.51, 9.04]
Glycine	1.76 (0.0055) [1.76 - 1.77]	1.71 (0.0055) [1.70 - 1.73]	0.049 (0.0078) [0.030 - 0.064]	0.015, 0.083	0.024	(1.61 - 1.86) [1.39, 2.05]
Histidine	1.07 (0.0021) [1.06 - 1.07]	1.03 (0.0021) [1.03 - 1.04]	0.033 (0.0021) [0.031 - 0.037]	0.024, 0.042	0.003	(0.98 - 1.13) [0.86, 1.27]
Isoleucine	1.87 (0.024) [1.84 - 1.90]	1.75 (0.024) [1.70 - 1.80]	0.11 (0.018) [0.095 - 0.15]	0.035, 0.19	0.025	(1.62 - 2.00) [1.34, 2.28]

Table E-10. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from OH Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.16 (0.015) [3.14 - 3.18]	3.05 (0.015) [3.02 - 3.07]	0.10 (0.014) [0.080 - 0.13]	0.046, 0.16	0.016	(2.86 - 3.37) [2.45, 3.76]
Lysine	2.62 (0.0092) [2.61 - 2.63]	2.56 (0.0092) [2.54 - 2.58]	0.063 (0.0091) [0.045 - 0.074]	0.024, 0.10	0.020	(2.42 - 2.78) [2.13, 3.06]
Methionine	0.59 (0.0038) [0.59 - 0.60]	0.59 (0.0038) [0.58 - 0.59]	0.0064 (0.0027) [0.0028 - 0.012]	-0.0052, 0.018	0.141	(0.52 - 0.61) [0.48, 0.66]
Phenylalanine	2.12 (0.0068) [2.12 - 2.13]	2.05 (0.0068) [2.04 - 2.06]	0.076 (0.0096) [0.053 - 0.099]	0.034, 0.12	0.015	(1.92 - 2.29) [1.61, 2.55]
Proline	2.05 (0.0089) [2.03 - 2.06]	1.95 (0.0089) [1.94 - 1.96]	0.097 (0.010) [0.085 - 0.12]	0.052, 0.14	0.011	(1.81 - 2.16) [1.53, 2.45]
Serine	2.16 (0.028) [2.08 - 2.21]	2.13 (0.028) [2.11 - 2.14]	0.029 (0.028) [-0.023 - 0.075]	-0.092, 0.15	0.412	(1.97 - 2.27) [1.75, 2.51]
Threonine	1.57 (0.0094) [1.56 - 1.58]	1.56 (0.0094) [1.54 - 1.58]	0.013 (0.013) [-0.012 - 0.032]	-0.043, 0.068	0.432	(1.45 - 1.65) [1.30, 1.82]
Tryptophan	0.46 (0.0059) [0.45 - 0.47]	0.45 (0.0059) [0.45 - 0.46]	0.0084 (0.0084) [-0.011 - 0.026]	-0.028, 0.045	0.421	(0.43 - 0.52) [0.35, 0.59]

Table E-10. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from OH Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.40 (0.032) [1.36 - 1.44]	1.34 (0.032) [1.28 - 1.41]	0.059 (0.044) [-0.024 - 0.13]	-0.13, 0.25	0.312	(1.21 - 1.49) [1.03, 1.67]
Valine	1.96 (0.030) [1.94 - 2.01]	1.84 (0.030) [1.78 - 1.90]	0.12 (0.021) [0.096 - 0.16]	0.033, 0.21	0.027	(1.70 - 2.11) [1.42, 2.41]
Fatty Acid (% Total FA)						
16:0 Palmitic	11.74 (0.11) [11.56 - 12.02]	11.63 (0.11) [11.58 - 11.71]	0.11 (0.15) [-0.080 - 0.44]	-0.53, 0.75	0.544	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic	4.46 (0.056) [4.32 - 4.53]	4.37 (0.056) [4.32 - 4.44]	0.088 (0.048) [0.0019 - 0.17]	-0.12, 0.29	0.208	(3.68 - 4.89) [2.87, 5.85]
18:1 Total 18:1	18.10 (0.49) [16.73 - 18.80]	20.65 (0.49) [20.41 - 20.79]	-2.54 (0.57) [-3.68 - -1.95]	-5.00, -0.084	0.046	(16.70 - 23.16) [12.56, 27.98]
Linoleic	25.37 (0.16) [25.06 - 25.75]	54.50 (0.16) [54.38 - 54.67]	-29.13 (0.22) [-29.61 - -28.70]	-30.07, -28.19	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:3 Linolenic	11.63 (0.11) [11.41 - 11.75]	8.02 (0.11) [7.85 - 8.21]	3.61 (0.15) [3.19 - 3.89]	2.95, 4.28	0.001	(6.95 - 10.58) [3.72, 13.46]
20:0 Arachidic	0.36 (0.0043) [0.35 - 0.36]	0.33 (0.0043) [0.32 - 0.33]	0.028 (0.0031) [0.024 - 0.034]	0.015, 0.041	0.011	(0.27 - 0.36) [0.20, 0.45]

Table E-10. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from OH Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
20:1 Eicosenoic	0.18 (0.0029) [0.17 - 0.19]	0.17 (0.0029) [0.17 - 0.18]	0.0072 (0.0032) [0.0019 - 0.013]	-0.0064, 0.021	0.150	(0.071 - 0.19) [0, 0.31]
22:0 Behenic	0.29 (0.015) [0.28 - 0.30]	0.33 (0.015) [0.31 - 0.37]	-0.037 (0.018) [-0.071 - -0.014]	-0.11, 0.039	0.172	(0.30 - 0.41) [0.22, 0.49]
Fiber (% DW)						
Acid Detergent Fiber	17.96 (0.36) [17.78 - 18.31]	17.65 (0.36) [16.69 - 18.15]	0.31 (0.51) [-0.35 - 1.62]	-1.88, 2.51	0.601	(14.57 - 18.85) [10.36, 22.77]
Neutral Detergent Fiber	16.70 (0.75) [15.06 - 18.13]	17.25 (0.75) [16.10 - 17.88]	-0.55 (1.06) [-2.82 - 2.03]	-5.13, 4.02	0.655	(15.03 - 18.92) [10.91, 22.59]
Proximate (% DW, unless noted)						
Ash	5.83 (0.040) [5.78 - 5.93]	5.71 (0.040) [5.68 - 5.77]	0.12 (0.019) [0.099 - 0.16]	0.041, 0.20	0.022	(5.59 - 6.20) [5.16, 6.64]
Carbohydrates	34.40 (0.53) [33.55 - 35.89]	36.24 (0.53) [36.17 - 36.33]	-1.83 (0.70) [-2.67 - -0.43]	-4.86, 1.20	0.121	(33.50 - 40.22) [26.76, 45.99]
Moisture (% FW)	7.59 (0.15) [7.20 - 7.87]	7.27 (0.15) [7.15 - 7.43]	0.32 (0.18) [-0.040 - 0.56]	-0.47, 1.11	0.222	(6.68 - 8.16) [5.23, 9.56]
Protein	41.37 (0.24) [40.92 - 41.70]	39.54 (0.24) [39.11 - 39.96]	1.83 (0.17) [1.54 - 2.14]	1.09, 2.57	0.008	(37.52 - 42.37) [33.37, 46.00]

Table E-10. Summary of the Statistical Comparison of MON 87769 vs. the Conventional Control Soybean Seed from OH Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Proximate						
Total Fat	18.40 (0.42) [17.26 - 18.97]	18.51 (0.42) [18.20 - 18.80]	-0.12 (0.59) [-1.54 - 0.76]	-2.67, 2.44	0.860	(13.99 - 20.56) [11.04, 25.03]
Vitamin						
Vitamin E (mg/100g DW)	2.06 (0.18) [1.68 - 2.33]	1.90 (0.18) [1.64 - 2.22]	0.17 (0.092) [0.040 - 0.34]	-0.23, 0.56	0.213	(0.27 - 2.93) [0, 4.65]
Antinutrient						
Lectin (H.U./mg DW)	4.17 (1.51) [2.76 - 6.67]	4.15 (1.51) [2.08 - 7.56]	0.016 (2.13) [-4.80 - 4.60]	-9.14, 9.18	0.994	(0.81 - 9.73) [0, 16.00]
Phytic Acid (% DW)	1.10 (0.039) [1.02 - 1.20]	1.01 (0.039) [0.97 - 1.04]	0.085 (0.033) [0.049 - 0.15]	-0.058, 0.23	0.124	(0.81 - 1.27) [0.51, 1.59]
Raffinose (% DW)	0.35 (0.020) [0.32 - 0.39]	0.35 (0.020) [0.32 - 0.38]	0.0081 (0.028) [-0.067 - 0.069]	-0.11, 0.13	0.801	(0.31 - 0.42) [0.19, 0.52]
Stachyose (% DW)	2.74 (0.10) [2.61 - 3.01]	2.64 (0.10) [2.54 - 2.70]	0.099 (0.14) [-0.087 - 0.47]	-0.52, 0.72	0.562	(2.23 - 3.29) [1.61, 4.05]
Trypsin Inhibitor (TIU/mg DW)	31.30 (2.41) [26.11 - 36.42]	28.18 (2.41) [25.71 - 31.34]	3.12 (3.41) [-5.23 - 8.93]	-11.55, 17.79	0.456	(24.29 - 46.29) [8.09, 57.27]
Isoflavone (µg/g DW)						
Daidzein	1125.54 (39.70) [1094.38 - 1183.11]	1668.07 (39.70) [1583.20 - 1750.03]	-542.53 (26.89) [-571.84 - -488.82]	-658.22, -426.83	0.002	(783.49 - 1691.97) [0, 2594.50]

Table E-10. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Seed from OH Site for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Isoflavone (µg/g DW)						
Genistein	760.07 (20.50) [750.00 - 773.91]	1143.19 (20.50) [1110.39 - 1199.09]	-383.12 (21.06) [-425.19 - -360.39]	-473.72, -292.52	0.003	(741.53 - 1580.48) [254.31, 1976.30]
Glycitein	82.62 (3.92) [74.89 - 90.42]	96.62 (3.92) [90.63 - 101.88]	-14.00 (5.55) [-26.98 - -0.22]	-37.86, 9.86	0.127	(74.87 - 189.64) [0, 243.40]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-11. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Forage for Fiber and Proximates

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	31.06 (1.36)	30.13 (1.36)	0.93 (0.93)	-1.06, 2.92	0.332	(24.31 - 31.73)
	[24.91 - 36.39]	[24.39 - 37.06]	[-9.14 - 5.67]			[19.24, 38.36]
Neutral Detergent Fiber	33.89 (1.37)	33.22 (1.37)	0.67 (1.27)	-2.05, 3.39	0.605	(24.37 - 38.09)
	[28.06 - 42.44]	[25.48 - 42.62]	[-9.33 - 7.60]			[19.01, 46.73]
Proximate (% DW, unless noted)						
Ash	6.58 (0.24)	6.56 (0.24)	0.020 (0.081)	-0.20, 0.24	0.814	(5.33 - 7.77)
	[5.29 - 7.30]	[5.38 - 7.75]	[-0.49 - 0.53]			[3.73, 9.32]
Carbohydrates	67.41 (1.32)	67.04 (1.32)	0.37 (0.61)	-1.32, 2.06	0.575	(64.94 - 72.18)
	[62.88 - 72.79]	[61.18 - 71.51]	[-3.30 - 4.45]			[60.10, 76.68]
Moisture (% FW)	72.28 (0.64)	72.20 (0.64)	0.080 (0.46)	-0.90, 1.06	0.863	(71.10 - 74.90)
	[69.50 - 75.90]	[70.10 - 74.70]	[-2.20 - 3.10]			[67.41, 78.15]
Protein	20.71 (1.05)	20.67 (1.05)	0.040 (0.34)	-0.69, 0.77	0.908	(16.96 - 21.65)
	[17.05 - 24.53]	[18.09 - 24.98]	[-2.01 - 2.21]			[13.69, 25.14]
Total Fat	5.30 (0.29)	5.72 (0.29)	-0.43 (0.30)	-1.26, 0.41	0.226	(4.15 - 7.02)
	[3.80 - 6.90]	[4.02 - 6.72]	[-2.09 - 0.96]			[1.46, 9.88]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-12. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Soybean Seed for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.78 (0.0084) [1.76 - 1.84]	1.74 (0.0084) [1.68 - 1.81]	0.044 (0.011) [-0.018 - 0.11]	0.021, 0.066	0.001	(1.63 - 1.86) [1.45, 2.02]
Arginine	3.23 (0.056) [3.00 - 3.61]	2.95 (0.056) [2.80 - 3.15]	0.28 (0.037) [0.0027 - 0.68]	0.20, 0.35	<0.001	(2.61 - 3.15) [2.13, 3.62]
Aspartic Acid	4.54 (0.035) [4.41 - 4.73]	4.36 (0.035) [4.21 - 4.60]	0.18 (0.036) [-0.034 - 0.43]	0.077, 0.28	0.007	(4.01 - 4.71) [3.45, 5.29]
Cystine	0.62 (0.0098) [0.56 - 0.65]	0.60 (0.0098) [0.56 - 0.64]	0.019 (0.0038) [-0.024 - 0.037]	0.011, 0.027	<0.001	(0.55 - 0.62) [0.49, 0.68]
Glutamic Acid	7.63 (0.059) [7.42 - 7.90]	7.29 (0.059) [7.03 - 7.71]	0.34 (0.055) [-0.0052 - 0.76]	0.22, 0.46	<0.001	(6.67 - 7.99) [5.51, 9.04]
Glycine	1.79 (0.012) [1.76 - 1.87]	1.73 (0.012) [1.67 - 1.81]	0.062 (0.0098) [0.0039 - 0.14]	0.035, 0.090	0.003	(1.61 - 1.86) [1.39, 2.05]
Histidine	1.09 (0.0073) [1.06 - 1.14]	1.05 (0.0073) [1.02 - 1.10]	0.036 (0.0074) [-0.013 - 0.090]	0.021, 0.051	<0.001	(0.98 - 1.13) [0.86, 1.27]
Isoleucine	1.87 (0.018) [1.75 - 1.97]	1.78 (0.018) [1.70 - 1.86]	0.088 (0.018) [-0.052 - 0.24]	0.050, 0.13	<0.001	(1.62 - 2.00) [1.34, 2.28]

Table E-12. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Soybean Seed for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (continued)

			Difference (Test minus Control)			
Analytical Component (Units)[1]	**MON 87769 Mean (S.E.) [Range]**	**A3525 Mean (S.E.) [Range]**	**Mean (S.E.) [Range]**	**95% CI (Lower, Upper)**	**p-Value**	**Commercial (Range) [99% Tolerance Interval[2]]**
Amino Acid (% DW)						
Leucine	3.19 (0.017) [3.13 - 3.32]	3.09 (0.017) [3.01 - 3.19]	0.10 (0.020) [-0.019 - 0.26]	0.059, 0.14	<0.001	(2.86 - 3.37) [2.45, 3.76]
Lysine	2.67 (0.020) [2.61 - 2.75]	2.60 (0.020) [2.51 - 2.73]	0.070 (0.016) [-0.059 - 0.19]	0.036, 0.10	<0.001	(2.42 - 2.78) [2.13, 3.06]
Methionine	0.60 (0.0067) [0.54 - 0.62]	0.58 (0.0067) [0.56 - 0.60]	0.017 (0.0057) [-0.030 - 0.038]	0.0015, 0.033	0.038	(0.52 - 0.61) [0.48, 0.66]
Phenylalanine	2.14 (0.011) [2.08 - 2.24]	2.06 (0.011) [1.99 - 2.15]	0.075 (0.015) [-0.016 - 0.20]	0.038, 0.11	0.002	(1.92 - 2.29) [1.61, 2.55]
Proline	2.09 (0.018) [2.03 - 2.19]	1.99 (0.018) [1.91 - 2.09]	0.10 (0.015) [0.016 - 0.25]	0.070, 0.13	<0.001	(1.81 - 2.16) [1.53, 2.45]
Serine	2.20 (0.016) [2.08 - 2.25]	2.14 (0.016) [2.00 - 2.29]	0.055 (0.023) [-0.11 - 0.21]	0.0020, 0.11	0.043	(1.97 - 2.27) [1.75, 2.51]
Threonine	1.60 (0.0095) [1.54 - 1.65]	1.57 (0.0095) [1.49 - 1.62]	0.030 (0.013) [-0.058 - 0.15]	0.0022, 0.057	0.035	(1.45 - 1.65) [1.30, 1.82]
Tryptophan	0.47 (0.0064) [0.45 - 0.51]	0.46 (0.0064) [0.44 - 0.50]	0.010 (0.0053) [-0.019 - 0.059]	-0.00097, 0.022	0.069	(0.43 - 0.52) [0.35, 0.59]

Table E-12. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Soybean Seed for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

			Difference (Test minus Control)			
Analytical Component (Units)[1]	**MON 87769 Mean (S.E.) [Range]**	**A3525 Mean (S.E.) [Range]**	**Mean (S.E.) [Range]**	**95% CI (Lower, Upper)**	**p-Value**	**Commercial (Range) [99% Tolerance Interval[2]]**
Amino Acid (% DW)						
Tyrosine	1.40 (0.016) [1.27 - 1.50]	1.34 (0.016) [1.26 - 1.44]	0.059 (0.021) [-0.072 - 0.21]	0.014, 0.10	0.013	(1.21 - 1.49) [1.03, 1.67]
Valine	1.98 (0.019) [1.84 - 2.08]	1.88 (0.019) [1.78 - 1.96]	0.096 (0.021) [-0.063 - 0.26]	0.053, 0.14	<0.001	(1.70 - 2.11) [1.42, 2.41]
Fatty Acid (% Total FA)						
16:0 Palmitic	12.06 (0.13) [11.53 - 12.54]	11.77 (0.13) [11.14 - 12.08]	0.29 (0.052) [-0.080 - 0.75]	0.19, 0.40	<0.001	(9.88 - 12.33) [7.28, 14.20]
18:0 Stearic	4.19 (0.10) [3.73 - 4.53]	4.15 (0.10) [3.85 - 4.44]	0.042 (0.031) [-0.16 - 0.19]	-0.044, 0.13	0.245	(3.68 - 4.89) [2.87, 5.85]
18:1 Oleic	15.18 (0.95) [12.66 - 18.80]	19.19 (0.95) [17.24 - 21.17]	-4.02 (0.52) [-5.38 - -1.95]	-5.46, -2.57	0.001	(16.70 - 23.16) [12.56, 27.98]
18:2 Linoleic	22.78 (1.64) [16.46 - 30.81]	54.93 (1.64) [54.05 - 56.04]	-32.16 (2.32) [-38.90 - -23.68]	-37.50, -26.81	<0.001	(53.36 - 57.39) [50.46, 59.96]
18:3 Linolenic	11.18 (0.46) [10.20 - 11.80]	9.20 (0.46) [7.42 - 10.66]	1.98 (0.50) [0.82 - 3.89]	0.60, 3.36	0.016	(6.95 - 10.58) [3.72, 13.46]
20:0 Arachidic	0.34 (0.0090) [0.31 - 0.37]	0.31 (0.0090) [0.28 - 0.34]	0.031 (0.0027) [0.017 - 0.058]	0.025, 0.036	<0.001	(0.27 - 0.36) [0.20, 0.45]

Table E-12. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Soybean Seed for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

			Difference (Test minus Control)			
Analytical Component (Units)[1]	**MON 87769 Mean (S.E.) [Range]**	**A3525 Mean (S.E.) [Range]**	**Mean (S.E.) [Range]**	**95% CI (Lower, Upper)**	**p-Value**	**Commercial (Range) [99% Tolerance Interval[2]]**
Fatty Acid (% Total FA)						
20:1 Eicosenoic	0.14 (0.023) [0.075 - 0.20]	0.13 (0.023) [0.069 - 0.19]	0.012 (0.0094) [-0.058 - 0.072]	-0.014, 0.038	0.282	(0.071 - 0.19) [0, 0.31]
22:0 Behenic	0.29 (0.0069) [0.26 - 0.31]	0.32 (0.0069) [0.28 - 0.37]	-0.026 (0.0087) [-0.11 - 0.022]	-0.047, -0.0050	0.023	(0.30 - 0.41) [0.22, 0.49]
Fiber (% DW)						
Acid Detergent Fiber	16.77 (0.42) [14.38 - 18.31]	16.90 (0.42) [13.80 - 18.15]	-0.14 (0.50) [-1.91 - 2.65]	-1.51, 1.24	0.794	(14.57 - 18.85) [10.36, 22.77]
Neutral Detergent Fiber	16.84 (0.38) [15.06 - 19.15]	17.18 (0.38) [14.43 - 19.37]	-0.34 (0.41) [-2.82 - 2.32]	-1.19, 0.50	0.411	(15.03 - 18.92) [10.91, 22.59]
Proximate (% DW, unless noted)						
Ash	5.72 (0.092) [5.23 - 6.17]	5.63 (0.092) [5.24 - 6.07]	0.098 (0.057) [-0.34 - 0.62]	-0.024, 0.22	0.106	(5.59 - 6.20) [5.16, 6.64]
Carbohydrates	36.45 (0.99) [33.23 - 39.93]	38.68 (0.99) [35.30 - 42.60]	-2.24 (0.53) [-6.24 - 2.23]	-3.32, -1.15	<0.001	(33.50 - 40.22) [26.76, 45.99]
Moisture (% FW)	7.47 (0.17) [6.71 - 8.21]	7.41 (0.17) [6.84 - 8.11]	0.063 (0.10) [-0.66 - 0.84]	-0.22, 0.35	0.572	(6.68 - 8.16) [5.23, 9.56]
Protein	41.92 (0.27) [40.92 - 43.36]	39.75 (0.27) [38.22 - 41.58]	2.18 (0.31) [0.90 - 5.07]	1.51, 2.84	<0.001	(37.52 - 42.37) [33.37, 46.00]

Table E-12. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Soybean Seed for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

			Difference (Test minus Control)			
Analytical Component (Units)[1]	**MON 87769 Mean (S.E.) [Range]**	**A3525 Mean (S.E.) [Range]**	**Mean (S.E.) [Range]**	**95% CI (Lower, Upper)**	**p-Value**	**Commercial (Range) [99% Tolerance Interval[2]]**
Proximate						
Total Fat (% DW)	15.91 (1.05) [12.95 - 19.03]	15.94 (1.05) [12.73 - 18.80]	-0.037 (0.61) [-3.01 - 3.27]	-1.74, 1.66	0.955	(13.99 - 20.56) [11.04, 25.03]
Vitamin						
Vitamin E (mg/100g DW)	1.56 (0.26) [0.86 - 2.54]	1.43 (0.26) [0.70 - 2.22]	0.13 (0.10) [-0.35 - 0.55]	-0.16, 0.42	0.271	(0.27 - 2.93) [0, 4.65]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg DW)	3.55 (0.80) [0.55 - 8.07]	3.73 (0.80) [0.71 - 11.32]	-0.18 (0.81) [-7.01 - 5.13]	-2.43, 2.07	0.836	(0.81 - 9.73) [0, 16.00]
Phytic Acid	1.05 (0.059) [0.81 - 1.34]	1.02 (0.059) [0.75 - 1.26]	0.031 (0.030) [-0.13 - 0.16]	-0.052, 0.11	0.357	(0.81 - 1.27) [0.51, 1.59]
Raffinose	0.37 (0.019) [0.32 - 0.45]	0.35 (0.019) [0.29 - 0.45]	0.022 (0.011) [-0.067 - 0.099]	-0.00076, 0.045	0.057	(0.31 - 0.42) [0.19, 0.52]
Stachyose	2.83 (0.11) [2.28 - 3.27]	2.75 (0.11) [2.43 - 3.21]	0.081 (0.070) [-0.46 - 0.47]	-0.064, 0.23	0.259	(2.23 - 3.29) [1.61, 4.05]
Trypsin Inhibitor (TIU/mg DW)	33.81 (2.81) [24.30 - 54.80]	31.10 (2.81) [21.34 - 41.69]	2.71 (2.01) [-10.58 - 29.14]	-1.44, 6.87	0.190	(24.29 - 46.29) [8.09, 57.27]

Table E-12. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Soybean Seed for Amino Acids, Fatty Acids, Fiber, Proximates, Vitamin E, Antinutrients and Isoflavones (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Isoflavone (µg/g DW)						
Daidzein	1187.81 (188.32) [957.23 - 1838.91]	1807.36 (188.32) [1380.05 - 2775.08]	-619.55 (120.04) [-1385.89 - -186.51]	-952.84, -286.26	0.006	(783.49 - 1691.97) [0, 2594.50]
Genistein	733.64 (114.81) [576.70 - 1118.40]	1136.52 (114.81) [770.81 - 1706.74]	-402.88 (80.11) [-821.84 - -86.03]	-625.30, -180.45	0.007	(741.53 - 1580.48) [254.31, 1976.30]
Glycitein	82.73 (5.66) [65.37 - 106.72]	102.18 (5.66) [65.51 - 158.73]	-19.45 (6.25) [-93.36 - 1.49]	-32.35, -6.55	0.004	(74.87 - 189.64) [0, 243.40]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table E-13. Trans-ALA, GLA, trans-SDA and and SDA Levels in MON 87769 Individual-Site Summary

Analytical Component (Units)[1]	MON 87769 Mean (S.E.)[2]	MON 87769 (Range)
Site IA-1 Seed Fatty Acid (% Total FA)		
18:3 9c,12c,15t (trans-ALA)	0.47 (0.0087)	[0.46 - 0.48]
18:3 Gamma Linolenic (GLA)	7.26 (0.010)	[7.25 - 7.28]
18:4 6c,9c,12c,15t (trans-SDA)	0.24 (0.0081)	[0.23 - 0.26]
18:4 Stearidonic (SDA)	31.93 (0.23)	[31.51 - 32.28]
Site IA-2 Seed Fatty Acid (% Total FA)		
18:3 9c,12c,15t (trans-ALA)	0.43 (0.012)	[0.41 - 0.45]
18:3 Gamma Linolenic (GLA)	7.99 (0.033)	[7.92 - 8.03]
18:4 6c,9c,12c,15t (trans-SDA)	0.20 (0.010)	[0.19 - 0.22]
18:4 Stearidonic (SDA)	28.77 (1.09)	[26.60 - 30.14]
Site IL Seed Fatty Acid (% Total FA)		
18:3 9c,12c,15t (trans-ALA)	0.38 (0.0054)	[0.38 - 0.39]
18:3 Gamma Linolenic (GLA)	6.22 (0.062)	[6.15 - 6.34]
18:4 6c,9c,12c,15t (trans-SDA)	0.061 (0.0022)	[0.058 - 0.065]
18:4 Stearidonic (SDA)	17.11 (0.26)	[16.83 - 17.64]

[1]FA = fatty acid; [2]S.E. = standard error.

Table E-13. Trans-ALA, GLA, trans-SDA and SDA Levels in MON 87769 Individual- Site Summary (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.)[2]	MON 87769 (Range)
Site MI Seed Fatty Acid (% Total FA)		
18:3 9c,12c,15t (trans-ALA)	0.47 (0.0081)	[0.45 - 0.48]
18:3 Gamma Linolenic (GLA)	7.64 (0.14)	[7.36 - 7.78]
18:4 6c,9c,12c,15t (trans-SDA)	0.24 (0.0090)	[0.22 - 0.25]
18:4 Stearidonic (SDA)	31.91 (1.03)	[30.49 - 33.92]
Site OH Seed Fatty Acid (% Total FA)		
18:3 9c,12c,15t (trans-ALA)	0.43 (0.0025)	[0.43 - 0.44]
18:3 Gamma Linolenic (GLA)	6.33 (0.24)	[6.07 - 6.82]
18:4 6c,9c,12c,15t (trans-SDA)	0.14 (0.0074)	[0.14 - 0.16]
18:4 Stearidonic (SDA)	20.95 (0.65)	[20.14 - 22.25]

[1]FA = fatty acid; [2]S.E. = standard error.

Table E-14. Literature and ILSI Database Values for Components in Conventional Soybean Seed and Forage

Analytical Component (Units)[1]	Literature Range	ILSI Range[2]
SEED		
Fiber (% DW)		
Acid Detergent Fiber	9.0 – 11.1[i]	7.81 – 18.61
Neutral Detergent Fiber	10.0 – 14.9[i]	8.53 – 21.25
Proximate (% DW, unless noted)		
Ash	4.61 – 5.94[b]	3.89 – 6.99
Carbohydrates	29.3 – 41.3[c]	29.6 – 50.2
Moisture (% FW)	5.3 – 8.73[c]	4.7 – 34.4
Protein	36.0 – 48.4[e]	33.19 – 45.48
Total Fat	16.0 – 23.1[e]	8.10 – 23.56
	19.8 – 26.7[d]	
Amino Acids (% DW)		
Alanine	1.60 – 1.86[c]	1.513 – 2.104
Arginine	2.56 – 3.46[c]	2.285 – 3.400
Aspartic acid	4.18 – 4.99[c]	3.808 – 5.122
Cystine	0.54 – 0.66[c]	0.370 – 0.808
Glutamic acid	6.64 – 8.16[c]	5.843 – 8.201
Glycine	1.60 – 1.87[c]	1.458 – 1.997
Histidine	0.98 – 1.16[c]	0.878 – 1.175
Isoleucine	1.65 – 1.95[c]	1.539 – 2.077
Leucine	2.81 – 3.37[c]	2.590 – 3.622
Lysine	2.47 – 2.84[c]	2.285 – 2.839
Methionine	0.51 – 0.59[c]	0.431 – 0.681
Phenylalanine	1.78 – 2.19[c]	1.632 – 2.346
Proline	1.86 – 2.23[c]	1.687 – 2.284
Serine	1.96 –2.28[c]	1.106 – 2.484
Threonine	1.51 – 1.73[c]	1.139 – 1.862
Tryptophan	0.56 – 0.63[c]	0.356 – 0.502
Tyrosine	1.35 – 1.59[c]	1.016 – 1.613
Valine	1.71 – 2.02[c]	1.597 – 2.204
Isoflavones (ug/g DW)		
Daidzein	219 – 1190[c]	60.0 – 2453.5
Genistein	286 – 1380[c]	144.3 – 2837.2
Glycitein	42.2 – 204[a]	15.3 – 310.4
Vitamin (mg/100g DW)		
Vitamin E	1.09 – 2.84[g]	0.19 – 6.17

Table E-14. Literature and ILSI Database Values for Components in Conventional Soybean Seed and Forage (Continued)

Analytical Component (Units)[1]	Literature Range	ILSI Range[2]
Fatty Acid (% Total FA)		
16:0 Palmitic	10.63 – 11.69[c]	9.55 – 15.77
18:0 Stearic	3.85 – 4.55[c]	2.70 – 5.88
18:1 Oleic	15.02 – 31.19[c]	14.3 – 32.2
18:2 Linoleic	44.03 – 54.96[c]	42.3 – 58.8
18:3 Linolenic	5.08 – 10.26[c]	3.00 – 12.52
20:0 Arachidic	0.31 – 0.43[c]	0.163 – 0.482
22:0 Behenic	0.46 – 0.59[c]	0.277 – 0.595
Antinutrient (% DW, unless noted)		
Phytic acid	1 – 2.74[i]	0.634 – 1.960
Raffinose	0.4 – 1.8[h]	0.212 – 0.661
Stachyose	3.08 – 4.13[f]	1.21 – 3.50
Trypsin inhibitor (TIU/mg DW)	33.2 – 54.5[c]	19.59 – 118.68
Lectin (H.U./mg FW)	0.8 – 2.4[c]	0.105 – 9.038
FORAGE		
Fiber (% DW)		
Acid Detergent Fiber	32-38[i]	not available
Neutral Detergent Fiber	34-40[i]	not available
Crude Fiber	not available	13.58-31.73
Proximate (% DW, unless noted)		
Ash	8.8-10.5[i]	6.718-10.782
Carbohydrates	not available	59.8-74.7
Moisture (% FW)	74-79[i]	73.5-81.6
Protein	11.2-17.3[i]	14.38-24.71
Total Fat	3.1-5.1[i]	1.302-5.132

[1]DW = dry weight; DM = dry matter; FW = fresh weight; FA = fatty acid; TIU = trypsin inhibitor unit; H.U. = hemaglutinating unit.

[2] ILSI-CCD, 2008. International Life Sciences Institute Crop Composition Database. Version 3.0 http://www.cropcomposition.org. Search criteria soybean seed, all locations, all years, all proximates, amino acids, fatty acids, bio-actives, fiber, dry weight other than moisture [Accessed June 3, 2008].

[a]Isoflavones Database (United States Department of Agriculture - Iowa State University, Version 1.3, 2002); [b](Taylor et al., 1999); [c](Padgette et al., 1996); [d](Maestri et al., 1998); [e](Hartwig et al., 1991); [f](Grieshop et al., 2003); [g](Guzman et al., 1986); [h](Jacorzynski and Barylko-Pikielna, 1983); [i](OECD, 2001).

Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw; g/100g dw x 10 = mg/g.

E.6. Materials and Methods Used for the Compositional Analysis of MON 87769 Processed Fractions

E.6.1. Acid Detergent Fiber (ADF)

The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash removed the fats and pigments. Lignocellulose fraction was collected on the frit and determined gravimetrically. The limit of quantitation was 0.100%.

Reference

USDA. 1970. Forage Fiber Analysis, Agriculture Handbook No. 379. United States Department of Agriculture, Washington, D.C.

E.6.2. Amino Acid Composition

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantitated using an automated amino acid analyzer. The limit of quantitation for each amino acid was 0.100 mg/g.

Reference Standards

- Thermo Scientific/Pierce, K18, 2.5 µmol/mL per constituent (except cystine 1.25 µmol/mL), Lot Number IJ115731
- Sigma, L-Tryptophan, 100%, Lot Number 076K0075
- Fluka, L-Cysteic Acid Monohydrate, >99.9% (used as 100%), Lot Number 1157629
- Sigma, L-Methionine Sulfone, >99% (used as 100%), Lot Number 012H3349

Reference

AOAC. 2005. Method 982.30 in Official Methods of Analysis. AOAC International, Gaithersburg, MD.

E.6.3. Ash

The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The limit of quantitation was 0.100%.

Reference

AOAC. 2005. Method 923.03 in Official Methods of Analysis, 18th ed. AOAC International, Gaithersburg, MD.

E.6.4. Carbohydrates

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

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

The limit of quantitation was 0.100%.

Reference

USDA. 1973. Energy value of foods. Agriculture Handbook No. 74. United States Department of Agriculture, Washington, D.C.

E.6.5. Fat by Soxhlet Extraction

The sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed. The limit of quantitation was 0.100%.

Reference

AOAC. 2005. Method 96.039 in Official Methods of Analysis. AOAC International, Gaithersburg, MD.

E.6.6. Fatty Acid Profile

The lipid was extracted, saponified with 0.5N methanolic sodium hydroxide, and methylated with 14% BF_3-methanol. The resulting methyl esters of the fatty acids were extracted with heptane. An internal standard was added prior to the lipid extraction. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The limit of quantitation was 0.005%-0.06% depending on the soybean fraction.

Reference Standards

- Nu Chek Prep GLC Reference Standard Hazelton No. 1, Lot Number JY19-R
- Nu Chek Prep GLC Reference Standard Hazelton No. 2, Lot Number M13-O
- Nu Chek Prep GLC Reference Standard Hazelton No. 3, Lot Number MA18-S
- Nu Chek Prep GLC Reference Standard Hazelton No. 4, Lot Number JY19-R
- Nu Chek Prep Methyl Gamma Linolenate, used as 100%, Lot Number U-63M-JY12-R
- Nu Chek Prep Methyl Tridecanoate, used as 100%, Lot Number N-13M-F5-S
- Nu Chek Prep Methyl Butyrate, used as 100%, Lot Number N-4M-J20-R
- Nu Chek Prep Methyl Hexanoate, used as 100%, Lot Number N-6M-A25-R
- Nu Chek Prep Methyl Erucate, used as 100%, Lot Number U-79M-AU3-Q
- Nu Chek Prep Methyl Lignocerate, used as 100%, Lot Number N-24M-F5-S
- Nu Chek Prep Methyl Docosapentaenoate, used as 100%, Lot Number U-101M-F18-S
- Nu Chek Prep Methyl Docosahexaenoate, used as 100%, Lot Number U-84M-AU28-R

- Nu Chek Prep Methyl Eicosapentaenoate, used as 100%, Lot Number U-99M-JY17-R
- Cayman Chemicals Stearidonic Acid Methyl Ester, used as 100%, Lot Number 182102-186015
- Nu Chek Prep Methyl Elaidate, used as 100%, Lot Number U-47M-JA18-R
- Nu Chek Prep Methyl Linoelaidate, used as 100%, Lot Number U-60M-F27-R
- Nu Chek Prep Methyl Palmitelaidate, used as as 100%, Lot Number U-41M-O26-R
- Monsanto Mono Trans SDA, 99%, Lot Number GLP-0707-18858-A
- Monsanto Mono Trans ALA, 96%, Lot Number GLP-0707-18857-A
- Larodan Methyl 6(z), 9(z)-Octadecadienoate, used as 100%, Lot Number LS-113

Reference

AOCS. Method Ce 1-63 in Official Methods and Recommended Practices of the AOCS, 5th ed. American Oil Chemists' Society, Champaign, IL.

E.6.7. Isoflavones Analysis

The sample was extracted using a solution of hydrochloric acid and reagent alcohol heated on steam baths or hot plates. The extract was brought to volume, diluted, and centrifuged. An aliquot of the supernatant was 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 sample was analyzed on a high-performance liquid chromatography system with ultraviolet spectrophotometric quantitation and was compared against an external standard curve of known standards. The limit of quantitation for each component was 10.0 µg/g.

Reference Standards
Indofine, Daidzein, 99% , Lot Number 020508146
Indofine, Genistein, ≥99% (used as 100% in calculations), Lot Number 0604043
- Indofine, Glycitein, 99%, Lot Number 0704034

References

Seo, A. and C.V. Morr. 1984. Improved High-Performance Liquid Chromatographic Analysis of Phenolic Acids and Isoflavonoids from Soybean Protein Products. Journal of Agricultural and Food Chemistry, 32(3):530-533.

Pettersson, H. and K-H Kiessling, K.-H. 1984. Liquid Chromatographic Determination of the Plant Estrogens Coumestrol and Isoflavones in Animal Feed. Association of Official Analytical Chemists Journal, 67(3):503-506.

E.6.8. Lectin

The sample was 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 sample and lectin control was read by 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. The limit of quantitation was 0.10 H.U./mg.

References

Klurfeld, D.M. and D. Kritchevsky. 1987. Isolation and Quantitation of Lectins from Vegetable Oils. Lipids 22:667-668.

Klurfeld, D. M., Personal communication.

Liener, I. E. 1955. The photometric determination of the hemagglutinating activity of soyin and crude soybean extracts. Archives of Biochemistry and Biophysics, 54:223-231.

E.6.9. Moisture

The sample was dried in a vacuum oven at approximately 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture. The limit of quantitation was 0.100%.

Reference

AOAC. 2005. Methods 926.08 and 925.09. AOAC International, 18th ed. AOAC Press, Gaithersburg, MD.

E.6.10. Neutral Detergent Fiber, Enzyme Method

The sample was placed in a fritted vessel and washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. Hemicellulose, cellulose, and lignin fractions were collected on the frit and determined gravimetrically. The limit of quantitation was 0.100%.

References

AACC. 1998. Methods 32.20 in Approved Methods of the American Association of Cereal Chemists, 9th ed.

USDA. 1970. Forage Fiber Anlyses. Agriculture Handbook No. 379. United States Department of Agriculture, Washington, D.C.

E.6.11. Phytic Acid

The sample was extracted using 0.5M HCl with ultrasonication. Purification and concentration was done on a silica based anion exchange (SAX) column. Sample analysis was done on a polymer HPLC column PRP-1, 5µm (150 x 4.1mm) and a refractive index detector. The limit of quantitation was 0.100%.

Reference Standard

- Aldrich, Phytic Acid Dodecasodium Salt Hydrate, 97%, Lot Number 035K0590
- Sigma-Aldrich, Phytic Acid Dodecasodium Salt Hydrate from Rice, 95%, Lot Number 077K0693

References

Lehrfeld, J. 1989. High-performance liquid chromatography analysis of phytic acid on a ph-stable, macroporous polymer column. Cereal Chemistry 66(6):510-515.

Lehrfeld, Jacob, "HPLC Separation and Quantitation of Phytic Acid and Some Inositol Phosphates in Foods: Problem and Solutions," *Journal of Agricultural Food Chemistry*, *42*:2726-2731, (1994).

E.6.12. Protein

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a standard acid. The percent nitrogen was calculated and converted to protein using the factor 6.25. The limit of quantitation was 0.100%.

References

AOAC. 2005. Method 955.04 and 979.09 in Official Methods of Analysis of AOAC International, 18th ed. AOAC International, Gaithersburg, MD.

Bradstreet, R.B. 1965. The Kjeldahl Method for Organic Nitrogen. Academic Press, New York.

Kolthoff, I.M., and E.B. Sandell. 1948. Acidimetry and Alkalimitry in Quantitative Inorganic Analysis. The MacMillan Company, New York.

E.6.13. Phosphatides

The sample was extracted with a 98% $CHCl_3$ 2% MeOH solvent. The extract is then analyzed on an HPLC system equipped with an evaporative light-scattering detector (ELSD). A calibration curve is used for quantification. The Limits of Quantitation for these assays were as follows: L-alpha-Phosphatidic Acid 0.70%, L-alpha-Phosphatidycholine 1.90%, L-alpha-Phosphatidylethanolamine 2.00%, and L-alpha-Phosphatidylinositol 1.20%.

Reference Standard

- (PA) – Avanti Polar Lipids, L-alpha-Phosphatidic Acid (sodium salt), 100%, Lot Number SPA-19
- (PC) – Avanti Polar Lipids, L-alpha-Phosphatidylcholine, 100%, Lot Number PPC-116
- (PE) – Avanti Polar Lipids, L-alpha-Phosphatidylethanolamine, 100%, Lot Number PPE-132b
- (PI) – Avanti Polar Lipids, L-alpha-Phosphatidylinositol, 100%, Lot Number PPI-149

Reference

International Lecithin and Phospholipid Society, Analytical Method AM-101 HPLC, Determination of Lecithins, 1997.

E.6.14. Raffinose and Stachyose

The sample was extracted with deionized water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The limit of quantitation was 0.0500%.

Reference Standards

- Sigma, Raffinose Pentahydrate, 99% / 84.0% after correction for degree of hydration, Lot Number 035K1371
- Sigma, Stachyose, 98% / 96.4% after correction for degree of hydration, Lot Number 065K3775

References

Mason, B. S., and H.T. Slover. 1971. A gas chromatographic method for the determination of sugars in foods. J. Agric. Food Chem. 19(3):551-554.

Brobst, K. M. 1972. Gas-liquid chromatography of trimethylsilyl derivatives in Methods in Carbohydrate Chemistry, Vol. 6. Academic Press, New York.

E.6.15. Trypsin Inhibitor

The sample was ground and defatted with petroleum ether. A sample of matrix was extracted for 3 hours with 0.01N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine-p-nitroanalide hydrochloride. The sample was allowed to react for 10 minutes at 37 °C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solution was centrifuged, then the absorbance was determined at 410 nm. Trypsin inhibitor activity as measured by trypsin inhibitor units (TIU) was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DL-arginine-p-nitroanalide hydrochloride. The limit of quantitation was 1.00 TIU/mg.

Reference

AOCS. 1997. Method Ba12-75 in Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th ed. Chemists' Society. AOCS Press, Champaign, IL.

E.6.16. Vitamin E

The sample was saponified to break down any fat and release any vitamin E. The saponified mixture was extracted with ethyl ether and then quantitated directly by HPLC on a silica column. The limit of quantitation was 0.500 mg/100g.

Reference Standard
USP, Alpha Tocopherol, 100%, Lot Number M

References

Cort, W.M., T.S. Vincente, E.H. Waysek, and B.D. Williams. 1983. Vitamin E content of feedstuffs determined by high-performance liquid chromatographic Fluorescence. Journal of Agricultural Food Chemistry, 31:1330-1333.

Speek, A.J., J. Schijver, and W.H.P. Schreurs. 1985. Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorometric quantitation. Journal of Food Science, 50(1):121-124.

McMurray, C.H., W.J. Blanchflower, and D.A. Rice. 1980. Influence of extraction techniques on determination of α-tocopherol in animal feedstuffs. Journal of the Association of Official Analytical Chemists, 63(6):1258-1261.

Statistical Summary from the Combined Site Analysis of MON 87769 Processed Fractions

Table E-15. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Meal Fraction from Combined-Site for Amino Acids, Fiber, Proximates and Antinutrients

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Amino Acid (% DW)							
Alanine	2.28 (0.025) [2.24 - 2.32]	2.28 (0.025) [2.24 - 2.31]	0.0053 (0.013)	-0.029, 0.039	0.703	(2.23 - 2.39) [2.05, 2.58]	2.18 - 2.59[a]
Arginine	4.34 (0.13) [4.18 - 4.51]	4.17 (0.13) [4.06 - 4.31]	0.16 (0.067)	-0.68, 1.01	0.245	(3.80 - 4.32) [3.36, 4.94]	3.29 - 4.49[a]
Aspartic Acid	6.10 (0.030) [6.05 - 6.13]	6.00 (0.030) [5.94 - 6.04]	0.10 (0.022)	0.044, 0.16	0.006	(5.80 - 6.30) [5.21, 6.97]	5.18 - 6.83[a]
Cystine	0.77 (0.0080) [0.77 - 0.78]	0.77 (0.0080) [0.75 - 0.79]	0.0036 (0.0084)	-0.10, 0.11	0.740	(0.71 - 0.80) [0.61, 0.93]	0.6[b] - 0.92[a]
Glutamic Acid	9.79 (0.031) [9.78 - 9.81]	9.58 (0.031) [9.50 - 9.68]	0.22 (0.043)	0.11, 0.32	0.002	(9.15 - 10.05) [8.21, 11.33]	8.05 - 11.21[a]
Glycine	2.31 (0.023) [2.28 - 2.34]	2.28 (0.023) [2.23 - 2.32]	0.038 (0.015)	-0.0017, 0.077	0.057	(2.23 - 2.36) [2.06, 2.55]	2.02 - 2.40[a]
Histidine	1.42 (0.010) [1.41 - 1.43]	1.40 (0.010) [1.38 - 1.42]	0.021 (0.0059)	0.0054, 0.036	0.017	(1.39 - 1.46) [1.28, 1.57]	1.32 - 1.63[a]
Isoleucine	2.53 (0.050) [2.46 - 2.60]	2.51 (0.050) [2.38 - 2.59]	0.023 (0.046)	-0.096, 0.14	0.642	(2.47 - 2.68) [2.11, 2.98]	2.11 - 2.74[a]

Table E-15. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Meal Fraction from Combined-Site for Amino Acids, Fiber, Proximates and Antinutrients (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Amino Acid (% DW)							
Leucine	4.13 (0.037) [4.07 - 4.19]	4.08 (0.037) [4.01 - 4.15]	0.047 (0.030)	-0.030, 0.12	0.178	(4.04 - 4.29) [3.64, 4.68]	3.62 - 4.72[a]
Lysine	3.34 (0.026) [3.29 - 3.38]	3.31 (0.026) [3.26 - 3.36]	0.027 (0.022)	-0.030, 0.084	0.284	(3.20 - 3.49) [2.90, 3.82]	2.97 - 3.69[a]
Methionine	0.77 (0.0079) [0.75 - 0.78]	0.76 (0.0079) [0.75 - 0.78]	0.0038 (0.011)	-0.044, 0.052	0.767	(0.74 - 0.82) [0.62, 0.92]	0.5 - 0.9[b]
Phenylalanine	2.78 (0.023) [2.75 - 2.81]	2.74 (0.023) [2.69 - 2.78]	0.042 (0.020)	-0.0099, 0.094	0.091	(2.70 - 2.90) [2.44, 3.15]	2.39 - 3.19[a]
Proline	2.66 (0.033) [2.64 - 2.68]	2.62 (0.033) [2.50 - 2.71]	0.034 (0.047)	-0.17, 0.24	0.543	(2.50 - 2.75) [2.15, 3.14]	2.32 - 3.05[a]
Serine	2.75 (0.034) [2.71 - 2.80]	2.68 (0.034) [2.58 - 2.77]	0.070 (0.048)	-0.048, 0.19	0.197	(2.53 - 2.84) [2.23, 3.27]	1.97[a] - 3.3[b]
Threonine	2.00 (0.016) [1.98 - 2.01]	1.97 (0.016) [1.91 - 2.01]	0.023 (0.022)	-0.035, 0.080	0.361	(1.93 - 2.06) [1.73, 2.28]	0.80 - 2.24[a]
Tryptophan	0.68 (0.020) [0.66 - 0.70]	0.67 (0.020) [0.65 - 0.69]	0.012 (0.0026)	0.0049, 0.019	0.006	(0.65 - 0.71) [0.57, 0.77]	0.60[c] - 2.08[a]

Table E-15. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Meal Fraction from Combined-Site for Amino Acids, Fiber, Proximates and Antinutrients (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Amino Acid (% DW)							
Tyrosine	1.81 (0.016) [1.78 - 1.83]	1.78 (0.016) [1.73 - 1.82]	0.027 (0.022)	-0.027, 0.081	0.265	(1.65 - 1.93) [1.39, 2.25]	1.68 - 2.17[a]
Valine	2.67 (0.047) [2.59 - 2.74]	2.64 (0.047) [2.50 - 2.71]	0.031 (0.055)	-0.11, 0.17	0.596	(2.61 - 2.79) [2.37, 3.01]	2.29 - 2.92[a]
Fiber (% DW)							
Acid Detergent Fiber	5.18 (0.14) [4.93 - 5.46]	4.52 (0.14) [4.22 - 4.96]	0.66 (0.19)	0.18, 1.13	0.014	(4.00 - 5.66) [1.79, 7.28]	5.2 - 6.7[f]
Neutral Detergent Fiber	6.36 (0.13) [6.19 - 6.56]	5.58 (0.13) [5.36 - 5.82]	0.78 (0.19)	-0.017, 1.58	0.052	(4.41 - 6.10) [2.46, 8.06]	7.4 - 12.2[f]

Table E-15. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Meal Fraction from Combined-Site for Amino Acids, Fiber, Proximates and Antinutrients (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Proximate (% DW, unless noted)							
Ash	6.62 (0.27) [6.19 - 6.91]	6.46 (0.27) [5.96 - 6.83]	0.16 (0.14)	-0.20, 0.52	0.299	(6.37 - 7.07) [5.63, 7.91]	5.2 - 9.1[f]
Carbohydrates	38.00 (0.29) [37.49 - 38.38]	40.05 (0.29) [39.44 - 41.00]	-2.06 (0.39)	-3.05, -1.06	0.003	(36.26 - 41.61) [28.99, 48.21]	NA
Moisture (% FW)	2.63 (0.56) [1.68 - 3.69]	2.45 (0.56) [1.76 - 4.12]	0.18 (0.64)	-1.46, 1.81	0.794	(2.36 - 4.40) [0, 7.38]	5.58 - 11.7[d]
Protein	54.22 (0.50) [53.28 - 55.50]	52.64 (0.50) [52.11 - 53.19]	1.57 (0.70)	-7.28, 10.42	0.265	(50.03 - 55.23) [44.73, 62.02]	47.4 - 59.5[a]
Total Fat	1.17 (0.39) [0.50 - 2.00]	0.84 (0.39) [0.61 - 1.13]	0.32 (0.47)	-5.70, 6.35	0.619	(0.82 - 2.38) [0, 3.87]	0.5[e] - 3.30[f]
Antinutrient (% DW, unless noted)							
Phytic Acid	1.33 (0.051) [1.19 - 1.52]	1.33 (0.051) [1.30 - 1.36]	0.0034 (0.072)	-0.17, 0.18	0.964	(1.22 - 1.51) [0.87, 1.88]	1.3 - 4.1[d]
Trypsin Inhibitor (TIU/mg DW)	2.03 (1.23) [0.52 - 3.56]	4.82 (1.23) [2.31 - 9.10]	-2.78 (1.75)	-7.05, 1.49	0.162	(0.52 - 6.63) [0, 16.57]	3.8 - 17.9[d]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.
[3] Literature/Historical range references: [a] Karr-Lilienthal et al., 2004, [b] Grieshop et al., 2003, [c] Soybean Meal Information Center, 2001.
[d] Padgette et al., 1996. [e] Orthoefer, 1978, [f] OECD, 2001.

Table E-16. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean RBD Oil from Combined-Site for Fatty Acids and Vitamin E

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Fatty Acid (% Total FA)							
14:0 Myristic	0.083 (0.0039) [0.078 - 0.088]	0.082 (0.0039) [0.078 - 0.089]	0.00064 (0.0016)	-0.0036, 0.0049	0.713	(0.067 - 0.084) [0.042, 0.11]	NA
16:0 Palmitic	12.10 (0.050) [11.98 - 12.23]	11.48 (0.050) [11.42 - 11.61]	0.61 (0.071)	0.44, 0.79	<0.001	(9.80 - 11.73) [7.68, 13.21]	NA
16:1 Palmitoleic	0.085 (0.0015) [0.083 - 0.087]	0.091 (0.0015) [0.088 - 0.095]	-0.0065 (0.0021)	-0.016, 0.0027	0.093	(0.079 - 0.11) [0.044, 0.14]	NA
17:0 Heptadecanoic	0.10 (0.0094) [0.090 - 0.11]	0.096 (0.0094) [0.088 - 0.11]	0.0053 (0.0031)	-0.035, 0.045	0.337	(0.080 - 0.10) [0.057, 0.12]	NA
18:0 Stearic	4.18 (0.019) [4.13 - 4.20]	4.08 (0.019) [4.04 - 4.12]	0.098 (0.027)	0.033, 0.16	0.010	(3.91 - 4.45) [3.00, 5.17]	NA
18:1 Oleic	16.02 (0.80) [14.49 - 17.34]	19.25 (0.80) [19.02 - 19.74]	-3.23 (0.89)	-14.51, 8.04	0.170	(19.57 - 23.22) [16.11, 26.19]	NA
18:2 6c,9c (Isolinoleic Acid)	0.091 (0.0055) [0.089 - 0.094]	0.075 (0.0055) [0.063 - 0.085]	0.015 (0.0078)	-0.018, 0.049	0.184	(0.030 - 0.077) [0, 0.15]	NA
18:2 9c,12c Linoleic	25.66 (3.53) [20.66 - 30.92]	55.38 (3.53) [54.82 - 55.87]	-29.72 (4.54)	-87.36, 27.92	0.096	(54.06 - 56.98) [50.27, 60.17]	NA

Table E-16. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean RBD Oil from Combined-Site for Fatty Acids and Vitamin E (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Fatty Acid (% Total FA)							
18:3 9c,12c,15t (Trans-ALA)	0.51 (0.020) [0.47 - 0.54]	0.14 (0.020) [0.10 - 0.16]	0.37 (0.020)	0.11, 0.63	0.035	(0.072 - 0.15) [0, 0.24]	NA
18:3 Linolenic	10.61 (0.55) [10.34 - 10.95]	8.31 (0.55) [7.42 - 9.07]	2.31 (0.47)	-3.71, 8.32	0.128	(6.44 - 8.61) [3.48, 11.79]	NA
18:3 (Other 18:3 Trans)	0.064 (0.0091) [0.031 - 0.078]	0.084 (0.0091) [0.069 - 0.098]	-0.019 (0.013)	-0.075, 0.036	0.271	(0.031 - 0.083) [0, 0.16]	NA
20:0 Arachidic	0.35 (0.0072) [0.34 - 0.35]	0.31 (0.0072) [0.30 - 0.33]	0.034 (0.0035)	-0.011, 0.079	0.065	(0.30 - 0.35) [0.23, 0.42]	NA
20:1 Eicosenoic	0.18 (0.020) [0.16 - 0.20]	0.17 (0.020) [0.14 - 0.19]	0.014 (0.0016)	-0.0061, 0.033	0.072	(0.15 - 0.22) [0.043, 0.31]	NA
22:0 Behenic	0.32 (0.019) [0.27 - 0.35]	0.33 (0.019) [0.29 - 0.36]	-0.0086 (0.022)	-0.065, 0.048	0.709	(0.32 - 0.40) [0.21, 0.51]	NA
24:0 Lignoceric	0.093 (0.015) [0.076 - 0.11]	0.12 (0.015) [0.10 - 0.14]	-0.030 (0.0024)	-0.036, -0.023	<0.001	(0.088 - 0.18) [0, 0.26]	NA

Table E-16. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean RBD Oil from Combined-Site for Fatty Acids and Vitamin E (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Vitamin (mg/100g FW)							
Vitamin E	8.61 (1.98) [6.56 - 10.90]	7.14 (1.98) [5.14 - 9.27]	1.48 (0.16)	1.06, 1.90	<0.001	(3.56 - 15.35) [0, 29.82]	0.9 - 35.2[a]

[1]FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.
[3] Literature/Historical range references: [a] Codex, 2005.

Table E-17. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Protein Isolate from Combined-Site for Amino Acids and Moisture

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Amino Acid (% DW)							
Alanine	3.70 (0.037) [3.63 - 3.81]	3.83 (0.037) [3.77 - 3.90]	-0.13 (0.052)	-0.36, 0.091	0.124	(3.56 - 3.82) [3.33, 4.12]	-
Arginine	7.78 (0.11) [7.61 - 8.09]	7.85 (0.11) [7.61 - 8.01]	-0.072 (0.13)	-0.41, 0.27	0.607	(7.54 - 8.08) [6.76, 8.87]	6.67[a]
Aspartic Acid	10.98 (0.14) [10.70 - 11.34]	11.07 (0.14) [10.67 - 11.41]	-0.087 (0.20)	-0.59, 0.41	0.683	(10.72 - 11.48) [9.79, 12.23]	-
Cystine	1.14 (0.010) [1.12 - 1.16]	1.12 (0.010) [1.09 - 1.15]	0.022 (0.014)	-0.013, 0.058	0.173	(1.09 - 1.18) [0.98, 1.27]	1.05[a]
Glutamic Acid	18.80 (0.28) [18.42 - 19.65]	18.83 (0.28) [18.12 - 19.34]	-0.024 (0.37)	-0.99, 0.94	0.951	(18.16 - 19.56) [16.29, 21.34]	-
Glycine	3.93 (0.033) [3.88 - 3.97]	4.00 (0.033) [3.90 - 4.06]	-0.069 (0.046)	-0.18, 0.044	0.184	(3.71 - 4.01) [3.45, 4.38]	-
Histidine	2.40 (0.021) [2.37 - 2.44]	2.45 (0.021) [2.39 - 2.51]	-0.048 (0.030)	-0.12, 0.025	0.158	(2.33 - 2.50) [2.15, 2.72]	2.3[a]
Isoleucine	4.43 (0.044) [4.33 - 4.50]	4.57 (0.044) [4.47 - 4.61]	-0.13 (0.062)	-0.40, 0.13	0.161	(4.39 - 4.68) [3.98, 4.96]	4.25[a]

Table E-17. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Protein Isolate Fraction from Combined-Site for Amino Acids and Moisture (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Amino Acid (% DW)							
Leucine	7.28 (0.053) [7.13 - 7.37]	7.47 (0.053) [7.38 - 7.59]	-0.19 (0.075)	-0.37, -0.0062	0.044	(7.08 - 7.61) [6.62, 8.11]	6.78[a]
Lysine	5.78 (0.040) [5.70 - 5.86]	5.91 (0.040) [5.80 - 5.98]	-0.13 (0.056)	-0.27, 0.0028	0.053	(5.68 - 5.95) [5.41, 6.21]	5.33[a]
Methionine	1.21 (0.011) [1.20 - 1.23]	1.22 (0.011) [1.19 - 1.25]	-0.014 (0.015)	-0.051, 0.022	0.368	(1.13 - 1.25) [1.02, 1.38]	1.13[a]
Phenylalanine	5.00 (0.046) [4.88 - 5.10]	5.10 (0.046) [5.02 - 5.19]	-0.096 (0.065)	-0.25, 0.063	0.189	(4.88 - 5.24) [4.54, 5.55]	4.59[a]
Proline	4.89 (0.13) [4.64 - 5.22]	4.90 (0.13) [4.57 - 5.08]	-0.014 (0.18)	-0.46, 0.43	0.939	(4.30 - 5.09) [3.46, 6.04]	-
Serine	4.96 (0.058) [4.86 - 5.08]	5.01 (0.058) [4.82 - 5.11]	-0.048 (0.082)	-0.40, 0.30	0.614	(4.78 - 5.10) [4.49, 5.54]	-
Threonine	3.25 (0.052) [3.10 - 3.35]	3.30 (0.052) [3.21 - 3.39]	-0.053 (0.074)	-0.23, 0.13	0.497	(2.96 - 3.31) [2.67, 3.78]	3.14[a]
Tryptophan	1.09 (0.011) [1.07 - 1.11]	1.10 (0.011) [1.09 - 1.12]	-0.014 (0.0070)	-0.032, 0.0043	0.107	(1.05 - 1.13) [0.92, 1.25]	1.12[a]

Table E-17. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Protein Isolate Fraction from Combined-Site for Amino Acids and Moisture (Continued)

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Amino Acid (% DW)							
Tyrosine	3.43 (0.040) [3.33 - 3.52]	3.52 (0.040) [3.44 - 3.58]	-0.087 (0.042)	-0.19, 0.020	0.091	(3.33 - 3.60) [3.03, 3.89]	-
Valine	4.48 (0.061) [4.44 - 4.56]	4.63 (0.061) [4.51 - 4.74]	-0.14 (0.086)	-0.51, 0.23	0.236	(4.34 - 4.65) [4.01, 5.03]	4.1[a]
Proximate							
Moisture (% FW)	3.22 (0.28) [2.56 - 3.64]	2.73 (0.28) [2.03 - 3.56]	0.50 (0.40)	-0.47, 1.47	0.256	(1.97 - 3.28) [0.47, 4.66]	3.9 - 7.0[c]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.
[3] Literature/Historical range references: [a] OECD, 2001, [b] Mattil, 1974.

Table E-18. Summary of the Compositional Comparison of MON 87769 vs. the Conventional Control Soybean Lecithin Fraction from Combined-Site for Phosphatides

Analytical Component (Units)[1]	MON 87769 Mean (S.E.) [Range]	A3525 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.)	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]	Literature /Historical Range[3]
Phosphatide (%FW)							
L-alpha-Phosphatidic Acid	3.90 (0.43) [3.16 - 4.54]	3.54 (0.43) [2.74 - 4.12]	0.36 (0.30)	-0.40, 1.12	0.278	(3.40 - 5.65) [0.71, 8.07]	0.2 - 14.0[a]
L-alpha-Phosphatidylcholine	7.16 (1.16) [6.37 - 8.16]	7.28 (1.16) [5.37 - 10.25]	-0.12 (1.02)	-13.06, 12.83	0.926	(5.66 - 8.54) [2.16, 11.62]	12.0 - 46.0[a]
L-alpha-Phosphatidylethanolamine	5.66 (0.67) [5.43 - 6.14]	5.48 (0.67) [4.40 - 7.42]	0.18 (0.72)	-9.01, 9.38	0.843	(4.73 - 5.97) [2.98, 7.63]	8.0 - 34.0[a]
L-alpha-Phosphatidylinositol	4.98 (0.37) [4.44 - 5.47]	4.67 (0.37) [4.00 - 5.89]	0.31 (0.53)	-1.96, 2.57	0.621	(3.93 - 5.01) [2.68, 6.61]	1.7 - 21.0[a]

[1]FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.
[3] Literature/Historical range references: [a] Szuhaj, 2005.

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Appendix F. Safety and Nutritional Impact Resulting from the Intended Fatty Acid Change in MON 87769

F.1. Safety and Nutritional Assessment of the Intended Changes in MON 87769

Beside the components typically present in soybean, MON 87769 is expected to contain SDA, GLA, and small amounts of trans-ALA and trans-SDA from the intended change. As expected, the intended change also decreased the levels of LA in MON 87769. The safety and nutritional assessment of MON 87769 will not be complete without a discussion of the safety and nutritional impact resulting from the intended changes. The following sections describe the safety and nutritional impact of SDA, GLA, and the two trans-isomers as well as the nutritional impact from the reduced levels of LA in MON 87769.

F.2. Safety and Nutritional Impact Assessment of SDA in MON 87769

SDA is an *in vivo* intermediate in the metabolism of ALA to long chain omega-3 fatty acids in mammals. There are many natural sources of SDA in the food supply. Fish oils contain levels of SDA ranging from 0.9 to 3.0 wt% (salmon, mackerel, cod, menhaden, herring, boal fish, and sardine). Many fish oils that contain SDA have previously received a Generally Recognized as Safe (GRAS) status (salmon, menhaden, sardine, and tuna oil). SDA is also present in certain edible algae species including *Undaria pinnatifida* and *Ulva pertusa* (16.3 to 26.3% of total fatty acids) and the seed oil of several plant species, notably black currant (*Ribes nigrum*) (2 to 4% of total fatty acids) and Echium (*Echium plantagineum*) (8 to 15% of total fatty acids). Echium oil, containing not less than 10% SDA, was recently authorized in the European Union as a novel food ingredient (EC, 2008). Many dietary supplements made with fish oil, algae or plant species such as black currant are rich in SDA and are consumed as a source of omega-3 fatty acids. Thus, SDA has a history of safe consumption in human foods across a range of sources.

The safety of SDA in MON 87769 is based on: 1) its occurrence as an *in vivo* intermediate in the metabolism of ALA to long chain omega-3 fatty acids in mammals, 2) a long-standing history of safe consumption of SDA from several marine and plant sources, 3) the GRAS status of four fish oils containing SDA, and 4) the positive confirmation from the European Food Safety Agency on the safety of Echium oil containing SDA. Furthermore, the safety of SDA was confirmed by several human, as well as animal studies, conducted with SDA and SDA soybean oil. These studies were conducted with SDA intake levels ranging from 0.8 to 62 mg/kg body weight/day for the human studies and up to 1.04 g/kg body weight/day for the rat studies with no adverse effects reported. Therefore, SDA is concluded to be safe for human and animal consumption.

F.3. Safety and Nutritional Impact Assessment of GLA in MON 87769

GLA is an *in vivo* metabolite in the conversion of LA to arachidonic acid in mammals (Fan and Chapkin, 1998; Horrobin, 1992). It is essential that LA or GLA be present in

the human diet (Fan and Chapkin, 1998; Horrobin, 1992). GLA is present in oats, barley, and human breast milk (Horrobin, 1992). Small concentrations of GLA are found in meats, fish, and a variety of other foods (Horrobin, 1992). Human breast milk contains approximately 50 mg/l of GLA and breast feeding infants appear to have the highest dietary intake among age groups (Kankaanpaa et al., 2001; Stoney et al., 2004; Thijs et al., 2000; Villamor et al., 2007). Breast fed infants will consume 5-10 mg/kg/day of GLA, equivalent to an intake of 0.35-0.7 grams/day GLA intake by a 70 kg adult. GLA is also found in large concentrations in certain plant oils (borage, echium, hemp, evening primrose, and black currant) (Horrobin, 1992) at levels which may exceed those found in MON 87769.

The safety of GLA in MON 87769 is based on: 1) its occurrence as an intermediate in the *in vivo* metabolism of LA to arachidonic acid in mammals, 2) a long history of consumption of foods that contain GLA, and 3) many human and animal studies using GLA-containing materials. A number of human clinical studies have examined chronic consumption of GLA with no safety issues reported. In published human studies, supplementation with GLA at doses of 1 to 5 g/day for periods of one to six months was well tolerated and without reports of serious adverse effects. A number of animal studies have also demonstrated the safety of large doses of GLA consumption. There are no identified concerns at this time regarding excessive dietary consumption of GLA (Health Canada, 2006; Horrobin, 1992). Thus, GLA is concluded to be safe for human and animal consumption.

F.4. Safety and Nutritional Impact of trans-SDA and trans-ALA in MON 87769

The primary source of trans-fatty acids (TFAs) in the human diet is the consumption of hydrogenated vegetable oils in liquid or solid form resulting in food products that may contain in excess of 30% TFA (Chardigny et al., 1996; Ledoux, 2007). Naturally occurring trans-fats also arise as a result of bacterial reduction of unsaturated fatty acids in the gut of ruminant animals, with intake resulting from consumption of meat and dairy products (Chardigny et al., 1996; Ledoux, 2007). The TFA levels in non-hydrogenated vegetable oils are generally low by comparison, typically less than 1% of total fatty acids. The TFAs arise during oil refining, particularly during heat-requiring processes such as deodorization (Chardigny et al., 1996; Ledoux, 2007). The total TFA levels in MON 87769 are approximately 0.6% of total fatty acids. Total TFAs in MON 87769 are well within the range of the total TFA content in samples of commercial soybean oil (Chardigny et al., 1996). The contribution of the TFA from MON 87769 to the overall human dietary TFA intake will be minimal relative to commonly experienced human dietary TFA intakes. Thus, the trans-SDA and trans-ALA are concluded to not raise safety concerns.

F.5. Nutritional Impact from Reduced LA Levels in MON 87769

Due to the conversion of LA to ALA and GLA in MON 87769, the LA concentration is expected to be lower in MON 87769 (Figure VII-1). LA is considered to be an essential fatty acid, and is necessary in the human diet to support health. However, the only

recognized role of LA in mammalian species is as a precursor to GLA (Horrobin, 1992) and as a source of energy. In mammalian species, LA is first converted to GLA and subsequently GLA to arachidonic acid (Horrobin, 1992). Daily human LA intake greatly exceeds the amount of LA needed to support health. Furthermore, LA can be replaced by GLA in the diet (Horrobin, 1992) and, due to the increased GLA concentration in MON 87769, it is anticipated that any decrease in LA intake will be compensated without any nutritional effect to humans or animals.

F.6. Safety and Nutrition Assessment of SDA Soybean Oil

The safety of SDA soybean oil is supported by the results of a published 90-day/one-generation reproductive rat toxicity study in which a no observable adverse effect level (NOAEL) of 1 g SDA/kg body weight/day (4 g SDA soybean oil/kg body weight/day) was determined (Hammond et al., 2008). Rats were fed diets supplemented with 1.5 or 4.0 g SDA soybean oil/kg body weight/day. Diets containing control soybean oil derived from conventional soybean or menhaden oil (4.0 g/kg body weight/day) were provided to control groups. No statistically significant dose-dependent test article-related adverse effects were reported in any of the parameters evaluated, including clinical signs, behavior, mortality, body weight, organ weights, macroscopic appearance of tissues, and histopathology. Statistically significant differences observed between the control and SDA-treated groups included: increased food consumption at Weeks 1 and 2 (low-dose SDA, females); increased basophils (high-dose SDA, females); decreased alanine transferase levels (low-dose SDA, females); decreased cholesterol levels (high-dose SDA, females); increased phosphorus levels (low-dose SDA, males); increased blood urea nitrogen levels (high-dose SDA, males); decreased triglycerides (high-dose SDA, males); and urine urobilinogen (low-dose SDA, males). Given that these changes were slight, within historical limits of the testing laboratory, not dose-dependent, and/or also were observed in the menhaden oil-treated group (*i.e.,* considered typical responses for rats fed high doses of long-chain polyunsaturated fatty acids), they were not considered to be of toxicological significance. Therefore, the NOAEL was determined to be 4 g SDA soybean oil/kg body weight/day (providing 1,051 and 1,073 mg SDA/kg body weight/day in male and female rats, respectively), the highest dose tested. Additional many published studies on SDA and GLA from other sources corroborate the safety of SDA soybean oil. The safety of SDA soybean oil is further supported by the regular dietary consumption of fats and oils containing ALA, LA, and palmitic acid, as well as by the permitted uses of LA and palmitic acid in food in the U.S.

Additionally, based on the known SDA:EPA conversion ratio of approximately 3:1 in humans (James et. al., 2003), the total intake of EPA and DHA (i.e., resulting from the intake of SDA from the proposed food uses of SDA soyeban oil plus the baseline per capita daily intake) would not exceed the maximum FDA established intake level of 3 g/day per person combined EPA and DHA.[13,14] Therefore, SDA soybean oil is safe for human and animal consumption.

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[13] http://www.cfsan.fda.gov/~dms/ds-ltr11.html

When viewed in its entirety, the scientific evidence presented above does not indicate any potential for adverse effects in humans following the consumption of SDA soybean oil under the conditions of intended use in foods. Following a critical evaluation of the scientific data generally available in the public domain that pertain to the safety of SDA soybean oil, a panel of experts, qualified by scientific training and experience to evaluate the safety of SDA soybean oil as a component of food, unanimously concluded that the proposed uses of SDA soybean oil are safe and suitable and are GRAS, based on scientific procedures. Therefore, Monsanto has concluded that SDA soybean oil is GRAS under the intended conditions of use on the basis of scientific procedures.

F.7. Safety and Nutrition Assessment Conclusion

In conclusion, having demonstrated: (i) the compositional equivalence of MON 87769 seed (except for the intended fatty acid change) and forage to seed and forage from conventional soybean already on the market, (ii) the safety of SDA soybean oil and the expected fatty acids resulting from the intended change, (iii) the history of safe use of the introduced proteins, and (iv) familiarity of the host organism from which the genes are derived, MON 87769 is as safe and nutritious as conventional soybean for food and feed use with the added nutritional improvement of SDA, an omega-3 fatty acid. No additional information was considered to be necessary to support the safety and nutritional assessment of MON 87769.

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Appendix G. Materials, Methods, and Individual-Site Results of the Plant Phenotypic and Agronomic Assessment of MON 87769 from the 2006 and 2007 Field Trials

G.1. Materials

The materials for the 2006 and 2007 plant phenotypic assessments included MON 87769, the conventional soybean control (variety A3525), and 20 commercial reference soybean varieties. The list of the soybean materials planted in each site is presented in Tables G-1 and G-2. The presence or absence of MON 87769 in the test and control seed was verified by event-specific polymerase chain reaction. The results of these analyses were as expected.

G.2. Field Sites and Experimental Design

Phenotypic and agronomic data were collected from 21 field locations over two consecutive years: 17 locations in 2006 and four locations in 2007. Table VIII-4 provides a list of the field site locations (including site codes). These locations provided a diverse range of environmental and agronomic conditions representative of major U.S. soybean-production regions. The researchers at each field site were familiar with the growth, production, and evaluation of the soybean characteristics.

In 2006, the field trial at each of the 17 sites was established in a randomized complete block design with three replications. At the IA2, IL2, IN1, IN2, KS, and MO1 sites, each plot consisted of four 20 ft long rows spaced approximately 30 inches apart. Phenotypic and agronomic data were collected from the second and third row of each plot. The plots were planted adjacent to each other and the entire plot area was surrounded by a four-row (approximately 10 ft) border of a commercial soybean variety. At the IA1, IL1, MO2, and NE sites, each plot consisted of eight 30 ft long rows spaced approximately 30 inches apart. Phenotypic and agronomic data were collected from the second and third row of each plot. Each plot was surrounded by four rows (approximately 10 ft) of a commercial soybean variety. At the IA3, IA4, IL3, MI, NE2, OH, and WI sites, each plot consisted of six 20 ft long rows spaced approximately 30 inches apart. Phenotypic and agronomic data were collected from the fourth and fifth row of each plot. The plots within each replicate were separated by approximately two rows (approximately 5 ft) of a commercial soybean variety, and the entire plot area was surrounded by a four row (approximately 10 ft) border of a commercial soybean variety.

In 2007, the field trial at each of the four sites was established in a randomized complete block design with three replications. At the IL, IN, MI, and WI sites, each plot consisted of eight 30 ft long rows spaced approximately 30 inches apart. Phenotypic and agronomic data were collected from the second and third row of each plot. Each plot was surrounded by at least four rows (approximately 10 ft) of a commercial soybean variety.

G.3. Planting and Field Operations

Planting information, soil description, and cropping history of the study area at each site are listed in Table G-7. Agronomic practices used to prepare and maintain the trial at each site were characteristic of the respective region. All maintenance operations were performed uniformly over the entire study area.

G.4. Data Collection and Statistical Analysis

The following 14 phenotypic and agronomic characteristics were evaluated at each site in 2006 and 2007: early stand count, seedling vigor, plant growth stages, days to 50% flowering, flower color, plant pubescence, plant height, lodging, pod shattering, final stand count, seed moisture, 100 seed weight, test weight, and yield. The description of the characteristics measured and the designated developmental stages where observations occurred are listed in Table VIII-1 of Section VIII.A.

Analysis of the phenotypic and agronomic data from the 2006 and 2007 field trials were conducted separately. An analysis of variance was conducted on the data from each year according to a randomized complete block design using SAS (Release 9.1, 2002-2003 for the 2006 data; Release 9.2, 2002-2008 for the 2007 data). The level of statistical significance was set at the 5% probability level ($p = 0.05$). For each year, MON 87769 was compared to the control substance within each site (individual-site analysis) and in a combined-site analysis, in which the data were pooled across all sites, for early stand count, seedling vigor, days to 50% flowering, plant height, lodging, shattering, final stand count, seed moisture, 100 seed weight, seed test weight, and yield. Growth stage, flower color, and plant pubescence data were categorical and were not statistically analyzed. For each assessed characteristic, the minimum and maximum means were determined from among the reference soybean varieties (reference range) planted at the sites to provide values that are representative of commercial soybean varieties. The following is a summary of the results from the 2006 and 2007 individual-site analyses of phenotypic and agronomic evaluations. Results from the 2006 and 2007 combined-site analyses are presented in Section VIII.D.2.1.

G.5. Individual Site Phenotypic and Agronomic Results

In the individual-site analysis of the data from the 2006 field trials, a total of 22 statistically significant differences were detected out of 165 site × characteristic comparisons between MON 87769 and the control (Table G-3). These significant differences were distributed among eleven phenotypic characteristics. None of the significant differences detected in the individual-site analysis were detected in the combined-site analysis. This suggests these significant differences were not indicative of a consistent plant response associated with the trait and are unlikely to be biologically meaningful in terms of increased plant pest or weediness potential of MON 87769 compared to the control. All plants of MON 87769 and the control had purple flowers and hairy pubescence at each site as expected (Table G-3). Furthermore, no differences

in plant growth stage were observed between MON 87769 and the control for any of the 126 observations conducted among the sites (Table G-4).

In the individual-site analysis of the data from the 2007 field trials, one statistically significant difference was detected out of 39 site × characteristic comparisons between MON 87769 and the control (Table G-5). Yield was lower for MON 87769 compared to the control at the MI site (43.0 vs. 52.5 bu/ac). Since the statistical difference detected in the individual-site analysis was not detected in the combined-site analysis, this suggests the difference was not indicative of a consistent response associated with the trait and is unlikely to be biologically meaningful in terms of increased plant pest or weediness potential of MON 87769 compared to the control. All plants of MON 87769 and the control had purple flowers and hairy pubescence at each site as expected (Table G-5). Furthermore, no differences in plant growth stage were observed between MON 87769 and the control for any of the 27 observations conducted among the sites (Table G-6).

Table G-1. Test, Control, and Reference Materials Assessed in the 2006 Field Trials

Material	Material type[1]	Sites[2,3]
MON 87769	Test	All
A3525	Control	All
A3244	Reference	IA1, IA3, IL2, KS, NE, NE2, OH, WI
ST3600	Reference	IA1, IL2, IL3, KS
Stewart SB3454	Reference	IA1, IL2, MO1
DKB34-51	Reference	IA1, IN1, MO1
ST3608	Reference	IA2, IN1, MO1
Pioneer 93M50	Reference	IA2, IN1, MO1
Pioneer 93B82	Reference	IA2, IL3, IN1, MO2
Lewis 372	Reference	IA2, IA4, IN2, MI, MO2, NE2, OH
AG3505	Reference	IL1, IN2, MO2
CST3461 (STS)	Reference	IL1, IN2, MO2
ST3300	Reference	IA3, IA4, IL1, IN2, NE, OH
CST37002	Reference	IL1, KS, NE
ST3870	Reference	IL2, KS, NE
A2869	Reference	IA3, NE2
ST2788	Reference	IA4, WI
Lewis 392	Reference	IL3
A2804	Reference	MI
A2553	Reference	MI, WI

[1] The commercial reference materials were all conventional soybean varieties with the exception of DKB34-51, Pioneer 93M50, and AG3505, which were Roundup Ready soybean 40-3-2 varieties.

[2] The test and control materials were planted at all field sites; the soybean reference varieties were planted at specific sites.

[3] IA1 = Jefferson County, IA; IA2 = Benton County, IA; IA3 = Jefferson County, IA; IA4 = Guthrie County, IA; IL1 = Stark County, IL; IL2 = Warren County, IL; IL3 = Clinton County, IL; IN1 = Parke County, IN; IN2 = Boone County, IN; KS = Pawnee County, KS; MI = Ottawa County, MI; MO1 = Macon County, MO; MO2 = Lincoln County, MO; NE = York County, NE; NE2 = York County, NE; OH = Fayette County, OH; WI = Walworth County, WI.

Table G-2. Test, Control, and Reference Materials Assessed in the 2007 Field Trials

Materials	Material type[1]	Sites[2,3]
MON 87769	Test	All
A3525	Control	All
DKB34-51	Reference	IN, MI,
Hoegemeyer 333	Reference	IN, MI,
CST3461 (STS)	Reference	IN, MI,
ST3600	Reference	IN, MI,
AG3505	Reference	IL
ST3300	Reference	IL
Stewart SB3454	Reference	IL
CST37002	Reference	IL
Pioneer 93M50	Reference	WI
Midland 363	Reference	WI
A3244	Reference	WI
ST3608	Reference	WI

[1] The commercial reference materials were all conventional soybean varieties with the exception of DKB34-51, AG3505, and Pioneer 93M50, which were Roundup Ready soybean 40-3-2 varieties.
[2] The test and control materials were planted at all field sites; the reference varieties were planted at specific sites.
[3] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.

Table G-3. Phenotypic and Agronomic Comparison of MON 87769 to the Conventional Control in the Individual-Site Analysis for the 2006 Field Study

	Phenotypic Characteristic (units)									
	Early stand count (#/plot)		Seedling vigor (1-9 scale)		Days to 50% flowering		Flower color[1]		Plant pubescence[1,2]	
Site	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
IA1	241.3	263.8	2.0	2.0	201.7	202.0	Purple	Purple	Hairy	Hairy
IA2	274.0	279.3	2.0†	2.0	192.0	192.0	Purple	Purple	Hairy	Hairy
IA3	225.7*	193.7	2.3	2.3	205.0	206.0	Purple	Purple	Hairy	Hairy
IA4	106.3	106.0	4.0	4.0	197.0†	197.0	Purple	Purple	Hairy	Hairy
IL1	197.5	203.6	1.0†	1.0	183.0†	183.0	Purple	Purple	Hairy	Hairy
IL2	272.0	286.7	1.0	1.0	189.0	189.0	Purple	Purple	Hairy	Hairy
IL3	195.3	201.7	3.0	3.3	199.0	199.0	Purple	Purple	Hairy	Hairy
IN1	302.3	301.0	2.0†	2.0	200.3	201.0	Purple	Purple	Hairy	Hairy
IN2	232.7	213.3	5.0	5.7	200.0†	200.0	Purple	Purple	Hairy	Hairy
KS	212.7	202.7	6.0	6.3	188.7	188.3	Purple	Purple	Hairy	Hairy
MI	189.3	184.0	4.7	4.3	206.0†	206.0	Purple	Purple	Hairy	Hairy
MO1	321.0	316.3	4.7	4.7	200.0*	202.3	Purple	Purple	Hairy	Hairy
MO2	134.5	128.0	3.3	3.0	193.0*	194.0	Purple	Purple	—	—
NE	226.2*	216.0	3.0	3.0	179.3	179.3	Purple	Purple	Hairy	Hairy
NE2	132.7	139.7	3.3	3.0	189.0	187.0	Purple	Purple	Hairy	Hairy
OH	191.0	216.0	2.7*	1.3	191.7	192.3	Purple	Purple	Hairy	Hairy
WI	234.0*	282.7	5.0	4.3	207.0†	207.0	Purple	Purple	Hairy	Hairy

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and conventional control (A3525).
† No statistical comparisons were made due to lack of variability in the data.
[1] Flower color and plant pubescence data were categorical and were not statistically analyzed.
[2] Plant pubescence data from the MO2 site was eliminated from the study because the data were collected incorrectly.

Table G-3 (continued). Phenotypic and Agronomic Comparison of MON 87769 to the Conventional Control in the Individual-Site Analysis for the 2006 Field Study

	Phenotypic Characteristic (units)									
	Plant height (in)		Lodging (0-9 scale)		Pod Shattering (0-9 scale)		Final stand count (#/plot)		Seed moisture (%)	
Site	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
IA1	35.6*	37.3	0.3	1.3	0.0†	0.0	188.7*	210.0	11.1	11.2
IA2	46.5	44.7	3.0	3.7	0.0†	0.0	245.3	253.7	11.9	12.0
IA3	33.3	34.1	0.7*	1.7	0.0†	0.0	202.7	204.0	11.2	11.1
IA4	30.5	30.5	1.3	1.0	0.3	0.0	91.7	90.0	10.8	11.3
IL1	45.2	43.7	1.7	1.7	0.0	0.0	287.8	298.7	13.2	12.9
IL2	43.4	43.2	0.3	0.7	0.0†	0.0	242.0	263.7	12.2	12.1
IL3	27.2	29.5	0.0†	0.0	0.0†	0.0	190.3	196.3	11.1	11.2
IN1	40.8	39.2	3.0	2.8	0.0†	0.0	273.0	267.7	11.8*	11.4
IN2	37.6	36.5	0.7	1.0	0.0†	0.0	176.0	136.0	12.6	12.4
KS	30.9	31.8	2.3	2.7	0.0*	0.7	146.7	136.7	8.7	9.0
MI	39.6	38.5	1.3	1.3	0.0†	0.0	166.0	170.7	17.2	17.5
MO1	26.1	26.8	1.3	1.3	0.3	0.0	298.7	284.3	15.3	15.1
MO2	28.4	28.6	0.0†	0.0	0.0†	0.0	148.4	163.8	12.0*	11.3
NE	45.1	45.3	1.3	1.3	2.0	3.0	224.9	214.4	11.4	10.8
NE2	39.7	39.6	1.3	1.3	1.0	1.0	128.3	143.3	10.7	10.8
OH	28.1	26.1	0.0*	0.7	1.0	0.7	199.0	209.0	14.2	14.0
WI	37.5	38.0	0.0	0.3	0.0†	0.0	183.3*	215.3	12.3*	13.6

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and conventional control (A3525).
† No statistical comparisons were made due to lack of variability in the data.

Table G-3 (continued). Phenotypic and Agronomic Comparison of MON 87769 to the Conventional Control in the Individual-Site Analysis for the 2006 Field Study

	Phenotypic Characteristic (units)					
	100 seed weight (g)		Test weight (lb/bu)[1]		Yield (bu/ac)[1]	
Site	Test	Control	Test	Control	Test	Control
IA1	12.7	12.3	59.7*	57.3	37.3	44.0
IA2	13.9	14.2	57.0	56.3	52.0	57.5
IA3	14.2	14.3	57.7	56.3	50.9	52.6
IA4	14.6	14.6	—	—	—	—
IL1	15.4	15.8	53.7	54.0	58.2	59.2
IL2	13.4*	14.4	57.0	56.8	65.6	68.8
IL3	13.0	12.7	54.0	55.0	28.2	28.9
IN1	15.4	15.7	61.0*	60.1	59.9	65.4
IN2	15.1*	16.4	60.0	61.1	61.3	65.0
KS	15.2	14.5	57.9	57.7	60.0*	70.0
MI	17.7	18.4	49.9	50.0	60.0	54.7
MO1	15.3	15.7	52.0	51.7	47.2	47.2
MO2	18.7	16.7	59.8	60.1	35.8	39.7
NE	15.0	15.3	56.3	55.7	52.4	51.2
NE2	14.0*	15.0	57.3	57.2	64.8	67.7
OH	18.2	18.8	54.0	52.3	47.7	48.6
WI	15.3	16.7	57.0	56.3	61.9*	70.1

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and conventional control (A3525).

[1] Test weight and yield data from the IA4 site were excluded from the data analysis because an inaccurate measuring device was used to collect the data.

Table G-4. Plant Growth Stages of MON 87769, the Conventional Control, and Reference Varieties at 17 U.S. Sites in 2006

		Date and Range of Growth Stages Observed[2]									
Site	**Substance[1]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**	**Obs. 5**	**Obs. 6**	**Obs. 7**	**Obs. 8**	**Obs. 9**	**Obs. 10**
IA1		06/28/2006	07/17/2006	07/31/2006	08/14/2006	09/05/2006	09/14/2006	09/29/2006	10/14/2006	—	—
	MON 87769	V2	R1	R2	R3	R5	R6	R8	R8	—	—
	Control	V2	R1	R2	R3	R5	R6	R8	R8	—	—
	References	V2	R1	R2	R3	R5	R6	R7-R8	R8	—	—
IA2		06/03/2006	06/20/2006	07/11/2006	07/29/2006	08/18/2006	09/05/2006	09/20/2006	10/10/2006	—	—
	MON 87769	VC	V3	V6-R1	R3	R5	R6	R7	R8	—	—
	Control	VC	V3	V6	R3	R5	R6	R7	R8	—	—
	References	VC	V3	V6-R1	R3	R5	R6	R6-R7	R8	—	—
IA3		07/03/2006	07/18/2006	07/27/2006	08/07/2006	08/23/2006	09/05/2006	09/19/2006	10/03/2006	—	—
	MON 87769	V2	V6-V7	V10	V14	R4	R5	R6	R8	—	—
	Control	V2	V6-V7	V10	V14	R4	R5	R6	R8	—	—
	References	V2	V6-V7	V10	V14	R4	R5	R6	R8	—	—
IA4		06/23/2006	07/07/2006	07/25/2006	08/12/2006	08/28/2006	09/12/2006	09/27/2006	10/17/2006	—	—
	MON 87769	V2	V5-V6	V14	R4	R5	R6	R6	R8	—	—
	Control	V2	V6	V14	R4	R5	R6	R6-R7	R8	—	—
	References	V2	V5-R1	V14	R3-R4	R5-R6	R6	R6-R7	R8	—	—
IL1		06/14/2006	06/28/2006	07/14/2006	08/3/2006	08/23/2006	09/07/2006	09/28/2006	10/19/2006	—	—
	MON 87769	V2	V6	R1-R2	R2	R4	R6	R7	R8	—	—
	Control	V2	V6	R1-R2	R2	R4	R6	R7	R8	—	—
	References	V2	V6	R1-R2	R2	R4	R6	R7	R8	—	—

Obs. = Observation number.
[1] Control = conventional variety A3525.
[2] Month-day-year.
— Information not available.

Table G-4 (continued). Plant Growth Stages of MON 87769, the Conventional Control, and Reference Varieties throughout the Growing Season at 17 Sites in 2006

		Date and Range of Growth Stages Observed[2]									
Site	**Substance[1]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**	**Obs. 5**	**Obs. 6**	**Obs. 7**	**Obs. 8**	**Obs. 9**	**Obs. 10**
IL2		06/12/2006	06/30/2006	07/21/2006	08/03/2006	08/30/2006	09/15/2006	10/06/2006	—	—	—
	MON 87769	V2-V3	V5-V6	R2-R3	R5	R6	R7	R8	—	—	—
	Control	V2-V3	V5-V6	R2-R3	R5	R6	R7	R8	—	—	—
	References	V2-V3	V5-V6	R2-R3	R5	R6	R6-R7	R8	—	—	—
IL3		06/29/2006	07/19/2006	08/09/2006	08/25/2006	09/15/2006	10/05/2006	—	—	—	—
	MON 87769	V2	R1-R2	R3	R6	R7	R8	—	—	—	—
	Control	V2	R1-R2	R3	R6	R7	R8	—	—	—	—
	References	V2	R1-R2	R3	R6	R7	R8	—	—	—	—
IN1		06/27/2006	07/14/2006	08/03/2006	08/22/2006	09/06/2006	09/20/2006	09/26/2006	10/04/2006	10/18/2006	10/24/2006
	MON 87769	V3	V6	R2	R4-R5	R6	R6-R7	R7	R7-R8	R8	R8
	Control	V3	V6	R2	R4-R5	R6	R6-R7	R7	R7-R8	R8	R8
	References	V3	V5-V6	R2	R4-R5	R6	R6-R7	R6-R7	R7-R8	R8	R8
IN2		06/26/2006	07/20/2006	08/18/2006	09/18/2006	—	—	—	—	—	—
	MON 87769	V2	R1-R2	R3	R7	—	—	—	—	—	—
	Control	V2	R1-R2	R3	R7	—	—	—	—	—	—
	References	V2	R1-R2	R3	R7	—	—	—	—	—	—
KS		06/19/2006	07/12/2006	07/27/2006	08/11/2006	08/28/2006	09/11/2006	09/26/2006	—	—	—
	MON 87769	V2	R2	R3	R5	R6	R7	R8	—	—	—
	Control	V2	R2	R3	R5	R6	R7	R8	—	—	—
	References	V2	R2	R3	R5	R6	R6-R7	R8	—	—	—

Obs. = Observation number.
[1] Control = conventional variety A3525.
[2] Month-day-year.
— Information not available.

Table G-4 (continued). Plant Growth Stages of MON 87769, the Control, and Reference Varieties throughout the Growing Season at 17 Sites in 2006

		Date and Range of Growth Stages Observed[2]									
Site	**Substance[1]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**	**Obs. 5**	**Obs. 6**	**Obs. 7**	**Obs. 8**	**Obs. 9**	**Obs. 10**
MI		06/23/2006	07/07/2006	07/21/2006	08/04/2006	08/18/2006	09/04/2006	09/17/2006	09/29/2006	10/14/2006	10/22/2006
	MON 87769	V2-V3	V5-V6	R1	R3	R5	R6	R6	R7	R8	R8
	Control	V2-V3	V6	R1	R3	R5	R6	R6	R7	R8	R8
	References	V2-V3	V6-V7	R1	R3	R5	R6	R6	R6-R8	R7-R8	R8
MO1		07/12/2006	08/02/2006	08/21/2006	09/15/2006	10/05/2006	—	—	—	—	—
	MON 87769	V3	R2	R5	R6	R7	—	—	—	—	—
	Control	V3	R2	R5	R6	R7	—	—	—	—	—
	References	V3	R2	R5	R6	R7-R8	—	—	—	—	—
MO2		06/12/2006	06/27/2006	07/10/2006	07/26/2006	08/09/2006	08/24/2006	09/13/2006	09/30/2006	—	—
	MON 87769	VC	V3	R1	R3	R4	R5	R6	R8	—	—
	Control	VC	V3	R1	R3	R4	R5	R6	R8	—	—
	References	VC	V3	V6-R1	R1-R3	R4	R5	R6	R8	—	—
NE		06/09/2006	06/23/2006	07/10/2006	08/03/2006	08/18/2006	09/08/2006	09/25/2006	—	—	—
	MON 87769	V2	V5-V6	R2	R5	R6	R7	R8	—	—	—
	Control	V2	V5-V6	R2	R5	R6	R7	R8	—	—	—
	References	V2	V5-V6	R2	R5	R6	R6-R7	R8	—	—	—

Obs. = Observation number.
[1] Control = conventional variety A3525.
[2] Month-day-year.
— Information not available.

Table G-4 (continued). Plant Growth Stages of MON 87769, the Conventional Control, and Reference Varieties throughout the Growing Season at 17 Sites in 2006

		Date and Range of Growth Stages Observed[2]									
Site	**Substance[1]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**	**Obs. 5**	**Obs. 6**	**Obs. 7**	**Obs. 8**	**Obs. 9**	**Obs. 10**
NE2		06/13/2006	06/28/2006	07/14/2006	08/04/2006	08/25/2006	09/08/2006	09/25/2006	—	—	—
	MON 87769	V2	V5-V6	R2	R4	R6	R6	R8	—	—	—
	Control	V2	V5-V6	R2	R4	R6	R6	R8	—	—	—
	References	V2	V5-V6	R2	R3-R5	R6	R6-R7	R8	—	—	—
OH		06/23/2006	07/12/2006	08/02/2006	08/29/2006	09/20/2006	10/24/2006	—	—	—	—
	MON 87769	V2	V6-V7	R3	R6	R6-R7	R8	—	—	—	—
	Control	V2	V6-V7	R3	R6	R6-R7	R8	—	—	—	—
	References	V2	V6-V7	R3	R6	R6-R7	R8	—	—	—	—
WI		06/28/2006	07/12/2006	07/26/2006	08/07/2006	08/22/2006	09/07/2006	09/22/2006	10/18/2006	11/01/2006	—
	MON 87769	V2	R1	R2	R3	R5	R5	R6	R8	R8	—
	Control	V2-V3	R1	R1-R2	R3	R5	R5-R6	R6	R8	R8	—
	References	V2-V3	R1	R1-R2	R3	R5	R5-R6	R6-R7	R8	R8	—

Obs. = Observation number.
[1] Control = conventional variety A3525.
[2] Month-day-year.
— Information not available.

Table G-5. Phenotypic and Agronomic Comparison of MON 87769 to the Conventional Control in the Individual-Site Analysis for the 2007 Field Study

	Phenotypic Characteristic (units)									
	Early stand count (#/plot)		Seedling vigor (1-9 scale)		Days to 50% flowering		Flower color[1]		Plant pubescence[1]	
Site	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
IL	458.0	463.3	1.0[†]	1.0	204.7	204.7	Purple	Purple	Hairy	Hairy
IN	214.7	217.0	4.0	3.7	207.0[†]	207.0	Purple	Purple	Hairy	Hairy
MI	408.3	438.0	3.7	3.3	198.0	198.0	Purple	Purple	Hairy	Hairy
WI	350.3	298.3	2.3	2.0	212.0	211.7	Purple	Purple	Hairy	Hairy

	Phenotypic Characteristic (units)									
	Plant height (in)		Lodging (0-9 scale)		Pod Shattering (0-9 scale)		Final stand count (#/plot)		Seed moisture (%)	
Site	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
IL	46.4	45.6	5.0	5.0	0.0[†]	0.0	415.7	410.7	11.6	11.7
IN	30.8	31.7	0.3	0.3	0.7	0.0	261.7	320.3	11.2	10.9
MI	32.0	38.1	0.3	1.3	0.0[†]	0.0	380.0	390.0	13.6	13.4
WI	43.8	41.8	1.0	0.7	0.0	0.0	269.0	237.3	10.7	10.7

[1] Flower color and plant pubescence data were categorical and were not statistically analyzed.
[†] No statistical comparisons were made due to lack of variability in the data.

Table G-5 (continued). Phenotypic and Agronomic Comparison of MON 87769 to the Conventional Control in the Individual-Site Analysis for the 2007 Field Study

	Phenotypic Characteristic (units)					
	100 seed weight (g)[1]		Test weight (lb/bu)		Yield (bu/ac)	
Site	Test	Control	Test	Control	Test	Control
IL	15.3	15.3	53.7	53.7	50.2	48.3
IN	16.0	15.6	49.9	51.4	50.0	58.2
MI	20.6	20.2	56.7	56.0	43.0*	52.5
WI	—	—	56.7	57.0	42.4	42.4

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and conventional control (A3525).

[1] 100 seed weight data from the WI site were excluded from the statistical analysis because the balance used to weigh the samples did not have sufficient precision.

Table G-6. Plant Growth Stages of MON 87769, the Conventional Control, and Reference Varieties at Four U.S. Sites in 2007

		Date and Range of Growth Stages Observed[2]									
Site	**Substance[1]**	**Obs. 1**	**Obs. 2**	**Obs. 3**	**Obs. 4**	**Obs. 5**	**Obs. 6**	**Obs. 7**	**Obs. 8**	**Obs. 9**	**Obs. 10**
IL		6/27/07	7/11/07	7/24/07	8/8/07	8/27/07	9/17/07	10/8/07	—	—	—
	MON 87769	V2	V3-V4	R1-R2	R3-R4	R5	R7	R7-R8	—	—	—
	Control	V2	V3-V4	R1-R2	R3-R4	R5	R7	R7-R8	—	—	—
	References	V2	V3-V4	R1-R2	R3-R4	R5	R7	R7-R8	—	—	—
IN		6/25/07	7/16/07	7/24/07	8/17/07	9/12/07	10/17/07	—	—	—	—
	MON 87769	V3	R1	R1-R2	R3-R4	R5-R6	R8	—	—	—	—
	Control	V2-V3	R1	R1-R2	R3-R4	R6	R8	—	—	—	—
	References	V2-V3	R1	R1-R2	R3-R4	R5-R6	R8	—	—	—	—
MI		6/20/07	7/5/07	7/18/07	8/1/07	8/14/07	8/29/07	9/12/07	9/26/07	10/9/07	—
	MON 87769	V2	V5-V6	R2	R3	R5	R6	R6	R7	R8	—
	Control	V2	V5-V6	R2	R3	R5	R6	R6	R7	R8	—
	References	V2	V5-V6	R1-R2	R2-R3	R5	R6	R6	R7	R8	—
WI		7/3/07	8/3/07	8/16/07	9/4/07	9/26/07	—	—	—	—	—
	MON 87769	V2	R1-R2	R2[3]	R4	R6	—	—	—	—	—
	Control	V2	R2	R2	R4	R6	—	—	—	—	—
	References	V2	R1-R2	R2-R3	R4	R6	—	—	—	—	—

Obs. = Observation number.
[1]Control = conventional variety A3525.
[2] Month-day-year.
[3] This value is representative of two of the three replicates as data for one of the replicates was inadvertently not collected or recorded.
—Information not available.

Table G-7. Field Site Planting Information, Soil Description, and Cropping History

2006 Field Study								
							Cropping history	
Site	Planting date[1]	Planting rate (seeds/ft)	Planting depth (in)	Plot size (ft)[2]	Rows/plot	Soil series, organic matter, pH	2005	2004
IA1	06/09/2006	9.0	1.5	20 × 30	8	Taintor loam, 3.7%, 6.7	Corn	Soybean
IA2	05/23/2006	9.0	1.5	10 × 20	4	Tama-Muscatine silty clay loam, 3-4%, 6.3	Corn	Soybean
IA3	06/10/2006	9.1	1.0	15 × 20	6	Taintor silty clay loam, 3.5%, 7.7	Soybean	—
IA4	05/29/2006	8.0	1.5	15 × 20	6	Carion loam, 4.0%, 6.5	Corn	—
IL1	05/24/2006	9.0	1.5	20 × 30	8	Plano silt loam, 3.3%, 6.7	Corn	Corn
IL2	05/23/2006	9.0	2.0	10 × 20	4	Tama silt loam, 4.5%, 6.2	Weeds[3]	Weeds[3]
IL3	06/08/2006	8.0	1.0	15 × 20	6	Silt loam, 2.8%, 6.6	Corn	—
IN1	06/07/2006	9.0	1.0	10 × 20	4	Reesville silt loam, 2.0%, 7.4	Corn	Corn
IN2	06/01/2006	9.0	1.5	10 × 20	4	Crosby silty clay loam, 2.9%, 6.4	Corn	Weeds[3]
KS	05/23/2006	9.0	1.5	10 × 20	4	Farnum loam, 1.2%, 7.9	Sorghum	Soybean
MI	05/26/2006	6.0	1.5	15 × 20	6	Nester loam, 1.9%, 6.2	Soybean	—
MO1	06/15/2006	9.0	1.0	10 × 20	4	Gorin silt loam, 4.2%, 5.8	Fescue	Fescue
MO2	05/29/2006	9.0	1.5	20 × 30	8	Hammond silt loam, 2.4%, 6.8	Corn	Soybean
NE	05/19/2006	9.0	1.0	20 × 30	8	Hastings silt loam, 3.0%, 6.6	Soybean	Soybean
NE2	05/23/2006	7.0	1.0	15 × 20	6	Silty clay loam, 2.7%, 6.5	Soybean	—
OH	05/29/2006	8.0	1.0	15 × 20	6	Crosby silt loam, 2.0%, 6.6	Soybean	—
WI	05/29/2006	10.5	1.0	15 × 20	6	Radford silt loam, 2-4%, 5.6-7.8	Soybean	—

[1] Month/day/year.
[2] Width × length.
[3] This study area was previously used for weed research.
— Information not available.

Table G-7 (continued). Field Site Planting Information, Soil Description, and Cropping History

2007 Field Study

Site	Planting date[1]	Planting rate (seeds/ft)	Planting depth (in)	Plot size (ft)[2]	Rows/plot	Soil series, organic matter, pH	Cropping history	
							2006	2005
IL	5/31/07	9.0	1.5	20 × 30	8	Plano silt loam, 3.8%, 7.1	Corn	Corn
IN	5/25/07	9.0	1.75	20 × 30	8	Crosby silt loam, 2.9%, 6.4	Corn	Soybean
MI	5/23/07	8.9	1.5	20 × 30	8	Nester loam, 2.1%, 6.5	Corn	Soybean
WI	6/11/07	9.0	1.0	20 × 30	8	Radford silt loam, 1.9%, 6.7	Winter wheat/turnips	Soybean

[1] Month/day/year.
[2] Width × length.

Appendix H. Materials, Methods, and Individual-Site Results from the Seed Dormancy and Germination Assessment of MON 87769

H.1. Materials

Seed from MON 87769, the conventional soybean control, and commercial reference soybean varieties were produced in Jefferson County, IA; Stark County, IL; and Boone County, IN in 2006.

Material Type[1]	Seed Materials Produced at Each Site		
	IA	IL	IN
Test	MON 87769	MON 87769	MON 87769
Control	A3525	A3525	A3525
Reference	A3244	AG3505	AG3505
Reference	ST3600	CST3461 (STS)	CST3461 (STS)
Reference	Stewart SB3454	ST3300	ST3300
Reference	DKB34-51	CST37002	Lewis 372

[1] The commercial references were all conventional soybean varieties with the exception of DKB34-51and AG3505, which were Roundup Ready soybean 40-3-2 varieties.

H.2. Characterization of the Materials

The presence or absence of MON 87769 in the test, control, and reference seed samples was verified by event-specific polymerase chain reaction. The results of these analyses were as expected with a few exceptions. Two out of nine control seed samples and five out of 36 reference seed samples across the three seed production sites contained between 0 and 1.84% MON 87769. In addition, one of the reference seed samples from the IN site contained between 0 and 5.65% MON 87769. Nevertheless, it was determined that the levels of MON 87769 in the control and reference seed samples from the isolated plots were low and did not negatively affect the quality of the study or interpretation of the results.

H.3. Performing Facility and Experimental Methods

Dormancy and germination evaluations were conducted at BioDiagnostics, Inc. in River Falls, WI. The principal investigator was certified to conduct seed dormancy and germination testing consistent with the standards established by the Association of Official Seed Analysts (AOSA), an internationally recognized seed testing organization (AOSA, 2000; AOSA, 2006).

Six germination chambers were used in the study and each chamber was maintained dark under one of the following six temperature regimes: constant temperature of approximately 10, 20, or 30 ^{o}C or alternating temperatures of approximately 10/20, 10/30, or 20/30 ^{o}C. The alternating temperature regimes were maintained at the lower temperature for 16 h and the higher temperature for 8 h. The temperature inside each

germination chamber was monitored and recorded throughout the duration of the study. Germination towels for MON 87769, the control, and reference materials were prepared per the facility SOPs. Each germination towel represented one replication. The types of data collected depended on the temperature regime. Each rolled germination towel in the AOSA-recommended temperature regime (i.e., 20/30 °C) was assessed periodically during the study for normal germinated, abnormal germinated, hard (viable and nonviable), dead, and firm-swollen (viable and nonviable) seed as defined by AOSA guidelines (AOSA, 2006). Each rolled germination towel in the additional temperature regimes (i.e., 10, 20, 30, 10/20, and 10/30 ^{o}C) was assessed periodically during the study for germinated, hard (viable and nonviable), dead, and firm-swollen (viable and nonviable) seed.

H.4. Statistical Analysis

Statistical analyses were performed by the Monsanto Statistics Technology Center. Analysis of variance was conducted according to a split-plot design using SAS® (SAS Release 9.1, 2002-2003). The whole-plot treatment was the seed production site arranged in a randomized complete block design. The sub-plot was the seed material arranged in a completely randomized design. The data were pooled across seed production sites and MON 87769 was compared to the control for the following germination characteristics from each temperature regime: percent germinated (categorized as percent normal germinated and percent abnormal germinated for the AOSA-recommended 20/30 °C temperature regime), percent viable hard, percent dead, and percent viable firm-swollen seed. The level of statistical significance was set at the 5% probability level ($p = 0.05$). Seed from the three production sites were tested within the same germination chamber for each temperature regime; thus, an analysis of the data pooled across sites was more appropriate than an analysis within each site. However, if an interaction between seed production site and seed material (i.e., MON 87769 and control material) had been detected, MON 87769 would have been compared to the control within sites. The means of MON 87769 and the control (across sites and within sites) and the results of the analysis of variance are reported. MON 87769 was not statistically compared to the reference varieties. The minimum and maximum means were determined from among the nine reference varieties (reference range) to provide seed germination values representative of commercial soybean grown in the three locations.

H.5. Individual Site Seed Dormancy and Germination Results

MON 87769, the control, and reference seed materials were produced at three sites to assess germination characteristics of seed grown under various environmental conditions. The individual site data in Table H-1 indicate that germination of MON 87769 and the control seed was greater than 99% across all production sites and temperature regimes. MON 87769 and control seed from the IA and IL sites had relatively higher percent abnormal germination at the 20 ^{o}C/30 ^{o}C temperature regime than MON 87769 and control seed from the IN site. The values, however, are not uncommon for soybean. A

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total of two viable hard seeds were observed in this study (i.e., one in each of the 10 °C and 10/20 °C temperature regimes for MON 87769 produced from the IL site). In soybean, it is not uncommon to observe low levels of hard seed (Mullin and Xu, 2001; Potts et al., 1978). Thus, the observance of two hard seed was not unexpected. In the analysis of the data, no seed production site × seed material interactions were detected for any characteristic in any temperature regime. Therefore, MON 87769 was comparable to the control material across sites (Section VIII.D.1., Table VIII-3).

References

Association of Official Seed Analysts. 2000. Tetrazolium Testing Handbook. AOSA, Lincoln, NE.

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Potts, H.C., J. Duangpatra, W.G. Hairston, and J.C. Delouche. 1978. Some influences of hardseededness on soybean seed quality. Crop Sci. 18:221-224

Table H-1. Germination Characteristic Mean for MON 87769 and the Conventional Control at Each Seed Production Site

Temperature Regime	Germination Category[1]	Mean %					
		IA		IL		IN	
		MON 87769	Control	MON 87769	Control	MON 87769	Control
10 °C	Germinated	100.0	100.0	99.5	100.0	100.0	100.0
	Viable Hard	0.0	0.0	0.3	0.0	0.0	0.0
	Dead	0.0	0.0	0.3	0.0	0.0	0.0
	Viable Firm-swollen	0.0	0.0	0.0	0.0	0.0	0.0
20 °C	Germinated	100.0	100.0	100.0	100.0	100.0	100.0
	Viable Hard	0.0	0.0	0.0	0.0	0.0	0.0
	Dead	0.0	0.0	0.0	0.0	0.0	0.0
	Viable Firm-swollen	0.0	0.0	0.0	0.0	0.0	0.0
30 °C	Germinated	99.8	99.3	99.8	99.8	99.5	99.8
	Viable Hard	0.0	0.0	0.0	0.0	0.0	0.0
	Dead	0.3	0.8	0.3	0.3	0.5	0.3
	Viable Firm-swollen	0.0	0.0	0.0	0.0	0.0	0.0

Note: Seed were produced in Jefferson County, IA; Stark County, IL; and Boone County, IN in 2006. No production site × seed material (MON 87769 and control material) interactions were detected; thus, MON 87769 was only compared to the conventional control (A3525) across sites.
[1] Germinated seed in the AOSA temperature regime were categorized as either normal germinated or abnormal germinated seed.
[2] In some instances, the total percentage of both MON 87769 and the control equaled 100.1% due to numerical rounding of the means.

Table H-1 (continued). Germination Characteristic Mean for MON 87769 and the Conventional Control at Each Seed Production Site

Temperature Regime[1]	Germination Category[2]	Mean %					
		IA		IL		IN	
		MON 87769	Control	MON 87769	Control	MON 87769	Control
10/20 °C	Germinated	99.5	99.5	99.8	100.0	100.0	100.0
	Viable Hard	0.0	0.0	0.3	0.0	0.0	0.0
	Dead	0.5	0.5	0.0	0.0	0.0	0.0
	Viable Firm-swollen	0.0	0.0	0.0	0.0	0.0	0.0
10/30 °C	Germinated	100.0	100.0	100.0	100.0	100.0	100.0
	Viable Hard	0.0	0.0	0.0	0.0	0.0	0.0
	Dead	0.0	0.0	0.0	0.0	0.0	0.0
	Viable Firm-swollen	0.0	0.0	0.0	0.0	0.0	0.0
20/30 °C	Normal Germinated	81.5	82.3	88.8	94.5	97.0	99.5
(AOSA)	Abnormal Germinated	18.0	17.5	11.3	5.0	2.8	0.5
	Viable Hard	0.0	0.0	0.0	0.0	0.0	0.0
	Dead	0.5	0.3	0.0	0.3	0.3	0.0
	Viable Firm-swollen	0.0	0.0	0.0	0.3	0.0	0.0

Note: Seed were produced in Jefferson County, IA; Stark County, IL; and Boone County, IN in 2006. No production site × seed material (MON 87769 and control material) interactions were detected; thus, MON 87769 was only compared to the conventional control (A3525) across sites.
[1] In the alternating temperature regimes, the lower temperature was maintained for 16 hours and the higher temperature for eight hours.
[2] Germinated seed in the AOSA temperature regime were categorized as either normal germinated or abnormal germinated seed.
[3] In some instances, the total percentage of both MON 87769 and the control equaled 100.1% due to numerical rounding of the means.

Appendix I. Methods and Results from the Assessment of MON 87769 for Plant Interactions with Abiotic Stressors, Diseases, and Arthropods under Field Conditions in 2006 and 2007

I.1. Abiotic stress response, disease damage, and arthropod damage

Plant response to abiotic stressors, disease damage, and arthropod damage were assessed at each of the 17 field sites in 2006 and each of the four field sites in 2007. Three abiotic stressors, three diseases, and three arthropod pests were evaluated at approximately the V2 – V4, R1 – R2, R3 – R5, and R6 – R8 growth stages. The observed abiotic stressors, diseases, and arthropod pests were "natural" (i.e., no artificial infestation or interference was used) and, therefore, often varied between observations at a site and between sites. During each observation, each plot was evaluated for the severity of injury caused by the abiotic stressor, disease, or arthropod pest. Plots were rated numerically using a continuous rating scale of increasing symptomology but the results are reported categorically (none, slight, moderate, or severe). Results from the 2006 and 2007 evaluations are summarized in Section VIII.D.2.2, while the data are presented in Tables I-1 through I-6.

The following 0 – 9 scale was used to rate plant response to abiotic stressors and disease damage in the 2006 and 2007 field trials and to rate arthropod damage in the 2006 field trials.

Rating	Extent of plant damage
0	none (no symptoms observed)
1 – 3	slight (symptoms not damaging to plant development)
4 – 6	moderate (intermediate between slight and severe)
7 – 9	severe (symptoms damaging to plant development)

In the 2007 field trials, arthropod damage was assessed on the upper four nodes of ten representative plants per plot using one of the following 0 – 5 rating scales specific to the arthrodpod being evaluated:

Defoliating arthropods (e.g., corn earworm, bean leaf beetle, Japanese beetle, soybean looper)		
Rating	**Defoliation (%)**	**Extent of plant damage**
0	none	none (no symptoms observed)
1	1 – 20 %	slight (symptoms not damaging to plant development)
2	21 – 40%	moderate (intermediate between slight and severe)
3	41 – 60%	
4	61 – 80%	severe (symptoms damaging to plant development)
5	> 80%	

Pod feeding arthropods (e.g., corn earworm, bean leaf beetle, stink bugs, Lygus bugs on reproductive plant parts)		
Rating	**Damaged pods (%)**	**Extent of plant damage**
0	none	none (no symptoms observed)
1	1 – 20 %	slight (symptoms not damaging to plant development)
2	21 – 40%	moderate (intermediate between slight and severe)
3	41 – 60%	
4	61 – 80%	severe (symptoms damaging to plant development)
5	> 80%	

Leafhoppers (e.g., potato leafhopper)		
Rating	**Foliar damage (%)**	**Extent of plant damage**
0	none	none (no symptoms observed)
1	1 – 50% of foliage with leaf yellowing; no leaf puckering or leaf margin necrosis	slight (symptoms not damaging to plant development)
2	1 – 50% of foliage with leaf yellowing, leaf puckering and/or leaf margin necrosis	moderate (intermediate between slight and severe)
3	> 50% of foliage with leaf yellowing; no leaf puckering or leaf margin necrosis	
4	> 50% of foliage with leaf yellowing, leaf puckering, and/ or leaf margin necrosis	severe (symptoms damaging to plant development)
5	> 50% of foliage with necrotic leaves (leaves dead due to leafhopper damage)	

Aphids (e.g., soybean aphid)		
Rating	**Aphids present**	**Extent of plant damage**
0	none	none (no symptoms observed)
1	1 – 100 aphids per plant; no leaf puckering	slight (symptoms not damaging to plant development)
2	101 – 250 aphids per plant; no leaf puckering	moderate (intermediate between slight and severe)
3	≥ 250 aphids per plant with leaf puckering	
4	≥ 250 aphids per plant with leaf puckering and leaf yellowing and/or necrosis	severe (symptoms damaging to plant development)
5	≥ 250 aphids per plant with plant stunting	

I.2. Arthropod abundance

The abundance of specific pest and beneficial arthropods were quantified at the IA1, IL1, MO2, and NE sites in the 2006 field trials and the IL, IN, MI, and WI sites in the 2007 field trials. At these sites, arthropods were collected from the plants in each replicate plot three times during the growing season at approximately the R1 – R2, R3 – R5, and R6 – R8 growth stages using a beat sheet sampling method (Kogan and Pitre, 1980). The beat sheet was a 36 × 42 inch vinyl sheet that was spread between the plants of two adjacent rows. Plants were shaken vigorously along the length of each side of the beat sheet to dislodge arthropods from the plants. A total of four sub-samples were collected in this way from each plot. Specifically, two sub-samples were collected from Rows # 5 and 6 of each plot (sub-samples 1 and 2) and two sub-samples were collected from Rows # 6 and 7 of each plot (sub-samples 3 and 4). The sub-samples collected from the same row were at least 3 ft from the edge of each plot. The four sub-samples were combined into one container that was either filled with alcohol or was placed on dry ice to preserve the integrity of the collection. The samples were then shipped to the laboratory for arthropod identification and enumeration.

A maximum of the six most abundant pest and six most abundant beneficial arthropods were determined for each collection from each individual site. These arthropods were then enumerated across all samples (i.e., one sample per plot) from a given collection at each individual site. The arthropods assessed often varied between collections from a site and between sites due to differences in temporal activity and geographical distribution of the taxa. Results from the 2006 and 2007 arthropod abundance evaluations are summarized in Section VIII.D.2.2, while the data are presented in Tables I-7 through I-10.

Table I-1. Abiotic Stressor Evaluations Using an Observational Severity Scale for MON 87769 and the Conventional Control in the 2006 Field Trials

Abiotic stressor	Number of observations across the 17 sites [1]	Number of observations where no differences were detected
Total	**162**	**161**
Cold stress	7	7
Compaction	7	7
Drought	40	40
Excess moisture[2]	18	17*
Frost	3	3
Hail	16	16
Heat	28	28
Mineral toxicity	1	1
Nutrient deficiency	7	7
Wind	35	35

* Indicates an observed difference between MON 87769 and the conventional control (A3525) for symptom severity caused by excess moisture at the WI site (slight vs. none; Observation 1). Data were not subjected to statistical analysis.
Note: The experimental design was a randomized complete block with three replications. Observational data were collected at four crop development stages: Observation 1 = V2-V4, Observation 2 = R1-R2, Observation 3 = R3-R5 and Observation 4 = R6-R8.
[1] Data were collected from the following field sites: Jefferson County, IA; Benton County, IA; Jefferson County, IA; Guthrie County, IA; Stark County, IL; Warren County, IL; Clinton County, IL; Parke County, IN; Boone County, IN; Pawnee County, KS; Ottawa County, MI; Macon County, MO; Lincoln County, MO; York County, NE (2 sites); Fayette County, OH; Walworth County, WI.
[2] Including wet soil and flooding.

Table I-2. Abiotic Stressor Evaluations Using an Observational Severity Scale for MON 87769 and the Conventional Control in the 2007 Field Trials

Abiotic stressor	Number of observations across the four sites [1]	Number of observations where no differences were detected
Total	**34**	**34**
Cold stress	2	2
Compaction	2	2
Drought	8	8
Flood	3	3
Frost	4	4
Hail	7	7
Heat	4	4
Wind	4	4

Note: The experimental design was a randomized complete block with three replications. Observational data were collected at four crop development stages: Observation 1 = V2-V4, Observation 2 = R1-R2, Observation 3 = R3-R5 and Observation 4 = R6-R8. No differences were observed between MON 87769 and the conventional control (A3525) for plant response to any of the assessed abiotic stressors. Data were not subjected to statistical analysis.

[1] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.

Table I-3. Disease Damage Evaluations Using an Observational Severity Scale for MON 87769 and the Conventional Control in the 2006 Field Trials

Disease	Number of observations across the 17 sites [1]	Number of observations where no differences were detected
Total	**208**	**207**
Alternaria leaf spot	11	11
Anthracnose	11	11
Asian rust	4	4
Bacterial blight	13	13
Bacterial pustule	4	4
Brown spot	3	3
Brown stem rot	11	11
Cercospora leaf blight[2]	4	4
Charcoal rot	3	3
Downy mildew	15	15
Frogeye leaf spot	14	13*
Fusarium	3	3
Phytophthora[3]	17	17
Powdery mildew	6	6
Pythium	7	7
Rhizoctonia	5	5
Sclerotinia	4	4
Septoria[4]	28	28
Soybean mosaic virus	10	10
Soybean rust	13	13
Stem canker	1	1
Sudden death	11	11
White mold	10	10

* Indicates an observed difference between MON 87769 and the conventional control (A3525) for disease susceptibility to frogeye leaf spot at the IA4 site (none vs. slight; Observation 4). Data were not subjected to statistical analysis.

Note: The experimental design was a randomized complete block with three replications. Observational data were collected at four crop development stages: Observation 1 = V2-V4, Observation 2 = R1-R2, Observation 3 = R3-R5, and Observation 4 = R6-R8.

[1] Data were collected from the following field sites: Jefferson County, IA; Benton County, IA; Jefferson County, IA; Guthrie County, IA; Stark County, IL; Warren County, IL; Clinton County, IL; Parke County, IN; Boone County, IN; Pawnee County, KS; Ottawa County, MI; Macon County, MO; Lincoln County, MO; York County, NE (2 sites); Fayette County, OH; Walworth County, WI.

[2] Including Cercospora leaf disease.

[3] Including Phytophthora root rot.

[4] Including Septoria brown spot and Septoria leaf spot.

Table I-4. Disease Damage Evaluations Using an Observational Severity Scale for MON 87769 and the Conventional Control in the 2007 Field Trials

Disease	Number of observations across the four sites [1]	Number of observations where no differences were detected
Total	**48**	**48**
Anthracnose	2	2
Asian rust	1	1
Bacterial blight	1	1
Brown stem rot	2	2
Cercospora leaf blight[2]	1	1
Charcoal rot	1	1
Downy mildew	3	3
Frogeye leaf spot	2	2
Phytophthora[3]	1	1
Powdery mildew	5	5
Pythium	2	2
Rhizoctonia	3	3
Sclerotinia	1	1
Septoria[4]	4	4
Soybean cyst nematode	1	1
Soybean mosaic virus	1	1
Soybean rust	5	5
Sudden death	5	5
White mold	7	7

Note: The experimental design was a randomized complete block with three replications. Observational data were collected at four crop development stages: Observation 1 = V2-V4, Observation 2 = R1-R2, Observation 3 = R3-R5 and Observation 4 = R6-R8. No differences were observed between MON 87769 and the conventional control (A3525) for plant damage caused by any of the assessed diseases. Data were not subjected to statistical analysis.

[1] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.

[2] Including Cercospora leaf disease.

[3] Including Phytophthora root rot.

[4] Including Septoria brown spot and Septoria leaf spot.

Table I-5. Arthropod Damage Evaluated Using an Observational Severity Scale for MON 87769 and the Conventional Control in the 2006 Field Trials

Arthropod	Number of observations across the 17 sites [1]	Number of observations where no differences were detected
Total	**203**	**203**
Aphid[2]	35	35
Bean leaf beetle	53	53
Blister beetle	2	2
Corn rootworm	2	2
Flea beetle	1	1
Grasshopper	34	34
Green cloverworm	16	16
Japanese beetle	22	22
Leafhopper[3]	15	15
Seed corn maggot	1	1
Soybean stem borer	3	3
Stink bug[4]	10	10
Thistle caterpillar	2	2
Thrips	4	4
Velvetbean caterpillar	1	1
Wireworm	1	1
Woolybear caterpillar	1	1

Note: The experimental design was a randomized complete block with three replications. Observational data were collected at four crop development stages: Observation 1 = V2-V4, Observation 2 = R1-R2, Observation 3 = R3-R5 and Observation 4 = R6-R8. No differences were observed between MON 87769 and the conventional control (A3525) for plant damage caused by any of the assessed arthropods. Data were not subjected to statistical analysis.

[1] Data were collected from the following field sites: Jefferson County, IA; Benton County, IA; Jefferson County, IA; Guthrie County, IA; Stark County, IL; Warren County, IL; Clinton County, IL; Parke County, IN; Boone County, IN; Pawnee County, KS; Ottawa County, MI; Macon County, MO; Lincoln County, MO; York County, NE (2 sites); Fayette County, OH; Walworth County, WI.

[2] Including soybean aphid.

[3] Including potato leafhopper.

[4] Including green and brown stink bugs.

Table I-6. Arthropod Damage Evaluated Using an Observational Severity Scale for MON 87769 and the Conventional Control in the 2007 Field Trials

Arthropod	Number of observations across the four sites [1]	Number of observations where no differences were detected
Total	**48**	**48**
Aphid[2]	10	10
Bean leaf beetle	8	8
Corn rootworm	1	1
Grasshopper	7	7
Green cloverworm	3	3
Japanese beetle	7	7
Leafhopper[3]	2	2
Leafroller	3	3
Mexican bean beetle	1	1
Spidermite	1	1
Stink bug[4]	2	2
Thrips	1	1
White fly	1	1
Wireworm	1	1

Note: The experimental design was a randomized complete block with three replications. Observational data were collected at four crop development stages: Observation 1 = V2-V4, Observation 2 = R1-R2, Observation 3 = R3-R5 and Observation 4 = R6-R8. No differences were observed between MON 87769 and the conventional control (A3525) for plant damage caused by any of the assessed arthropods. Data were not subjected to statistical analysis.

[1] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.

[2] Including soybean aphid.

[3] Including potato leafhopper.

[4] Including green and brown stink bugs.

Table I-7. Abundance of Pest Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2006

		Abundance of Pest Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Aphid	IA1	2.0	0.7	0.0 – 1.7	—	—	—	116.7	96.7	47.3 – 103.0
	IL1	8.7	13.3	2.0 – 10.3	39.7	25.3	16.7 – 52.0	62.7	63.3	38.3 – 64.0
	MO2	1.3	15.3	0.0 – 1.7	0.0	1.7	0.0 – 2.7	45.3	65.0	55.7 – 82.7
	NE	—	—	—	12.7	3.5	2.5 – 10.0	20.7	20.0	4.5 – 13.3
Bean leaf beetle	IA1	11.3	10.3	11.3 – 25.0	8.0	4.3	4.3 – 7.3	3.3	5.3	2.3 – 4.7
	IL1	13.7	12.3	10.0 – 16.3	27.3	17.7	26.0 – 31.3	22.3	14.0	16.3 – 22.0
	MO2	6.3	7.7	5.7 – 7.3	3.3	2.0	3.0 – 4.7	2.0	3.0	2.0 – 5.0
	NE	0.3	1.0	0.0 – 1.0	1.7	3.0	1.3 – 3.0	26.3	24.0	6.5 – 26.0
Garden fleahopper	IA1	—	—	—	—	—	—	2.0	1.3	0.7 – 2.0
	IL1	—	—	—	0.3	0.0	0.3 – 2.0	0.0[†]	0.0	0.0 – 0.0
	MO2	—	—	—	1.0	1.0	0.7 – 1.0	0.0	0.0	0.3 – 0.3
	NE	—	—	—	0.0	0.0	0.0 – 0.5	2.3	1.5	0.5 – 0.7
Green cloverworm	IA1	0.0	0.0	0.0 – 0.3	13.3	11.0	7.3 – 14.0	1.7	1.7	0.0 – 2.3
	IL1	0.7	0.0	0.0 – 1.3	31.3	22.0	19.3 – 31.7	1.7*	0.3	1.3 – 2.0
	MO2	5.0	3.0	2.0 – 4.0	4.3	4.7	2.0 – 5.7	10.7	9.3	9.3 – 13.0
	NE	0.0	0.0	0.0 – 0.3	0.3	0.0	0.0 – 0.5	1.3	0.0	0.0 – 0.5
Leafhopper	IA1	33.7	29.3	21.0 – 30.3	0.7	0.7	0.0 – 0.7	3.3	1.3	1.0 – 2.0
	IL1	55.0	94.7	58.3 – 86.3	0.3	0.7	1.0 – 2.7	0.3	0.0	0.0 – 0.7
	MO2	8.3	6.3	7.3 – 7.7	2.0*	0.3	0.3 – 2.0	0.0	0.3	0.0 – 0.7
	NE	12.3	7.0	4.0 – 8.0	8.7	1.0	2.3 – 7.7	0.7	1.0	0.0 – 1.0

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] IA1 = Jefferson County, IA; IL1 = Stark County, IL; MO2 = Lincoln County, MO; NE = York County, NE.
[†] No statistical comparisons were made due to lack of variability in the data.
— Arthropod was not evaluated.

Table I-7 (continued). Abundance of Pest Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2006

		Abundance of Pest Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Looper	IL1	0.0	0.0	0.0 – 0.7	—	—	—	—	—	—
	NE	—	—	—	0.0	0.0	0.0 – 0.3	0.0†	0.0	0.0 – 0.0
Stink bug	IA1	0.0	0.3	0.0 – 0.7	1.0	1.3	0.3 – 1.7	2.0	5.3	0.3 – 3.7
	IL1	0.0	0.3	0.0 – 0.3	1.0	2.0	2.0 – 6.0	0.7	1.0	0.3 – 1.3
	MO2	0.7	0.0	0.0 – 2.3	1.3	1.0	0.0 – 2.3	3.7	6.3	1.7 – 6.3
	NE	0.7	0.5	0.3 – 0.5	3.7	0.5	0.3 – 1.3	4.3	2.0	2.3 – 5.0
Tarnished plant bug	IA1	0.0	0.0	0.0 – 0.7	0.7	1.7	1.7 – 3.7	2.0	3.0	0.7 – 3.0
	IL1	1.0	0.0	0.0 – 0.7	6.3*	2.3	2.7 – 4.7	0.3	0.3	0.3 – 1.0
	MO2	0.0	1.3	0.3 – 4.0	0.0	0.0	0.0 – 1.0	0.0†	0.0	0.0 – 0.0
	NE	0.0†	0.0	0.0 – 0.0	0.7	1.5	0.0 – 1.3	0.0	0.0	0.0 – 1.0
Velvetbean caterpillar	IA1	0.0	0.0	0.0 – 0.3	—	—	—	1.3	0.3	0.0 – 0.7
	IL1	0.3	0.0	0.0 – 1.3	—	—	—	—	—	—
	MO2	—	—	—	—	—	—	0.0†	0.0	0.0 – 0.0

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] IA1 = Jefferson County, IA; IL1 = Stark County, IL; MO2 = Lincoln County, MO; NE = York County, NE.
† No statistical comparisons were made due to lack of variability in the data.
— Arthropod was not evaluated.

Table I-8. Abundance of Beneficial Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2006

		Abundance of Beneficial Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Big-eyed Bug	IA1	0.0	0.0	0.0 – 0.7	0.3	0.3	0.0 – 0.3	—	—	—
	MO2	8.0	7.0	3.0 – 10.3	24.3	12.7	13.3 – 18.7	5.7	6.0	7.0 – 12.7
	NE	1.3	0.0	0.0 – 0.5	—	—	—	—	—	—
Ladybird beetle	IA1	0.0	0.0	0.0 – 0.7	—	—	—	0.0	0.3	0.0 – 0.3
	IL1	0.3	1.3	0.3 – 1.0	0.0	0.3	0.0 – 0.3	4.3	7.7	1.3 – 7.7
	MO2	0.3	0.0	0.0 – 1.3	—	—	—	0.7	0.7	1.0 – 1.7
	NE	—	—	—	0.7	0.5	0.0 – 2.0	0.3	0.5	0.0 – 0.5
Micro parasitic hymenoptera	IA1	—	—	—	2.0	0.7	0.7 – 1.0	4.0	5.3	2.7 – 8.7
	IL1	1.3	0.7	0.0 – 0.7	3.7	3.0	1.0 – 2.7	0.0	0.0	0.0 – 0.3
	MO2	—	—	—	0.0	1.0	0.0 – 1.7	4.3	3.7	4.7 – 14.3
	NE	0.0	0.5	0.0 – 0.7	1.7	0.0	0.7 – 3.3	0.7	0.0	0.7 – 3.0
Nabis	IA1	0.7	1.3	1.3 – 2.3	2.3	3.3	2.7 – 5.0	8.7	12.0	12.3 – 19.7
	IL1	0.3	0.0	0.7 – 3.3	17.3*	6.3	11.3 – 21.7	1.3*	0.0	0.0 – 1.3
	MO2	2.7	3.0	0.7 – 4.3	2.0	4.0	2.0 – 7.7	1.7	0.3	1.0 – 5.0
	NE	2.0	1.0	0.7 – 3.0	8.7	6.5	6.7 – 9.5	1.7	1.0	2.0 – 5.0
Orius	IA1	2.7	3.0	1.3 – 3.7	7.3	5.3	4.3 – 7.7	16.0	16.3	16.7 – 29.0
	IL1	8.7	13.3	11.0 – 15.3	52.7*	33.0	42.0 – 62.3	8.7	4.0	3.7 – 6.0
	MO2	2.3	0.3	0.0 – 0.7	0.3	0.0	0.3 – 2.0	—	—	—
	NE	0.0	0.0	0.0 – 0.7	5.0	3.0	1.0 – 5.0	4.0	4.0	1.5 – 3.3

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] IA1 = Jefferson County, IA; IL1 = Stark County, IL; MO2 = Lincoln County, MO; NE = York County, NE.
— Arthropod was not evaluated.

Table I-8 (continued). Abundance of Beneficial Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2006

		Abundance of Beneficial Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Spider	IA1	1.0	0.7	0.7 – 2.0	4.0	2.3	1.7 – 3.7	12.7	16.0	17.0 – 17.3
	IL1	0.7	1.3	1.0 – 2.0	8.7	6.3	7.7 – 9.7	4.0	1.3	1.3 – 4.0
	MO2	3.7	4.7	4.0 – 6.7	11.0	7.0	3.3 – 15.7	8.3	5.3	8.0 – 12.0
	NE	4.7	2.0	1.0 – 2.7	4.3	1.0	2.7 – 5.3	22.7	15.0	7.3 – 23.0

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.

[2] IA1 = Jefferson County, IA; IL1 = Stark County, IL; MO2 = Lincoln County, MO; NE = York County, NE.

Table I-9. Abundance of Pest Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2007

		Abundance of Pest Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Aphid	IL	7.0	11.0	9.7– 23.0	—	—	—	0.0	0.7	0.0 – 1.0
	MI	50.3	14.7	12.0– 20.7	1540.0	3050.0	650.0 – 1520.0	—	—	—
	WI	2950.0	1383.3	983.3 – 4283.3	—	—	—	—	—	—
Bean leaf beetle	IL	11.0	12.0	6.3 – 16.0	6.3	4.7	2.3 – 8.0	24.7	25.3	2.0 – 20.0
	IN	2.0	2.0	0.7 – 2.0	0.3	0.3	0.7 – 1.3	23.7	23.0	20.3 – 40.7
	MI	0.3	0.7	0.0 – 0.7	0.0†	0.0	0.0 – 0.0	1.3	0.3	0.0 – 2.7
	WI	2.3	3.0	2.0 – 2.7	0.0	0.7	0.0 – 0.7	6.0	3.3	5.0 – 8.0
Corn flea beetle	IN	—	—	—	—	—	—	0.3	1.0	0.0 – 2.0
Garden fleahopper	WI	—	—	—	10.3	3.7	7.0 – 12.3	2.7	1.3	0.3 – 3.7
Green cloverworm	IL	0.0	0.0	0.0 – 0.7	1.3	1.3	0.7 – 1.7	1.0	0.3	0.0 – 0.3
	IN	0.0†	0.0	0.0 – 0.0	3.3	6.7	3.0 – 7.0	0.7	0.7	0.0 – 1.7
	MI	0.0	0.3	0.0 – 0.3	2.7	3.0	2.3 – 3.0	0.0	0.3	0.0 – 2.3
	WI	14.3	12.3	6.3 – 9.0	3.0	1.0	1.7 – 4.3	0.0*	1.0	0.0 – 0.3
Japanese beetle	IN	0.7	0.7	0.0 – 1.3	2.7	2.7	0.3 – 5.3	—	—	—
	MI	1.7	2.0	3.0 – 4.3	0.3	0.3	0.3 – 3.0	—	—	—
Leafhopper	IL	57.0	70.0	39.0 – 119.0	—	—	—	—	—	—
	IN	2.7	0.7	0.3 – 2.3	5.7	7.3	3.7 – 14.0	—	—	—
	MI	18.0	9.3	7.7 – 21.3	5.7*	0.3	2.3 – 10.0	—	—	—

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.
† No statistical comparisons were made due to lack of variability in the data.
— Arthropod was not evaluated.

Table I-9 (continued). Abundance of Pest Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2007

		Abundance of Pest Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Stink bug	IL	0.0	0.3	0.0 – 0.7	2.3	2.0	1.3 – 3.7	2.3	2.7	1.0 – 2.0
	IN	0.0[†]	0.0	0.0 – 0.0	0.0	1.3	0.0 – 2.0	5.0	2.0	2.0 – 4.0
	MI	3.0*	0.0	0.0 – 2.7	3.0	1.0	1.3 – 2.0	11.3	11.0	7.3 – 12.7
	WI	0.0	0.3	0.0 – 0.7	0.7	0.3	0.0 – 0.7	1.7	1.3	0.0 – 1.3
Thrips	IL	105.7	139.3	135.0 – 174.3	—	—	—	—	—	—
Tarnished plant bug	IN	—	—	—	—	—	—	0.0	0.7	0.0 – 0.7
	MI	—	—	—	—	—	—	11.0	10.0	4.7 – 13.7
	WI	4.7	4.0	1.7 – 4.7	3.7	3.3	2.0 – 3.7	0.7	0.7	0.0 – 1.0
Western corn rootworm	IN	1.3	1.7	1.0 – 2.3	—	—	—	—	—	—

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.
[†] No statistical comparisons were made due to lack of variability in the data.
— Arthropod was not evaluated.

Table I-10. Abundance of Beneficial Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2007

		Abundance of Beneficial Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Carabid beetle	IL	—	—	—	—	—	—	1.0	1.0	0.0 – 4.0
Lacewing	IL	0.3	0.3	0.0 – 1.0	0.0	0.0	0.3 – 3.3	0.0	0.3	0.0 – 0.7
	IN	—	—	—	0.0	0.0	0.0 – 0.7	—	—	—
	MI	0.3	1.0	0.0 – 1.0	3.0	3.0	2.0 – 7.7	2.3	4.7	1.0 – 5.7
	WI	5.3	3.0	2.0 – 4.7	0.7	1.3	0.3 – 2.0	—	—	—
Ladybird beetle	IL	—	—	—	0.0	0.3	0.0 – 3.7	2.0	0.7	0.3 – 1.0
	IN	—	—	—	0.0	0.3	0.0 – 1.7	0.0	0.0	0.0 – 0.7
	MI	0.0	0.0	0.0 – 0.7	7.3	3.7	2.3 – 5.0	32.7*	8.7	11.3 – 15.3
	WI	24.3	39.0	25.0 – 41.7	2.0	2.0	2.0 – 4.0	1.0	1.0	0.7 – 3.0
Micro-parasitic hymenoptera	IL	0.3	1.3	0.0 – 0.7	—	—	—	—	—	—
	MI	0.7	1.3	0.3 – 1.3	2.0	1.3	0.3 – 2.0	1.0	1.0	0.3 – 3.0
	WI	5.3	7.0	2.3 – 4.3	2.0	3.0	0.7 – 2.3	3.7	1.7	2.7 – 8.3
Nabis	IL	0.7	1.3	0.7 – 1.7	0.7	0.7	0.0 – 2.3	0.7	1.0	0.3 – 2.0
	IN	0.0	0.0	0.0 – 1.0	0.7	1.0	0.7 – 1.3	2.7	0.7	1.0 – 3.7
	MI	0.0*	1.7	0.0 – 1.0	3.7	2.0	1.7 – 5.7	9.3	9.0	3.0 – 14.0
	WI	3.3	4.7	2.0 – 5.7	0.7	3.3	2.3 – 7.3	6.7	4.0	5.7 – 9.7
Orius	IL	16.3	15.3	14.3 – 29.3	14.7	16.0	14.3 – 42.3	1.3	8.7	3.7 – 21.3
	IN	0.0	1.3	0.0 – 3.0	3.7	5.7	3.0 – 10.0	4.7	4.7	2.3 – 16.7
	MI	1.0	1.7	0.3 – 3.0	36.7	15.3	15.3 – 29.3	63.0	73.0	51.3 – 79.7
	WI	83.0	89.7	38.7 – 78.7	66.3	70.3	50.7 – 92.7	62.0	29.7	39.7 – 109.7

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.
— Arthropod was not evaluated.

Table I-10 (continued). Abundance of Beneficial Arthropods in Beat Sheet Samples Collected from MON 87769, the Conventional Control, and the Reference Varieties in U.S. Field Trials in 2007

		Abundance of Beneficial Arthropod[1]								
		Collection 1			Collection 2			Collection 3		
Arthropod	Sites[2]	Test	Control	Reference Range	Test	Control	Reference Range	Test	Control	Reference Range
Spined soldier bug	IL	0.0	0.3	0.0 – 0.7	—	—	—	—	—	—
	IN	—	—	—	—	—	—	0.7	0.3	0.0 – 0.3
Spider	IL	1.7	2.7	1.0 – 2.7	1.3	1.0	0.0 – 1.0	0.7	0.7	0.0 – 1.3
	IN	0.7	0.3	0.3 – 1.3	0.7	2.3	0.3 – 1.0	0.0	0.0	0.7 – 1.7
	MI	0.0[†]	0.0	0.0 – 0.0	0.3	1.3	0.3 – 1.3	0.7	0.7	0.0 – 1.7
	WI	9.0	8.3	3.3 – 5.3	9.0	6.7	1.7 – 8.0	4.3	6.3	4.7 – 11.3

* Indicates a statistically significant difference ($p \leq 0.05$) between the test (MON 87769) and the conventional control (A3525).
Collections 1, 2, and 3 occurred at the R1 – R2, R3 – R5, and R6 – R8 growth stages, respectively.
[1] Test and control values represent mean number of arthropods collected across three replications.
[2] Data were collected from the following field sites: Stark County, IL; Boone County, IN; Ottawa County, MI; Walworth County, WI.
[†] No statistical comparisons were made due to lack of variability in the data.
— Arthropod was not evaluated.

References

Kogan, M., and H.N. Pitre. 1980. General Sampling Methods for Above-Ground Populations of Soybean Arthropods. Pages 34-37 in Sampling Methods in Soybean Entomology. M. Kogan and D.C. Herzog (eds.). Springer-Verlag, New York, United States of America.

Appendix J. Materials and Methods from the Pollen Viability and Morphology Assessment of MON 87769

J.1. Materials and Methods

Plant Production

Plants of MON 87769, the conventional soybean control, and four commercial reference soybean varieties were grown under similar agronomic conditions in a randomized complete block design with three replications at a field site in Lincoln County, Missouri. Each plot consisted of eight 30 ft long rows spaced approximately 30 inches apart.

Material Type	Material Name[1]
Test	MON 87769
Control	A3525
Reference	Pioneer 93B82
Reference	Lewis 372
Reference	AG3505
Reference	CST3461

[1]The commercial references were all conventional soybean varieties with the exception of AG3505, which was a Roundup Ready soybean 40-3-2 variety.

Flower Collection

Twenty flowers were collected from the fourth row of each plot. One flower from the bottom of the plant, two flowers from the middle, and one flower from the top were collected from each of five representative plants per plot. Flowers were collected from all plots of the first replication on July 18, 2006 and from all plots of the second and third replication on July 19, 2006. Upon collection, all flowers from a plot were placed into a clean, uniquely labeled container. The containers were kept on wet ice for less than eight hours until the pollen was extracted and stained.

Pollen Sample Preparation

Pollen samples were prepared in a laboratory. Clean microscope slides were labeled with the plot number. A circle of approximately 1 cm diameter was drawn in the center of the slide with a pap hydrophobic barrier pen. Tweezers were used to open each of the collected flowers from a plot and brush the pollen into the circle on the slide. The utensil was cleaned between extractions of the pollen from the flowers of another plot. Approximately 20 µl of Alexander 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. All pollen grains from a replicate sample were stained and evaluated on the same day. Viability data were collected from a minimum of approximately 75-100 pollen grains per plot.

J.2. Data Collection

All pollen samples were viewed under an Olympus Provis AX70 light/fluorescence microscope equipped with an Olympus DP70 digital color camera. The microscope and camera were connected to a computer running Microsoft Windows 2000 Professional (© 1981-1999, Microsoft Corp.) and installed with associated camera software [DP Controller v1.2.1.108 and DP Manager v1.2.1.107(© 2001-2003, Olympus Optical Co., Ltd.)] and imaging software [Image-Pro Plus v4.5.1.27 (© 1993-2002, Media Cybernetics, Inc.)].

Pollen Viability
Pollen viability was evaluated for each sample by counting the number of viable and non-viable pollen grains. When exposed to the stain solution, viable pollen grains were stained red to purple due to the presence of living cytoplasm. Non-viable pollen grains were stained blue to green and may have appeared round to collapsed in shape, depending on the degree of hydration. At least 75 individual pollen grains per plot were evaluated for viability from a random field of view under the microscope. Dense clusters of pollen or pollen grains adhering to flower parts were not counted because they may not have absorbed the stain solution uniformly.

Pollen Diameter
Micrographs (400X magnification for replication 1 and 500X magnification for replications 2 and 3) of ten representative pollen grains from each plot were taken 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 plot was calculated from the 20 total measurements (i.e., two axes per each of ten representative pollen grains from each plot).

General Pollen Morphology
General pollen morphology was observed from micrographs of MON 87769, the control, and reference materials that were also used for pollen diameter measurements.

J.3. Statistical Analysis

An analysis of variance was conducted by the Monsanto Statistics Technology Center using SAS (SAS Release 9.1, 2002-2003) according to a randomized complete block design with three replications. Statistical significance was set at the 5% probability level ($p = 0.05$). MON 87769 was compared to the control for percent viable pollen and pollen diameter. No statistical comparisons were made between MON 87769 and the reference varieties. A reference range for each measured characteristic was determined from the minimum and maximum mean values from among the four reference soybean varieties. General pollen morphology was qualitative; therefore, no statistical analysis was conducted on these observations. A summary of the results is provided in Section VIII.D.3.

Reference

Alexander, M.P. 1980. A versatile stain for pollen fungi, yeast and bacteria. Stain Technology. 55(1): 13-18.

Appendix K. Materials and Methods for the Assessment of MON 87769 for Plant Interactions with Nitrogen-fixing Soil Symbionts

K.1. Initial Study

K.1.1. Materials

Seed of MON 87769, the conventional soybean control (variety A3525), and three commercial reference soybean varieties were used to assess the symbiotic association between *Bradyrhizobium japonicum* and MON 87769 compared to that of conventional soybean.

Material Type	Seed Materials Tested	Phenotype
Test	MON 87769	SDA trait
Control	A3525	Conventional
Reference	Stewart SB3454	Conventional
Reference	ST3300-0	Conventional
Reference	CST37002	Conventional

K.1.2. Characterization of Materials

The presence or absence of MON 87769 in the test and control starting seed was verified by event-specific polymerase chain reaction. The results of these analyses were as expected. The identity of the reference substances was maintained via chain-of-custody documentation and seed bag labels.

K.1.3. Experimental Methods

Plants of each material were grown in a greenhouse with a 14-hour photoperiod, a target day-time temperature of 27 ^{o}C, and a target night-time temperature of 22 ^{o}C. At planting, eight replications (for each of two sampling periods) of 10-inch pots were filled with a nitrogen-deficient potting medium composed of primarily peat, vermiculite, and perlite. Four seeds of MON 87769, the control, or reference variety were inoculated with approximately 1 x 10^8 cells of *B. japonicum* in phosphate-buffered saline and planted in each pot. An additional eight replications (for each of two sampling periods) of pots were planted with uninoculated control material seed and included in the study to validate the test system. Pots were arranged in eight replicated blocks in the greenhouse for 4- and 6-week sampling periods using a randomized strip-plot design.

One week after emergence, all pots were thinned to one seedling per pot and re-inoculated with approximately 1 x 10^8 cells of *B. japonicum*, with the exception of the uninoculated control pots. A solution of nitrogen-free nutrient solution (target of 250

mL) was added weekly to each pot after plant emergence. The growth stage of each plant was evaluated and documented in the study notebook at least once per week.

Four and six weeks after thinning, plants were excised at the surface of the potting medium and shoot and root materials were removed from the 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 nodules were later removed by hand from the roots of each plant and placed in weighing tins. Nodules from each plant were enumerated and the fresh weight determined. The nodules were dried for a minimum of 48 hours at approximately 65 °C to determine dry weight. The fresh weight of the remaining root and shoot mass was determined for each plant. Root and shoot material from each plant was dried for a minimum of 48 hours at approximately 65 °C to determine dry weight. After drying, the shoot tissue was ground with a mortar and pestle prior to shipping to Agvise Laboratories (Northwood, ND) for total nitrogen analysis. Shoot total nitrogen was determined by combustion using a nitrogen analyzer.

K.1.4. Statistical Methods

An analysis of variance was conducted using a randomized strip-plot design with eight replications for each test, control, and reference material at each sampling period. Data were analyzed using SAS (SAS Release 9.1, 2002-2003), and statistical significance was set at the 5% probability level ($p = 0.05$). MON 87769 was compared to the conventional control for five measurement endpoints from each of the two sampling periods (4- and 6-week): nodule number, nodule dry wt (g), shoot dry wt (g), root dry wt (g), and shoot total nitrogen (percent dry weight). The commercial reference varieties provided a range of values for each measured endpoint that are representative of commercial soybean varieties. A summary of the results is provided in Section VIII.D.4. The nodule number and shoot total nitrogen from the inoculated and uninoculated control plants were also compared to each other and demonstrated that in the absence of the nitrogen-fixing symbiont, plant growth was limited in the nitrogen-deficient test system (Table K-1).

Table K-1. Comparison of Uninoculated Conventional Control (A3525-UN) to the Inoculated Conventional Control (A3525) for Nodule Number and Shoot Total Nitrogen

Measurement Endpoint	Sampling Period	A3525 (Mean)	A3525-UN (Mean)	Standard Error	*p* value	Treatments SSD[1]
Nodule Number (per plant)	4 week	68.29	3.25	0.46	<0.0001	S
	6 week	140.50	2.38	0.70	<0.0001	
Shoot Total Nitrogen (% dry wt)	4 week	2.74	1.00	0.14	<0.0001	S
	6 week	2.80	1.11	0.12	<0.0001	

[1]SSD, statistical significance of differences: S, significant at 5% level ($p < 0.05$).

K.2. Subsequent Analysis Using Refined Methodology

K.2.1. Materials

Seed of MON 87769, the conventional soybean control (variety A3525), the negative isoline control, and six commercial reference soybean varieties were used to assess the symbiotic association between *B. japonicum* and MON 87769 compared to that of conventional soybean.

Material Type	Seed Materials Tested	Phenotype
Test	MON 87769	SDA trait
Control	MON 87769 negative isoline	Negative isoline
Control	A3525	Conventional
Reference	Williams 82	Conventional
Reference	A2553	Conventional
Reference	3585N	Conventional
Reference	CST 37002	Conventional
Reference	A4324	Conventional
Reference	5989	Conventional

K.2.2. Characterization of Materials

The presence or absence of MON 87769 in the test and control starting seed was verified by event-specific polymerase chain reaction. The results of these analyses were as expected. The identity of the reference substances was maintained via chain-of-custody documentation and seed bag labels.

K.2.3. Experimental Methods

Plants of each material were grown in a greenhouse with a 14-hour photoperiod, a target day-time temperature of 27 ^{o}C, and a target night-time temperature of 22 ^{o}C. At planting, eight replications of 6-inch pots were filled with a nitrogen-deficient potting medium composed of primarily peat, vermiculite, and perlite. Three seeds of MON 87769, the conventional control, negative isoline, or reference soybean variety were inoculated with approximately 1 x 10^7 cells of *B. japonicum* in phosphate-buffered saline and planted in each pot. Pots were arranged in the greenhouse in a randomized complete block design with eight replications for the 6-week sampling period (plants were not sampled at four weeks).

One week after emergence, all pots were thinned to one seedling per pot. A solution of nitrogen-free nutrient solution (target of 250 mL) was added weekly to each pot after plant emergence. The growth stage of each plant was evaluated and documented in the study notebook at least once per week.

Six weeks after thinning, plants were excised at the surface of the potting medium and shoot and root plus nodule material were removed from the 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 nodules were later removed by hand from the roots of each plant and placed in weighing tins. Nodules from each plant were enumerated and the fresh weight determined. The nodules were dried for a minimum of 48 hours at approximately 65 °C to determine dry weight. The fresh weight of the remaining root and shoot mass was determined for each plant. Root and shoot material from each plant was dried for a minimum of 48 hours at approximately 65 °C to determine dry weight. After drying, the shoot tissue was ground prior to shipping to A&L Great Lakes Laboratory (Fort Wayne, IN) for total nitrogen analysis. Shoot total nitrogen was determined by thermal conductance using a nitrogen analyzer.

K.2.4. Statistical Methods

An analysis of variance was conducted using a randomized complete block design with eight replications. Data were analyzed using SAS (SAS Release 9.1, 2002-2003), and statistical significance was set at the 5% probability level ($p = 0.05$). MON 87769 was compared to the conventional control and the negative isoline control for six measurement endpoints in separate pairwise comparisons: nodule number, nodule dry wt (g), shoot dry wt (g), root dry wt (g), and shoot nitrogen (percent and total dry weight).

The commercially-released reference soybean varieties provided a range of values for each measured endpoint that are representative of commercial soybean varieties. A summary of the results is provided in Section VIII.D.4.