Application to Food Standards Australia New Zealand for the Inclusion of Soybean MON 87769 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

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Andre Silvanovich and Haidi Tu. Updated Bioinformatics Evaluation of Δ6 and Δ15 Desaturases Utilizing the AD_2009, TOX_2009 and PRT_2009 Databases. Study Number: RAR-09-520

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GM CHECKLIST

	Data Provided	Part No.	Data Not Provided	Omission Explained
Executive Summary				
Separately bound document	☒		☐	☐
Part 1: General Information				
1.1 Applicant				
(a) Company / Organisation Name	☒	1.1	☐	☐
(b) Address	☒	1.1	☐	☐
(c) Contact	☒	1.1	☐	☐
(d) Nature of business	☒	1.1	☐	☐
(e) Sole or joint application	☒	1.2	☐	☐
(f) Co-applicants	☐	1.2	☐	☒
Part 2: Specific Data Requirements				
2.1 General Details				
(a) Description of GM organism	☒	2.1.a.	☐	☐
(b) Name / Number of new line / strain	☒	2.1.b.	☐	☐
(c) Marketing name	☒	2.1.c.	☐	☐
(d) Product list	☒	2.1.d.	☐	☐
2.2 History of Use				
(a) Donor	☒	2.2.a.	☐	☐
(b) Host	☒	2.2.b.	☐	☐
2.3 Nature of Genetic Modification				
(a) Method used	☒	2.3.a.	☐	☐
(b) Bacteria used	☒	2.3.a.	☐	☐
(c) Gene Construct and transformation event	☒	2.3.b.	☐	☐
(d) Molecular characterisation	☒	2.3.c.	☐	☐
(e) Derivation of line or strain	☒	2.3.d.	☐	☐
(f) Evidence of stability	☒	2.3.d.	☐	☐
2.4 Antibiotic Resistance Genes				
(a) Clinical / veterinary importance	☐	2.4.a.(NA)	☒	☐
(b) Viability	☐	2.4.b.(NA)	☒	☐
(c) Presence in food	☐	2.4.c.(NA)	☒	☐
2.5 Characterisation of Novel Protein				
(a) Description	☒	2.5.a.	☐	☐
(b) Site of expression	☒	2.5.d.	☐	☐
(c) Non-expression	☐	2.5.e.(NA)	☒	☐
(d) History of human consumption	☒	2.5.f.	☐	☐
(e) Oral toxicological studies	☒	2.5.f.	☐	☐
(f) Amino acid sequence	☒	2.5.f.	☐	☐
(g) Known allergenicity of source	☒	2.5.h.	☐	☐
(h) Unknown allergenicity information	☒	2.5.i.	☐	☐

GM CHECKLIST (cont'd.)

	Data Provided	Part No.	Data Not Provided	Omission Explained
2.6 Characterisation of Other Novel Substances				
(a) Identification	☐	2.6.a.(NA)	☒	☐
(b) Toxicity	☐	2.6.b.(NA)	☒	☐
2.7 Comparative Analyses				
(a) Key nutrients etc.	☒	2.7.a.	☐	☐
(b) Other constituents	☒	2.7.b.	☐	☐
(c) Allergenic proteins	☐	2.7.c.(NA)	☒	☐
2.8 Nutritional Impact				
(a) Animal feeding studies	☒	2.8a	☐	☐
(b) Nutritional changes	☒	2.8	☐	☐
2.9 Other Technical Information				
(a) Detection methodology	☒	2.9.a.	☐	☐
(b) Market penetration	☒	2.9.b.	☐	☐
Part 3: Regulatory / Legislative Implications				
3.1 Other approvals				
(a) Relevant overseas approvals	☒	3.1.a.	☐	☐
(b) Approval refusal	☒	3.1.b.	☐	☐
Part 4: Statutory Declaration	☒		☐	☐

PART 1 GENERAL INFORMATION

1.1 Applicant Details

Company Name:	Monsanto Australia Limited
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Contact:	Amanda Forster, +61 3 9522 7109
Nature of Your Business:	Technology Provider to the Agricultural and Food Industries.

1.2 Nature of Application

This application is submitted to Food Standards Australia New Zealand by Monsanto Australia Limited and is not made on behalf of any other party.

The purpose of this submission is to make an application to vary **Standard 1.5.2** – *Food Produced Using Gene Technology* to seek the addition of soybean MON 87769 that produces stearidonic acid (SDA; 18:4) and products containing soybean MON 87769 (hereafter referred to as MON 87769) to the Table to Clause 2 (see below).

Column 1 **Food derived from gene technology**	**Column 2** **Special requirements**
Food derived from Soybean MON 87769	None

PART 2 SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT

2.1 General Details

a) Description of the Nature and Purpose of the Introduced Trait

Soybean is the most commonly grown oilseed in the world. In 2007/08, approximately 218.8 million metric tons (MMT) of harvested soybean seed were produced, representing 56% of the world's oilseed production. Monsanto has developed biotechnology-derived soybean MON 87769 that contains stearidonic acid (SDA), a sustainable alternate source of an omega-3 fatty acid to help meet the need for increased dietary intake of long chain omega-3 fatty acids. SDA is an eighteen carbon fatty acid with four double bonds (18:4) and as such is found in fish and fish/algal oil products. In mammals, SDA is a metabolic intermediate in the production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from alpha linolenic acid (ALA), a common dietary constituent. 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.

Refined oil produced from MON 87769 contains approximately 20 to 30% SDA (wt% of total fatty acids) 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 either 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 consumers. Fish and plant oils rich in omega-3 fatty acids are used currently in feed applications such as aquaculture and poultry feeds. SDA soybean oil from MON 87769 may be used in aquaculture and feed applications as an alternative to fish oil and other omega-3 rich feed components. MON 87769 soybean meal is compositionally similar to other commodity soybean meal and will be used in a manner similar to conventional soybean meal.

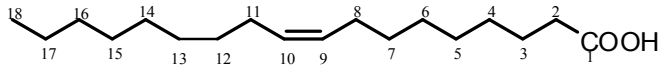
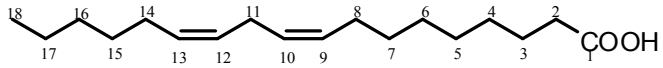
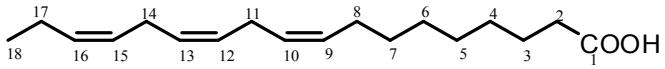
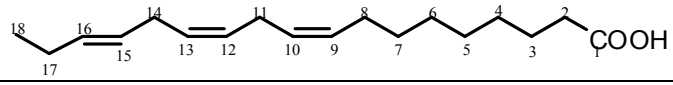
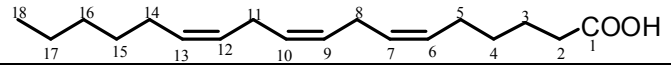
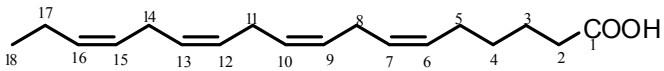
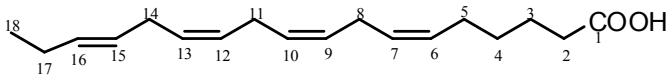
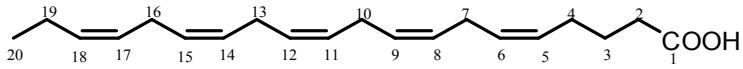
b) Identity and Intended Function of the Genetic Modification

MON 87769 was created by introducing a Δ6 desaturase gene from *Primula juliae* and a Δ15 desaturase gene from *Neurospora crassa,* into conventional soybean, A3525. The introduction of these two genes results in the seed-specific production of the PjΔ6D and NcΔ15D proteins. These proteins desaturate certain endogenous fatty acids resulting in the production of SDA at approximately 20-30% of total fatty acids. SDA is the product of Δ6 desaturation of ALA in some plants and animals (Figure 1 and Table 1). Since soybean lacks a Δ6 desaturase, the minimal requirement for production of SDA in soybean would be the introduction of a gene encoding Δ6 desaturase. However, Δ6 desaturase may also convert linoleic acid to α-linolenic acid (Figure 1 and Table 1). Addition of a Δ15 desaturase with temporal expression similar to the Δ6 desaturase increases ALA levels, allowing greater flux to SDA accumulation in MON 87769. The

Δ15 desaturase also lowers LA levels, and hence lowers the substrate pool for GLA production. The oil derived from MON 87769 can serve as an alternate sustainable source of omega-3 fatty acid and help meet the need for increased dietary intake of long chain omega-3 fatty acids.

Figure 1. Fatty Acid Biosynthesis in Plants and the Introduced Changes to Produce MON 87769

Table 1. Common Name, Acronym and Chemical Structure of SDA and Related Fatty Acids

Common Name	Acronym	Chain Length & Unsaturation	Chemical Structure
Oleic acid	O	9c-18:1	
Linoleic acid	LA	9c,12c-18:2	
α-linolenic acid	ALA	9c,12c,15c-18:3	
trans α-linolenic acid	*t*-ALA	9c,12c,15t-18:3	
γ-linolenic acid	GLA	6c,9c,12c-18:3	
Stearidonic acid	SDA	6c,9c,12c,15c-18:4	
trans Stearidonic acid	*t*-SDA	6c,9c,12c,15t-18:4	
Eicosapentaenoic acid	EPA	5c,8c,11c,14c,17c-20:5	

c) Application and Proposed Product Name

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 US 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 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. There are currently no plans to produce in Australia and New Zealand. A commercial trade name for the product has not been determined at the time of this submission and will be available prior to commercial launch of the product.

d) Soybean as a Food Source

Soybean has the remarkable ability to produce more edible protein per acre of land than any other known crop (Liu, 2004a). On average, dry soybean contains roughly 40% protein and 20% oil. It has the highest protein content among cereals and other legume species, and has the second-highest oil content among all food legumes. Soybean is highly versatile and can be processed into a wide variety of food products. In general, soyfoods can be roughly classified into four major categories:

1. Traditional soyfoods: As discussed above, traditional soyfoods are primarily made from whole soybean. The nonfermented traditional soyfoods include soymilk, tofu, and soybean sprouts, whereas the fermented soyfoods include soybean paste (miso), soy sauce, natto, and tempeh.

2. Soybean oil: Soybean oil constitutes approximately 71% of global consumption of edible fats and oil (ASA 2008b), and is the second largest source of vegetable oil worldwide (Soyatech 2008). Refined, bleached, and deodorized soybean can be further processed to produce cooking oils, shortening, margarine, mayonnaise, salad dressings, and a wide variety of products that are either based entirely on fats and oils or contain fat or oil as a principal ingredient (Liu, 2004b).

3. Soybean protein products: Soybean protein products are made from defatted soybean flakes, and include soybean flour, soybean protein concentrate, and soybean protein isolate. Soybean flour has a protein content of ~50% and is used mainly as an ingredient in the bakery industry. Soybean protein concentrate has a protein content of ~70% and is used widely in the meat industry as a key ingredient of meat alternative products such as soybean burgers and meatless “meatballs.” Soybean protein isolate has a protein content of 90%, and possesses many functional properties such as gelation and emulsification. As a result, it can be used in a wide range of food applications, including soups, sauce bases, energy bars, nutritional beverages, infant formula, and dairy replacements (Liu, 2004b).

4. Dietary supplements: Soybean is a rich source of certain phytochemicals used as dietary supplements, which include isoflavones and tocopherols. Isoflavones have been shown to inhibit the growth of cancer cells, lower cholesterol levels, and inhibit bone resorption

(Messina, 1999). Tocopherols have long been recognized as a classic free radical scavenging antioxidant whose deficiency impairs mammalian fertility. In addition, new biological activities have been reported for the desmethyl tocopherols, such as γ-tocopherol, to possess anti-inflammatory, antineoplastic, and natriuretic functions (Hensley et al., 2004; IFIC, 2005; Schafer et al., 2003). Detailed reviews of soybean as functional foods have been recently reviewed in detail and can be found at IFIC (IFIC, 2005; Liu, 2004b).

e) Soybean as a Feed Source

Soybean meal is the most valuable component obtained from processing the soybean, accounting for roughly 50-75% of its overall value (USDA-ERS, 2005). Soybean meal is produced by solvent extraction of the dehulled soybean flakes, and the spent flakes (soybean flakes with the oil removed) are conveyed to a desolventizer-toaster for removal of the hexane. The process involves heating the spent flakes to evaporate the hexane and utilizing steam to carry away hexane vapors. This process also provides toasting of the meal to inactivate enzymes like urease and trypsin inhibitors that may reduce the digestibility and nutritional value of the meal. Subsequently, the meal is dried to about 13 to 14 percent moisture, and is screened and ground to produce a uniform particle size prior to shipment to the end user. The finished meal from dehulled soybean will contain less than 1.5% crude fat and approximately 48% protein, and is referred to as high protein meal (SMIC, 2006).

Soybean meal is the premier supplemental protein source in U.S. livestock and poultry rations due to its nutrient composition, availability, and price. Typically, soybean meal is used to meet the animal's requirement for limiting amino acids, as it is the most cost-effective source of amino acids. Soybean meal is also one of the best protein sources for complementing the limiting amino acid profile of corn protein (Kerley and Allee, 2003). Due to the high value and versatility, approximately two-thirds of the total protein meal use in the world is derived from soybean, with the remainder divided between rapeseed, cottonseed, sunflower, peanut, and other meals (ASA, 2008b). Poultry and swine account for most of the soybean meal utilized in the U.S., with poultry consuming 50%, swine 27%, cattle 17%, and 3% for companion animals and the reminder to other feed uses (ASA, 2008b).

Dairy and livestock producers need an inexpensive, readily available, on-farm source of high-quality, high-protein forage adapted to growth during the summer months when other forage legume species typically are restricted in growth. Soybean forage can provide livestock and dairy producers with a source of high-protein feed for their livestock (USDA-ARS, 2006). Harvested forage can be used as hay or to produce silage.

2.2 History of Use

a) Donor Organism

Safety of the PjΔ6D Donor Organism *Primula juliae*

The donor organism of the PjΔ6D protein, *P. juliae*, is a member of a large genus of plants commonly known as 'Primrose' and is not known to be pathogenic or allergenic; however, it is not generally consumed as food. Desaturases, including Δ6 desaturase, are ubiquitous in nature, being present in many common plants, yeast, animals, and some bacteria. All desaturases show strong conservation of the three histidine box motifs required for binding two iron atoms at the catalytic center. The PjΔ6D protein shares a relatively high percent identity with Δ6 desaturases present in *Echium plantagineum* and *Borago officinalis,* which are used to produce oils for human consumption. It also shares high identity with Δ8 desaturase from *Brassica napus,* which is a major food crop worldwide. The PjΔ6D shares 20-30% identity and demonstrates similar functions and specify with Δ6 desaturases from two widely consumed fresh water fish species, trout and carp. Δ6 desaturases have been consumed for many years with no history of adverse health effects, have no known toxicity or allergenicity, and are not associated with pathogenicity. Taken together, these data demonstrate that PjΔ6D homologous proteins share a long history of safe consumption.

Safety of NcΔ15D Donor Organism: *Neurospora crassa*

The fungus, *Neurospora,* is a long standing, widely used model organism in genetics and biological research, with many years of familiarity. Thousands of scientific publications related to *Neurospora* are available. Perkins and Davis (2000b) have reviewed the contributions of *Neurospora* to genetics and biology and they have also reviewed the evidence for the general safety of *Neurospora* (Perkins and Davis, 2000a) suggesting that the genus of *Neurospora* is well qualified to be recognized under U.S. Food and Drug Administration (FDA) regulations as a generally recognized as safe (GRAS) organism. *Neurospora crassa,* while ubiquitous in the environment (Turner et al., 2001) is not associated with any adverse effects to human health. *Neurospora crassa* has been used for food preparation in a number of world regions. It is a major constituent of onchom, a soybean-based press cake, which is daily consumed in Indonesia (Matsuo, 1997). It is used in Brazil to process cassava in preparing a fermented beverage (Park et al., 1982) and present in Roquefort cheese prepared by traditional methods in southern France (Perkins and Davis, 2000a). There is no evidence of food allergy due to oral consumption of *Neurospora crassa* and, therefore, it is considered a non-pathogenic and non-allergenic organism. Thus, the safety of the donor organism for the Δ15 desaturase protein in MON 87769 is well established.

b) Host Organism

Taxonomic Classification

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 2.

Table 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 (Hymowitz, 2004).

Reproduction and Life Cycle of Soybean

Glycine max (L.) Merr, the cultivated soybean, is an annual crop that is planted in late spring from April to May in the north hemisphere, and from November to February in the southern hemisphere. Soybean seed germinates when the soil temperature reaches 10°C and emerges in a 5-7 day period under favourable conditions (OECD, 2000). The system of soybean growth stages divides plant development into vegetative (V) and reproductive (R) stages (Pedersen, 2008). 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 R8. The vegetative development phase lasts about 40 days, during which time the root nodules develop slowly, but do not become fully functional. Soybeans grow most rapidly when air temperatures are between 25 and 35°C (Beversdorf, 1993). Pods typically develop in late summer, and harvest occurs in the autumn. The life cycle of soybean is approximately 100 to 160 days, depending on the variety and the region it is cultivated. Harvesting may begin when the plants are completely dry and the seeds are liberated within the pods.

Soybean is 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. 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 only can take place during the short time when the pollen is viable. As a result, soybeans exhibit a high percentage of self-fertilization, and cross-pollination is usually less than one percent (Caviness, 1966).

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

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 could only 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 approximately 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 throughout 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 farmers throughout the U.S., and soybean has been cultivated ever since and subsequently has become a key source of nutrient for food and feed use in the U.S. (Hymowitz, 1987). Based on this long history of cultivation and use of soybean and soybean processed fractions in the U.S. and elsewhere in the world, soybean is considered to have a history of safe use.

Soybean Processing

Soybeans are grown primarily for meal, and oil is a secondary product. There is no food use for unprocessed soybeans, since they contain anti-nutrient factors, such as trypsin inhibitors and lectins (OECD, 2001). Adequate heat processing inactivates these factors.

There are three main methods for processing soybeans; these are hydraulic processing, expeller processing and solvent extraction (SMIC, 2006). The description below on the soybean processing is adopted from Snyder and Wilson (2003).

Dehulling and flaking soybean

Soybeans are cleaned and cracked into several pieces (meats). The hulls are removed by aspiration and the meats are conditioned by warming and by adding moisture. Conditioning is necessary to make a cohesive flake. The conditioned meats are put through smooth rollers that make flakes of approximately 0.025 cm thickness. Making flakes is advantageous for uniform penetration of solvent in deep beds (minimal channelling) and for disruption of the soybean tissue, so that solvent can penetrate and dissolve the oil. Additionally, crushers may put the flakes through a cooking extruder to yield collets. This process gives a porous, but still high-density collet, that extracts more readily than flakes. Also, the collet holds less solvent than flake, thereby minimizing the energy needed for removing the solvent.

Oil extraction

The full-fat flakes (or collets) are loaded into the extractor to make beds over which the solvent flows countercurrently to the movement of the beds. The temperature of extraction is about 60ºC to speed up diffusion of solvent and to lower the miscella viscosity, both of which enhance the extraction of oil. Solvent extraction is capable of reducing the residual oil in the soybean flakes to less than 1%.

Removal of solvent and generation of crude oil and meal

Upon completion of the extraction, the solvent will be removed from both the oil and flakes. The full-fat miscella contains 25-30% oil and solvent is removed by two stages of rising-film vacuum evaporators followed by a third-stage stripping column. The flakes are treated in a desolventizer-toaster by direct contact with steam first to remove the solvent and second to heat treat the flakes for trypsin inhibitor destruction.

After leaving the desolventizer-toaster, the flakes are cooled and ground to a meal for use as a high-protein feed ingredient. The protein content is 44% with hulls added or 47.5-49% protein without hulls. If flakes are to be used to produce soybean food products, the solvent has to be removed with minimal heat to maintain protein solubility. Flash desolventizers are available in which superheated solvent is used as the heat-transfer medium to evaporate the solvent. With this system, the flakes are kept dry and protein solubility is preserved.

Oil refining

After extraction and removal of solvent, the crude soybean oil needs to be refined to convert it to edible products. Before going to refining, crude oil may undergo alkali refining (to remove free fatty acids), degumming (to remove phospholipids) or bleaching (to lighten the colour of the oil). Hydrogenation and deodorisation made it possible to substitute vegetable oils for animal fats in human diets in the early 1900s. Hydrogenation controlled the texture and stability of the oil, while deodorization improves the flavour of vegetable oils.

The gummy material that is removed during the oil refinement is further processed into lecithin, which is typically used as an emulsifier to keep water and fats from separating in foods such as margarine, peanut butter, chocolate candies, ice cream, and infant formulas.

Soy protein isolate

Defatted white flakes are extracted first with sodium hydroxide at ~pH 9 to remove fibre, and the remaining solution is acidified to ~pH 4 to precipitate the protein. The precipitated protein in slurry form can be spray-dried directly to produce an isoelectric soybean protein isolate (pH 4), or can be neutralized and spray-dried to produce soy proteinate (~pH 7).

Resulting soybean products

- Soybean oil products: after sufficient refining, soybean oil is used to produce shortening, margarines, salad dressings and cooking oils of various types.
- Soybean meal: the majority of the defatted soybean flakes is heated to produce soybean meal as animal feeds.
- Soybean protein products: a variety of food products such as soybean flour, soybean protein concentrates and isolates can be used in a variety of food products including simulating meats, nutrition bars and protein-fortified drinks. See Figure 2.

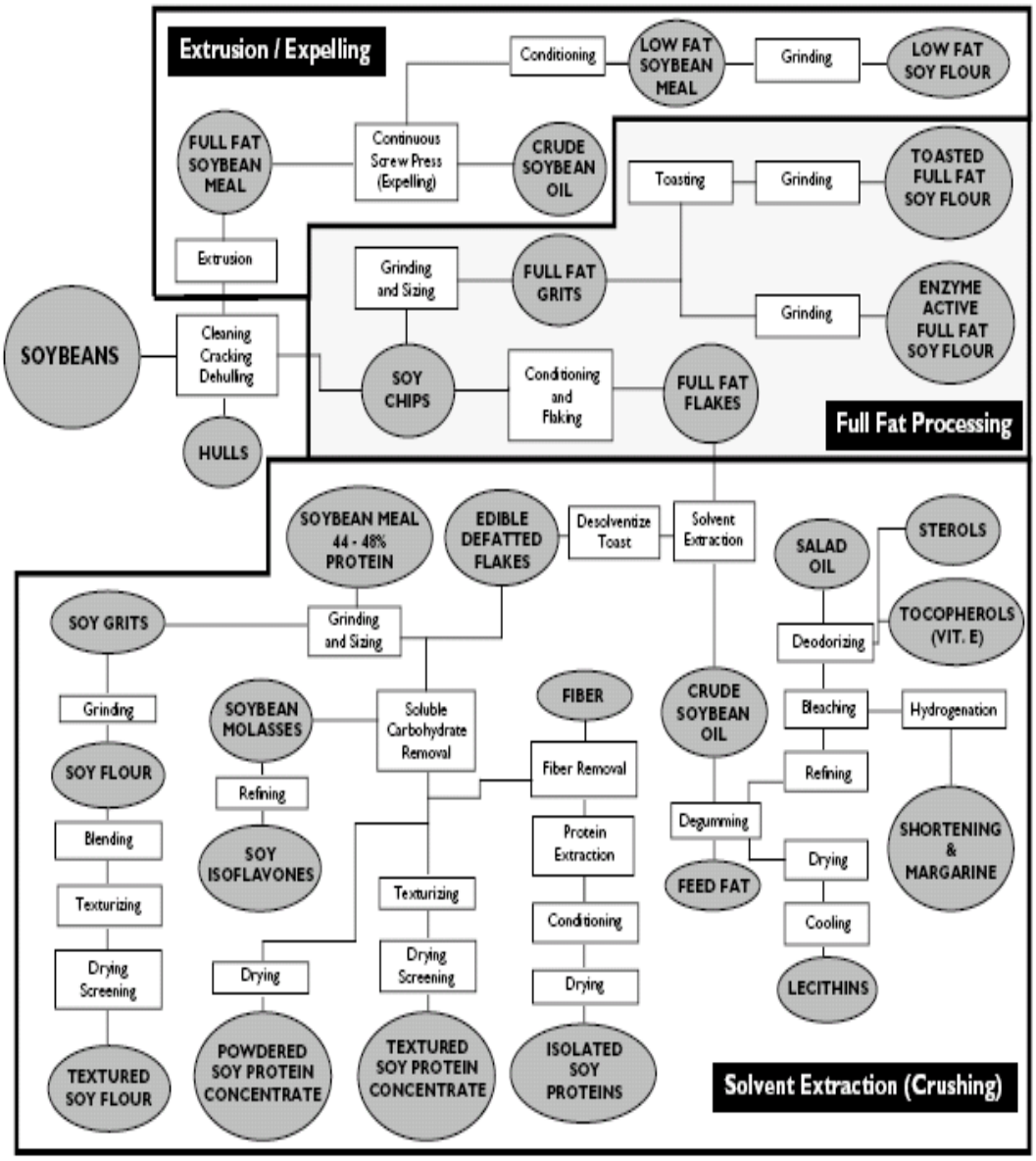


Figure 2. Soybean Processing Using Extrusion or Solvent Extraction

Figure is adapted from Soya and Oilseeds Bluebook, 2004.

2.3 Nature of Genetic Modification

a) Transformation Method

MON 87769 was developed through *Agrobacterium*-mediated transformation of soybean meristem tissue using plasmid vector PV-GMPQ1972 (Figure 3 and Table 3). *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 herbicide.

The use of the 2T-DNAs 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, and antibiotics carbenicilin and claforan to inhibit the growth of untransformed plant cells and excess *Agrobacterium*, respectively. The meristems were then placed on media conducive to shoot and root development, and only rooted plants

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

The R0 plants generated through the above transformation 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. This R1 plant was self-pollinated to generate a population of R2 plants which 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 4.

b) Gene Construct and Transformation Event

The plasmid vector PV-GMPQ1972 used to transform soybean meristems to produce MON 87769 is shown in Figure 3. 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' non-translated sequence. The *Nc.Fad3* expression cassette consists of the *Nc.Fad3* coding sequence under the regulation of the *7Sα* promoter and the *E9* 3' non-translated 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′ non-translated 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 3.

i) T-DNA I

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

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 5. The PjΔ6D protein is a Δ6 desaturase which creates a double bond at the 6th position from the carboxyl end of a fatty acid.

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' non-translated 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.

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 6**.** The NcΔ15D protein is a Δ15 desaturase which creates a double bond at the 15th position from the carboxyl end of a fatty acid.

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' non-translated 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.

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 3 and Table 3) were derived from *Agrobacterium tumefaciens* plasmids (Barker et al., 1983; Depicker et al., 1982).

ii) 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.

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).

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).

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' non-translated *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' non-translated 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 2.3.c.iii.

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 3 and Table 3) were derived from *Agrobacterium tumefaciens* plasmids (Barker et al., 1983; Depicker et al., 1982).

iii) 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 et al., 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).

As 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 2.3.c.ii.

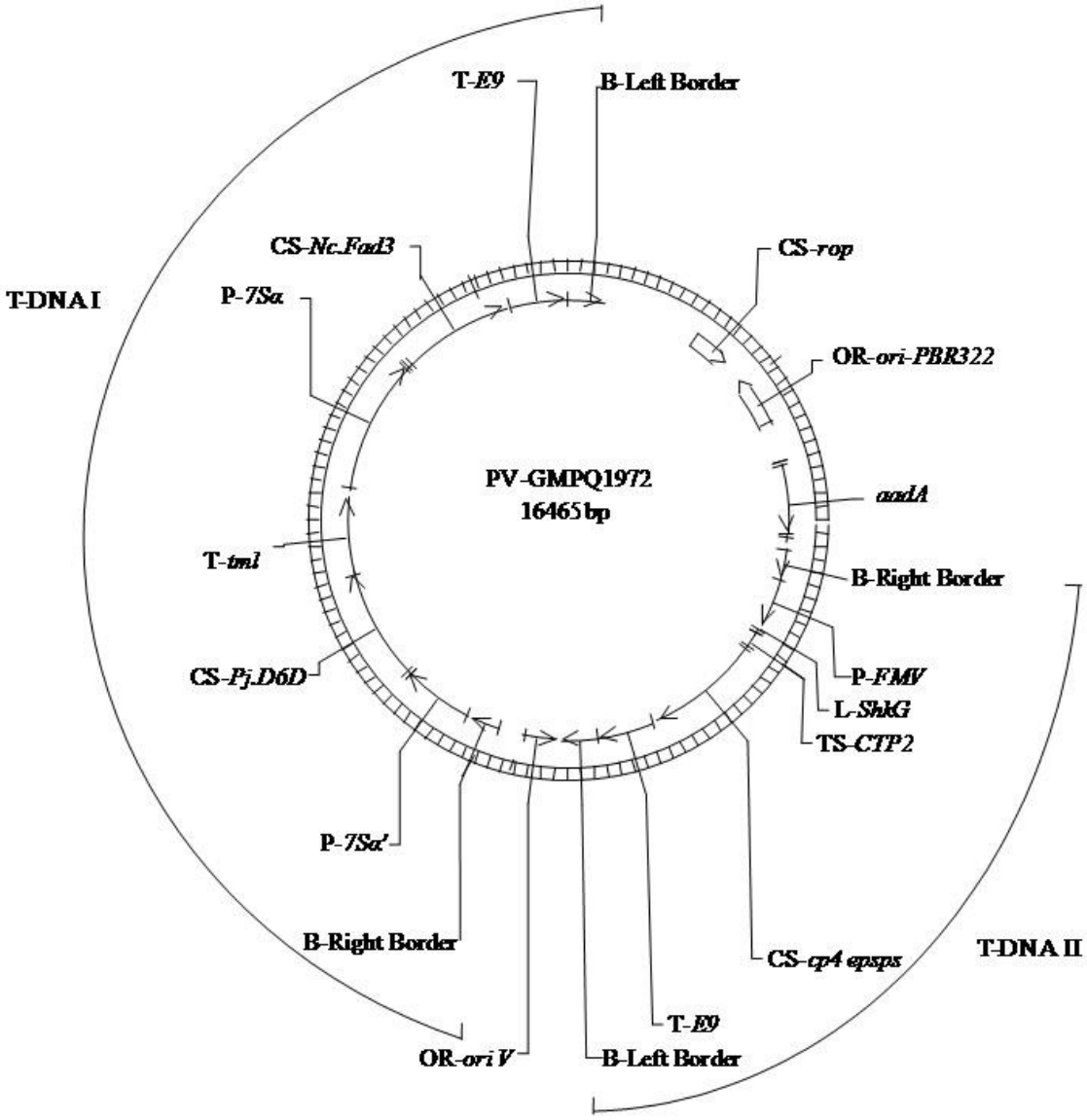

Figure 3. Plasmid Map of Vector PV-GMPQ1972

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 3. 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' non-translated 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

Table 3 (continued). Summary of Genetic Elements in Plasmid Vector PV-GMPQ1972		
T-DNA II (cont.)		
T[7]-*E9*	7197-7839	A 3' non-translated 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 delta 6 desaturase from *Primula juliae* (Ursin et al., 2008)
Intervening Sequence	11679-11686	Sequence used in DNA cloning
T-*tml*	11687-12636	3' non-translated 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 delta 15 desaturase (Ursin et al., 2006)
Intervening Sequence	15736-15787	Sequence used in DNA cloning
T-*E9*	15788-16430	3' non-translated 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

[1]B – Border; [2]CS – Coding Sequence; [3]OR – Origin of Replication; [4]P – Promoter; [5]L-Leader; [6]TS – Targeting Sequence; [7]T – Transcript Termination Sequence and polyadenylation signal sequences.

Table 4. Summary of Genetic Elements in MON 87769

Genetic Element[1,2]	Location in Sequence[2]	Function (Reference)
Sequence flanking 5' end of the insert	1-933	Soybean genomic DNA
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
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)
Intervening Sequence	1869-1884	Sequence used in DNA cloning
CS-*Pj.D6D*	1885-3225	Coding region for the fatty acid delta-6 desaturase from *Primula juliae* (Ursin et al., 2008)
Intervening Sequence	3226-3233	Sequence used in DNA cloning
T-*tml*	3234-4183	3′ non-translated region of the *tml* gene from *Agrobacterium tumefaciens* octopine-type Ti plasmid (Kemp et al., 2000)
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).
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′ non-translated region of the *Pisum sativum 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 (Depicker et al., 1982)
Sequence flanking 3' end of the insert	8301-9131	Soybean genomic DNA

[1] B – Border; P – Promoter; CS – Coding Sequence; T – Transcript Termination Sequence and polyadenylation signal sequences.

[2] 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.

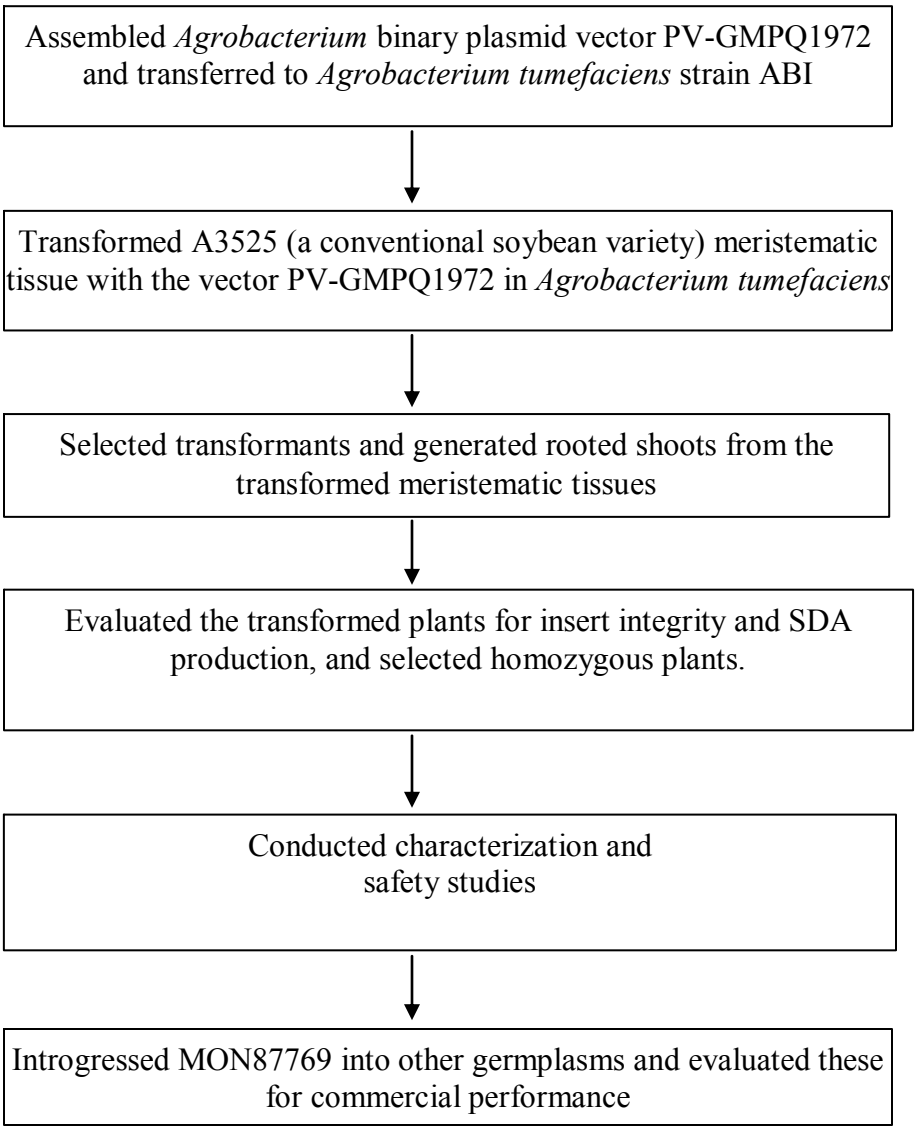

Figure 4. Schematic of the Development of MON 87769

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  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 5. 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 6. 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.

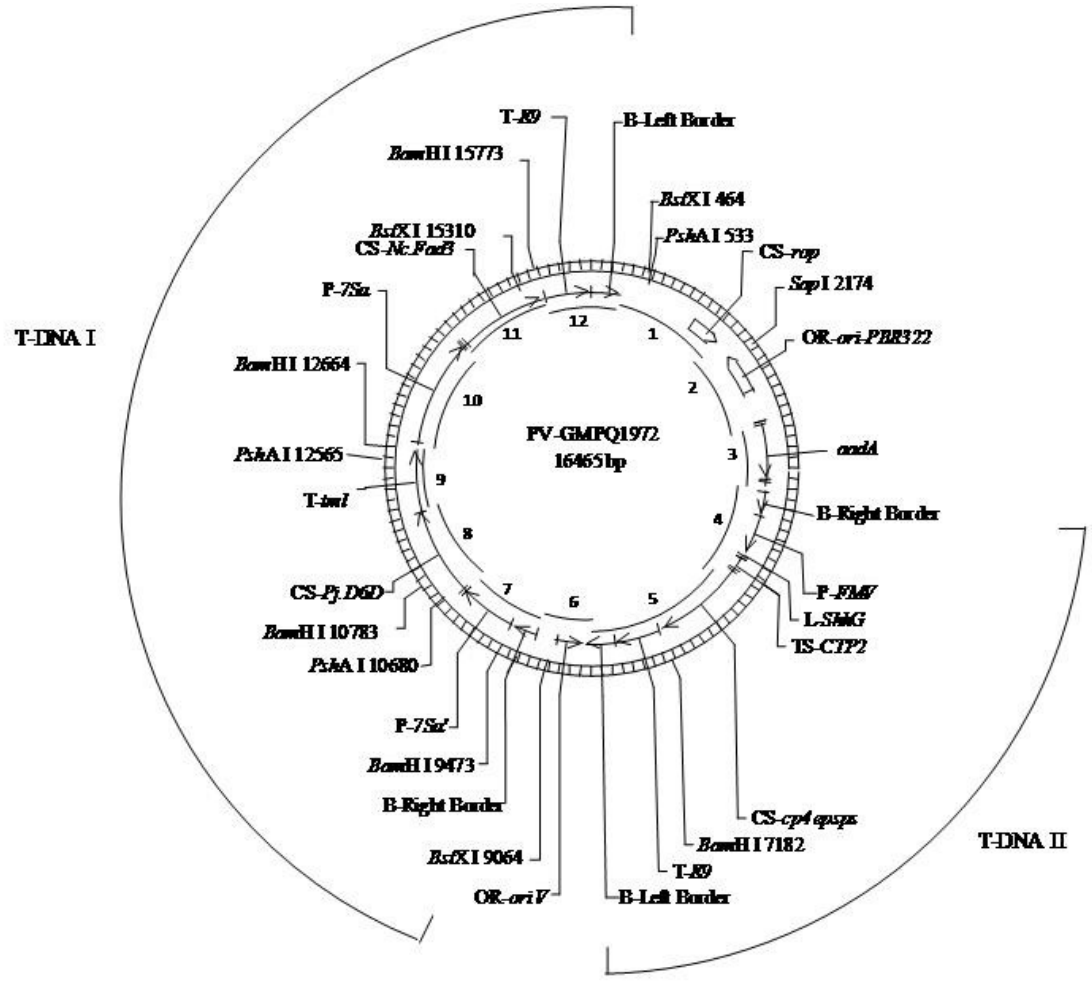
c) Molecular Characterization

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 lack of plasmid backbone elements and absence of T-DNA II, 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. 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 controls for Southern blots hybridized. 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 4 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 Figure 7 and Figure 8, respectively. The information and results derived from the molecular analyses 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 9. A table based on the insert linear map and the plasmid map, summarizing the expected DNA fragments for Southern analyses, is presented in Table 5.

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 insert segregates as expected indicating that the *Pj.D6D* and *Nc.Fad3* gene cassettes segregate

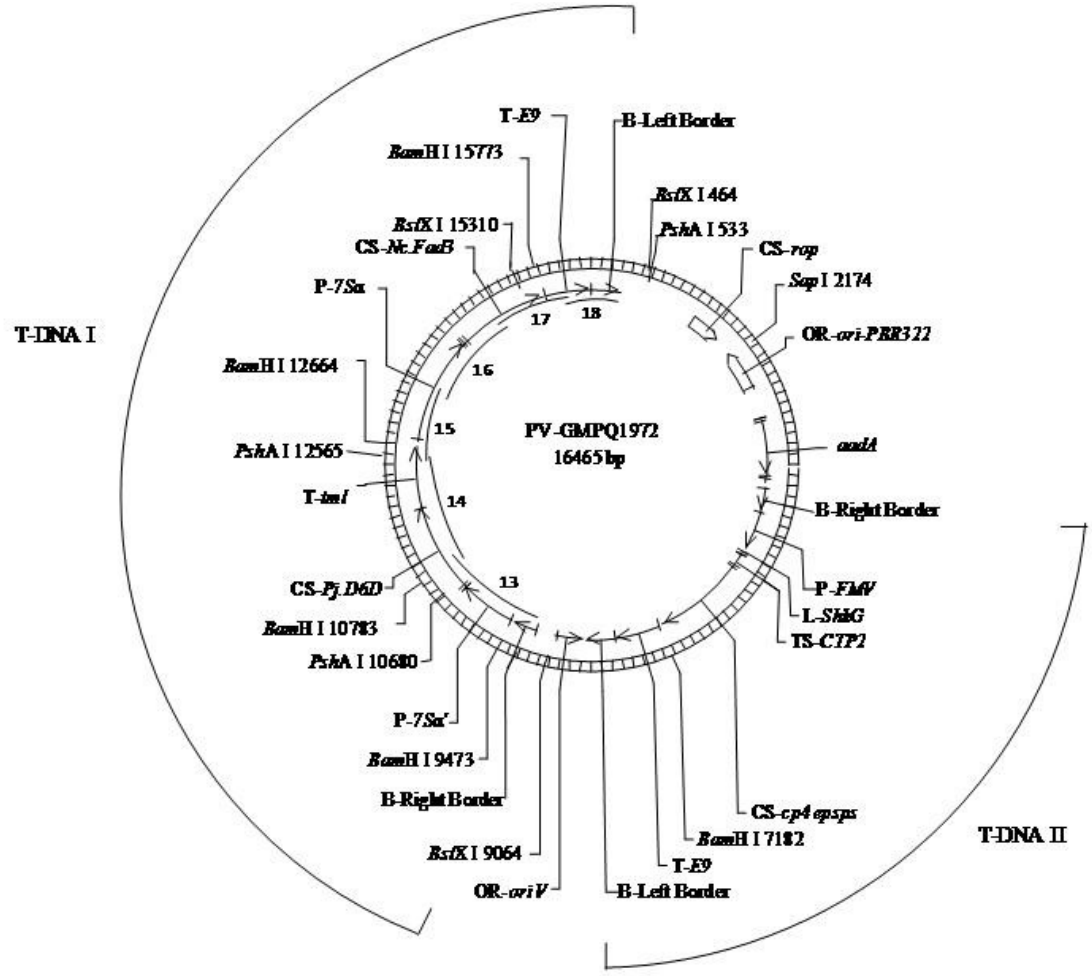
according to Mendel's laws of genetics. All these results indicate a single detectable chromosomal insertion of the T-DNA in MON 87769.



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 7. 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 5. 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 8. 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 5. PV-GMPQ1972 contains two T-DNA regions designated as T-DNA I and T-DNA II.

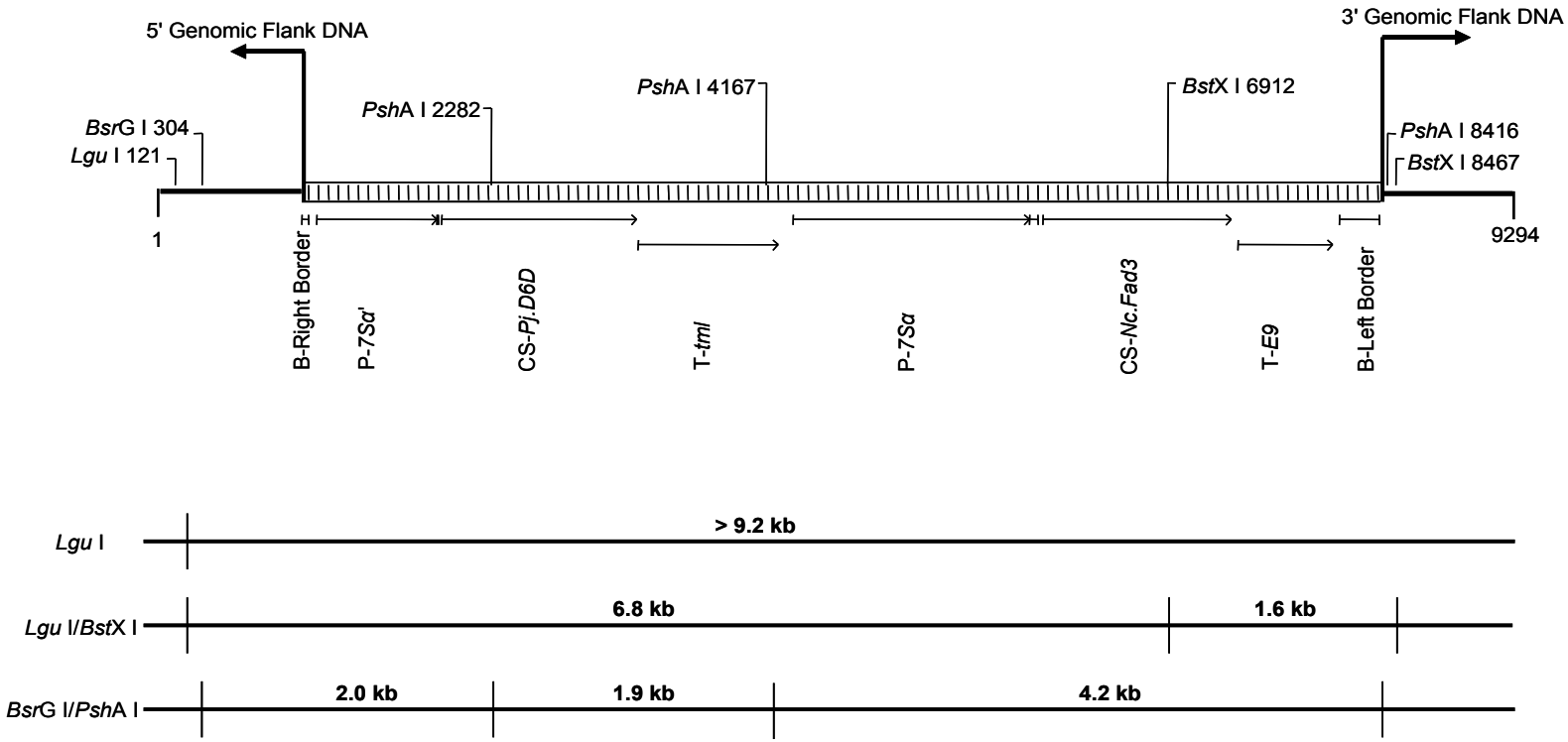


Figure 9. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87769

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 (thick 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 5. 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 5. 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	10a and 21a*	10b and 21b*	13	14	15	16	17	18	11 and 22*	12 and 23*
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	2.0 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.

i) 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 9). 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 9). 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 8) 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 10a for probes 13-15 and Figure 10b for probes 16-18. The expected DNA fragments are summarized in Table 5.

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 10, 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 as 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 pre-digested with *Lgu* I and *Bst*X I, and analyzed using probes 13-15 (lane 9, Figure 10a) 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 5). The probe template controls (probes 13-15) mixed with pre-digested 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 pre-digested conventional soybean DNA and analyzed with probes 16-18 (lane 9, Figure 10b) 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 5). 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 10a and probes 16-18, Figure 10b). 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 10a) 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 10b) 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.

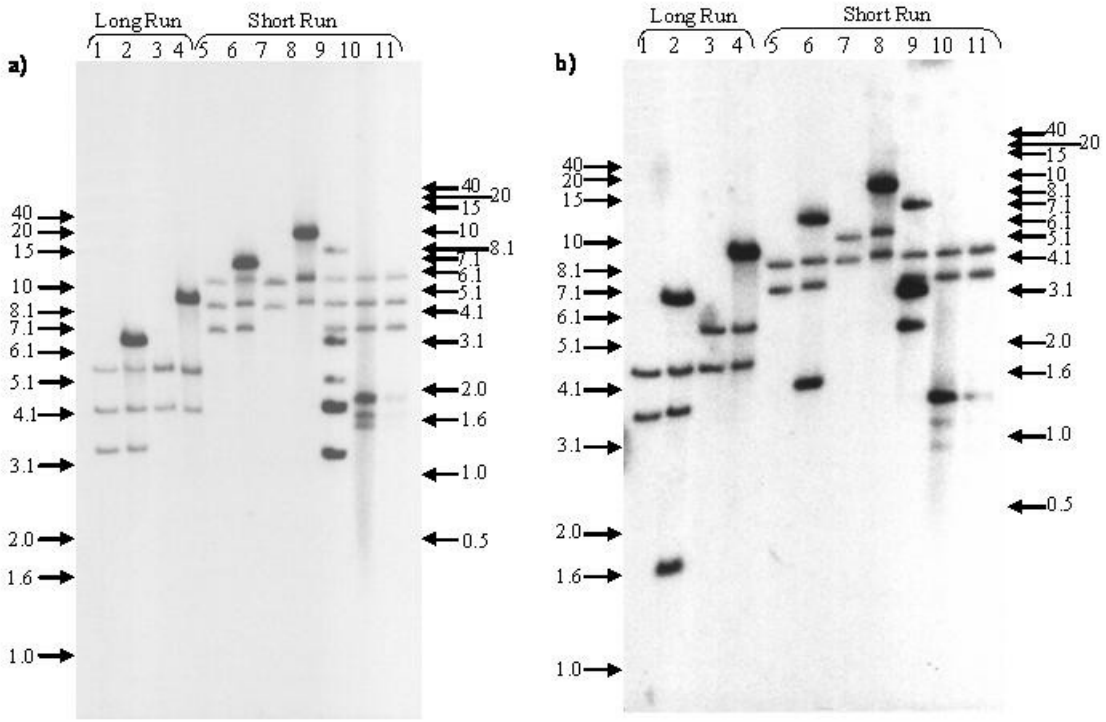


Figure 10. Southern Blot Analysis of MON 87769 with Insert and Copy Number Probes

Each blot was hybridized simultaneously with three overlapping ^{32}P-labeled T-DNA probes (probes 13-18, Figure 8). Figure 10a was hybridized with probes 13-15 and Figure 10b 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.

ii) 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 7). 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 5.

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 11). 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 simultaneously with the backbone probes (probes 1-3 and 6, Figure 7) 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 (pre-digested with *Lgu* I and *Bst*X I) and included on the Southern blot (Figure 11). PV-GMPQ1972 DNA digested with *Bam*H I, mixed with pre-digested conventional soybean DNA, and hybridized simultaneously with probes 1-3 and 6 (Figure 7) 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 non-specific 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 (Figure 11) showed no detectable hybridization signal. This indicates that MON 87769 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ1972.

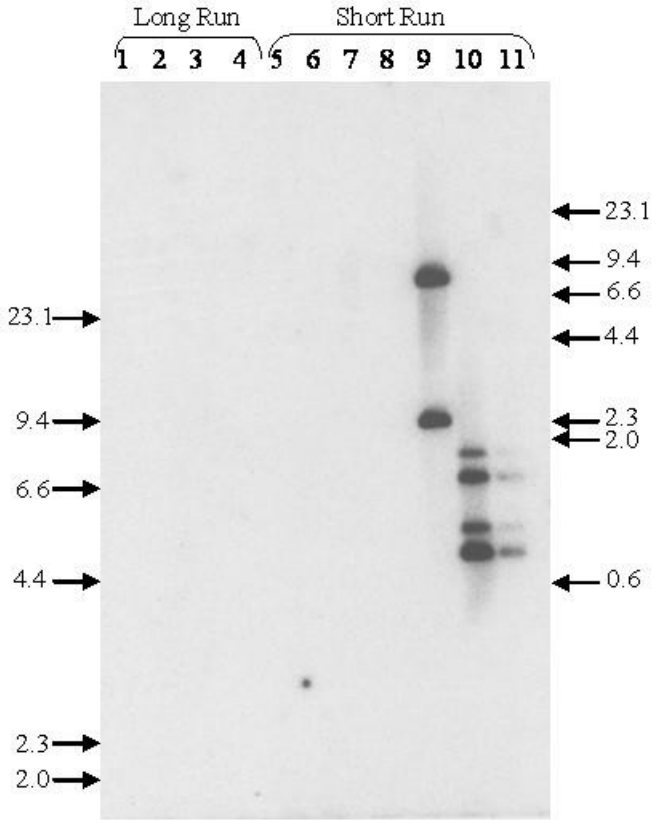


Figure 11. Southern Blot Analysis of MON 87769 with PV-GMPQ1972 Backbone Probes

The blot was hybridized simultaneously with four overlapping ^{32}P-labeled probes that span the entire backbone sequence (probes 1-3, and 6, Figure 7) 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.

iii) 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 12) using probes 4 and 5 (Figure 7). The results are summarized in Table 5.

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 12). 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 (pre-digested with *Lgu* I and *Bst*X I) and used on the Southern blot (Figure 12). 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 7) 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′ non-translated 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′ non-translated region + left border sequence (lanes 2 and 6, Figure 18). 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 12. 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′ non-translated region + left border sequence (lanes 4 and 8, Figure 18). 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 12. 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. These results indicate that MON 87769 does not contain any additional detectable T-DNA II elements other than those associated with T-DNA I.

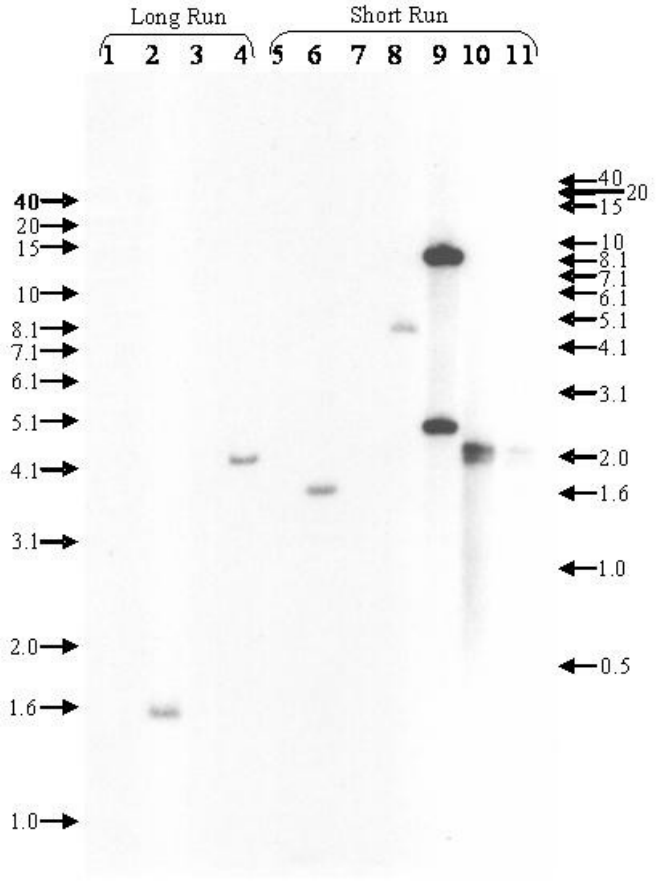


Figure 12. Southern Blot Analysis of MON 87769 with T-DNA II Probes

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 7) 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.

iv) 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 5.

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 9). 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′ non-translated 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′ non-translated 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 9). 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′ non-translated region. The 4.2 kb fragment contains the remaining portion of the *tml* 3′ non-translated region, *7Sα* promoter sequence, *Nc.Fad3* coding sequence, *E9* 3′ non-translated 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′ non-translated region probe, *7Sα* promoter probe, *Nc.Fad3* coding sequence probe, or *E9* 3' non-translated region + left border probe (probes 7-12, Figure 7). The expected DNA fragments identified by probes 7-12 are summarized in Table 5. 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 pre-digested conventional soybean DNA was used as a positive hybridization control.

Right Border/7Sα

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 13). 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 7) 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 13). 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).

***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 14). 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 7) 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 (pre-digested 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.

***tml* 3′ Non-translated 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' non-translated region (Figure 15). 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' non-translated region probe (probe 9, Figure 7) showed no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the *tml* 3' non-translated region probe.

As a positive hybridization control, plasmid PV-GMPQ1972 DNA was digested with *Bam*H I, mixed with conventional soybean DNA (pre-digested with *Lgu* I and *Bst*X I) and included on the *tml* 3' non-translated 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′ non-translated region probe. This indicates that MON 87769 contains no additional, detectable *tml* 3′ elements other than those associated with the *Pj.D6D* gene cassette.

***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 16). 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 7), 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 (pre-digested 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.

***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 17). 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 7) 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)[see earlier comment], 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.

***E9* 3′ Non-translated 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′ non-translated region and left border sequence (Figure 18). 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′ non-translated region + left border probe (probe 12, Figure 7) produced no detectable hybridization bands. This indicates that there are no endogenous soybean DNA sequences that hybridized to the *E9* 3′ non-translated 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′ non-translated region and + left border Southern blot analysis (Figure 18). 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 *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 additional bands detected using the *E9* 3′ non-translated region + left border probe. This indicates that MON 87769 contains no additional detectable *E9* 3′ non-translated region or Left Border elements other than those associated with the *Nc.Fad3* gene cassette.

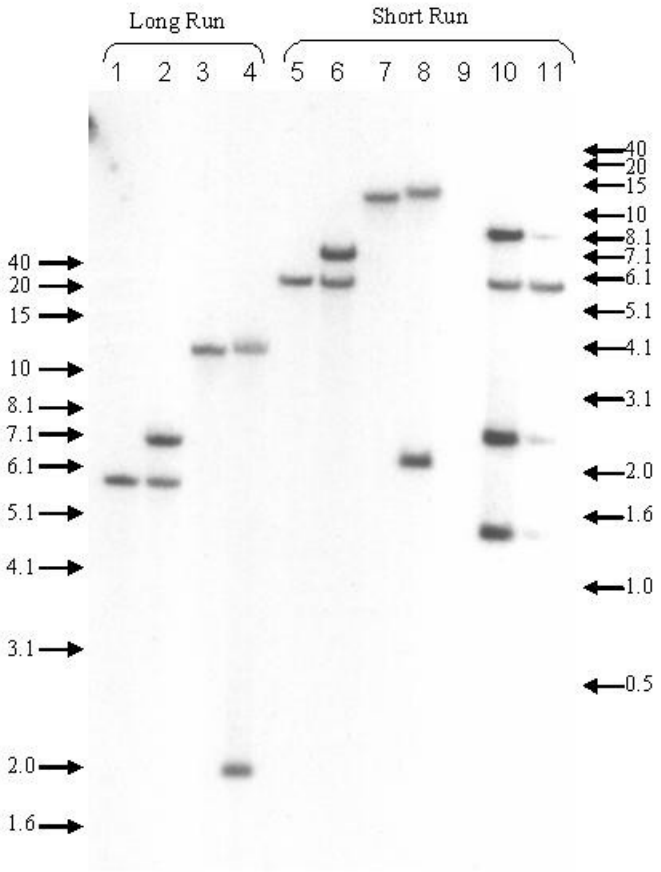


Figure 13. Southern Blot Analysis of MON 87769 with Right Border/P-7sα′ Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the right border and *7sα′* promoter sequence (probe 7, Figure 7). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane 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.**

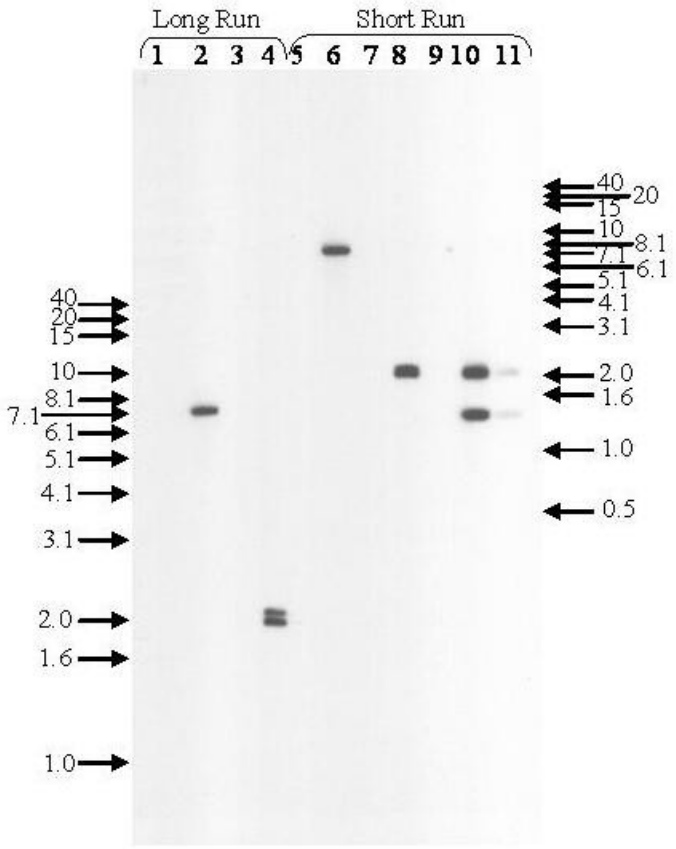


Figure 14. Southern Blot Analysis of MON 87769 with CS-*Pj.D6D* Probe

The blot was hybridized with a ^{32}P-labeled probe that spanned the *Pj.D6D* coding sequence (probe 8, Figure 7). 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.**

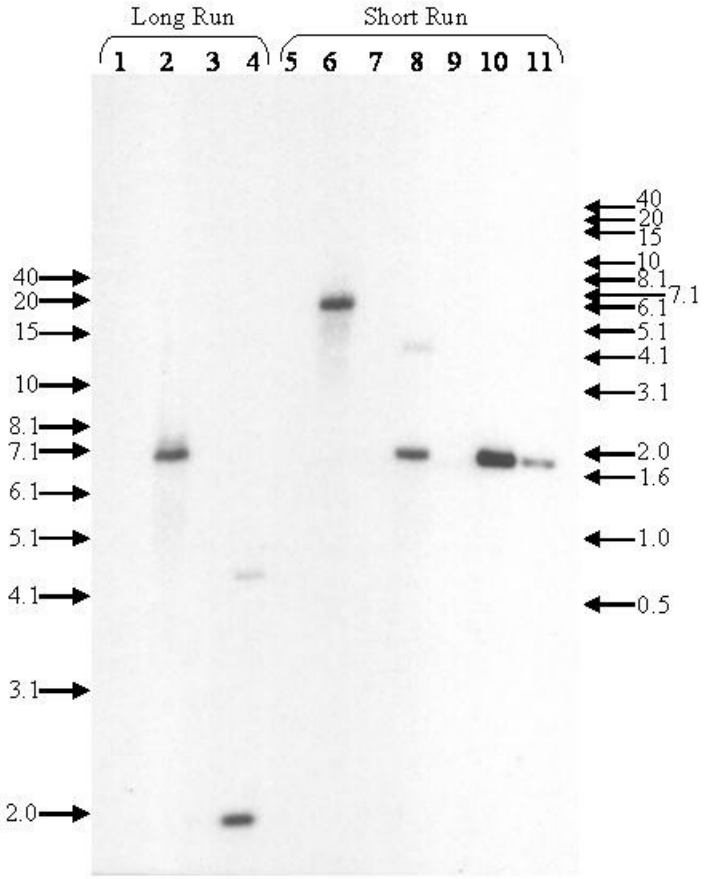


Figure 15. Southern Blot Analysis of MON 87769 with T-*tml* Probe

The blot was hybridized with a ^{32}P-labeled probe that spanned the *tml* non-translated region (probe 9, Figure 7). 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.**

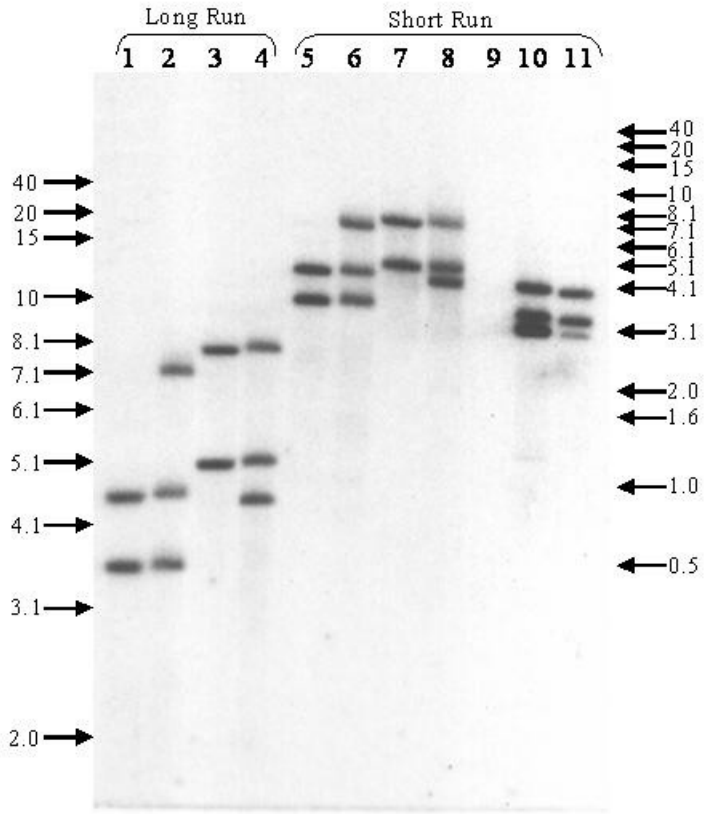


Figure 16. Southern Blot Analysis of MON 87769 with P-*7sα* Probe
The blot was hybridized with a ^{32}P-labeled probe that spanned the *7sα* promoter sequence (probe 10, Figure 7). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

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.

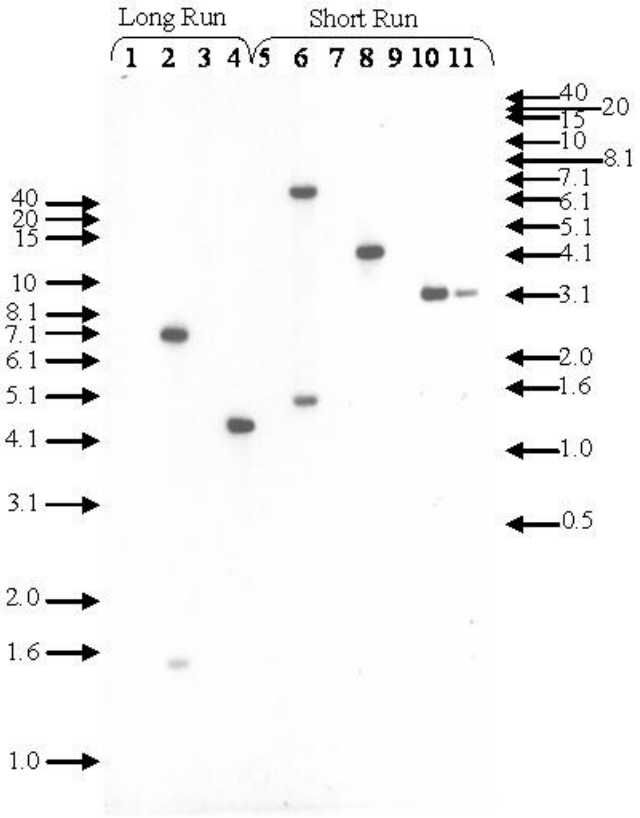


Figure 17. Southern Blot Analysis of MON 87769 with CS-*Nc.Fad3* Probe

The blot was hybridized with a ^{32}P-labeled probe that spanned the *Nc.Fad3* coding sequence (probe 11, Figure 7). 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.**

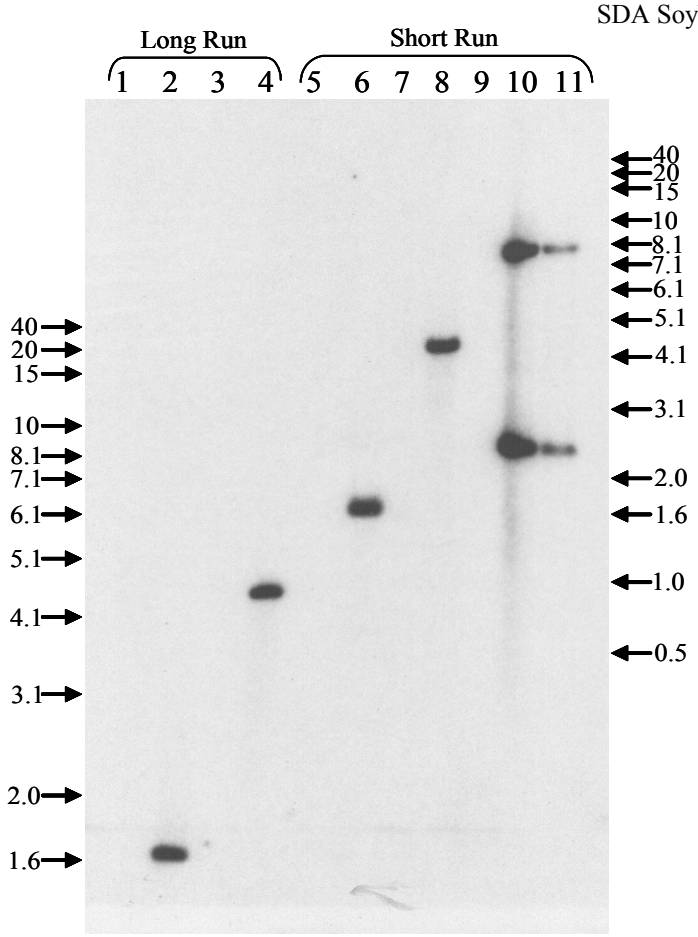


Figure 18. Southern Blot Analysis of MON 87769 with T-*E9*/Left Border Probe

The blot was hybridized with a ^{32}P-labeled probe that spanned the *E9* non-translated region and left border (probe 12, Figure 7). 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.

v) 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 (Figure 19). 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. 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 9. The DNA sequence of the insert and the adjacent genomic regions is presented in Appendix 1 as confidential business information (CBI).

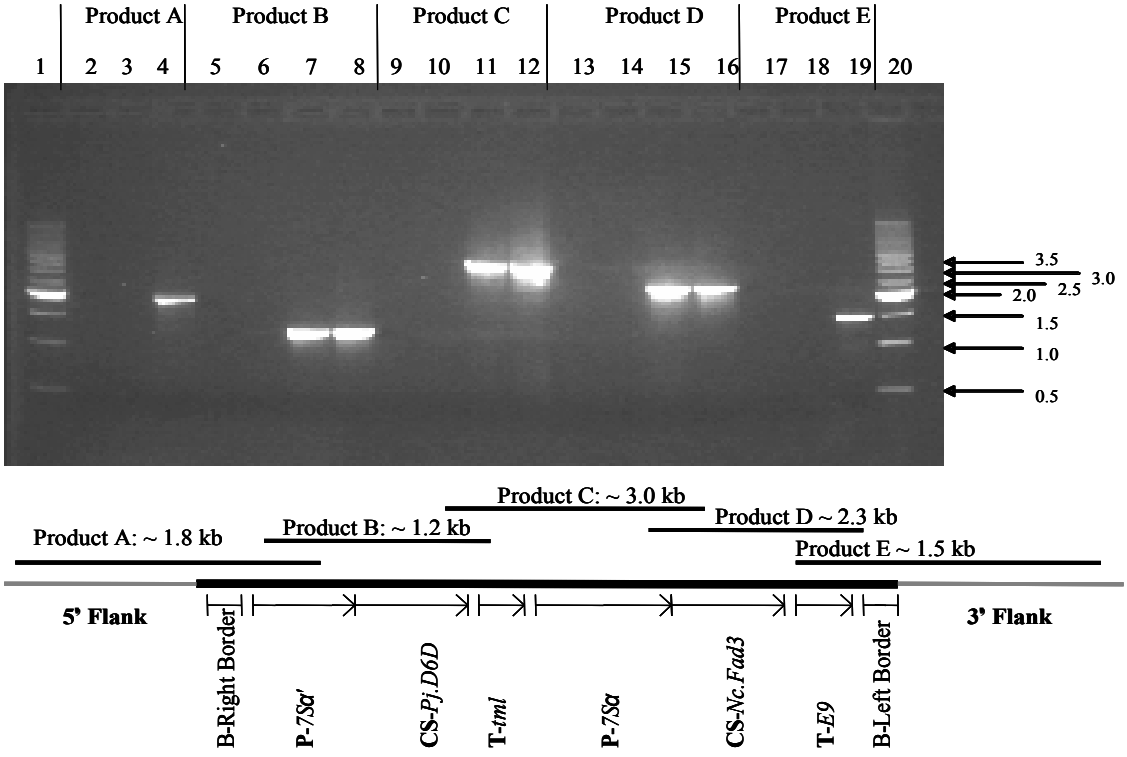


Figure 19. PCR Analysis Across the Insert in MON 87769

PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 87769 were performed on MON 87769 genomic DNA extracted from leaf tissue (Lanes 4, 7, 11, 15, and 19). Lanes 3, 6, 10, 14, and 18 contain reactions with conventional soybean DNA while lanes 2, 5, 9, 13, and 17 are reactions containing no template DNA. Lanes 8, 12, and 16 contain reactions with PV-GMPQ1972 control DNA. Lane 1 and 20 contain Invitrogen 500 bp DNA ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87769 that appears at the bottom of the figure. Three to ten microliters of each of the PCR products were loaded on the gel. Lane designations are as follows:

Lane
1: Invitrogen 500 bp DNA ladder
2: No template DNA control
3: Conventional soybean DNA
4: MON 87769
5: No template DNA control
6: Conventional soybean DNA
7: MON 87769
8: PV-GMPQ1972 plasmid
9: No template DNA control
10: Conventional soybean DNA
11: MON 87769
12: PV-GMPQ1972 plasmid
13: No template DNA control
14: Conventional soybean DNA
15: MON 87769
16: PV-GMPQ1972 plasmid
17: No template DNA control
18: Conventional soybean DNA
19: MON 87769 genomic DNA
20: Invitrogen 500 bp DNA ladder

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

vi) Insert Junction Open Reading Frame Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5′ and 3′ junctions of the MON 87769 inserted DNA were performed using a bioinformatic comparison strategy. The purpose of the assessment is to evaluate the potential for novel open reading frames that may produce a protein(s) with similarity to known allergens and toxins. DNA sequence spanning the 5′ and 3′ junctions of the MON 87769 insertion site was analyzed for translational stop codons (TGA, TAG, TAA) and all open reading frames originating or terminating within the MON 87769 insertion site were translated using the standard genetic code from stop codon to stop codon. Five sequences of eight amino acids or greater in length spanning the 5′ junction, and six sequences of eight amino acids or greater in length spanning the 3′ junction were identified and used as search sequences for FASTA comparisons against the AD_2009, TOX_2009 and PRT_2009 databases. In addition, the eleven sequences were searched for eight amino sequences that match proteins in the AD_2009 database.

Results of the FASTA sequence alignments demonstrated that no inferred structurally-relevant similarity was observed between any known allergen, toxin, or bioactive protein and the eleven putative polypeptides. Results from the eight amino acid sliding window search also demonstrated the lack of immunologically-relevant matches between any of the putative polypeptides in the AD_2009 database. Bioinformatic analyses performed using the eleven query sequences support the conclusion that even in the highly unlikely occurrence that any of the putative junction polypeptides were translated they would not share a sufficient degree of sequence similarity with known allergens or toxins. Therefore, there is no evidence for concern regarding health implications of the cross-junction putative polypeptides in MON 87769.

vii) Assessment of Open Reading Frames Contained in the *Pj.D6D* and *Nc.Fad3* Coding Sequences

Although DNA replication, DNA transcription and mRNA translation are of extremely high fidelity, a mutation in rare circumstances may lead to the potential translation of mRNA on reading frames other than those defined by the intended translation start codon. In such instances, a novel protein may be produced. Due to the spontaneous nature of mutations, it is not possible to determine when or where in a coding sequence such a mutation may occur. In order to assess potential risks, bioinformatic analyses were performed using the AD_2009, TOX_2009 and PRT_2009 databases on the entire T-DNA sequence in MON 87769. Results demonstrate that putative proteins derived from alternative open reading frames of the *Pj.D6D* and *Nc.Fad3* coding sequences as well as all other sequence contained in the MON 87769 T-DNA are unlikely to be allergenic, toxic or otherwise exhibit adverse biological activity.

Using the translation of frames 1 through 6 of the MON 87769 T-DNA for FASTA search queries of the AD_2009 database, no alignment met or exceeded the Codex Alimentarius (2003) FASTA alignment threshold of 35% identity over an 80 amino acid sequence. Furthermore, when translation of frames 1 through 6 of the MON 87769 T-

DNA were used for searches of the TOX_2009 and PRT_2009 databases, no significant sequence alignments were observed with toxins or other biologically-active proteins.

Using an eight amino acid sliding window search, a single eight amino acid polyserine tract was found in frame 2 of the T-DNA translation. This polyserine mitif is contained in the *Nc.Fad3* coding sequence and is described in Section 2.5 i) ii.

None of the possible open reading frames in the MON 87769 T-DNA which includes the *Pj.D6D* and *Nc.Fad3* coding produced significant sequence alignments with the AD_2009, TOX_2009 and PRT_2009 databases, confirming that any putative proteins are unlikely to be allergenic, toxic or biologically-active

d) Derivation of Line and Generational Stability

i) 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 8). For reference, the breeding history of MON 87769 is presented in Figure 20. The expected Southern hybridization DNA banding pattern for these analyses is summarized in Table 5.

Generational Stability of the Insert

Conventional soybean DNA was used as a control to determine the endogenous background hybridization of MON 87769. As shown in Figure 21, conventional soybean DNA digested with a combination of *Lgu* I and *Bst*X I (lane 4) produced several hybridization signals. This is expected as 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 were 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 21a) 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 5). 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

21b) 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 5). 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 21a) 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 21b) 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 non-specific hybridization spots exists in lanes 4, 8, and 9. These non-specific hybridization spots are faint and do not affect the results of the Southern blot. The observed results are consistent with the results presented in Figure 10a and 10b, and confirm that the single copy of the insert in MON 87769 is stably maintained across multiple generations of its breeding history.

Confirmation of the Absence of PV-GMPQ1972 Backbone Sequence

The four generations of MON 87769 utilized to assess generational stability were also 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 7).

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 22). 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 7) 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 22). 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 7) produced the expected size bands of approximately 2.3 and 7.9 kb (lane 1; refer to Table 5). 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 7) 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 11. This indicates that MON 87769 does not contain any detectable backbone sequence from the transformation vector PV-GMPQ1972.

Confirmation of the Absence of T-DNA II Sequence

The four generations of MON 87769 material utilized to assess generational stability were also 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 7).

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 23). 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 23). 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 5). 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 which is 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 7) 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′ non-translated region sequence, and the left border sequence that are also 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′ non-translated region + left border sequence (lanes 2 and 6, Figure 18). 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. There were no additional fragments observed. The observed results

are consistent with the results presented in Figure 12, and confirm that all five generations of MON 87769 do not contain any additional detectable T-DNA II elements other than those associated with T-DNA I.

ii) Inheritance of the Introduced Traits

The genotypic stability and segregation of T-DNA I in MON 87769, initially demonstrated by Southern blot analysis, 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 24. The R0 plant was self-pollinated to produce R1 seed, which is expected to segregate 1:2:1 (1 homozygote:2 hemizygous: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 hemizygous progeny, the F3 and F4 hemizygous progeny were each predicted to segregate 1:2:1 (1 homozygote:2 hemizygote:1 null segregant) for the insert.

The Chi-square ($\chi 2$) 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 6 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.

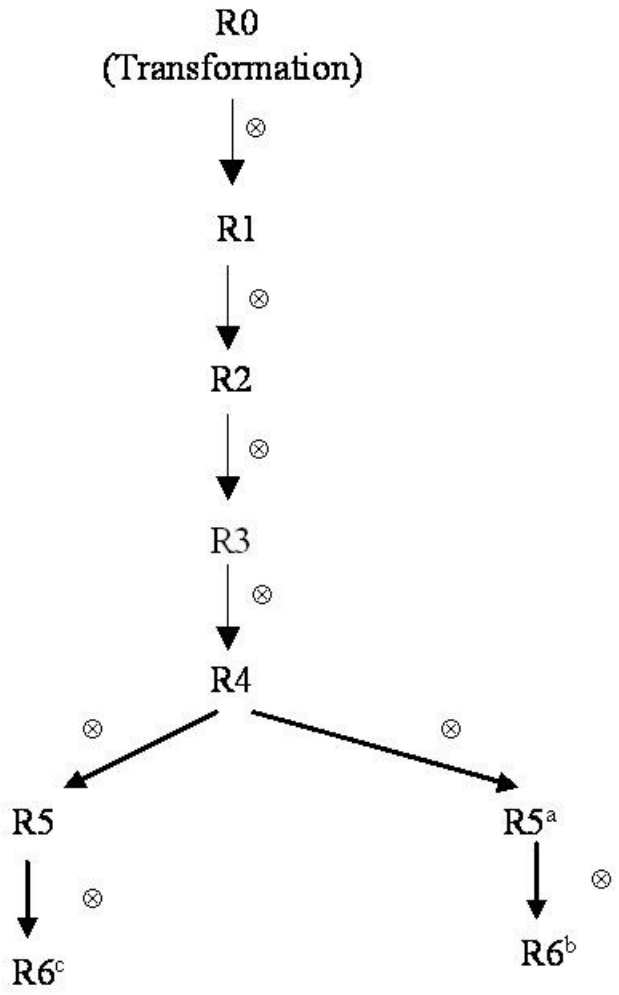


Figure 20. MON 87769 Breeding Diagram.

All generations shown were self-pollinated (⊗). The R1 generation was used for segregation analysis and the selection of plants homozygous for the T-DNA insert. 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.

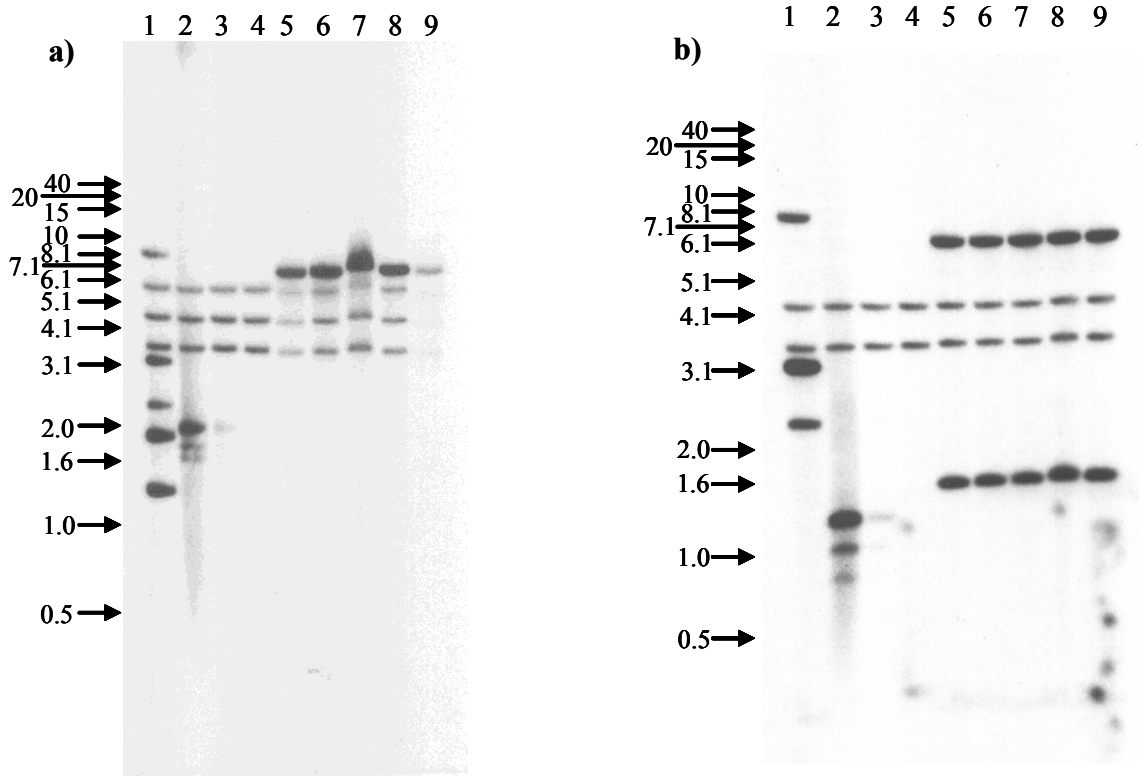


Figure 21. Generational Stability of MON 87769 with T-DNA I Probes

The blot was hybridized with overlapping ^{32}P-labeled probes that spanned the T-DNA (probes 13-18, Figure 8). Each blot was hybridized simultaneously with three of the overlapping probes. Figure 21a was hybridized with probes 13-15 and Figure 21b 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 20. 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: R5a: MON 87769 (*Lgu* I and *Bst*X I)
8: R6c: MON 87769 (*Lgu* I and *Bst*X I)
9: R6b: 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.

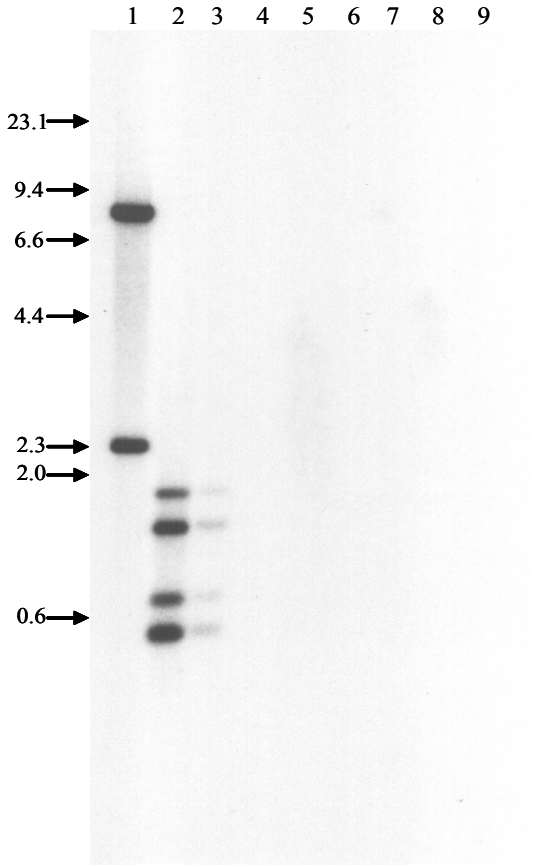


Figure 22. Generational Stability of MON 87769 with PV-GMPQ1972 Backbone Probes

The blot was hybridized with four overlapping ^{32}P-labeled probes that span the entire backbone sequence (probes 1, 2, 3, and 6, Figure 7) 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 20. 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.

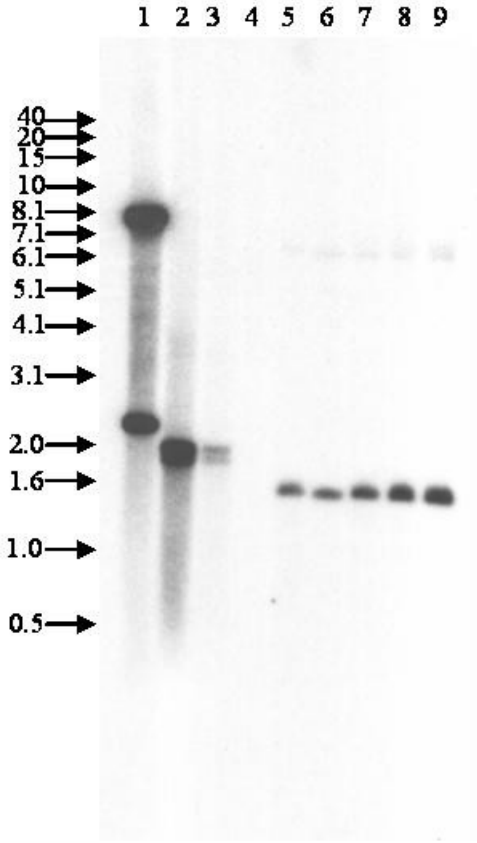


Figure 23. Generational Stability of MON 87769 with T-DNA II Probes

The blot was hybridized with two overlapping 32P-labeled probes that span the entire T-DNA II (probes 4 and 5, Figure 7) 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 20. 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: R5a: MON 87769 (*Lgu* I and *Bst*X I)
8: R6c: MON 87769 (*Lgu* I and *Bst*X I)
9: R6b: 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.

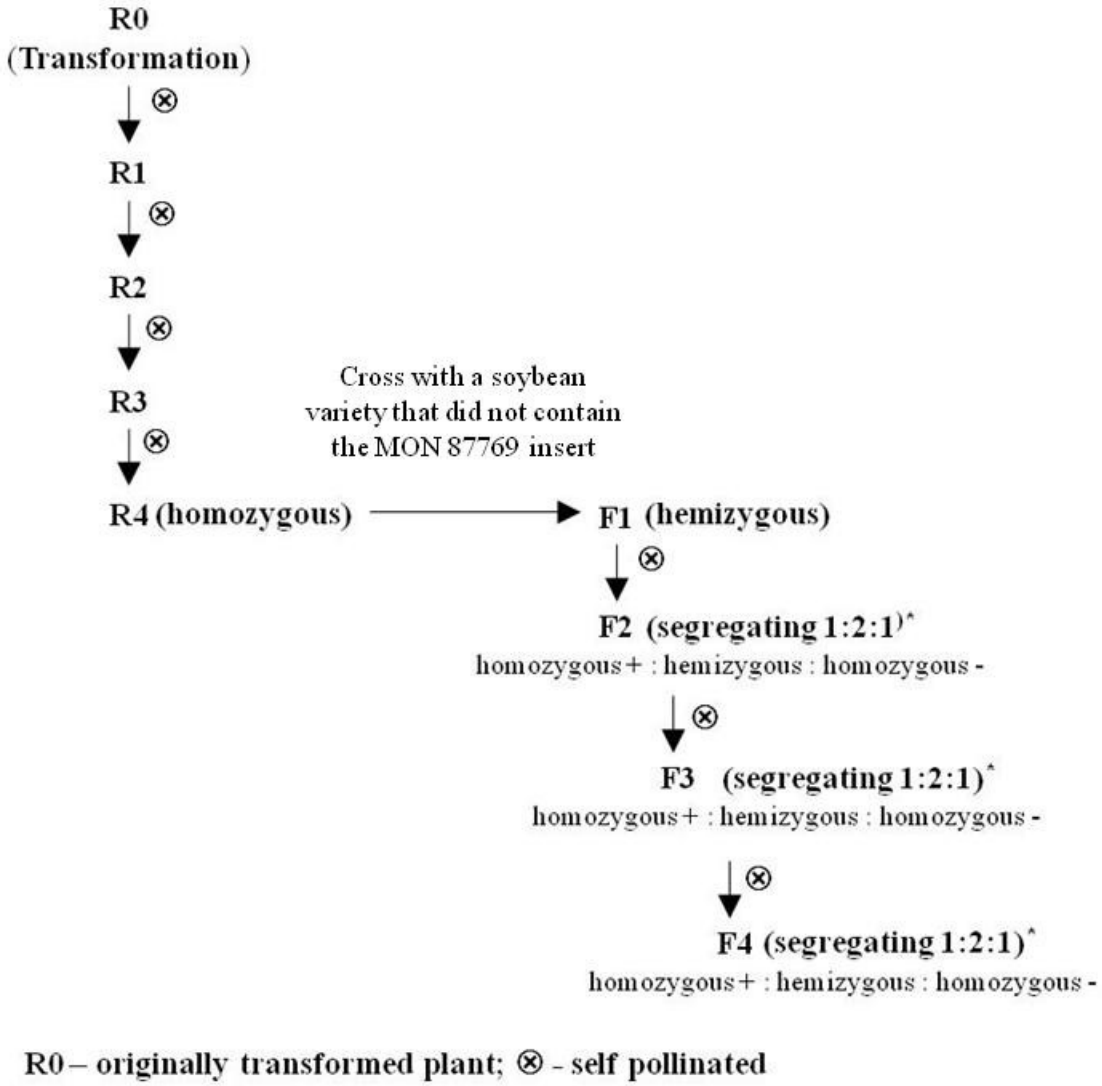


Figure 24. Breeding path for generating segregation data on MON 87769

Table 6. 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.

2.4 Antibiotic Resistance Marker Genes

MON 87769 does not contain gene(s) that encode resistance to any antibiotic marker(s). Molecular characterization data presented demonstrate the absence of the *aad*A antibiotic resistant marker in MON 87769.

a) Clinical / Veterinary Importance

Not applicable.

b) Viability

Not applicable.

c) Presence in Food

Not applicable.

2.6 Characterisation of Novel Proteins

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. 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 (Table 9 and 10). 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 their 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 is 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.

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

a) Description 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 is often 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 is usually studied in the context of an intact membrane system (Reed et al., 2000; Sayanova et al., 2001). Due to the tremendous difficulty 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. Our understanding of the biochemistry of the integral membrane desaturases is largely inferred from spectroscopic and structural studies conducted with proteins that share a common conserved chemical mechanism such as the soluble Δ9 acyl-ACP desaturase proteins found in plant plastids (Lindqvist et al., 1996).

Confirmation of the function of the NcΔ15D and PjΔ6D proteins *in vivo* has been provided by several pieces of evidence. MON 87769 accumulates SDA as well as GLA. These fatty acids are novel in the context of the soybean lipid biosynthetic pathways and confirm a metabolic signature for the activity of the NcΔ15D and PjΔ6D in MON 87769. Furthermore, a yeast expression system was utilized to functionally characterize the NcΔ15D and PjΔ6D proteins. The *Pj.D6D* and *Nc.Fad3* genes were cloned into a yeast expression vector and transformed into *Saccharomyces cerevisiae* for heterologous expression and whole-cell substrate feeding studies. Yeast was identified as a good system for functional evaluation of desaturases (Reed et al., 2000; Sayanova et al., 2001) for several reasons: 1) yeast contain an extensive ER membrane system and they express cytb5 and b5R enzymes, 2) there are commercially available yeast expression vectors and cell strains, and 3) the fatty acid composition of *S. cerevisiae* is composed of only four fatty acids (palmitic acid, stearic acid, palmitoleic acid, and oleic acid). Results of the characterization of the NcΔ15D and PjΔ6D proteins in yeast is described below. In addition, the functionality of the PjΔ6D and NcΔ15D proteins has also been tested *in vitro* using crude extracts of immature SDA soybean seed, as described below.

PjΔ6D Protein

The PjΔ6D desaturase protein expressed in MON 87769 is identical to the native protein produced by *Primula juliae*. The protein consists of 446 amino acids with a calculated molecular mass of 50,985 Daltons 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 25). 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 (Figure 25 and 26). As in other Δ6 desaturases the first His residue of the third His-box is substituted in PjΔ6D with glutamine (QXXHH instead of HXXHH) and therefore, 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 26) 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 25. Deduced Amino Acid Sequence of PjΔ 6D from PV-GMPQ1972

Three histidine-motifs are represented by black shaded blocks while the heme binding motif is shaded in light gray. Notice that the first histidine in the third histidine motif has been replaced by glutamine (position 371).

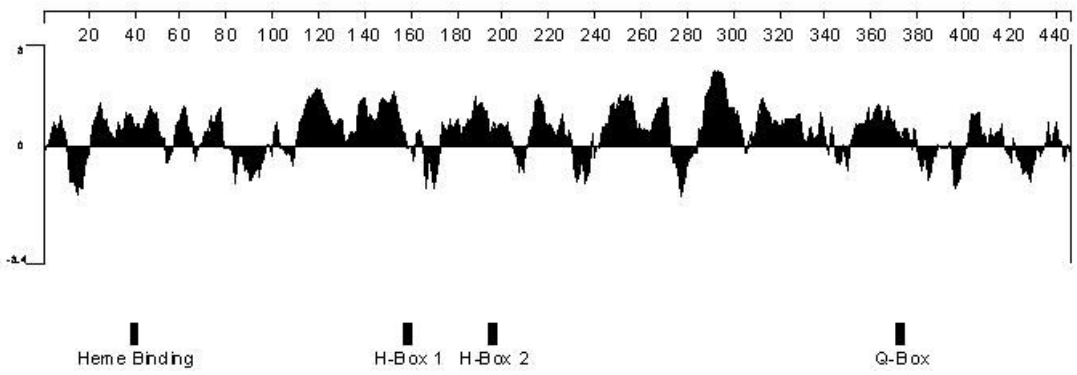


Figure 26. Hydropathy plot of the Deduced Amino Acid Sequence of PjΔ6D

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 compose 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), palmitoelic 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 composed 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.

NcΔ15D Protein

The NcΔ15D desaturase 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 Daltons 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 27). 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 the PjΔ6D, the NcΔ15D proteins shows multiple membrane-spanning regions (Figure 28) 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 27. Deduced Amino Acid Sequence of NcΔ15D from PV GMPQ1972

Three histidine-motifs are represented by shaded blocks.

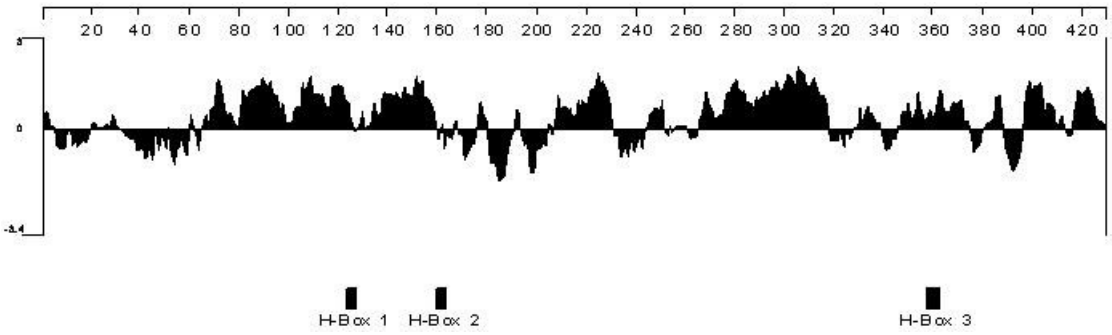


Figure 28. Hydropathy Plot of the Deduced Amino Acid Sequence of NcΔ15D

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 above. *S. cerevisiae* was grown in the absence or presence of various 18 and 20 carbon endogenous fatty acids. In the absence of the endogenous fatty acids, the yeast cells produced ALA from oleic acid. The NcΔ15D protein is the only known enzyme capable of catalyzing this reaction. Results also demonstrated that all of the 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 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 contains an endogenous Δ15 desaturase; therefore, this Δ15 desaturation reaction occurs to some extent in the conventional control soybean seed. The fatty acid composition of yeast containing *Nc.Fad3* vector and MON 87769 seed demonstrate the function of the NcΔ15D protein encoded by the *Nc.Fad3* gene.

b) Characterization of the MON 87769-Produced PjΔ6D Protein

i) 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 31) and in immunoblot analysis with PjΔ6D antibody (Figure 30) resulted in the expected sequence for the PjΔ6D protein (Table 7). 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 the removal of the N-terminal methionine 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.

ii) 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 Dalton (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 29), 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.

iii) 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 30, 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 30 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.

Table 7. 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.
[3]The N-terminal methionine was removed by the action of methionine aminopeptidase during co-translation, 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 29. 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. 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.

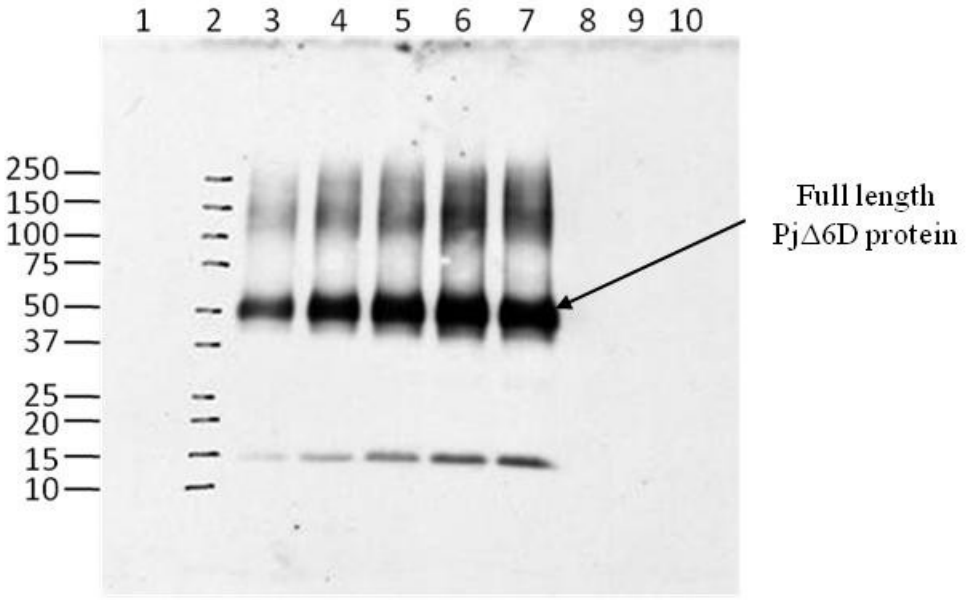


Figure 30. Western Blot Analysis of MON 87769-Produced PjΔ6D Protein

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 enhanced chemiluminescence (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	-

iv) PjΔ6D Protein Molecular Weight

The molecular weight of the MON 87769-produced PjΔ6D protein was determined using SDS-PAGE (Figure 31). 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%.

v) 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 polyvinylidene fluoride (PVDF) membrane, and glycosylation analysis was performed to detect carbohydrate moieties on the proteins (Figure 31). The positive control, transferrin, was detected at the expected molecular weights of ~75 kDa in a concentration-dependent manner (Figure 32, Lanes 2-5). No detectable signal was observed at the expected molecular weight of approximately 46 kDa for the PjΔ6D protein (Figure 32, 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 32) 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.

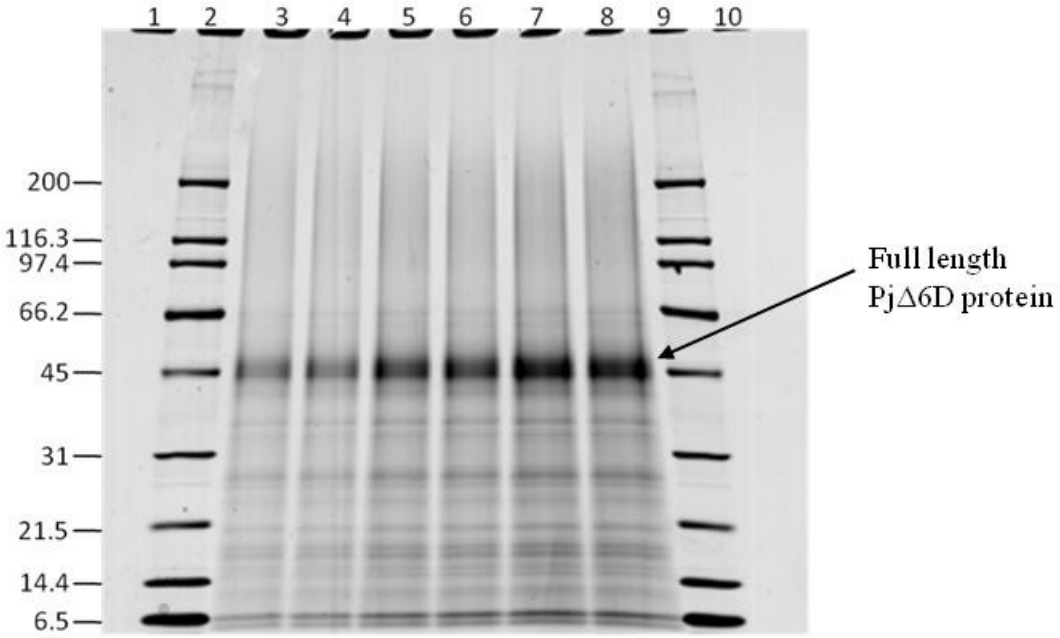


Figure 31. SDS-PAGE of the MON 87769-Produced PjΔ6D Protein

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

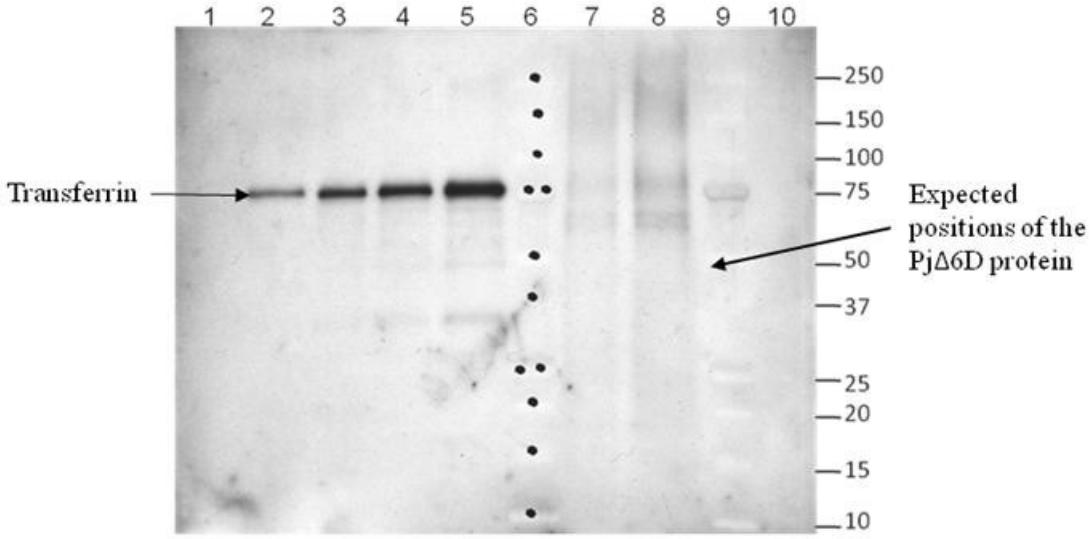


Figure 32. Glycosylation Analysis of the MON 87769-Produced PjΔ6D Protein

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) Characterization of the MON 87769-Produced NcΔ15D Protein

i) 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 gels resulted in the sequence expected for the NcΔ15D protein (Table 8). 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.

ii) 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 Dalton (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 33), 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.

iii) 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 in Figure 34, a predominant immuno-reactive band was observed at the expected molecular weight of approximately 46 kDa (Figure 34, lanes 2-6). As expected, the immuno-reactive signal increased with increasing amount of protein loaded.

The immuno-reactive profiles shown in Figure 34 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 NcΔ15D protein; as the smear area is immuno-reactive with anti- NcΔ15D antibody.

In addition, two minor bands with molecular weights of approximately 27 kDa and 30 kDa were recognized by the antibody (Figure 34, lanes 2-6). Most likely 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.

Table 8. 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 PjΔ6D Sequence[1,2]	→	M	A	V	T	T	R	S	H	K	A	A	A	A	T	E	P
			|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence	→		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.
[3]The N-terminal methionine was removed by the action of methionine aminopeptidase during co-translation, therefore, was not observed.

```
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 33. 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. Boxed 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.

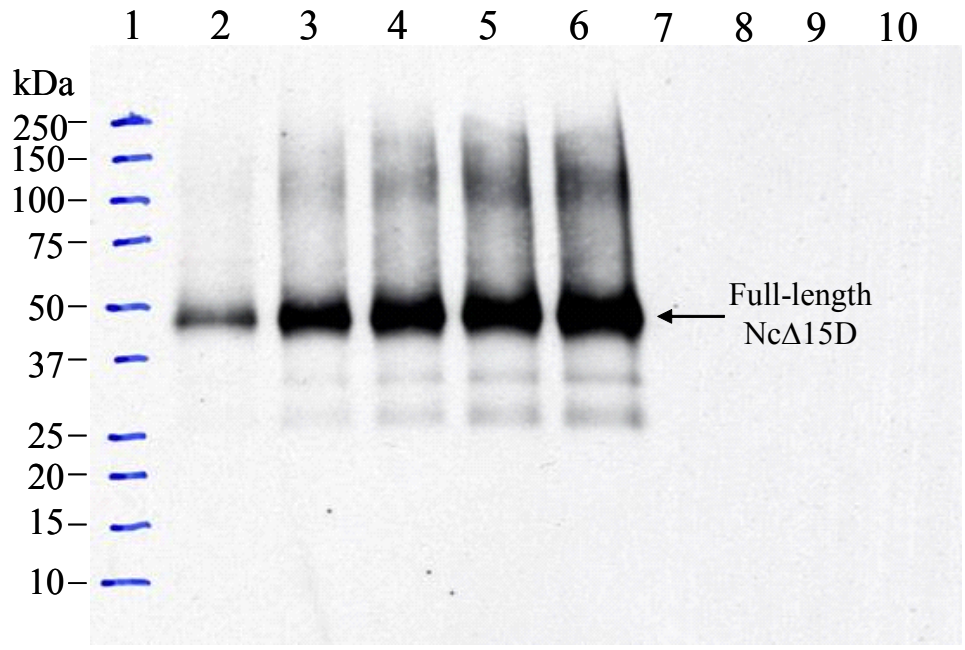


Figure 34. Western Blot Analysis of MON 87769-Produced NcΔ15D Proteins

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). 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	

iv) NcΔ15D Protein Molecular Weight

The molecular weight of the MON 87769-produced NcΔ15D protein was determined using SDS-PAGE (Figure 35). The MON 87769-produced NcΔ15D protein migrated with an apparent molecular weight of 46.2 kDa. The average purity of the full length NcΔ15D protein was estimated to be 74%.

v) 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 Veluraj, 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. 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 36). The positive control, transferrin, was detected at the expected molecular weights of ~75 kDa in a concentration-dependent manner (Figure 36, Lanes 2-5). No detectable signal was observed at the expected MW of approximately 46 kDa for the NcΔ15D protein (Figure 36, lanes 7-8).

Two faint bands were detected at ~70 kDa in lanes 7-8 containing purified NcΔ15D protein (Figure 36, 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 approximately 74%, that no signal was observed at the expected MW for NcΔ15D protein of ~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.

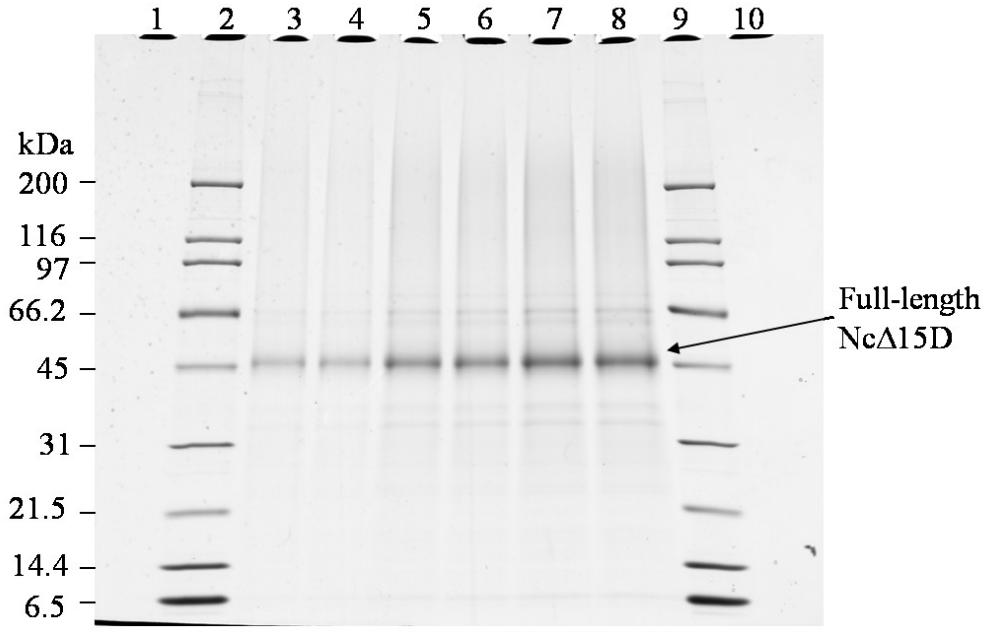


Figure 35. SDS-PAGE of the MON 87769-Produced NcΔ15D Protein

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	

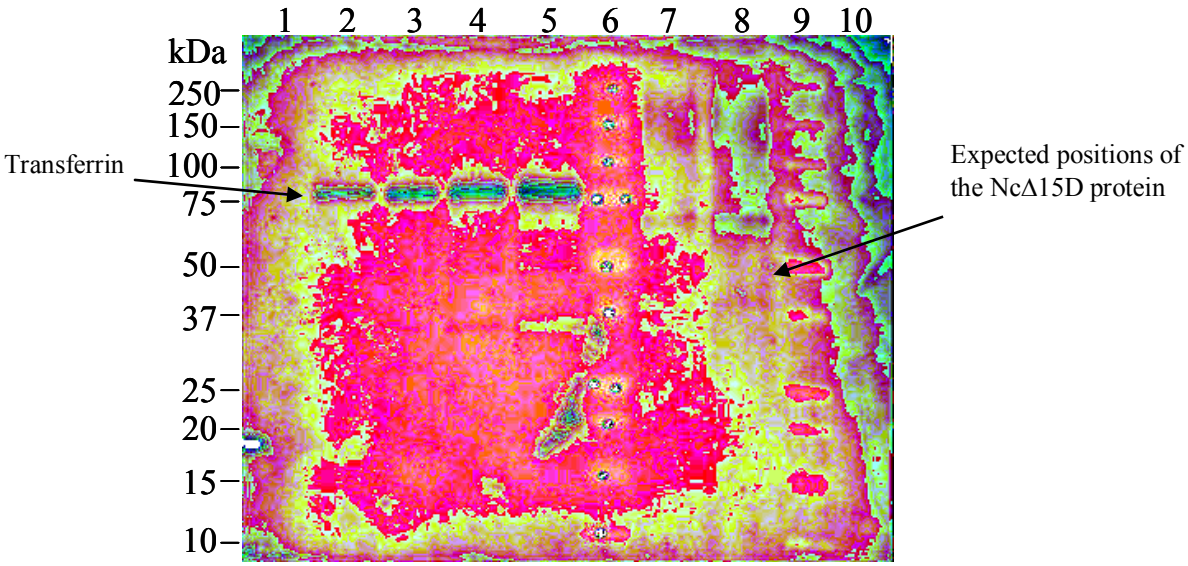


Figure 36. Glycosylation Analysis of the MON 87769-Produced NcΔ15D Protein

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. Chemi-luminescent 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	

d) Expression of PjΔ6D and NcΔ15D Proteins in MON 87769

PjΔ6D and NcΔ15D are integral membrane proteins and detection of such proteins 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, 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 blots. For western blot analysis, 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.

Protein Expression Study 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 aerial 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 or 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 grams of tissue. Mature seed was harvested from all plants of each plot yielding 3.0 to 8.1 lbs 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 procedure according to applicable standard operating procedures (SOP).

The levels of PjΔ6D and NcΔ15D proteins obtained from immunoblot analysis were determined in OSL-1 through OSL-4, forage, root, immature and mature harvested seed and the results are summarized in Table 9 and 10. 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 was also measured and protein levels were then 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 assay (Table 9 and 10). 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 9). 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 10). The PjΔ6D and NcΔ15D proteins were not detected in the conventional control soybean A3525.

Table 9. Summary of PjΔ6D Protein levels in Tissue 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, arithmetic mean and standard deviation (SD) were calculated for each tissue type across all sites (n=15 for all tissue).

[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 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 10. Summary of NcΔ15D Protein Levels in Tissue 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, arithmetic mean and standard deviation (SD) were calculated for each tissue type across all sites (n=15 for all tissue).

[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.

e) Non-expression

Not applicable

f) History of Human Consumption

i) PjΔ6D Protein

PjΔ6D possesses a strong safety profile. It's donor organism, *Primula*, is a common garden plant that is an ingredient in certain herbal formulations. PjΔ6D shares homology with a variety of proteins that themselves have a history of safe use. PjΔ6D does not share any meaningful homologies with known toxic or bioactive proteins. Finally PjΔ6D did not exhibit any signs of toxicity when administered to mice via oral gavage. This weight of evidence supports the conclusion that PjΔ6D is unlikely to exhibit toxic effects when consumed in food and feed prepared from MON 87769.

Safety of the PjΔ6D Donor Organism *Primula juliae*

Primula is a member of the large genus of plants commonly known as Primrose. They are familiar as a popular garden plant in colder climates. These plants contain significant levels of SDA in their leaves, presumably to improve the structure and function of chloroplast membranes in their native cold climates. A number of species of Primula (family *Primulaceae*) are well-known in folk medicinal practices in Bulgaria, Romania, the Czech Republic, Germany, and other European countries (Bown, 1995). The whole plant can be utilized for medicinal purposes, including flowers, seeds, leaves, and roots. As an expectorative, *Primula* species are widely used for bronchitis, blue cough, pneumonia, bronchial asthma, flu, rheumatic pains, etc. (Chiej, 1984). *Primula officinalis,* commonly known as Cowslip, has a long history of medicinal use and has been used to treat conditions involving spasms, cramps, paralysis and rheumatic pains (Bown, 1995). Cowslip root is high in saponin (Znidarsic et al., 1999) and is known for its anti-inflammatory, sedative, expectorant, and analgesic properties and probably is the biochemical basis for the herbal claims (Seigler, 1998). Primrose plants are also used as a food source. Young leaves, raw or cooked, are utilized in soups and fresh or dried leaves are used as a tea substitute (Facciola, 1990). Recently the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency authorized the use of flowers and roots of *Primula veris* (L.) and *Primula elatior* (L.) species for preparation of teas to treat cough associated with cold[1]. Reports of contact dermatitis have been published for several varieties of Primula (Aplin and Lovell, 2001; Higuchi et al., 2000). However, the allergic response is triggered by a non-protein allergen called primin that is secreted from leaf and stem trichomes (Horper and Marner, 1996). Thus, the safety of the donor organism for the Δ6 desaturase protein in MON 87769 is well established.

Similarity of PjΔ6D to Other Proteins with a History of Safe Use and Consumption

The Δ6 desaturase protein from *Primula* is similar to several proteins that are ubiquitous in the human and animal diet and directly consumed in many common foods. The PjΔ6D protein belongs to the subfamily of so-called "front-end desaturases" that introduce a

[1] http://www.emea.europa.eu/htms/human/hmpc/monograph/primulae_radix.htm

double bond between an existing double bond and the carboxyl end of the fatty acids. The subfamily of "front-end desaturases" include Δ4, Δ5, Δ6, and bifunctional Δ6/sphingolipid Δ8 desaturases (Hashimoto et al., 2008). A search of publicly available databases using the PjΔ6D protein sequence has shown that the PjΔ6D protein present in MON 87769 shares amino acid identity to other naturally occurring "front-end" desaturase proteins found in certain food crops (Table 11). Table 11 shows only sequences with the same or similar annotated regiospecificity to the PjΔ6D protein and amino acid identity ranges from 19.5 to 64.8%. The homologues of PjΔ6D are found in plant, fungi, vertebrate and cyanobacterial species. All integral membrane desaturases show strong conservation of the three histidine box motifs required for binding two iron atoms at the catalytic center (Hashimoto et al., 2008) and demonstrate similar functions and specificity. It is suggested that the integral membrane desaturase proteins should have a common overall topology as their histidine motifs must be brought into close proximity to form the di-iron cluster at the active site of these enzymes.

Desaturases that are highly similar to the PjΔ6D proteins (~64% identity) are found in the plant species *Echium plantagineum* (echium) and *Borago officinalis* (borage). Both of these species have been used to produce oils for human consumption. The oils produced by these species (that are relatively high in GLA and/or SDA) have been studied extensively for their anti-inflammatory effects on leukotriene and prostaglandin biosynthesis (Fan and Chapkin, 1998), and are sold as cold-pressed oils for use as dietary supplements (EU Food Laws Commission Decision, 2008/558/EC). Additionally, the flowers of *Echium* spp. have been consumed as medicinal plants in countries such as Iran (Hooper, 1937). Other plants that contain high levels of GLA in their oil, and, therefore, Δ6 desaturase, is *Oenothera* spp., commonly known as evening primrose (Sayanova et al., 1997). Evening primrose plants have been used as ornamental plants, a food source, and as medicinal herbs for a long period of time. *Oenothera* spp. was first introduced into gardens in the eighteenth century as a fleshy root-vegetable (yellow lamb's lettuce) and was cultivated for food in Germany (Facciola, 1990). Since then, it has also been used as a medicinal agent. The PjΔ6D protein shares ~57% amino acid identity with Δ8 desaturase from *Brassica napus* (Table 11), which includes vegetables such as rutabaga and kale as well as canola. Edible plants in the family *Brassicaceae* (also called *Cruciferae*) are termed cruciferous vegetables and represent a major food crop worldwide.

The PjΔ6D protein shows sequence homology to other fungal and cyanobacterial desaturases (Table 11). The PjΔ6D protein shows ~28% sequence identity to *Mortierella alpina* Δ6 desaturase. *M. alpina* is currently used for the commercial production of arachidonic acid for fortification of baby food. The PjΔ6D shows ~20% sequence identity to the Δ6 desaturases from two species of cyanobacteria (*Arthrospira platensis* and *Synechosystis* spp.). In non-western cultures, cyanobacteria have been part of the human diet for centuries (Gantar and Svircev, 2008). More recently, food products containing the cyanobacteria Spirulina (*A. platensis*) have been sold worldwide as supplements (Belay et al., 1993).

Finally, the PjΔ6D protein shows approximately 25% identity to the Δ6 desaturase from two widely consumed fresh water fish species: *Oncorhynchus mykiss* (rainbow trout) and *Cyprinus carpio* (common carp). Thus, consumption of related Δ6 desaturases has occurred from the consumption of fresh water fish species.

These data indicate that the PjΔ6D protein expressed in MON 87769 share sequence identity with desaturases found in common food sources. Homologues are found in plant, vertebrate, fungal and cyanobacterial food sources and have been consumed for many years with no history of adverse health effects establishing a history of safe exposure for this protein.

Structural Similarity of PjΔ6D to Known Toxins or Other Biologically-Active Proteins

Bioinformatic analyses of amino acid sequences provide an additional assessment of the potential for protein toxicity. The goal of the bioinformatic analysis is to ensure that the introduced protein does not share homology to known toxins or antinutritional proteins associated with adverse health effects. Results for PjΔ6D further confirm that this protein is not likely to exhibit toxic effects.

Potential structural similarities shared between the PjΔ6D protein and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the PjΔ6D protein sequence were performed with the TOX_2009 database to identify possible homology with proteins that may be harmful to human and animal health. The TOX_2009 database is a subset of 7,651 sequences derived from the protein database (PROTEIN) consisting of publicly available protein sequences from GenBank (GenBank protein database, release 169.0, December 16, 2008). The 7,651 sequence subset was selected using a keyword search of the public databases and filtered to remove non-toxin proteins. Initially, all sequence descriptions contained in header lines and the associated protein sequence derived from the PRT_2009 database were keyword screened using all possible combinations of upper and lower case characters spelling the words “toxic” and “toxin.” The resulting 11,151 sequences and their respective descriptions were then filtered to exclude several terms used in combination with “toxic” or ”toxin”; such as “synthetic,” “anti,” “putative,” “like,” etc. to remove non-toxin protein sequences.

An expectation score (E-score) acceptance criteria of $<1\times10^{-5}$ for any alignments were used to identify proteins from the TOX_2009 database with potential for significant shared structural similarity and function with PjΔ6D. The E-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger E-score indicates a lower degree of similarity between the query

sequence and the sequence from the database. Typically, alignments between two sequences will need to have an E-score of 1×10^{-5} or smaller to be considered to have significant homology. The results of the search produced no alignments below an E-score of 1.0 and therefore, there were no alignments with shared significant similarity with known toxins.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the PjΔ6D protein and any known toxic or other biologically active proteins that would be harmful to human or animal health.

Acute Oral Toxicity Studies with the PjΔ6D Protein

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond et al., 2008a; Hammond et al., 1998; Sjoblad et al., 1992). The few exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which typically require a short-term (2-4 week) feeding study to manifest toxicity (Liener, 1994). The amino acid sequence of the PjΔ6D desaturase protein produced in MON 87769 is not similar to any of these anti-nutritional proteins or to any other known mammalian protein toxin. Therefore, for the protein safety evaluation an acute administration of a single dose of the protein is considered to be an appropriate and sufficient test to confirm the lack of mammalian toxicity of the PjΔ6D protein and to establish a NOAEL (Pariza and Foster, 1983; Pariza and Johnson, 2001).

The protein used in these studies were extracted from MON 87769 immature seed and thoroughly characterized. Purification of integral membrane desaturases requires removal of membranes and replacement of the lipids surrounding the protein's hydrophobic membrane-spanning regions with the appropriate detergent that will keep the protein in solution. Membrane proteins tend to aggregate and are difficult to concentrate. To utilize PjΔ6D purified from immature soybean seed as the test material for an acute mouse gavage study, the level of the detergent was reduced by buffer exchange to the minimal level that is necessary to keep the protein in solution. After buffer exchange, the protein was concentrated to the highest attainable level. The dosage administered to mice during the study was the highest that could be practically given based on the protein's properties and its low expression in mature seed.

To assess acute toxicity, PjΔ6D was administered at a single dose of 4.7 mg/kg of body weight (BW) to 10 male and 10 female CD-1 mice. Following dosing, all mice were observed once daily (twice on day of dosing) for signs of mortality or toxicity. Food consumption was measured on Days 0, 7 and 14. Body weights were measured prior to dosing and on study Days 0, 7 and 14. All animals were sacrificed on Day 14 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the NOAEL for the PjΔ6D protein was considered to be 4.7 mg/kg BW, the highest possible dose tested. This dose was several orders of magnitude higher than conservative estimates for human exposure to the PjΔ6D protein from consumption of MON 87769 (Table 13).

Table 11. Amino Acid Sequence Identity Between MON 87769-Produced PjΔ6D and Other Δ6/Δ8 Desaturase Proteins Present in Consumed Foods or Used in Food Production

Sequences were extracted from publicly available databases. Sequences were aligned and sequence identity was calculated using the MegAlign function of the Lasergene suite of sequence analysis software [version 7.1.0 (44)] (DNASTAR Inc., Madison, WI, USA).

#	Proteins	Common Name	Sequence Identity (%)													
			1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	MON 87769 PjΔ6D	-	100	57.2	56.6	64.3	64.6	64.3	64.8	57.0	28.1	19.5	20.4	26.1	24.7	26.6
2	B_napus_3819708_Δ8	Oilseed rape		100	67.7	60.0	60.0	60.0	62.7	67.3	27.0	20.3	19.8	26.9	26.7	26.4
3	B_officinalis_12002282_Δ8	Borage			100	59.2	59.2	59.2	61.0	73.1	26.5	17.3	18.7	26.1	25.6	28.0
4	B_officinalis_2062403_Δ6	Borage				100	99.8	85.3	66.7	61.4	28.2	20.6	19.0	25.3	24.8	25.5
5	B_officinalis_4102021_Δ6	Borage					100	85.5	67.0	61.4	28.2	20.6	19.0	25.3	24.8	25.5
6	E_plantagin._70795233_Δ6	Echium						100	68.3	61.6	29.4	20.9	19.0	25.1	24.3	24.6
7	C_sinensis_37727301_Δ6	Chinese tea							100	61.6	26.4	16.7	17.2	26.2	25.1	26.2
8	H_annuus_1040729_Δ8	Sunflower								100	28.2	18.1	20.1	27.5	26.6	26.8
9	M_alpina_34221932_Δ6	Fungus									100	19.0	17.4	23.5	23.2	23.1
10	A_platensis_809110_Δ6	Spirulina										100	51.5	19.3	17.8	17.2
11	Synechosystis_L11421_Δ6	Cyanobacteria											100	18.9	17.4	19.2
12	D_rerio_AF309556_Δ6	Zybrafish												100	89.4	67.3
13	C_carpio_AF309557_Δ6	Common carp													100	66.4
14	O_mykiss_AF301910_Δ6	Rainbow trout														100

ii) NcΔ15D Protein

NcΔ15D possesses a strong safety profile. Its donor organism, *Neurospora crassa*, is ubiquitous in the environment and is used to manufacture food in a variety of different world areas. NcΔ15D shares homology with a variety of proteins that themselves have a history of safe use. NcΔ15D does not share any meaningful homologies with known toxic or bioactive proteins. Finally, NcΔ15D did not exhibit any signs of toxicity when administered to mice via oral gavage. This weight of evidence supports the conclusion that NcΔ15D is unlikely to exhibit toxic effects when consumed in food and feed prepared from MON 87769.

Safety of NcΔ15D Donor Organism: *Neurospora crassa*

The fungus, *Neurospora,* is a long standing, widely used model organism in genetics and biological research, with many years of familiarity. Thousands of scientific publications related to *Neurospora* are available. Perkin et al (Perkins and Davis, 2000b) have reviewed the contributions of *Neurospora* to genetics and biology and they have also reviewed the evidence for the general safety of *Neurospora* (Perkins and Davis, 2000a) suggesting that the genus of *Neurospora* is well qualified to be recognized under Food and Drug Administration (FDA) regulations as a GRAS organism. *Neurospora crassa,* while ubiquitous in the environment (Turner et al., 2001) is not associated with any adverse effects to human health. *Neurospora crassa* has been used for food preparation in a number of world regions. It is a major constituent of onchom, a soybean-based press cake, which is consumed daily in Indonesia (Matsuo, 1997). It is used in Brazil to process cassava in preparing a fermented beverage (Park et al., 1982) and present in Roquefort cheese prepared by traditional methods in southern France (Perkins and Davis, 2000a). There is no evidence of food allergy due to oral consumption of *Neurospora crassa* and, therefore, it is considered a non-pathogenic and non-allergenic organism. Thus the safety of the donor organism for the Δ15 desaturase protein in MON 87769 is well established.

Similarity of NcΔ15D to other Proteins with a History of Safe Use and Consumption

The Δ15 desaturase protein from *Neurospora crassa* is similar to several proteins that are ubiquitous in the human diet and directly consumed in many common foods. The NcΔ15D protein belongs to the subfamily of the so-called "omega" desaturases that introduce a double bond between an existing double bond and the acyl end of the fatty acids and include Δ12 and Δ15 desaturases. All members of the "omega" desaturase subfamily are involved in the synthesis of LA and ALA from oleic acid and are found mainly in fungi and plants. In contrast, vertebrates do not possess these desaturases and, therefore, have to consume LA and ALA as a part of the diet to survive (Hashimoto et al., 2008). All Δ12 and Δ15 desaturase proteins share nearly identical hydrophobicity profiles and show very strong conservation of the three histidine box motifs required for binding two iron atoms at the catalytic center (Hashimoto et al., 2008). Because histidine motifs should be in close proximity to form the di-iron cluster at the active site, it suggests a common overall membrane topology for these proteins.

A search of publicly available databases using the NcΔ15D protein sequence has demonstrated that the NcΔ15D protein present in MON 87769 shares amino acid identity

to many naturally occurring Δ12 and Δ15 desaturase proteins contained in a large variety of food crops (Table 12). The identity scores ranged from 20% to 44% with the highest score for the Δ12 desaturase from *Brassica oleracea* and lower scores for cyanobacterial homologues. *B. oleracea* (broccoli, cabbage and cauliflower) is a member of one of the most common plant families (*Brassicaceae*) consumed. The *Brassicaceae* family also includes other most common vegetables eaten by people such as collard greens, Brussels sprout, radish, and horseradish (McNaughton and Marks, 2003). The NcΔ15D protein also shares high sequence identities to Δ12 desaturase proteins contained in other food species of the *Brassicaceae*. These include the Δ12 desaturases from *B. rapa* (43% identity), *B. napus* (38%), and *B. juncea* (38%). Cruciferous vegetables are one of the dominant food crops worldwide and widely considered to be healthful foods (Zhang, 2004).

The NcΔ15D protein shares sequence identities between 29% and 38% with other Δ12 or Δ15 desaturase proteins in some of the world's most important food crops (Table 12). These major crops include *G. max* (soybean), *O. sativa* (rice), *T. aestivum* (wheat), *S. tuberosum* (potato) and *A. hypogaea* (peanut). Similar identity is observed between the NcΔ15D protein and Δ12 or Δ15 desaturases found in highly consumed fruit species such as *M. domestica* (apple), *L. esculentum* (tomato), *M. charantia* (pear), *P. Americana* (plum) and *O. europaea* (olive). Furthermore, the NcΔ15D protein shows sequence homology to fungal and cyanobacterial desaturases (Table 12). For example, the NcΔ15D protein shares ~37% sequence identity to *Mortierella alpina* Δ15 desaturase. *M. alpina* is currently used for the commercial production of arachidonic acid for fortification of baby foods. The NcΔ15D protein shares ~20% sequence identity to the Δ12 desaturase from cyanobacteria (*A. platensis*). In non-western cultures, cyanobacteria have been part of the human diet for centuries, (Gantar and Svircev, 2008). More recently, cyanobacteria spirulina (*A. platensis*) have been sold worldwide as a food supplement to provide complete protein, essential fatty acids and vitamins (Belay, 2008). The consumption of spirulina is increasing and it is currently commercially produced and sold in United States, Thailand, India, Taiwan, China, Pakistan and Burma and production is growing worldwide (Pulz and Gross, 2004; Vonsha, 1997).

Thus, NcΔ15D functional and structural homologues are found in many common food sources. Virtually all plant species contain Δ12 or Δ15 desaturases, which are required for the synthesis of 18:2 or 18:3 unsaturated fatty acids. Consumption of foods containing these fatty acids is a dietary requirement for human and animal health since they cannot be synthesized in the body. Thus, proteins that are closely related and functionally similar to NcΔ15D have been consumed for many years with no history of adverse health effects establishing a history of safe exposure for this protein.

Estimated Consumption of Δ15 desaturases

Desaturases that are highly similar to NcΔ15D are consumed in a wide variety of foods that have been in use for long periods of time. Examples of foods containing Δ15 desaturases or its homologues include cruciferous vegetables (cabbage, cauliflower, broccoli, kale, mustard, etc.), olives, tomatoes, potato, many fruits and major food crops including soy, barley, rice, wheat, and corn. *Per capita* mean daily consumption of four

cruciferous vegetables in the U.S. including broccoli, brussels sprout, cabbage, and cauliflower was determined to be 10.8 g [calculated from the National Centers for Health Statistics National Health and Nutrition Examination Survey 1999-2002 (NHANES) data] and average daily consumption of all cruciferous vegetables combined is 13.8 g in the U.S. and approximately twice that in some other countries including Japan, Korea, and Eastern Europe (GEMS, http://www.who.int/foodsafety/chem/gems/). In addition, U.S. *per capita* daily consumption of soybean meal, soybean milk and soybean seed together was 2.7 g [food consumption data from the 1994-1996 and 1998 USDA Continuing Survey of Food Intakes by Individuals (CSFII)].

To estimate consumption of the desaturases that are homologous to the NcΔ15D from MON 87669, conventional soybean was used as an example. To quantify Δ15 desaturase consumption, the level of the protein was determined in conventional mature soybean seed grown in two different locations in the U.S. using a quantitative western blot analysis with a soybean Δ15 desaturase specific antibody. Immunoreactive bands corresponding to the Δ15 desaturase were quantified relative to a standard curve prepared using soybean Δ15 desaturase reference protein spiked into extracts lacking endogenous Δ15 desaturase. All tested seed contained measurable quantities of Δ15 desaturase protein with an average level of 1.2 ± 0.37 µg/g on a fresh weight basis with a range of 0.86 – 1.57 µg/g. Since total protein increases 1.35 fold in soybean meal and soybean milk as compared to seed, the average level of Δ15 desaturase was determined to be 1.6 µg/g. Estimates of dietary exposure to soybean endogenous Δ15 desaturase from consumption of soybean meal, milk, and seed was determined using the Dietary Exposure Evaluation Model (DEEM-FCID version 2.03, Exponent Inc.) and food consumption data from the 1994-1996 and 1998 USDA CSFII. Assuming a 0.042 g/kg BW per capita daily soybean meal, soybean milk and soybean seed consumption rate, and a Δ15 desaturase level of 1.2 µg/g, the average daily consumption of Δ15 desaturase from soybean meal, milk, and seed is approximately 0.05 µg Δ15 desaturase per kg BW. For the NcΔ15D from MON 87669, similar calculations (9.5 µg/g in soybean meal × 0.042 g/kg BW) were conducted under a very conservative assumption that all soybean meal consumed in U.S. will originate from MON 87769 soybean and there is no loss of the NcΔ15D during processing. Under this assumption, daily consumption of NcΔ15D was determined as 0.4 µg per kg BW. Given that MON 87769 is an identity preserved product targeted to be grown on relatively small acreage, the actual consumption of the NcΔ15D would be dramatically lower.

The consumption of the homologues of Δ15 desaturases from other foods will be significantly higher compared to the likely contribution of NcΔ15D from MON 87769. For example, cruciferous vegetables contain Δ15 and Δ12 desaturases that are homologous to the NcΔ15D from MON 87769. Assuming that expression levels of these desaturases is similar to the level of Δ15 desaturase detected in soybean (1.2 µg/g of fresh weight), per capita daily consumption of desaturases via four cruciferous vegetables (broccoli, brussels sprout, cabbage, and cauliflower) would be approximately 13 µg, which is significantly higher than the conservative consumption estimate for NcΔ15D from MON 87669. The level of consumed Δ15 and Δ12 desaturases from cruciferous vegetables will be higher for other countries where consumption of these vegetables is

higher than in the U.S. This hypothetical calculation of the level of desaturase consumption from only four cruciferous vegetables further demonstrates that the level of consumed desaturases is significantly higher than the level of consumption of NcΔ15D from MON 87769, thus, establishing a history of safe consumption for NcΔ15D.

Structural Similarity of NcΔ15D to Known Toxins or Other Biologically Active Proteins

The assessment of the potential for protein toxicity includes bioinformatic analysis of the amino acid sequence of the introduced protein(s). The goal of the bioinformatic analysis is to ensure that the introduced protein(s) do not share homology to known toxins or anti-nutritional proteins associated with adverse health effects.

Potential structural similarities shared between the NcΔ15D protein and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the NcΔ15D protein sequence were performed with the TOX_2009 database to identify possible homology with proteins that may be harmful to human and animal health. The TOX_2009 database is a subset of 7,651 sequences derived from the protein database (PROTEIN) consisting of publicly available protein sequences from GenBank (GenBank protein database, release 169.0, December 16, 2008). The 7,651 sequence subset was selected using a keyword search of the public databases and filtered to remove non-toxin proteins. Initially, all sequence descriptions contained in header lines and the associated protein sequence derived from the PRT_2009 database were keyword screened using all possible combinations of upper and lower case characters spelling the words “toxic” and “toxin.” The resulting 11,151 sequences and their respective descriptions were then filtered to exclude terms used in combination with “toxic” or ”toxin”; such as “synthetic,” “anti,” “putative,” “like,” etc. to remove non-toxin protein sequences.

An expectation score (E-score) acceptance criteria of $<1\times10^{-5}$ for any alignments was used to identify proteins from the TOX_2009 database with potential for significant shared structural similarity and function with NcΔ15D protein. The E-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger E-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an E-score of 1×10^{-5} or smaller to be considered to have significant homology. The results of the search produced no alignments below an E-score of 1.0 and therefore, there were no alignments with shared significant similarity with known toxins.

The results of the bioinformatic analyses demonstrated that no structurally-relevant similarity exists between the NcΔ15D protein and any known toxic or other biologically-active proteins that would be harmful to human or animal health.

Acute Oral Toxicity Studies with the NcΔ15D Protein

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond et al., 2008a; Hammond et al., 1998; Sjoblad et al., 1992). The few exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which typically require a short-term (2-4 week) feeding study to manifest toxicity (Liener, 1994). The amino acid sequence of the NcΔ15D desaturase proteins produced in MON 87769 is not similar to any of these anti-nutritional proteins or to any other known mammalian protein toxin. Therefore, for the protein safety evaluation an acute administration of a single dose of the protein is considered to be an appropriate and sufficient test to confirm the lack of mammalian toxicity for the NcΔ15D protein and to establish a NOAEL (Pariza and Foster, 1983; Pariza and Johnson, 2001).

The proteins used in these studies were extracted from MON 87769 immature seed and thoroughly characterized. Purification of integral membrane desaturases requires removal of membranes and replacement of the lipids surrounding the protein's hydrophobic membrane-spanning regions with the appropriate detergent that will keep the protein in solution. Membrane proteins tend to aggregate and are difficult to concentrate. To utilize NcΔ15D protein purified from immature soybean seed as the test material for acute mouse gavage study, the level of the detergent was reduced by buffer exchange to the minimal level which is necessary to keep the protein in solution. After buffer exchange, the protein was concentrated to the highest attainable level. The dosage administered to mice during the study was the highest that could be practically given based on protein's properties and its low expression in mature seed.

To assess acute toxicity, NcΔ15D was administered by gavage at a single dose of 37.3 mg/kg BW to 10 male and 10 female CD-1 mice. Following dosing, all mice were observed twice daily for signs of mortality and moribundity and once daily for signs of toxicity. Body weights were measured prior to randomization and on study Day 7. Food consumption was measured daily from study Days 1 to 7. A gross necropsy was performed on all animals at the end of the study (Day 7). There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the NOAEL for NcΔ15D proteins was considered to be 37.3 mg/kg BW, the only dose tested. This dose was several orders of magnitude higher than conservative estimates for human exposure to the NcΔ15D from consumption of MON 87769 (Table 13).

Table 12. Amino Acid Sequence Identity Between MON 87769-Produced NcΔ15D and Other Omega (Δ12/Δ15) Desaturase Proteins

Sequences were extracted from publicly available databases. Sequences were aligned and sequence identity was calculated using the MegAlign function of the Lasergene suite of sequence analysis software [version 7.1.0 (44)] (DNASTAR Inc., Madison, WI, USA).

	Proteins	Common Name	Sequence Identity (%)																																		
#			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	N_crassa_delta15	fungus	100	44	43	38	38	38	38	38	37	37	37	36	36	36	36	34	33	31	33	30	30	29	29	30	29	29	30	29	29	28	28	20	21
2	B_oleracea_6503049_delta12	kale, broccoli		100	99.5	99.5	99.5	97	81	77	78	47	77	81	75	85	42	40	49	40	62	40	36	41	38	42	39	39	37	40	39	39	35	22	26
3	B_rapa_delta12	Chinese cabbage, turnip			100	99.7	99.7	96	81	75	78	48	75	79	75	82	42	40	48	38	64	37	35	37	36	38	36	37	38	37	37	36	31	25	26
4	S_oleracea_24298827_delta12	kale, broccoli				100	100	95	77	71	74	44	71	75	73	78	39	40	45	35	60	35	32	34	33	35	33	34	37	34	34	34	30	23	22
5	B-napus_46399191_delta12	rutabaga					100	95	77	71	74	44	71	75	73	78	39	40	45	35	60	35	32	34	33	35	33	34	37	34	34	34	30	23	22
6	B_juncea_1212781_delta12	mustard cabbage						100	76	71	74	42	71	74	71	78	38	40	45	35	60	35	32	35	33	35	33	33	37	33	33	33	30	21	21
7	P_americana_23428547_delta12	Common wild plum							100	71	77	45	73	77	76	76	41	42	46	36	59	36	34	37	34	35	34	36	39	34	36	34	29	22	23
8	G_max_60594769_delta12	soybean								100	71	42	83	76	68	71	40	41	45	34	61	33	32	34	32	34	34	33	35	33	34	33	29	20	21
9	P_crispum_2501790_delta12	parsley									100	43	72	75	76	77	40	41	45	35	59	34	33	35	33	34	34	34	35	34	35	32	30	22	22
10	M_alpina_6448794_delta12	fungus										100	43	45	44	44	52	39	46	34	39	34	33	36	33	36	33	32	37	33	32	32	30	24	23
11	A_hypogaea_14572857_delta12	peanut											100	76	67	71	40	41	46	34	62	33	32	34	32	33	33	33	34	33	34	32	30	22	22
12	O_europaea_58013373_delta_12	olive												100	72	75	39	42	44	35	61	34	33	36	34	36	34	34	35	34	36	34	29	24	23
13	H_annuus_60392580_delta12	sunflower													100	75	41	40	43	35	58	35	32	35	33	34	34	35	36	33	34	32	28	22	21
14	B_officinalis_3417601_delta12	borage														100	42	40	46	37	60	36	33	34	33	34	33	33	36	34	34	32	31	23	24
15	M_alpina_62084356_delta15	fungus															100	37	42	32	36	31	32	32	32	34	32	31	31	32	32	31	28	20	22
16	B_rapa_15811218_delta15	Chinese cabbage, turnip																100	40	72	39	71	72	75	72	73	76	75	71	75	74	75	34	26	27
17	S_diclina_38426731_delta12	fungus																	100	36	41	37	35	36	35	35	35	34	36	34	35	35	28	23	25
18	O_sativa_1777376_delta15	rice																		100	34	80	68	66	68	65	67	68	74	68	67	67	32	24	24
19	M_charantia_6224716_delta12	balsam pear																			100	34	34	34	34	35	34	34	35	34	35	33	29	23	23
20	T_aestivum_3157460_delta15	wheat																				100	67	67	66	66	66	68	71	67	66	65	32	25	25
21	S_tuberosum_3550663_delta15	potato																					100	68	97	69	75	73	80	77	76	77	32	25	22
22	V_radiata_416638_delta15	mung bean																						100	68	74	68	70	69	70	67	68	30	25	25
23	L_esculentum_32479366_delta15	tomato																							100	69	75	73	79	76	75	75	32	26	22
24	G_max_34787276_delta15	soybean																								100	69	70	70	69	68	67	31	24	24
25	M_domestica_45385976_delta15	apple																									100	75	78	85	75	74	29	25	23
26	B_napus_47028567_delta15	rutabaga																										100	79	75	73	73	30	25	24
27	H_vulgare_6900316_delta15	barley																											100	82	79	81	34	20	27
28	P_persica_45385321_delta15	parsley																												100	76	73	29	24	22
29	B_juncea_7378667_delta15	mustard cabbage																													100	74	30	25	24
30	P_crispum_1786066_delta15	parsley																														100	31	25	21
31	S_diclina_38426733_delta15	fungus																															100	22	21
32	A_platensis_805064_delta12	spirolina (Blue-green algae)																																100	45
33	B_napus_49355354_delta12	rutabaga																																	100

Table 13. Acute (95th Percentile, "eater-only") Dietary Intake and Margins of Exposure for the PjΔ6D and NcΔ15D Proteins from Consumption of MON 87769 Soybean Meal-Derived Food Products in the U.S.[1]

Population	*Protein Intake[2] (mg/kg BW/day)*		*Margins of Exposure[3]*	
	PjΔ6D	*NcΔ15D*	*PjΔ6D*	*NcΔ15D*
General Population	0.00016	0.0008	29,000	47,000
Non-nursing Infants	0.006	0.036	800	1000

[1]Estimated using DEEM-FCID version 2.03, Exponent Inc., utilizing food consumption data from the 1994-1996 and 1998 USDA Continuing Surveys of Food Intakes by Individuals (CSFII). Includes soybean seed, flour, and oil. The 95th percentile soybean (seed, flour, soybean milk) consumption numbers used were 0.07 g/kg/day for the general U.S. population and 2.79 g/kg/day for non-nursing infants. Assumes 100% soybean consumed is derived from MON 87769 soybean meal.

[2]Based on average expression levels of 1.7 µg/g FW and 9.5 µg/g FW for PjΔ6D and NcΔ15D proteins, respectively, in whole seed (Table 9 and 10). To estimate levels of these proteins in processed meal used to make flour and soymilk, levels in seed were multiplied by a concentration factor of 1.35, derived from the ratio of total protein in processed meal/to total protein in seed based on composition studies with MON 89788 (Lundry et al., 2008). The desaturase intake was calculated by multiplying the level of desaturase protein in seed × 1.35 x consumption number, e.g., for PjΔ6D, General Population, 1.7 µg/g × 1.35 × 0.07 g/kg/day = 0.16 µg/kg/day or 0.00016 mg/kg/BW/day.

[3]Calculated by dividing NOAELs from acute mouse gavage studies (4.7 and 37.3 mg/kg for PjΔ6D and NcΔ15D proteins respectively) by protein intake. Rounded to two significant figures or nearest hundred.

g) Dietary Safety Assessment for PjΔ6D and NcΔ15D Proteins

Dietary exposure to PjΔ6D and NcΔ15D proteins is expected to mainly come from the consumption of MON 87769 soybean meal derived from soybean seed. Estimates of dietary exposure to PjΔ6D and NcΔ15D proteins from consumption of MON 87769 soybean meal was determined using the Dietary Exposure Evaluation Model (DEEM-FCID version 2.03, Exponent Inc.) and food consumption data from the 1994-1996 and 1998 USDA CSFII. DEEM-FCID differentiates soybean consumption into four fractions: seed, flour, milk and oil. However, since soybean oil contains negligible amounts of protein (Tattrie and Yaguchi, 1973), it would not be a significant source of dietary exposure to PjΔ6D and NcΔ15D proteins and was thus excluded from this assessment.

Soybean meal is a blended commodity that has been highly processed before being consumed by humans. Thus, most food products that might be derived from MON 87769 soybean meal and entering the human food supply would likely be blended with other commercial soybean meal or meal-derived products before being processed and consumed. However, estimating the percentage of consumed soybean products that would likely be derived from MON 87769 soybean meal is difficult. Therefore, for the purposes of this dietary risk assessment, the conservative assumption was made that

100% of all soybean products consumed in the U.S. will be derived from MON 87769 soybean meal, i.e., no adjustments were made for market share. Actual consumption of MON 87769 soybean meal is likely to be far less because MON 87769 will be grown on a limited number of acres.

Because soybean is a blended commodity, the mean level of the PjΔ6D and NcΔ15D proteins in each of the potentially consumed food fractions (seed, flour, and soybean milk) should be used when estimating total intake of these proteins from consumption of MON 87769 soybean meal. However, specific values for levels of PjΔ6D and NcΔ15D proteins in each of these fractions are not available. Thus, the concentrations of PjΔ6D and NcΔ15D proteins in each of these fractions were derived from mean concentrations in mature MON 87769 seed grown in the U.S. in 2006 (1.7 µg/g FW and 9.5 µg/g FW, respectively, Table 9 and 10) corrected for concentration in meal during processing of seed (see footnote 2, Table 13). This is a very conservative assumption since it assumes there is no loss of the two proteins during storage, processing and/or cooking. Soybean contains certain factors, such as trypsin inhibitors, which may act as anti-nutrients if soybean is not properly heated during preparation (Rackis, 1974). Thus, virtually all protein-containing soybean fractions are heated during processing prior to consumption by humans and most animals. However, PjΔ6D and NcΔ15D desaturase proteins are not heat stabile and are likely to be denatured during toasting and processing. Thus, the amounts of functionally-active protein present in consumed soybean products will be substantially lower than assumed for this evaluation.

Based on these assumptions, the 95th percentile acute intake (eater-only) for PjΔ6D and NcΔ15D proteins were estimated to be 0.00016 mg/kg BW/day and 0.0008 mg/kg BW/day, respectively, for the overall U.S. population. The 95th percentile estimates of acute intake (user-only) for non-nursing infants, the most highly exposed sub-population, were 0.006 mg/kg BW/day and 0.036 mg/kg BW/day, respectively (Table 13).

Margins of Exposure for the PjΔ6D and NcΔ15D Proteins Derived From MON 87769

A common approach used to assess potential health risks from chemicals or other potentially toxic products is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an appropriate animal toxicity study and an estimate of human exposure. No adverse health effects were observed when mice were administered an acute dose of 4.7 mg/kg BW of PjΔ6D protein and 37.3 mg/kg BW of NcΔ15D protein. Therefore, based on an apparent absence of hazard associated with exposure to these proteins and the extremely low expression levels of these proteins in MON 87769 meal, dietary risk assessments for these proteins would normally not be considered necessary. Nevertheless, dietary risk assessments for both proteins were conducted in order to provide further assurances of safety.

Potential health risks from acute dietary intake of the PjΔ6D and NcΔ15D proteins from consumption of food derived from MON 87769 soybean meal were evaluated by calculating MOEs based on the acute mouse NOAELs for each protein and the 95th

percentile "user-only" estimates of acute dietary exposure from DEEM-FCID. The MOEs for acute dietary intake of PjΔ6D protein were estimated to be approximately 29,000 and 800 for the general population and non-nursing infants, respectively. For NcΔ15D protein, the MOEs for acute dietary intake were estimated to be 47,000 and 1000 for the general population and non-nursing infants, respectively (Table 13). These very large MOEs indicate that there are no meaningful risks to human health from dietary exposure to the PjΔ6D and NcΔ15D proteins present in MON 87769 soybean meal.

Estimated Animal Exposure to the PjΔ6D and NcΔ15D Proteins Present in MON 87769

In 2006/2007, 236 million metric tons of soybeans were produced globally. The major producers included the U.S. (37%), Brazil (25%), Argentina (20%), China (7%), India (3%), Paraguay (3%), Canada (1%), and others (4%) (Soyatech, 2008). In the U.S. over 93% of the soybean grown are either crushed domestically or internationally with less than 7% of the soybean seed used as feed, seed or residual. During the crushing process soybean meal (SBM) is produced (approximately 0.74 kg of dehulled soybean meal from each kg of seed). Of the soybean meal produced in the United States, approximately 98% is consumed by the livestock industry (Soyatech, 2008). Poultry consumes 50%, swine 27%, cattle 17%, companion animals 3%, and the remainder to others[2]. Full-fat soybean contains a trypsin inhibitor that affects protein digestion in monogastrics and thus it must be heated-treated to deactivate the inhibitor before it can be fed to poultry and swine (Harris, 1990). Heat treatment also enhances the level of rumin undegradable protein that is beneficial to the ruminant as long as it is digestible in the lower GI tract. Typically, the feeding level of whole soybean to dairy cattle is 2.7 (2.3 -3.1) kg/cow/day (Harris, 1990; Hutjens, 1999) and is limited to a maximum of 20% of the swine diet due to its high oil content (Yacentiuk, 2007). Soybean forage can be fed to cattle and is limited to 50% of the total ration dry matter (Brown, 1999). However, MON 87769 is targeted for the production of the value added oil containing SDA and there are no intended uses for MON 87769 forage. Dehulled soybean meal consumption in: 1) the four week old broiler is 27.0 g dry matter/kg BW/day (30.2% SBM in the diet, 161 g intake/day, diet 89% dry matter, 1.6 kg BW) (Popescu and Criste, 2003); 2) the young pig is 10.9 g dry matter/kg BW/day (24.3% SBM in the diet, 2.02 kg intake/day, diet 89% dry matter, 40 kg BW) (Cromwell et al., 2002); 3) the older pig is 3.8 g dry matter/kg BW/day (14% SBM in the diet, 3.04 kg of intake/day, diet 89% dry matter, 100 kg BW) (Cromwell et al., 2002); and 4) in the lactating dairy cow producing 37.4 kg of fat-corrected milk is 7.8 g/kg BW/day (18.6% SBM in the diet, 27.4 kg of dry matter intake/day, 655 kg BW) (Bal et al., 2000).

The exposure of poultry and livestock to MON 87769 may primarily result from feeding soybean meal with some animals being fed the heat treated full-fat seed. For the following exposure calculations the intake of the soybean meal will be used since the diets contain a higher inclusion level of the protein from soybean meal than from the full-fat seed. For example, if we assume that in the crushing process full-fat seed yield 74% dehulled soybean meal then we can multiply the full-fat soybean inclusion levels

[2] http://www.soystats.com/2008/Default-frames.htmSoyStats

mentioned above by 74%. The resulting dehulled soybean meal equivalent is equal to or less than the dehulled soybean meal inclusion levels used in this analysis. Soybean meal bought for animal feed would be expected to have gone through a series of commingling steps with non-MON 87769 soybean meal as it makes its way through commerce. MON 87769 heat-treated and fed as full-fat seed may not have been commingled with non-MON 87769 soybean; therefore, livestock could be exposed to the highest anticipated levels of PjΔ6D and NcΔ15D proteins. For this assessment it will be assumed that MON 87769 soybean meal is the only source of soybean meal in the diet and has not been commingled with non-MON 87769 sources.

Estimated Dietary Intake of PjΔ6D and NcΔ15D Proteins from MON 87769

Animals will be exposed to the PjΔ6D and NcΔ15D proteins through dietary intake of feed derived from MON 87769. Based on the estimate of the quantity of soybean meal consumed on a daily basis by poultry and livestock, as well as the levels of PjΔ6D and NcΔ15D proteins in MON 87769 soybean meal, an estimate of daily dietary intake (DDI) can be derived. DDI is computed as follows:

DDI = Daily soybean meal consumption (g) × PjΔ6D or NcΔ15D protein concentration (µg/g)

The intake calculations make the conservative assumption that there is no loss of the PjΔ6D and NcΔ15D proteins during the processing of soybean into soybean meal. It also assumes that 100% of the soybean meal in animal feed is derived from MON 87769, which is an excessive and unlikely over estimation.

The potential dietary intake of PjΔ6D and NcΔ15D proteins from the consumption of MON 87769 soybean meal can be estimated by multiplying the consumption of each commodity by the levels of these two proteins in that commodity. For the purpose of this dietary intake calculation, both the highest and mean levels of the PjΔ6D and NcΔ15D proteins reported for MON 87769 soybean were used. The mean and high end range values of the PjΔ6D and NcΔ15D protein levels in soybean used in this assessment were from soybean varieties containing MON 87769 grown in the U.S. in 2006.

The mean level of the PjΔ6D protein in MON 87769 harvested seed is 1.8 µg/g DW (range 0.50 – 3.2 µg/g DW). Assuming a crushing yield of 74% (1 kg of soybeans yielding 0.74 kg of dehulled soybean meal), the mean level of the PjΔ6D protein in soybean meal derived from MON 87769 soybeans is 2.4 µg/g DW of soybean meal (range 0.68 – 4.3 µg/g DW).

The mean level of the NcΔ15D protein in MON 87769 harvested seed is 10.0 µg/g DW (range 4.8 – 25.0 µg/g DW). Assuming a crushing yield of 74% (1 kg of soybean yielding 0.74 kg of dehulled soybean meal), the mean level of the NcΔ15D protein in soybean meal derived from MON 87769 is 13.5 µg/g DW of soybean meal (range 6.5 – 33.8 µg/g DW).

The estimated mean and maximum daily intake of the PjΔ6D and NcΔ15D proteins by poultry and livestock are shown in Table 14. Mean and Maximum Daily Intakes of the PjΔ6D and NcΔ15D Proteins in Poultry and Livestock (g/kg body weight/day).

The broiler chicken, young pig, finishing pig, and lactating dairy would typically consume 18 g dietary protein/kg BW (NRC, 1994), 14 g dietary protein/kg BW (NRC, 1998), 4 g dietary protein/kg BW (NRC, 1998), and 6 g dietary protein/kg BW (NRC, 2001), respectively. The highest percentage of the PjΔ6D protein (g/kg BW) per total protein consumed was in the broiler, 0.00065% of the total dietary protein intake (0.000117 g PjΔ6D /kg BW/day divided by 18 g dietary protein which is the total dietary protein intake for the broiler × 100). The highest percentage of NcΔ15D protein (g/kg BW) per total protein consumed was in the broiler, 0.0051% of the total dietary protein intake (0.000914 g NcΔ15D /kg BW/day divided by 18 g dietary intake which is the total dietary protein intake for the broiler). The percentages of the PjΔ6D and NcΔ15D proteins consumed as part of the daily protein intake for the pig and dairy cow are less than the broiler. This dietary assessment shows that there is little risk to animals that are fed diets containing MON 87769.

Table 14. Mean and Maximum Daily Intakes of the PjΔ6D and NcΔ15D Proteins in Poultry and Livestock (g/kg body weight/day)

Species	Total SBM Consumption (g/kg BW /day) DW	Trait Protein Intake (g/kg BW/day DW)			
		PjΔ6D Protein		NcΔ15D Protein	
		Mean	Highest Level	Mean	Highest Level
Chicken broiler[1]	27.0	0.000066	0.000117	0.000365	0.000914
Young pig[1]	10.9	0.000027	0.000047	0.000146	0.000369
Finishing pig[1]	3.8	0.000009	0.000016	0.000051	0.000128
Lactating dairy cow[1]	7.8	0.000019	0.000034	0.000105	0.000264

[1]Soybean meal (SBM) consumed × concentration of PjΔ6D or NcΔ15D protein in the soybean seed.

h) Known Allergenicity of Source

The PjΔ6D protein is from *Primula juliae*, an organism that is not known to be allergenic. The NcΔ15D protein is from *Neurospora crassa*, a fungi that is ubiquitous in the environment, is not allergenic, and found in the digestive tracts of vertebrate species, including humans. 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 non-allergenic 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.

i) Unknown Allergenicity of Source

According to guidelines adopted by the Codex Alimentarius Commission (Codex, 2003) for the evaluation of the potential allergenicity of novel proteins, the allergenic potential of a novel protein is assessed by comparing the biochemical characteristics of the novel protein to characteristics of known allergens.(Codex Alimentarius 2003) A protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents only a very small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence, and 4) the protein is rapidly digested in mammalian gastrointestinal systems.

The above characteristics were assessed for PjΔ6D and NcΔ15D proteins produced in MON 87769 and the details for the assessment are described in the following sections.

i) PjΔ6D and NcΔ15D Proteins as a Proportion of Total Soybean Protein

The PjΔ6D and NcΔ15D proteins were detected at relatively low levels in immature and mature seed and seed-containing plant tissue, such as forage (Table 9 and 10). Among these tissues, mature seed is the most relevant to the assessment of food allergenicity. The mean levels of PjΔ6D and NcΔ15D protein in mature seed are 1.8 and 10 µg/g DW, respectively. The mean percent (%) dry weight of total protein in mature MON 87769 seed is 41.92% (Table 19) (or 419,200 µg/g). The percent of PjΔ6D protein in mature MON 87769 seed is calculated as follows:

(1.8 µg/g ÷ 419,200 µg/g) × 100% = 0.00043% of total mature soybean seed protein

Likewise, the percent of NcΔ15D protein in seed of MON 87769 is calculated as follows:

(10 µg/g ÷ 419,200 µg/g) × 100% = 0.00239% of total mature soybean seed protein

Therefore, the PjΔ6D and NcΔ15D proteins represent only a negligible portion of the total protein present in MON 87769 mature seed.

ii) Bioinformatics Analyses of Sequence Similarity of the PjΔ6D and NcΔ15D Proteins Produced in MON 87769 to Known Allergens

In 2003, the Codex Alimentarius Commission published guidelines for the evaluation of the potential allergenicity of novel proteins based on shared amino acid sequence identity (Codex, 2003). The guideline is based on the comparison of amino acid sequences between introduced proteins and allergens, where potential allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids. The Codex guideline also recommends that a sliding window search with a scientifically justified peptide size could be used to identify immunologically-relevant peptides in otherwise unrelated proteins.

The potential for allergenic cross-reactivity between the MON 87769 PjΔ6D and NcΔ15D proteins and known allergens, gliadins, and glutenins was assessed using two search algorithms: 1) a FASTA sequence alignment program was used to determine if any allergens shared at least 35% amino acid identity over at least an 80 amino acid segment with the PjΔ6D and NcΔ15D proteins, and 2) a sliding window search was used to identify any eight-amino acid matches to known allergens (ALLERGENSEARCH program) (Codex, 2003; Thomas et al., 2005). Both bioinformatic search algorithms were used in conjunction with the allergen, gliadin and glutenin sequence database (AD_2009) obtained from the Food Allergy Research and Resource Program Database (FARRP, 2009)[3]. The AD_2009 database was released January 2009 and contains 1,386 sequences.

[3] located at http://www.allergenonline.com

Based on the results of the FASTA comparisons, known allergens were ranked according to their degree of similarity to the PjΔ6D and NcΔ15D proteins. Alignments for full-length search sequences may be considered relevant for similarity to known allergens if the identity is equal to or greater than 35% and equal to or greater than an 80 amino acid segment in aligned sequence length (Codex, 2003). All alignments were inspected visually to determine if an alignment represented biologically relevant sequence similarity. None of the proteins in the AD_2009 database met or exceeded the threshold of 35% identity over 80 amino acids when compared to PjΔ6D or NcΔ15D. Although none of the obtained alignments satisfied minimum Codex standards, the quality of each alignment was also thoroughly evaluated for their percent identity and expectation score (E-score) as produced from the FASTA bioinformatic program (Pearson, 2000; Pearson and Lipman, 1988). The analysis of the shared percent identity, length of the alignment, as well as the E-score is intended to add additional information to the search for proteins that may have potentially significant homology. The E-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger E-score indicates a lower degree of similarity between the query sequence and the sequence from the database. There were no alignments that had an E-score below 1, therefore, no alignments between the PjΔ6D and NcΔ15D proteins and sequences in the AD_2009 database were observed that were considered relevant from an allergenic assessment perspective.

An eight-amino acid sliding window search was used to specifically identify short linear polypeptide matches to known allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain smaller immunologically significant epitopes. A query sequence may have allergenic potential if it has a shared sequence identity of at least eight linear and contiguous amino acids with a potential allergen epitope (Goodman et al., 2002; Hileman et al., 2002; Metcalfe et al., 1996). However, most allergen epitopes have not been confirmed and the amino acid length for those that have been identified can vary widely, thus the relevance of an exact match of eight amino acids may have limited immunological relevance (Thomas et al., 2005). The eight amino acid bioinformatic strategy is currently a theoretical search for shared epitopes and this bioinformatic search can produce alignment matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006).

A sliding eight-amino acid window search (ALLERGENSEARCH) was performed between the PjΔ6D or NcΔ15D amino acid sequences and proteins contained within the allergen database (AD_2009). Results show that the PjΔ6D protein does not share any eight amino acid matches with proteins found in the AD_2009 allergen database. The NcΔ15D protein sequence was found to have one match with a known allergen, a carboxypeptidase protein (GI number 66840994) found in the food crop, wheat. The eight amino acid match is a series of eight serine residue (SSSSSSSS). However, there is no evidence that 1) a series of eight serine residues confers unique protein structure and that this serine sequence is descriptive of protein allergens or 2) an eight serine sequence present in the wheat carboxypeptidase protein would act as an allergen IgE-binding epitope. In a previous version of the allergen database (AD7) the same series of eight serine residues was found in the putative egg allergen, vitellogenin. This allergen was removed from the AD8 database and subsequent database updates because an

international panel of allergy experts who compile the database judged that there was insufficient evidence to support its inclusion.

The polyserine sequence is found in many non-allergenic proteins that are present in all kingdoms, including many human food sources such as chicken, beef, grapes, rice, corn, and tomatoes. Proteins containing stretches of eight or more serine residue represent functionally diverse classes, including enzymes, signaling molecules, structural proteins and transport molecules (Faux et al., 2005). A polyserine stretch located in the N-terminal portion of some membrane associated proteins is thought to contribute to the overall stability of the proteins, but has no effect on protein functionality (Hasper et al., 1999; Huntley and Golding, 2006). Presence of the conserved poly-serine sequences is a typical feature of dehydrin proteins which are the most abundant plant proteins produced during late embryogenesis or in response to drought, low-temperature, and salt stresses (Rorat, 2006). Dehydrins are distributed in a wide range of organisms including higher plants, algae, yeast, and cyanobacteria. These proteins are found in such food sources as wheat, pea, rice, barley, soybean, and corn and have been shown to accumulate to relatively high levels in both vegetative tissue and seed (Danyluk et al., 1998; Lee et al., 2005). Although the exact function of dehydrin proteins remains uncertain, it is suggested that dehydrins stabilize membrane vesicles and membrane structures in rapidly developing or stressed plants (Koag et al., 2003; Rorat, 2006). There is no mention in the literature that members of dehydrin family of proteins have an association with allergy and none of the dehydrin proteins thus far has been identified as an allergen.

Given the frequent occurrence of polyserine sequences in non-allergenic proteins, it is highly unlikely that the poly-serine sequence contained in the NcΔ15D protein is an IgE-binding epitope. There is no evidence that eight-serine residues indicate a shared, unique allergen epitope sequence and no studies to date have mapped or otherwise shown IgE-binding specifically to epitopes containing polyserine sequences (Bannon and Ogawa, 2006). The bioinformatic analyses of the PjΔ6D and NcΔ15D proteins demonstrate that these proteins are not likely to share biologically-relevant amino acid sequence similarities with known allergens and, therefore, support the conclusion that both PjΔ6D and NcΔ15D are non-allergenic.

iii) Digestibility of the PjΔ6D and NcΔ15D Proteins in SGF and SIF

A factor that increases the likelihood of allergic oral sensitization to proteins is the stability of the proteins to gastrointestinal digestion. One characteristic of many protein allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999). When resistant to digestion, allergens, or their fragments, are presented to the intestinal immune system, which can lead to a variety of gastrointestinal and systemic manifestations of immune-mediated allergy.

One aspect of this allergenicity assessment includes analysis of the digestibility of the introduced protein(s) in a SGF assay containing pepsin. A correlation between the digestibility in SGF and the likelihood of being an allergen has been previously reported

with a group of proteins consisting of both allergens and non-allergens (Astwood et al., 1996), but this correlation is not absolute (Fu et al., 2002). Recently, the SGF assay protocol was standardized by the International Life Sciences Institute (ILSI) based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). The study showed that the results of *in vitro* pepsin digestion assays are reproducible when standard protocols were followed. Using this protocol, the pepsin digestion assay was used to assess the susceptibility of the PjΔ6D and NcΔ15D proteins to *in vitro* pepsin digestion.

The complete enzymatic degradation of an ingested protein by exposure to gastric pepsin and intestinal pancreatic proteases makes it highly unlikely that either the intact protein or protein fragment(s) reach the absorptive epithelial cell of the small intestine where antigen processing cells reside (Moreno et al., 2006). To reach the intestinal mucosa, protein or protein fragment(s) must first pass through the stomach where they are exposed to pepsin and then the duodenum where they are exposed to pancreatic fluid containing a mixture of enzymes called pancreatin. The digestion of a protein by pepsin in the gastric system greatly reduces the possibility that an intact protein or protein fragment(s) will reach the absorptive epithelium of the small intestine. In instances where transient stability of the protein or protein fragment(s) is observed in SGF, further degradation of these fragments in SIF can be evaluated to better understand the fate of the protein during *in vivo* digestion.

Finally, digestibility of a protein in SIF is also used as an independent test system to assess the *in vitro* digestibility of food components (Okunuki et al., 2002; Yagami et al., 2000). The relationship between protein allergenicity and protein stability in the stand-alone *in vitro* SIF assay is limited, because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case *in vivo* (Helm, 2001).

Digestibility of the PjΔ6D Protein in SGF and SIF

Digestibility of the PjΔ6D protein in SGF was assessed by SDS-PAGE and western blot methods. The extent of PjΔ6D protein digestion was evaluated by visual analysis of stained polyacrylamide gels (Figure 37) or by visual analysis of developed X-ray film (Figure 38). The LOD of the PjΔ6D protein by Colloidal Brilliant Blue G staining was 0.005 µg or approximately 0.6% of the total PjΔ6D protein loaded (0.005 µg divided by 0.8 µg of total protein loaded in each lane of the gel; Figure 37, panel B, lane 10). The LOD of the PjΔ6D protein by western blotting was 0.5 ng or approximately 3.3% of the total PjΔ6D protein loaded (0.5 ng divided by 15 ng of the protein loaded in each lane of the gel; Figure 38, panel B, lane 8).

Visual examination of the Colloidal Brilliant Blue G stained gel (Figure 37, panel A) showed that the full-length PjΔ6D protein was digested below the LOD within 30 s of digestion in SGF (Figure 37, panel A, lane 5). Therefore, more than 99% (100% - 0.6% = 99.4%) of the full-length PjΔ6D was digested within 30 s of incubation in SGF. Two diffuse, faint bands with approximate molecular weights of 5 kDa and 4 kDa were observed between 30 s and 60 min of exposure to SGF and a faint band with molecular weight of ~3.5 kDa was observed between 10 min and 60 min of exposure to SGF.

Western blot analysis with antibody developed against full-length PjΔ6D protein demonstrated that the PjΔ6D protein was digested below the LOD within 30 s of incubation in SGF (Figure 38, panel A, lane 5). Based on the western blot LOD for the PjΔ6D protein it was concluded that greater than 96% (100%-3.3%=96.7%) of the PjΔ6D protein was digested within 30 s. A fragment of ~10 kDa was observed only at the 30 s digestion time point (Figure 38, lane 5) that was completely digested in less than 2 min of incubation in SGF. No other bands were detected in the lanes corresponding to the 2 min through 60 min digestion time points (Figure 38, lanes 6-11).

Because the fragments observed between 5 kDa and 3.5 kDa in the SGF assay were not cross-reactive with PjΔ6D-specific antibodies raised against full-length PjΔ6D protein, an attempt was made to establish identities of these fragments by N-terminal sequencing. The sequence obtained for the ~4 kDa and ~3.5 kDa fragments did not match the predicted sequence of the PjΔ6D protein and their identities could not be established. The N-terminal sequencing of the ~5 kDa fragment did not yield enough amino acid residues to allow a definitive sequence comparison to the PjΔ6D predicted sequence or to a protein in the protein database. Since the PjΔ6D protein preparation was not highly pure, the fragments observed between 5 kDa and 3.5 kDa most likely originated from one of the endogenous soybean proteins co-purified with the PjΔ6D protein.

To better understand the fate of the PjΔ6D protein during gastrointestinal digestion, PjΔ6D was exposed to digestion in SGF followed by digestion in SIF containing pancreatin. After digestion in SGF for 2 min, the reaction mixture was quenched and protein and/or protein fragments were exposed to further digestion in SIF. The digestibility of the PjΔ6D protein was evaluated by visual analysis of Colloidal Brilliant Blue G stained polyacrylamide gel (Figure 39, panel A). The gel was loaded with ~0.9 µg total PjΔ6D protein (based on concentration of the protein prior to the digestion in SGF) for each of the SIF digestion time points. Visual examination of the stained gel demonstrated that transiently stable fragments with molecular weight of ~5 kDa and ~4 kDa observed after 2 min of digestion in SGF (Figure 39, panel A, lane 3) were rapidly digestible (<5 min) in SIF (Figure 39, panel A, Lane 9). The digestibility of PjΔ6D protein in SGF followed by SIF was also evaluated using Western blot (Figure 39, panel B). The gel was loaded with ~15 ng total protein (based on pre-digestion concentrations) for each of the digestion specimens. Visual examination of the Western blot showed that, as expected, the full-length PjΔ6D was observed only at the 0 min (Figure 39, panel B, lanes 2 and 3) time point in the SGF phase. No immune-reactive bands were observed at any other time points. The data clearly indicate that the fragments observed in SGF rapidly degrade upon short exposure to SIF.

SIF was also used as a stand alone test system to test digestibility of the PjΔ6D protein. The assay was performed according to methods described in the United States Pharmacopeia (USP, 1995). The digestion of the PjΔ6D protein in SIF was evaluated by Western blot method (Figure 40). A western blot to determine the LOD (Figure 40, panel B) of the PjΔ6D protein was performed concurrently with the SIF assay (Figure 39, panel A). The LOD was estimated to be 2.5 ng, which represented 16.7% of the total protein loaded in this experiment (2.5 ng divided by 15 ng of loaded protein). Western blot

analysis demonstrated that a band corresponding to the full-length PjΔ6D protein was digested below the LOD within 5 min of incubation in SIF (Figure 40, panel A, lane 5). Therefore, based on the LOD, more than 84% (100% - 16.7% = 84.3%) of the full-length PjΔ6D protein was digested within 5 min. No proteolytic fragments of the PjΔ6D protein were detected in any digestion time points. These data suggest that the full-length PjΔ6D protein degrades rapidly when exposed to pancreatin at neutral pH.

In conclusion, these results show that the integral membrane protein, PjΔ6D, is readily digestible in SGF and SIF. Rapid digestion of the full-length PjΔ6D protein and any PjΔ6D protein fragments in SGF and SIF indicates that it is highly unlikely that the PjΔ6D protein will pose any safety concern to human and animal health.

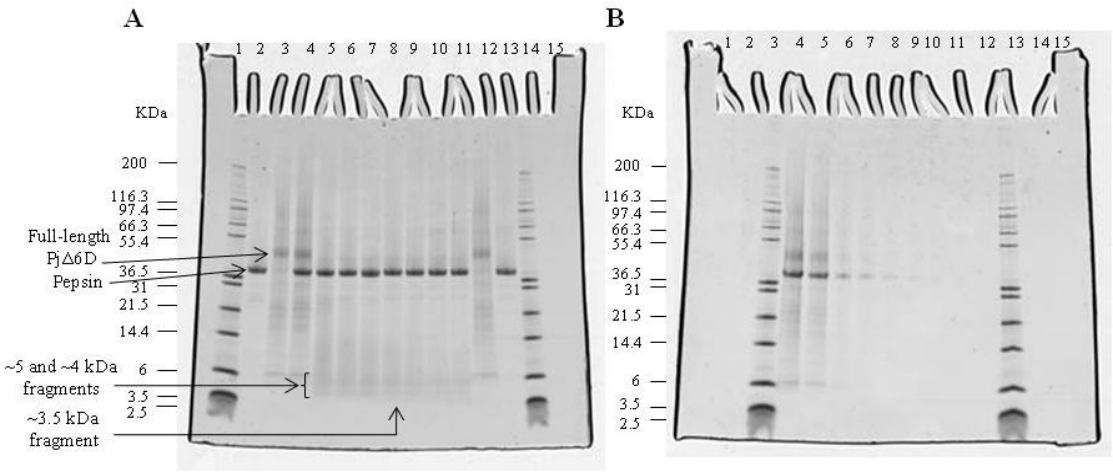


Figure 37. Colloidal Brilliant Blue G Stained SDS-PAGE Gels of PjΔ6D Protein Digestion in SGF

Panel A corresponds to the PjΔ6D protein digestion in SGF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded in each lane containing the PjΔ6D protein. **Panel B** corresponds to the limit of detection of the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, the PjΔ6D protein migrated to approximately 46 kDa and pepsin migrated to approximately 38 kDa (indicated by arrows on the left).

Lane assignment for Panel A:

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0[1]	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Mark 12 MWM	-
15	Blank	-

Lane assignment for Panel B:

Lane	Sample	Amount (µg)
1	Blank	-
2	Blank	-
3	Mark 12 MWM	-
4	T0, protein+SGF	0.8
5	T0, protein+SGF	0.5
6	T0, protein+SGF	0.1
7	T0, protein+SGF	0.05
8	T0, protein+SGF	0.02
9	T0, protein+SGF	0.01
10	T0, protein+SGF	0.005
11	T0, protein+SGF	0.0025
12	T0, protein+SGF	0.001
13	Mark 12 MWM	-
14	Blank	-
15	Blank	-

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0 and N7 are negative controls (no test protein); P0 and P7 are MON 87769-purified protein control (no pepsin); T0-T7are incubation time point in SGF.

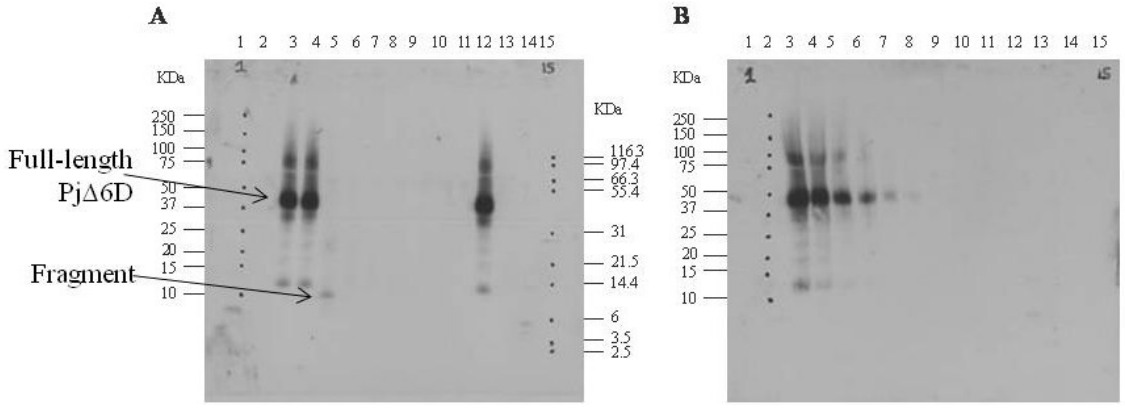


Figure 38. Western Blot Analysis of the Digestion of the PjΔ6D Protein in SGF

Panel A corresponds to the PjΔ6D protein digestion in SGF. Based on pre-digestion protein concentrations, 15 ng (total protein) was loaded in the lanes containing the PjΔ6D protein. **Panel B** corresponds to the limit of detection of the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and right of Panel A and only on the left in Panel B. In both gels, the PjΔ6D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

Lane assignment for Panel A:

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	SGF N0[1]	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	-
15	Mark 12 MWM	-

Lane assignment for Panel B:

Lane	Sample	Amount (ng)
1	Blank	-
2	Precision Plus MWM	-
3	T0, protein+SGF	15
4	T0, protein+SGF	10
5	T0, protein+SGF	5
6	T0, protein+SGF	2.5
7	T0, protein+SGF	1
8	T0, protein+SGF	0.5
9	T0, protein+SGF	0.2
10	T0, protein+SGF	0.1
11	T0, protein+SGF	0.05
12	Mark 12 MWM	0.025
13	Blank	-
14	Blank	-
15		-

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0 and N7 are negative controls (no test protein); P0 and P7 are protein controls (no pepsin); T0-T7 are incubation time point in SGF.

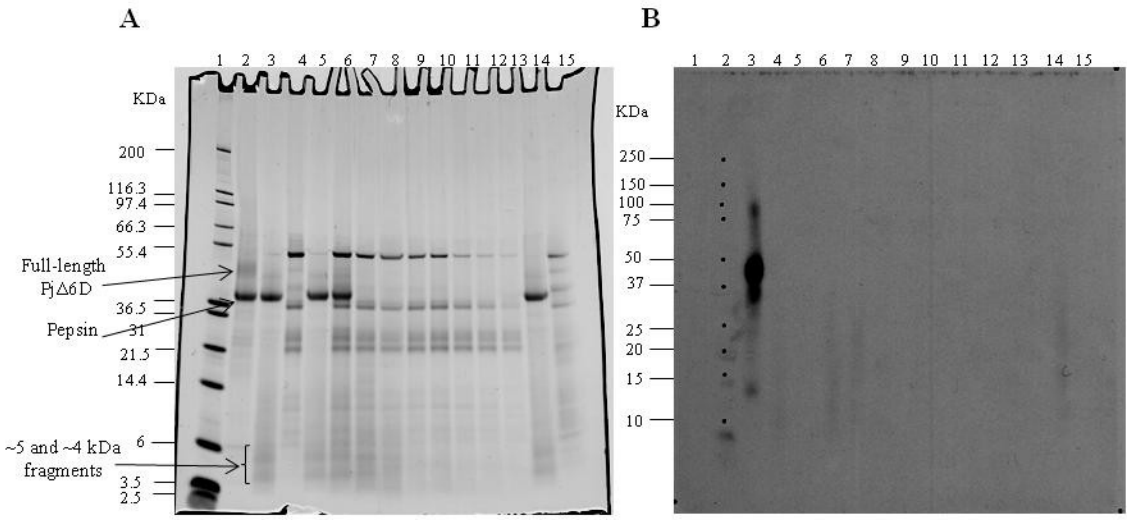


Figure 39. SDS-PAGE and Western Blot Analysis of the Digestion of the PjΔ6D Protein in SGF Followed by SIF

Panel A corresponds to the colloidal stained SDS-PAGE of the PjΔ6D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 0.9 µg of total protein was loaded per lane containing PjΔ6D protein. **Panel B** corresponds to the western blot of the PjΔ6D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 15 ng of total protein was loaded per lane containing the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the PjΔ6D protein migrated to approximately 46 kDa. A 5 min exposure is shown.

Lane	Sample	Incubation Time	Lane	Sample	Incubation Time
1	Mark 12 MWM	—	1	Precision Plus MWM	—
2	SEQ 0min	0	2	SEQ 0min	0
3	SEQ 2min	2 min	3	SEQ 2min	2 min
4	SEQ N0[1]	0	4	SEQ N0	0
5	SEQ P0	0	5	SEQ P0	0
6	SEQ T0	0	6	SEQ T0	0
7	SEQ T1	0.5 min	7	SEQ T1	0.5 min
8	SEQ T2	2 min	8	SEQ T2	2 min
9	SEQ T3	5 min	9	SEQ T3	5 min
10	SEQ T4	10 min	10	SEQ T4	10 min
11	SEQ T5	30 min	11	SEQ T5	30 min
12	SEQ T6	1 h	12	SEQ T6	1 h
13	SEQ T7	2 h	13	SEQ T7	2 h
14	SEQ P7	2 h	14	SEQ P7	2 h
15	SEQ N7	2 h	15	SEQ N7	2 h

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0 and N7 are negative controls (no test protein); P0 and P7are protein control (no pancreatin); T0-T7 are incubation time point in SGF.

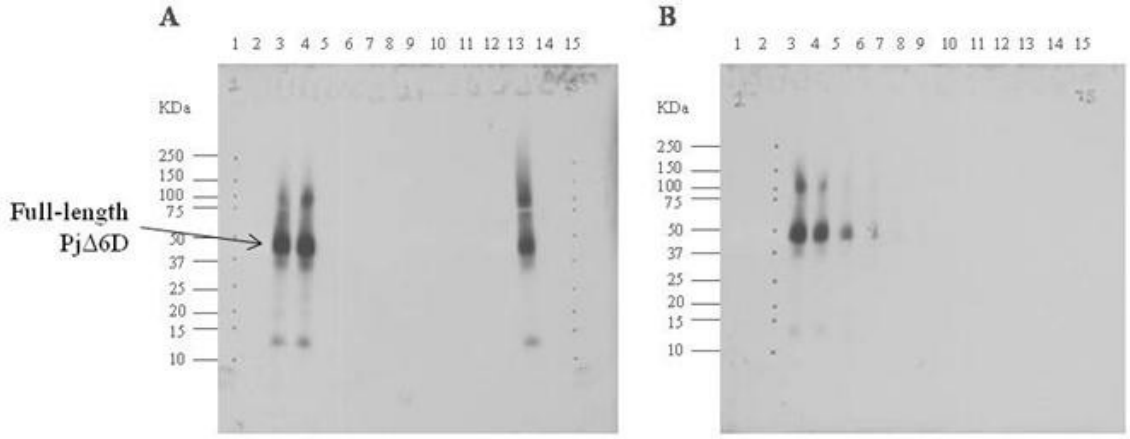


Figure 40. Western Blot Analysis of the Digestion of the PjΔ6D Protein in SIF

Panel A corresponds to the PjΔ6D protein digestion in SIF. Based on pre-digestion protein concentrations, 15 ng (total protein) was loaded in the lanes containing the PjΔ6D protein. **Panel B** corresponds to the limit of detection of the PjΔ6D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, the PjΔ6D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

Lane assignment for Panel A:

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
2	SIF N0[1]	0
3	SIF P0	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 h
9	SIF T5	2 h
10	SIF T6	4 h
11	SIF T7	8 h
12	SIF T8	24 h
13	SIF P8	24 h
14	SIF N8	24 h
15	Precision Plus MWM	-

Lane assignment for Panel B:

Lane	Sample	Amount (ng)
1	Blank	-
2	Precision Plus MWM	-
3	T0, protein+SIF	15
4	T0, protein+SIF	10
5	T0, protein+SIF	5
6	T0, protein+SIF	2.5
7	T0, protein+SIF	1
8	T0, protein+SIF	0.5
9	T0, protein+SIF	0.2
10	T0, protein+SIF	0.1
11	T0, protein+SIF	0.05
12	T0, protein+SIF	0.025
13	Precision Plus MWM	-
14	Blank	-
15	Blank	-

[1]A numerical code using the numbers 0 through 9 was used to distinguish incubation time points. N0 and N8 are negative controls (no test protein); P0 and P8are protein controls (no pancreatin); T0 and T7 are incubation time points in SIF.

Digestibility of the NcΔ15D Protein in SGF and SIF

Digestibility of the NcΔ15D protein in simulated gastric fluid (SGF) was assessed by SDS-PAGE and western blot methods. The extent of NcΔ15D protein digestion was evaluated by visual analysis of stained polyacrylamide gels (Figure 41) or by visual analysis of developed X-ray film (Figure 42). The LOD of the NcΔ15D protein by Colloidal Brilliant Blue G staining was 0.02 µg or approximately 2.5% of the total NcΔ15D protein loaded (0.02 µg divided by 0.8 µg of the protein loaded in each lane of the gel; Figure 41, panel B, lane 8). The LOD of the NcΔ15D protein by Western blotting was 0.5 ng or approximately 3.3% of the total NcΔ15D protein loaded (0.5 ng divided by 15 ng of the protein loaded in each lane of the gel; Figure 42, panel B, lane 9).

Visual examination of the Colloidal Brilliant Blue G stained gel (Figure 41, panel A) showed that the full-length NcΔ15D protein (~46 kDa) was digested below the LOD within 30 s of incubation in SGF (Figure 41, panel A, lane 5). Therefore, greater than 97% (100% - 2.5% = 97.5%) of the full-length NcΔ15D was digested within 30 s of incubation in SGF. Several fragments were observed in SGF for varying durations between the 30 s and 60 min time points. Fragments of ~17 and ~12 kDa were observed up to 5 min and 10 min, respectively (Figure 41, panel A, lanes 7 and 8). A minor band of ~7 kDa was observed only for 30 s and was digested after 2 min of exposure to SGF (Figure 41. panel A, lane 5 and 6). Two fragments, ~4 kDa and ~5 kDa, were observed for 20 min and 60 min, respectively (Figure 41, panel A, lanes 9 and 11).

Western blot analysis, using an antibody specific to full-length NcΔ15D protein, demonstrated that the full-length NcΔ15D protein was digested below the LOD within 30 s of incubation in SGF (Figure 42, panel A, lane 5). Based on the Western blot LOD for the NcΔ15D protein it was concluded that greater than 96% (100% - 3.3% = 96.7%) of the full-length NcΔ15D protein was digested within 30 s. Two fragments with apparent molecular weights of ~17 kDa and ~12 kDa were observed up to 5 min and 10 min of the digestion, respectively (Figure 42, panel A, lanes 7 and 8). These two fragments correspond to the ~17 kDa, and ~12 kDa fragments observed on the stained gel and represent products of proteolytic digestion of the NcΔ15D protein. The fragments with molecular weight of ~7 kDa, ~5 kDa and ~4 kDa were not observed on the Western blot indicating that either the NcΔ15D-specific antibodies raised against full-length protein do not recognize these fragments or that these fragments are not derived from NcΔ15D protein. To establish the identity of the ~5 kDa and ~4 kDa fragments, the N-terminal sequence of these fragments was evaluated. Sequence obtained for the fragment of ~4 kDa matched the expected sequence of the NcΔ15D protein to a region of NcΔ15D sequence starting at amino acid 376 (Table 15). The sequence obtained for the ~5 kDa fragment did not match the expected sequence of the NcΔ15D protein and its identity was not established. Since the protein has a purity of only approximately 74%, these fragments most likely originated from one of the endogenous soybean proteins co-purified with the NcΔ15D protein.

To better understand fate of the NcΔ15D protein during gastrointestinal digestion, it was exposed to digestion in SGF followed by digestion in SIF containing pancreatin. After digestion in SGF for 2 min, the reaction was quenched and the reaction mixture was subjected to further digestion in SIF. The digestibility in SIF of the NcΔ15D protein

fragments was evaluated by visual analysis of stained polyacrylamide gels (Figure 43). The gel was loaded with ~0.8 μg total protein (based on concentration of the protein prior to the digestion in SGF) for each of the SIF digestion time points. Visual examination of the stained gel demonstrated that all three low abundance NcΔ15D fragments (~17 kDa, ~12 kDa, ~4 kDa) were rapidly digested (<5 min) in SIF (Figure 43, Lane 7). The digestibility of NcΔ15D protein in SGF followed by SIF was also evaluated using Western blot (Figure 43, panel B). The gel was loaded with ~15 ng total protein (based on pre-digestion concentrations) for each of the digestion specimens. Two proteolytic fragments with molecular weight of ~17 kDa and ~ 12 kDa were observed at the 2 min time point in the SGF phase (Figure 43, panel B, lane 3). No fragments recognizable by NcΔ15D specific antibody were observed at the 30s digestion time point indicating their rapid degradation in SIF (Figure 43, panel B, lane 7).

SIF was also used as a stand-alone test system to test digestibility of the intact NcΔ15D protein. The assay was performed according to methods described in the United States Pharmacopeia (1995). The digestion of the NcΔ15D protein in SIF was evaluated by Western blot method (Figure 44). A Western blot to determine the LOD (Figure 44, panel B) of the NcΔ15D protein was performed concurrently with the SIF assay (Figure 44, panel A). The LOD was estimated to be 0.5 ng, which represented 3.3% of the total protein loaded in this experiment (0.5 ng divided by 15 ng of loaded protein). Western blot analysis demonstrated that the full-length NcΔ15D protein was digested below the LOD within 5 min of incubation in SIF (Figure 44, lane 5). Therefore, based on the LOD, greater than 96% (100% - 3.3% = 96.7%) of the full-length NcΔ15D protein was digested within 5 min. No proteolytic fragments of the NcΔ15D protein were detected at any time points suggesting that the NcΔ15D protein degrades rapidly when exposed to pancreatin at neutral pH.

In conclusion, these results show that the integral membrane protein, NcΔ15D, is readily digestible in SGF and SIF. Rapid digestion of the full-length NcΔ15D protein and any NcΔ15D protein fragments in SGF and SIF indicates that it is highly unlikely that the NcΔ15D protein will pose any safety concern to human and animal health.

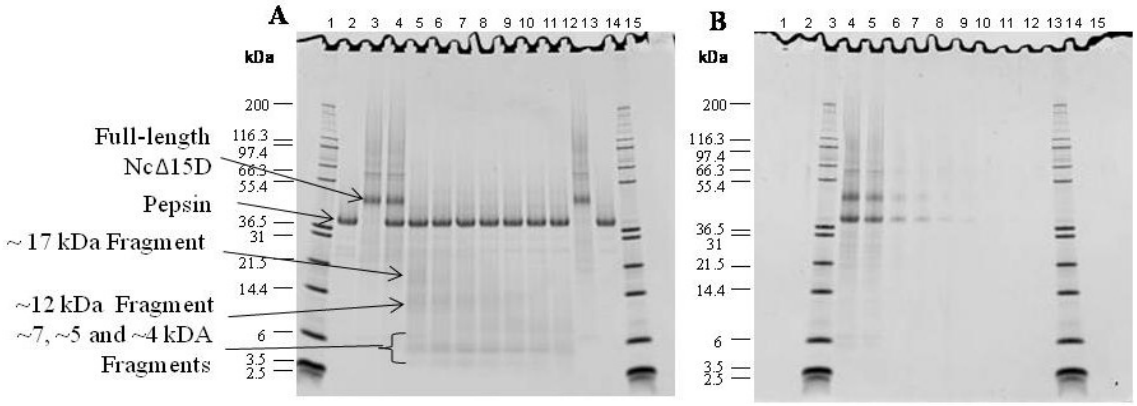


Figure 41. Colloidal Brilliant Blue G Stained SDS-gels of NcΔ15D Protein Digestion in SGF

Colloidal Brilliant Blue G stained SDS-PAGE were used to analyze the digestibility of the NcΔ15D protein in SGF. **Panel A** corresponds to the NcΔ15D protein digestion in SGF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded in each lane containing the NcΔ15D protein. **Panel B** corresponds to the limit of detection of the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa and pepsin migrated to approximately 38 kDa (indicated by arrows on the left).

Lane assignment for Panel A:			**Lane assignment for Panel B:**		
Lane	**Sample**	**Incubation Time (min)**	**Lane**	**Sample**	**Amount (ng)**
1	Mark 12 MWM	-	1	Blank	-
2	SGF N01	0	2	Blank	-
3	SGF P0	0	3	Mark 12 MWM	-
4	SGF T0	0	4	T0, protein+SGF	0.8
5	SGF T1	0.5	5	T0, protein+SGF	0.4
6	SGF T2	2	6	T0, protein+SGF	0.1
7	SGF T3	5	7	T0, protein+SGF	0.05
8	SGF T4	10	8	T0, protein+SGF	0.02
9	SGF T5	20	9	T0, protein+SGF	0.01
10	SGF T6	30	10	T0, protein+SGF	0.005
11	SGF T7	60	11	T0, protein+SGF	0.0025
12	SGF P7	60	12	T0, protein+SGF	0.001
13	SGF N7	60	13	Mark 12 MWM	—
14	Mark 12 MWM	-	14	Blank	-
15	Blank	-	15	Blank	-

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0 and N7 are negative controls (no test protein); P0 and P7are protein control (no pepsin); T0-T7 are incubation time points in sequential digestion assay (SEQ).

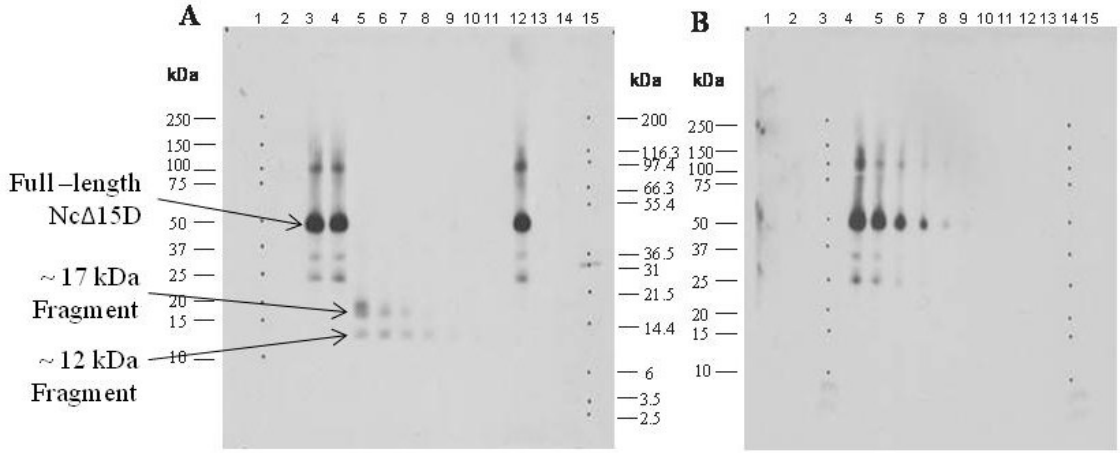


Figure 42. Western Blot Analysis of the Digestion of the NcΔ15D Protein in

Panel A corresponds to the NcΔ15D protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing the NcΔ15D protein. **Panel B** corresponds to the LOD of the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and right for Panel A and on left for Panel B, correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

Lane assignment for Panel A:

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	—
2	SGF N0[1]	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	—
15	Mark 12 MWM	—

Lane assignment for Panel B:

Lane	Sample	Amount (ng)
1	Blank	—
2	Blank	—
3	Precision Plus MWM	—
4	T0, protein+SGF	15
5	T0, protein+SGF	10
6	T0, protein+SGF	5
7	T0, protein+SGF	2.5
8	T0, protein+SGF	1
9	T0, protein+SGF	0.5
10	T0, protein+SGF	0.2
11	T0, protein+SGF	0.1
12	T0, protein+SGF	0.05
13	T0, protein+SGF	0.025
14	Precision Plus MWM	—
15	Blank	—

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0 and N7 are negative controls (no test protein). P0 and P7 are protein controls (no pepsin), T0 and T7 are incubation time point in sequential digestion assay (SEQ).

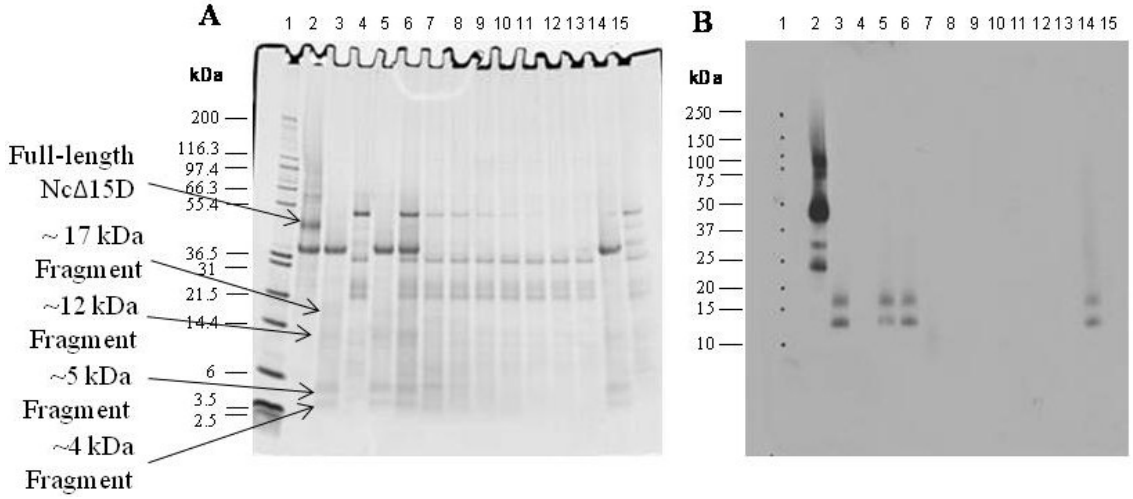


Figure 43. Colloidal Brilliant Blue G stained SDS-gel and Western Blot Analysis of the NcΔ15D Protein First Digested in SGF Followed by SIF

Panel A corresponds to the colloidal stained SDS-PAGE of the NcΔ15D protein first digested in SGF followed by SIF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded per lane containing NcΔ15D protein. **Panel B** corresponds to the Western blot of the NcΔ15D protein digestion in SGF followed by SIF. Based on pre-digestion protein concentrations, 15 ng of total protein was loaded per lane containing the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

Lane assignment for Panel A:			**Lane assignment for Panel B:**		
Lane	**Sample**	**Incubation Time**	**Lane**	**Sample**	**Incubation Time**
1	Mark 12 MWM	—	1	Precision Plus MWM	—
2	SEQ 0min	0	2	SEQ 0min	0
3	SEQ 2min	2 min	3	SEQ 2min	2 min
4	SEQ N0[1]	0	4	SEQ N0	0
5	SEQ P0	0	5	SEQ P0	0
6	SEQ T0	0	6	SEQ T0	0
7	SEQ T1	0.5 min	7	SEQ T1	0.5 min
8	SEQ T2	2 min	8	SEQ T2	2 min
9	SEQ T3	5 min	9	SEQ T3	5 min
10	SEQ T4	10 min	10	SEQ T4	10 min
11	SEQ T5	30 min	11	SEQ T5	30 min
12	SEQ T6	1 h	12	SEQ T6	1 h
13	SEQ T7	2 h	13	SEQ T7	2 h
14	SEQ P7	2 h	14	SEQ P7	2 h
15	SEQ N7	2 h	15	SEQ N7	2 h

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0 and N7 are negative controls (no test protein). P0 and P7 are protein controls (no pancreatin). T0 and T7 are incubation time points in sequential digestion assay (SEQ).

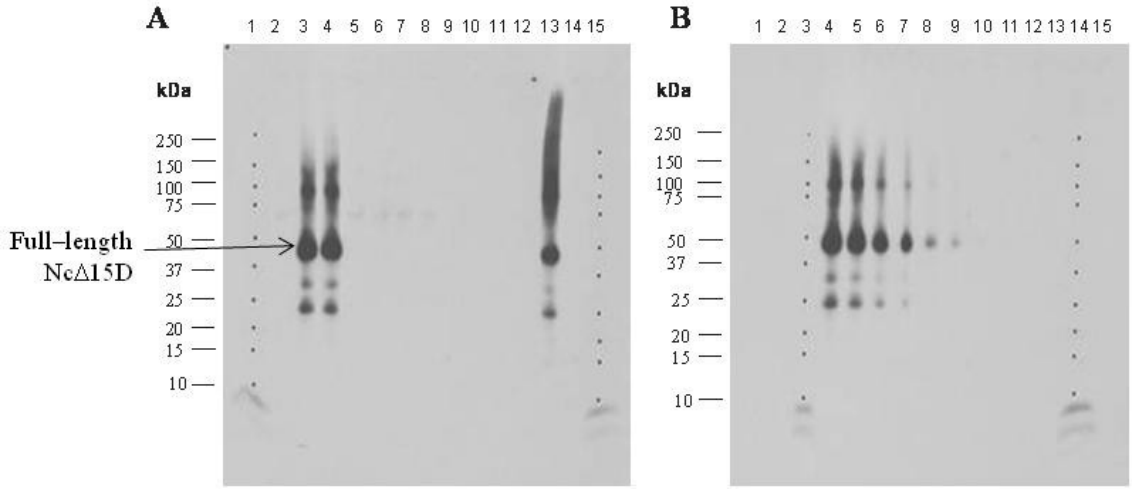


Figure 44. Western Blot Analysis of the Digestion of the NcΔ15D Protein in SIF

Panel A corresponds to the NcΔ15D protein digestion in SIF. Based on pre-digestion protein concentrations, 15 ng (total protein) was loaded in the lanes containing the NcΔ15D protein. **Panel B** corresponds to the LOD of the NcΔ15D protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded on each gel. In both gels, the NcΔ15D protein migrated to approximately 46 kDa. A 3 min exposure is shown.

Lane assignment for Panel A:

Lane	Sample	Incubation Time
1	Precision Plus MWM	—
2	SIF N0[1]	0
3	SIF P0	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 h
9	SIF T5	2 h
10	SIF T6	4 h
11	SIF T7	8 h
12	SIF T8	24 h
13	SIF P9	24 h
14	SIF N9	24 h
15	Precision Plus MWM	—

Lane assignment for Panel B:

Lane	Sample	Amount (ng)
1	Blank	—
2	Blank	—
3	Precision Plus MWM	—
4	T0, protein+SIF	15
5	T0, protein+SIF	10
6	T0, protein+SIF	5
7	T0, protein+SIF	2.5
8	T0, protein+SIF	1
9	T0, protein+SIF	0.5
10	T0, protein+SIF	0.2
11	T0, protein+SIF	0.1
12	T0, protein+SIF	0.05
13	T0, protein+SIF	0.025
14	Precision Plus MWM	—
15	Blank	—

[1]-A numerical code using the numbers 0 through 9 was used to distinguish incubation time points. N0 and N9 are negative controls (no test protein). P0 and P9 are protein controls (no pancreatin). T0 and T7 are incubation time points in SIF.

Table 15. N-terminal Amino Acid Sequence Analysis of the ~4 kDa Fragment Produced from the NcΔ15D Protein During Digestion in SGF

Amino acid residue # from the N-terminus	1	2	3	4	5	6	7	8	9	10
Predicted NcΔ15D Sequence[1,2]	A	T	N	S	I	R	P	M	L	G
	|	|	|	|	|	|	|	|	|	|
Observed Sequence[3]	A*	T	N	S	I	X	(P)	(M)	(L)	(G)

[1]The amino acid sequence of the ~4 kDa transiently stable fragment from the SGF T3 sample.
[2]The single letter IUPAC-IUB amino acid code is The single letter IUPAC-IUB amino acid code is A, alanine; T, threonine; N, asparagine; S, serine; I, isoleucine; R, arginine; P, proline; M, methionine; L, leucine; and G, glycine.
[3]The "X" refers to an undesignated call and ( ) denotes tenuous designation.
*The first amino acid corresponds to position 376 of the NcΔ15D protein sequence (Figure 27).

iv) Assessment of Human and IgE Binding to MON 87769, Control, and Reference Soybean Extracts SIF

One of the key elements in the safety assessment of the genetically improved plants is an evaluation of potential changes in their allergenic properties. Allergenic properties of the plant can potentially be altered if a known allergen or a protein that has high potential to become an allergen is introduced. In addition, the level of expression of endogenous allergens might be altered as a result of transformation and insertion of the new gene into the plant genome (Konig et al., 2004).

Soybean is one of eight allergenic foods that, in total, are responsible for approximately 90% of all food allergies (FAO, 1995). Soybean is less allergenic than other foods in this group and rarely is responsible for severe, life-threatening reactions (Cordle, 2004). Allergy to soybean is more prevalent in children than adults and is considered a transient allergy of infancy/childhood (Sicherer et al., 2000). Since soybean is a known allergenic food crop, there was a need to ensure that the introduction of the genes and production of the PjΔ6D and NcΔ15D proteins in soybean did not cause an unintended change in allergenic potential relative to conventional soybean. This question can be addressed by comparing levels of soybean-specific IgE in the biotechnology-derived soybean to a set of reference soybean varieties that are already on the market. Determining the levels of direct IgE binding using an enzyme-linked ELISA has been shown to be an appropriate method to perform such comparisons (Sten et al., 2004 ; Thomas et al., 2005), especially when the assay is validated and calibrated prior to data generation (Ahlstedt et al., 2006).

A study was conducted to determine the binding levels of IgE antibody collected from clinically documented, soybean allergic patients to protein extracts prepared from MON 87769, a conventional control variety, and 24 commercial soybean varieties that served to establish a range in IgE binding. The level of IgE binding provides an estimate

of the amount of endogenous soybean allergens present in the seed. The commercially available soybean reference varieties included Roundup Ready Soybean 40-3-2, conventional, high protein, high oil, and food-grade (tofu) varieties already on the market and used for human consumption.

Sera from 16 clinically documented, soybean allergic patients and six non-allergic patients were used to assess the range of IgE binding to each soybean seed extract. Only soybean allergic patients with a documented history of anaphylactic reactions to soybean and showed positive response to a Double-Blind Placebo Controlled Food Challenge (DBPCFC) were included in this study.

Aqueous extracts were prepared from the ground MON 87769 seed, conventional soybean control, and the commercial reference soybean varieties and analyzed by a validated ELISA for IgE binding. Each soybean extract was tested in triplicate wells at protein concentrations of 10 µg of total seed protein/ml of extract. Soybean-specific IgE binding was quantified by use of a soybean-specific IgE standard curve and expressed as ng of IgE/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163[4] containing a known amount of soybean-specific IgE into wells coated with internal reference soybean seed extract. The bound soybean-specific IgE was detected using biotin conjugated anti-human IgE polyclonal antibody from goat.

The IgE binding values obtained for reference soybean extracts were used to calculate a 99% tolerance interval for each patient's serum. The 99% tolerance interval represents the range of IgE binding for each patient's serum to the extracts prepared from the commercial soybean varieties such that 99% of the IgE binding values are expected to fall within this range with 95% confidence. The IgE binding levels obtained for protein extracts prepared from MON 87769 were compared to the calculated tolerance intervals. MON 87769 and control soybean IgE values fell within the established tolerance intervals obtained for each serum (Figure 45) with two exceptions. The IgE binding values for sera # 6 and #10 to extracts from MON 87769 were below the assay's limit of detection. None of the tested soybean varieties showed IgE binding to sera from non-allergic patients.

The results of this study demonstrate that the levels of endogenous soybean allergens in MON 87769 and conventional soybean control are comparable to the levels of endogenous soybean allergens in the soybean varieties that are currently on the market. Therefore, the MON 87769 has no greater allergenic potential than conventional soybean control or other soybean varieties that are currently on the market.

[4] PEI 163 – PEI is the designation given to all sera collected at Paul-Ehrlich-Institut. PEI 163 contained a known amount of soybean-specific IgE, i.e., 36 kU/l.

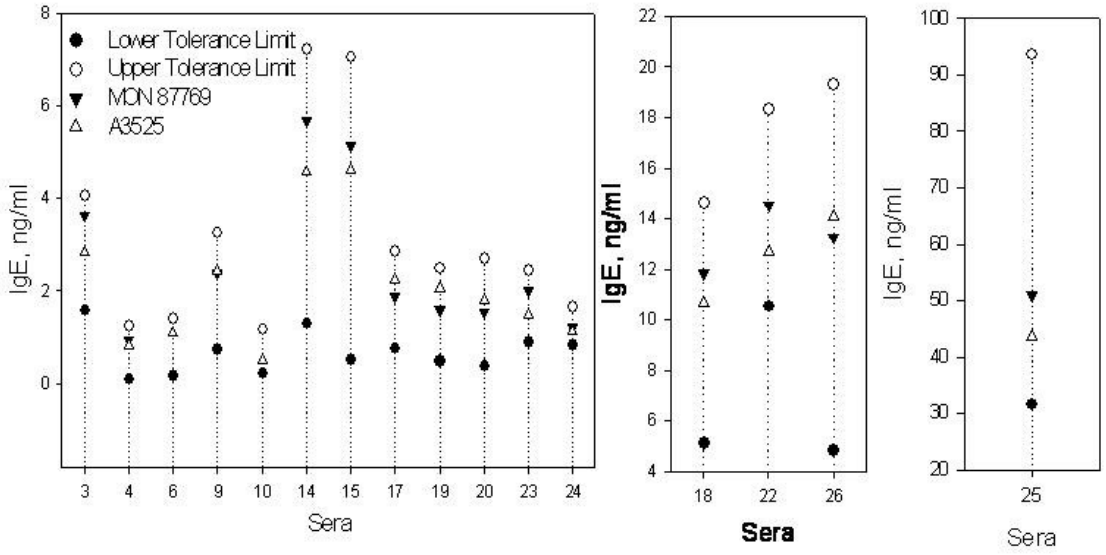


Figure 45. Comparison of the Levels of IgE Bound to Protein Extracts Obtained for MON 87769 and a Conventional Soybean Control A3525 to the 99% Tolerance Interval with a 95% Confidence Level Calculated for 24 Commercial Soybean Varieties

2.6 Characterisation of Other Novel Proteins

a) Identification

Not applicable

b) Toxicity

Not applicable

2.7 Comparative Analysis

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 of harvested soybean seed included proximates (moisture, fat, protein, ash, and carbohydrates by calculation), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids, fatty acids, trypsin inhibitors, phytic acid, lectin, isoflavones (daidzein, glycitein and genistein), vitamin E, raffinose, and stachyose. In addition, MON 87769 harvested seed was analyzed for SDA, GLA, trans-SDA and trans-ALA, as changes in the levels of these analytes were expected from the intended change. Compositional analysis on soybean forage included proximates and fiber.

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 United States, during the 2006 growing season. Ten different commercially available soybean varieties were included as references to provide data for the development of a 99% tolerance interval for each component analyzed. 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 harvested seed was collected at physiological maturity. The harvested seed and forage collected from MON 87769, the conventional control, and the reference varieties were analyzed for compositional components.

In all, 75 different analytical components, 68 in harvested seed and seven in forage were measured. Of the measured components, 26 in harvested seed had more than 50% of the observations below the assay limit of quantitation (LOQ). Thus, statistical analyses were conducted for 49 components (42 in harvested 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). Since 26 components had more than 50% of the observations below LOQ, they were excluded from the statistical analysis. 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 differences ($p<0.05$) 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 ILSI Crop Composition Database (International Life Sciences Institute Crop Composition Database Version 3.0).

Intended Changes to Fatty Acid Levels in MON 87769 Harvested 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 16). 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. 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) were also observed (Table 16). The formation of trans-ALA and trans-SDA is due to the known spontaneous trans-isomerization of unsaturated fatty acids, at rates that 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 present.

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 16.

Table 16. Combined-Site Summary of SDA, GLA, Trans-SDA and Trans-ALA Levels in MON 87769 Harvested 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.

a) Compositional Comparisons of Harvested Seed and Forage

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 17. Of the 28 detected differences in harvested 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 the ILSI Crop Composition Database. Therefore these differences were not considered to be biologically meaningful from a food/feed safety and/or nutritional perspective. The LA levels 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 are expected, since 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 harvested 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 17. The statistical summaries for the combined-site analysis data are presented in Table 18 (forage) and Table 19 (seed). Reported literature and ILSI Crop Composition Database ranges for the analytical components present in harvested seed are provided in Table 20.

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 17). In MON 87769 harvested 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 1), their concentrations are interdependent with that of other fatty acids, and therefore, were expected to be different in MON 87769 harvested 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 harvested seed were within the 99% tolerance interval for the population of the conventional reference varieties. Therefore the differences in fatty acid levels 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 1), LA levels were expected to be significantly lower across sites.

Nutrient Levels Other Than Fatty Acids in Harvested Seed

Harvested soybean seed was also analyzed for the following 26 nutrients: proximate (5), ADF, NDF, amino acids (18), and vitamin E. Nineteen nutrient analytes in harvested 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 and in more than one individual-site analyses (Table 17). Proline values in MON 87769 were found to be significantly different from the conventional soybean control in the combined-site 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 – 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 were very small (≤ 10%). The mean and range values for proline, arginine, cystine, glycine, and phenylalanine in MON 87769 harvested 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 and in one individual-site analyses. The magnitude of these differences were very small (<10%). For the remaining six of 19 nutrient analytes, significant differences were only found in the combined-site analysis (alanine, methionine, serine, threonine, tyrosine and carbohydrates). The magnitude of these differences were also 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 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 Crop Composition Database (Table 17 and 20 Therefore, these differences were not considered to be biologically meaningful from a food/feed safety and/or nutritional perspective.

Proximate and Fiber Levels in 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 analysis, 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.

b) Naturally Occurring Anti-Nutrient Levels in Soybean Seed

Soybean seed contains several well-described anti-nutritional factors according to OECD (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 17.

Trypsin inhibitors are heat-labile anti-nutrients 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 anti-nutrients. Compositional analyses of the harvested seed indicated that both trypsin inhibitors and lectins were present at similar levels in MON 87769 and the conventional soybean control, such that, no statistical differences were observed for all comparisons.

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 anti-nutrients as they have also been reported to have beneficial anti-carcinogenic effects (OECD, 2001). Genistein and daidzein levels in MON 87669 were found to be significantly different ($p<0.05$) from the conventional soybean control in the combined-site 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 and in each of four individual-site analyses were lower than the values observed in the conventional soybean control (Table 17). 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 Crop Composition Database ranges. Therefore, these differences do not raise any nutritional, anti-nutritional or other biological or toxicological concerns and are not considered biologically relevant.

Stachyose and raffinose are low molecular weight carbohydrates present in harvested seed that are considered to be anti-nutrients 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 17).

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 17).

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 anti-nutrients in harvested soybean seed.

Table 17. Summary of the Soybean Seed and Forage Analyte Difference (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 17. Summary of the Soybean Seed and Forage Analyte Difference (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 17. Summary of the Soybean Seed and Forage Analyte Difference (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)		
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)						
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]	

Table 17. Summary of the Soybean Seed and Forage Analyte Difference (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)		
18:1 Oleic Site IA-1	13.42	18.45	-27.29	0.001	[13.14 - 13.80]	[12.56, 27.98]
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]	

Table 17. Summary of the Soybean Seed and Forage Analyte Difference (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)		
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]	
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]	

Table 17. Summary of the Soybean Seed and Forage Analyte Difference (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)		
Statistical Differences Observed in One Site						
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]
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 Anti-nutrient						
Lectin (H.U./mg DW) Site MI	1.49	3.44	-56.79	0.019	[0.55 - 2.14]	[0, 16.00]

Table 17. Summary of the Soybean Seed and Forage Analyte Difference (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)		
Phytic Acid (% DW) Site IA-1	1.28	1.17	9.49	0.041	[1.22 - 1.34]	[0.51, 1.59]
Seed Isoflavone						
Glycitein (µg/g DW) Site IL	84.86	120.49	-29.57	0.044	[83.23 - 87.74]	[0, 243.40]
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.

Table 18. Statistical Summary from the Combined-Site Compositional Analysis of MON 87769 vs. the Conventional Control (A3525) Forage 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	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						
Ash (% DW)	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 (% DW)	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 (% DW)	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 (% DW)	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 19. 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, Vitamins, Anti-nutrients 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 (0.0084) [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 19. 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, Vitamins, Anti-nutrients 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 19. 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, Vitamins, Anti-nutrients 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 19. 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, Vitamins, Anti-nutrients 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						
Ash (% DW)	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 (% DW)	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 (% DW)	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 19. 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, Vitamins, Anti-nutrients 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]
Anti-nutrient						
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 (% DW)	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 (% DW)	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 (% DW)	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 19. 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, Vitamins, Anti-nutrients and Isoflavones (continued)

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 20. Literature and ILSI Database Values for Components in Conventional Soybean Seed and Forage

Forage Tissue/Component[1]	Literature Range[2]	ILSI Range[3]
Proximate (% dw)		
Ash	5.36 – 8.91	6.72 – 10.78
Carbohydrates	62.25 – 72.28	59.8 – 74.7
Moisture (% fw)	68.50 – 78.40	73.5 – 81.6
Protein	16.48 – 24.29	14.38 – 24.71
Total Fat	2.65 – 9.87	1.302 – 5.132
Fiber (% dw)		
Acid Detergent Fiber (ADF)	23.86 – 50.69	not available
Neutral Detergent Fiber (NDF)	19.61 – 43.70	not available
Seed Tissue Component[1]	**Literature Range[2,4]**	**ILSI Range[3]**
Proximates (% dw)		
Ash	4.61 – 6.32	3.89 – 6.99
Carbohydrates	32.75 – 40.98	29.6 – 50.2
Moisture (% fw)	6.24 – 11.10	4.7 – 34.4
Protein	34.78 – 43.35	33.19 – 45.48
Total Fat	14.62 – 20.68	8.10 – 23.56
Fiber (% dw)		
Acid Detergent Fiber (ADF)	9.22 – 26.26	7.81 – 18.61
Neutral Detergent Fiber (NDF)	10.79 – 23.90	8.53 – 21.25
Amino Acids (% dw)		
Alanine	1.62 – 1.89	1.51-2.10
Arginine	2.57 – 3.27	2.29-3.40
Aspartic acid	4.16 – 5.02	3.81-5.12
Cystine/Cysteine	0.52 – 0.69	0.37-0.81
Glutamic acid	6.52 – 8.19	5.84-8.20
Glycine	1.59 – 1.90	1.46-2.00
Histidine	0.96 – 1.13	0.88-1.18
Isoleucine	1.59 – 2.00	1.54-2.08
Leucine	2.79 – 3.42	2.59-3.62
Lysine	2.36 – 2.77	2.29-2.84
Methionine	0.45 – 0.63	0.43-0.68
Phenylalanine	1.82 – 2.29	1.63-2.35
Proline	1.83 – 2.23	1.69-2.28
Serine	1.95 – 2.42	1.11-2.48
Threonine	1.44 – 1.71	1.14-1.86
Tryptophan	0.30 – 0.48	0.36-0.50
Tyrosine	1.27 – 1.53	1.02-1.61
Valine	1.68 – 2.09	1.60-2.20

Table 20. Literature and ILSI Database Values for Components in Conventional Soybean Seed and Forage (Continued).

Seed Tissue Component[1]	Literature Range[2,4]	ILSI Range[3]
Fatty Acids (% Total FA)		
8:0 Caprylic	not available	0.148 – 0.148
10:0 Capric	not available	not available
12:0 Lauric	not available	0.082 – 0.132
14:0 Myristic	not available	0.071 – 0.238
14:1 Myristoleic	not available	0.121 – 0.125
15:0 Pentadecanoic	not available	not available
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	10.63-11.69	9.55 – 15.77
16:1 Palmitoleic	not available	0.086 – 0.194
17:0 Heptadecanoic	not available	0.085 – 0.146
17:1 Heptadecenoic	not available	0.073 – 0.087
18:0 Stearic	3.85-4.55	2.70 – 5.88
18:1 Oleic	15.02-31.19	14.3 – 32.2
18:2 Linoleic	44.03-54.96	42.3 – 58.8
18:3 Gamma Linolenic	not available	not available
18:3 Linolenic	5.08-10.26	3.00 – 12.52
20:0 Arachidic	0.31-0.43	0.163 – 0.482
20:1 Eicosenoic	not available	0.140 – 0.350
20:2 Eicosadienoic	not available	0.077 – 0.245
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.46-0.59	0.277 – 0.595
22:1 Erucic	not available	not available
24:0 Lignoceric	not available	not available
Vitamins (mg/100g dw)		
Vitamin E	1.29 – 4.80	0.19-6.17
Antinutrients		
Lectin (H.U./mg fw)	0.45 – 9.95	0.09 – 8.46
Trypsin Inhibitor (TIU/mg dw)	20.79 – 59.03	19.59 – 118.68
Phytic Acid (% dw)	0.41 – 1.92	0.63 – 1.96
Isoflavones	**(µg/g dw)**	**(mg/kg dw)**
Daidzein	224.03 – 1485.52	60.0 – 2453.5
Genistein	338.24 – 1488.89	144.3 – 2837.2
Glycitein	52.72 – 298.57	15.3 – 310.4
Bio-Actives (% dw)		
Raffinose	0.26 – 0.84	0.21 – 0.66
Stachyose	1.53 – 2.98	1.21 – 3.50

[1]fw=fresh weight; dw=dry weight; H.U. = hemagglutinating unit; TIU = trypsin inhibitor unit.
[2]Lundry et al. (2008). [3]ILSI Crop Composition Database at: http://www.cropcomposition.org. [4]Padgette et al. (1996).
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 dw

c) Compositional Comparisons of Processed Fractions from Soybean Seed

Harvested seed samples were collected for preparing soybean processed fractions 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 harvested 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). A summary of the significant differences observed between the processed fractions prepared from the harvested seed of MON 87769 and the conventional control are summarized in Table 21.

Table 21. Summary of the Soybean Processed Fraction Analyte Differences (p<0.05) in the Combined-Site 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) (% of A3525)	Signif. (p-Value)	Test Range	Commercial Tolerance Interval[2]
Meal Amino Acid (% DW)						
Aspartic Acid	6.10	6.00	1.68	0.006	[6.05 - 6.13]	[5.21, 6.97]
Glutamic Acid	9.79	9.58	2.25	0.002	[9.78 - 9.81]	[8.21, 11.33]
Histidine	1.42	1.40	1.46	0.017	[1.41 - 1.43]	[1.28, 1.57]
Tryptophan	0.68	0.67	1.75	0.006	[0.66 - 0.70]	[0.57, 0.77]
Meal Fiber						
Acid Detergent Fiber (% DW)	5.18	4.52	14.57	0.014	[4.93 - 5.46]	[1.79, 7.28]
Meal Proximate						
Carbohydrates (% DW)	38.0	40.05	-5.13	0.003	[37.49 - 38.38]	[28.99, 48.21]
RBD Oil Fatty Acid (% Total FA)						
16:0 Palmitic	12.1	11.48	5.34	<0.001	[11.98 - 12.23]	[7.68, 13.21]
18:0 Stearic	4.18	4.08	2.41	0.01	[4.13 - 4.20]	[3.00, 5.17]
18:3 9c,12c,15t (Trans ALA)	0.51	0.14	258.16	0.035	[0.47 - 0.54]	[0, 0.24]
24:0 Lignoceric	0.093	0.12	-24.19	<0.001	[0.076 - 0.11]	[0, 0.26]
RBD Oil Vitamin						
Vitamin E (mg/100g FW)	8.61	7.14	20.71	<0.001	[6.56 - 10.90]	[0, 29.82]
Protein Isolate Amino Acid (% DW)						
Leucine (% DW)	7.28	7.47	-2.54	0.044	[7.13 - 7.37]	[6.62, 8.11]

[1]DW = dry weight; FW = fresh weight; FA=fatty acid.
[2]With 95% confidence interval contains 99% of values expressed in the population of commercial soybean varieties.

i) Composition of DT Soybean Meal

Comparison of the composition of DT soybean meal from MON 87769 and the conventional soybean control showed that four amino acids (aspartic acid, glutamic acid, histidine, and tryptophan), ADF, and carbohydrate values were significantly different ($p<0.05$). The magnitude of differences were small, and the mean and range of values for the six analytes (four amino acids, ADF, and carbohydrates) in MON 87769 were all within the 99% tolerance interval for the population of conventional soybean varieties (Table 21). As expected, the DT soybean meal from MON 87769 contained a small amount (1.17%) of oil. DT soybean meal is expected to contain oil, and accordingly, the National Oil Processors Association has established a minimum oil content (0.5%) of defatted soybean meal that meets quality standards and guidelines for soybean meal for domestic and international shipping (NOPA, 2006). Therefore, the composition of DT soybean meal from MON 87769 is compositionally similar to conventional DT soybean meal.

ii) Composition of RBD Oil

Introduced Fatty Acid Levels in RBD Oil

RBD oil samples from MON 87769, the conventional soybean control and eight conventional soybean reference varieties were analyzed for fatty acids and vitamin E. SDA, GLA, and trans-SDA, which were not detected in conventional control RBD oil, were excluded from the statistical analysis and their values are reported in Table 22. In general, for the RBD oil the levels of SDA, GLA, and trans-SDA were similar, and in the same relative proportion, to those observed in harvested seed of MON 87769 (Table 16).

Table 22. Summary of the SDA, GLA, and Trans-SDA Levels in RBD Oil from MON 87769 in the Combined-Site Analysis

	(% Total FA[1])	
Analytical Component	**MON 87769 Mean (S.E.)[2]**	**MON 87769 Range**
18:4 Stearidonic (SDA)	22.62 (3.08)	[16.88 - 28.35]
18:3 γ-Linolenic (GLA)	6.68 (0.26)	[6.19 - 7.19]
18:4 6c,9c,12c,15t (Trans-SDA)	0.26 (0.052)	[0.17 - 0.39]

[1]FA=Fatty Acid; [2]S.E. = standard error.

Other Fatty Acids and Vitamin E Levels in RBD Oil

A summary of the differences ($p<0.05$) observed in comparing other fatty acids and vitamin E values in the RBD oil from MON 87769, the conventional soybean control, and eight conventional references are shown in Table 21. Unlike in harvested seed, trans-ALA was observed in the RBD oil of MON 87769 and the conventional soybean control. The presence of trans-ALA in RBD oil of the conventional soybean control is likely due to the effects of processing. As expected, the value for trans-ALA was significantly higher for MON 87769 (mean = 0.51% of total fatty acids) when compared to the conventional soybean control (mean = 0.14% of total fatty acids). Palmitic and stearic acids were observed to be significantly different in MON 87769 compared to the conventional soybean control; however the magnitude of these differences were low (<6% of the total fatty acids). Lignoceric acid, which occurs at very low levels in RBD oil (~0.1%), was observed to be significantly different in MON 87769 compared to the conventional soybean. Vitamin E value was observed to be 20% higher in MON 87769 compared to the conventional soybean control. While significant differences ($p<0.05$) were noted in some of the compositional comparisons between the RBD oil of MON 87769 and the conventional soybean control, the MON 87769 values (including vitamin E values) were within the calculated 99% tolerance interval for the population of eight conventional soybean reference varieties grown at the same time and in the same field trial as MON 87769.

Additionally, while the LA values for MON 87769 seed were significantly different from the conventional soybean control seed (Table 17), the LA values for MON 87769 RBD oil were not significantly different from the conventional soybean control RBD oil. However, the levels of LA were comparable in MON 87769 harvested seed and MON 87769 RBD oil. The mean LA values in MON 87769 harvested seed and RBD oil were 22.8 and 25.7 wt% of total fatty acids, respectively (Table 17).

Composition of Soybean Protein Isolate

Soybean protein isolate is prepared by removing all non-protein substances from defatted soybean flour and analyzed for amino acids and moisture. Leucine was observed to be significantly different ($p<0.05$) in the MON 87769 protein isolate compared to the protein isolate from conventional soybean control; however the magnitude of the difference was very small (<3%). The mean and range of values for amino acids and moisture obtained from MON 87769 protein isolate were comparable to the conventional soybean control values and were also within the 99% tolerance interval for the population of conventional reference varieties.

Composition of Crude Lecithin

The crude lecithin from MON 87769, the conventional soybean control, and conventional soybean reference varieties were analyzed for four individual phosphatides, i.e., α-phosphatidic acid, α-phosphatidyl-choline, α-phosphatidyl-etholamine, and α-phospha-tidylinositiol. The phosphatide values in crude lecithin from MON 87769 were comparable to the levels of phosphatide values in the conventional soybean control.

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 for the OECD list of analytes (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 frations 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.

Intended Changes to the Composition of Food and Feed

Through the introduction of PjΔ6 and NcΔ15 desaturases, the fatty acid composition of MON 87769 has been intentionally altered to produce the omega-3 fatty acid, SDA. As expected, the increased levels of SDA also resulted in increased levels of GLA and a slight increase in ALA levels. Since SDA, GLA, and ALA are produced at the expense of LA, the level of LA is expected to be lower in MON 87769 compared to the conventional soybean control. 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%. Associated with this change in SDA levels is the production of GLA from the Δ6 desaturation of LA by PjΔ6D. The GLA levels in MON 87769 ranged from 6.07 to 8.03% of total fatty acids, with a mean of 7.09%. It is expected that more ALA is produced in MON 87769 from the action of the NcΔ15D on LA. Trans-isomerization occurs spontaneously with unsaturated fatty acids at rates that increase with the increasing degree of unsaturation (Chardigny et al., 1996), and since SDA and ALA represent a significant proportion of total fatty acids in MON 87769 (approx. 35-40% in total), it is reasonable to expected that the trans-isomers of these fatty acids will be formed. Trans-SDA and trans-ALA are present at 0.18% and 0.44% of total fatty acids, respectively.

Given the intended shift in fatty acid metabolism, the fatty acid profile in MON 87769 harvested seed was expected to differ from conventional soybean seed. Except for the intended change in fatty acid composition, there were no biologically-relevant differences noted when MON 87769 was compared to the conventional control, A3525, although, for some of these comparisons, a significant difference ($p<0.05$) was noted. In the instances in which a statistically significant difference was noted, the composition values for MON 87769 were within the calculated 99% tolerance interval for the population of conventional reference varieties grown at the same time, and from the same fields, as MON 87769 and, therefore, should not be considered to be biologically meaningful from a food/feed safety and/or nutritional perspective.

Except for the fatty acid changes and the presence of the introduced proteins, PjΔ6D and NcΔ15D, there have been no biologically-relevant changes to the composition (including nutrients and anti-nutrients) of food or feed derived from MON 87769 compared to other conventional soybean varieties. Given this extensive compositional characterization, it is concluded that no pleiotropic changes have occurred in MON 87769, and that only the intended changes in fatty acid composition have occurred. Therefore, except for the intended changes, MON 87769 is concluded to be compositionally and nutritionally equivalent to conventional soybean varieties already on the market.

d) Allergenic Proteins

Not applicable

2.8 Nutritional Impact

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 LA levels in MON 87769.

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 (Harris et al., 2008; James et al., 2003; Diboune, 1992; Surette et al., 2004; Miles et al., 2004a; 2004b; Schubert, 2007; Hammond et al., 2008b; Harris et al., 2007; Hanson-Petrik et al., 2000; and Khan, 2007). 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.

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 (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 (Leventhal et al., 1993; Zurier et al., 1996; Middleton et al., 2002; Barre and Holub, 1992; Guivernau et al., 1994; Miles et al., 2004b; Fewtrell et al., 2004; Fiocchi et al., 1994; Stoney et al., 2004). A number of animal studies have also demonstrated the safety of large doses of GLA consumption (Karlstad et al., 1993; Palombo et al., 2001; 2000; Kaku et al., 2001; Engler et al., 1992; 1998; Peterson et al., 1999; Frenoux et al., 2001; Liu et al., 2004; Fukushima et al., 2001; Ingram et al., 1996; Everett et al., 1988a; Miller and Ziboh, 1988; Wainwright et al., 2003). 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.

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). TFAs in non-hydrogenated vegetable oils are generally low by comparison, typically less than 1% of total fatty acids. These TFAs arise during oil refining, particularly during heat-requiring processes such as deodorization (Chardigny et al., 1996; Ledoux, 2007). Measured values for 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 MON 87769 to the overall dietary TFA intake will be minimal relative to commonly experienced human dietary intakes. Thus, the trans-SDA and trans-ALA are concluded to not raise safety concerns.

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 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.

Safety and Nutrition Assessment of SDA Soybean Oil

Beside the fatty acids typically found in soybean oil, MON 87769 oil contains approximately 20 – 30% of SDA (wt% of total fatty acids) and ~7% of GLA (wt% of total fatty acids). Also seen in the oil are very small amounts of trans-SDA (<0.3 wt% of total fatty acids) and trans-ALA (<0.5 wt% of total fatty acids) from the spontaneous isomerizaton known to occur with polyunsaturated fatty acids (Chardigny et al., 1996). However, the total trans-fat content of MON 87769 oil remains well below the allowed level of trans-fat in vegetable oil. Additionally, commercial soybean oil contains trans-ALA at levels >0.5 wt% of total fatty acids in the oil (Chardigny et al., 1996). A number of reported human and animal studies have looked into the safety of SDA and GLA and have confirmed that SDA and GLA are safe for human and animal consumption. In addition to these published studies, Monsanto has confirmed the safety of SDA and GLA through a 28-day rodent study and a combined 90-day rodent study with one-generation reproduction study conducted with SDA soybean oil from MON 87769 as test material (Hammond et al., 2008).

Safety and Nutrition Assessment Conclusion

In conclusion, having demonstrated: (i) the compositional equivalence of MON 87769 harvested seed (except for the intended fatty acid changes) and forage to harvested 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 omega-3 fatty acid. No additional information was considered to be necessary to support the safety and nutritional assessment of MON 87769.

a) Other Information Relevant to Nutritional Assessment of MON 87769

i) 90-day Study in the Rat

A 90-day rat toxicology study was conducted with defatted meal from MON 87769, in order to further assess the safety of the soybean meal when fed to rats. The control for this study was processed meal from a conventional soybean variety, A3525 that has genetic background similar to that of MON 87769. In this study, processed soybean meal from MON 87769 was fed to rats for at least 90 days. Sprague-Dawley

(Crl:CD®[SD]) rats, following acclimation, were randomly assigned to three groups consisting of 20 males and 20 females/each following receipt from Charles River Laboratories, Inc. (Raleigh, NC) so that the body weights/group were ± 20% of the mean for each sex at the time of randomization at six weeks of age. All three diets were formulated to contain 15% (w/w) soybean meal: test (MON 87769), conventional control (A3525) with genetics similar to MON 87769, or a mixture of 5% (w/w) test (MON 87769) and 10% (w/w) control (A3525).

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. Clinical pathology evaluations (hematology, coagulation, serum chemistry and urinalysis) were performed on blood and urine samples collected from 10 randomly-selected, fasted animals/sex/group on the day of the scheduled necropsy (during study week 13). Complete necropsies were conducted on all animals, and selected organs were weighed at the scheduled necropsy. Approximately 23 tissues were examined microscopically from all animals fed the 15% MON 87769 test meal or 15% A3525 control meal diets.

Statistical analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 5% and 1%, comparing each test substance-treated group to the control group by sex. Body weight, cumulative body weight change, food consumption, clinical pathology and organ weight data were subjected to a parametric one-way analysis of variance (ANOVA) (Snedecor and Cochran, 1980) to determine intergroup differences. If the ANOVA revealed statistically significant ($p<0.05$) intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the test substance treated groups to the control group. Microscopic findings were compared using Fisher's exact test (Steel and Torrie, 1980).

Results

There were no treatment related deaths. One female from the group fed 5% MON 87769 meal was found dead on study day 60. Based on the absence of antemortem signs and lack of gross pathology that can be attributed to this death, the cause of death was undetermined, but is considered to be incidental and unrelated to test substance administration. There were no test substance-related clinical observations or differences in body weights, cumulative body weight gains, or food consumption. Isolated occurrences of significant increase in food consumption were noted in the male group fed 15% MON 87769, but based on the lack of consistency and lack of correlated finding in the 15% MON 87769 female group, these were not considered treatment-related events. No test-substance related effects on organ weights were noted. There were no test substance related effects on clinical pathology parameters. Urobilinogen of the females from the 5% MON 87769 group was significantly increased over control, but this was considered a spurious finding. There were no test substance related gross macroscopic or microscopic findings.

Conclusion

Administration of MON 87769 processed soybean meal to rats for at least 90 consecutive days at inclusions of up to 15% (w/w) in the diet (equivalent to

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10,915 mg/kg body weight/day for males and 12,597 mg/kg body weight/day for females) had no adverse effects on the growth or health of Sprague-Dawley rats.

ii) 42-day Study in Broiler

A 42-day broiler study was conducted to compare the nutritional value of defatted soybean meal derived from MON 87769, to a conventional control (A3525) with genetics similar to MON 87769, and six commercial reference soybean varieties when fed to rapidly growing Ross x Ross 308 broilers

Results
Performance of broilers fed diets containing defatted meal from MON 87769 was not different ($P \geq 0.05$) than that of broilers fed diets formulated with control soybean meal produced from conventional soybeans of similar genetic background (Table 23). Performance over the entire 42-day test period was also not different ($P \geq 0.05$) for birds fed diets containing defatted meal from MON 87769 compared to the population of birds fed conventional control and reference soybean meal (Table 23). A diet × sex interaction was detected ($P < 0.15$) for average Day 0 bird weight; however, within sex analyses detected no difference ($P \geq 0.05$) between MON 87769 and conventional control soybean meal for male or female birds. Bird performance measurements were of similar magnitude for test, control and reference groups. No unexpected effects on broiler performance were observed when broilers were fed diets formulated with defatted meal from MON 87769 compared to diets formulated with control or reference soybean meal.

Carcass yield measurements were not different ($P \geq 0.05$) for broilers fed diets containing defatted meal from MON 87769 compared to those fed conventional control soybean meal, with exception of fat pad weight (0.039 versus 0.044 kg/bird, and 1.50 versus 1.64 % of live weight, respectively) (Table 23). Similarly, carcass yield measures were not different ($P \geq 0.05$) for birds fed diets containing defatted meal from MON 87769 compared to the population of those fed diets containing conventional control and reference soybean meal, with exception of fat pad weight (0.039 versus 0.044 kg/bird, respectively) and wing weight (10.9 versus 10.6% of chilled carcass weight, respectively). The magnitude of these differences is quite small and is therefore not considered biologically meaningful. Average carcass measurements were of similar magnitude for test, control and reference groups.

Measurement of fat, moisture and protein content of skinless breast and thigh meat samples collected during bird processing showed no differences ($P \geq 0.05$) among treatments (Table 23). Meat analysis results were not different ($P \geq 0.05$) for birds fed diets containing defatted meal from MON 87769 versus those of birds fed diets containing control or reference soybean meal based on individual diet comparisons or comparison to the population of control and reference soybean meal diets.

Conclusions
There were no biologically relevant differences in broiler performance, carcass yield or meat composition between broilers fed diets containing defatted meal from MON 87769 and those fed diets containing genetically similar conventional control A3525 or reference soybean meal. The diets containing defatted meal from MON 87769

were as wholesome as the diets formulated with conventional control or reference soybean meal regarding their ability to support the rapid growth of broiler chickens. These data support the conclusion that defatted meal from MON 87769 is as nutritious as conventional soybean meal.

Table 23. Performance, Carcass Yield and Meat Quality of Broilers fed Diets Formulated with Soybean Meal versus that of the Population of Broilers fed Diets Formulated with Conventional Control and Reference Soybean Meal (means[1] ± SEM[2] combined across males and females).

Parameter	Soybean Meal Diets		Treatment p-value[3]	LSD[4] 5%
	MON 87769	Conventional Control and References		
Performance				
Average bird weight (g/bird), d 0	38.733 ± 0.2618	39.183 ± 0.0990	0.1339[8]	0.610
Average bird weight (kg/bird), d 42	2.699 ± 0.0317	2.754 ± 0.0120	0.1550	0.083
Average bird gain (kg/bird), d 42	2.661 ± 0.0317	2.715 ± 0.0120	0.1575	0.083
Feed intake (kg/bird), d 0 to 42	4.104 ± 0.0877	4.211 ± 0.0332	0.2784	0.204
Feed:gain (kg/kg), d 0 to 42	1.673 ± 0.0494	1.661 ± 0.0187	0.8263	0.115
Adj. feed:gain[5] (kg/kg), d 0 to 42	1.592 ± 0.0083	1.595 ± 0.0031	0.6961	0.022
Carcass Yield				
Processing live wt[6] (kg/bird)	2.643 ± 0.0300	2.693 ± 0.0114	0.1759	0.079
Chilled wt (kg/bird)	1.919 ± 0.0257	1.951 ± 0.0097	0.2934	0.067
Chilled wt (% of live wt.)	72.577 ± 0.1691	72.444 ± 0.0639	0.4900	0.442
Fat pad wt (kg/bird)	0.039 ± 0.0015	0.044 ± 0.0006	0.0357	0.004
Fat pad wt (% of live wt.)	1.496 ± 0.0567	1.636 ± 0.0214	0.0602	0.148
Breast wt (kg/bird)	0.547 ± 0.0088	0.552 ± 0.0033	0.5919	0.023
Breast wt (% of chilled wt)	28.597 ± 0.2127	28.328 ± 0.0804	0.2409	0.453
Thigh wt (kg/bird)	0.314 ± 0.0046	0.321 ± 0.0017	0.2474	0.012
Thigh wt (% of chilled wt)	16.416 ± 0.1076	16.417 ± 0.0407	0.9977	0.229
Drum wt (kg/bird)	0.263 ± 0.0042	0.266 ± 0.0016	0.5302	0.011
Drum wt (% of chilled wt)	13.762 ± 0.1018	13.597 ± 0.0385	0.1347	0.217
Wing wt (kg/bird)	0.207 ± 0.0024	0.206 ± 0.0009	0.9061	0.006
Wing wt (% of chilled wt)	10.855 ± 0.0843	10.577 ± 0.0319	0.0029	0.180
Breast Meat Analysis[7]				
Moisture (%)	75.199 ± 0.1664	75.211 ± 0.0629	0.9517	0.435
Protein (%, as is basis)	23.184 ± 0.1920	23.175 ± 0.0726	0.9640	0.502
Fat (%, as is basis)	1.087 ± 0.0731	0.980 ± 0.0276	0.1741	0.156
Thigh Meat Analysis[7]				
Moisture (%)	76.982 ± 0.1704	76.989 ± 0.0644	0.9718	0.446
Protein (%, as is basis)	20.675 ± 0.2115	20.712 ± 0.0800	0.8716	0.493
Fat (%, as is basis)	1.362 ± 0.1170	1.448 ± 0.0442	0.4929	0.249

[1] Each mean for MON 87769 represents 10 observations (1/pen) and the that for the population of control and references represents 70 observations (1/pen).

[2] SEM = standard error of the mean for respective parameter.

[3] MON 87769diet versus the population of the control and six reference diets

[4] LSD = least significant difference between two means ($P < 0.05$).

[5] Adjusted feed:gain is adjusted by adding the weight at removal of mortalities and culls to the weight of the live birds in a pen.

[6] Processing live weight = pre-processing weight on d 43 (males) or d 44 (females).

[7] Mean values for skinless breast and thigh meat analyses based on one bird per pen.

[8] A diet × sex interaction ($P < 0.15$) was detected, see statistical report, Tables 1 - 26 for within sex analysis

2.9 Other Techincal Information

a) Detection Methodology

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.

b) Projected Market Penetration

Soybean is the most commonly grown oilseed in the world. In 2007/08, approximately 218.8 million metric tons (MMT) of harvested soybean seed were produced, representing 56% of the world's oilseed production.

Approximately 50% of the world's soybean seed supply was crushed to produce soybean meal and oil in 2007 (ASA, 2008a; 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, the EU, Japan, and Mexico being the top soybean seed import geographies (ASA, 2008b). The remainder of the soybean seed produced was used as certified seed, feed, or stocks.

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 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 in the northern U.S. soybean growing region. The oil will be used in food and feed applications where omega-3 products are currently being used. The co-product, 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.

There are no current plans to produce MON 87769 commercially in Australia or New Zealand. However, soybean grains and products containing processed soybean may potentially be imported into Australia and New Zealand from countries where MON 87769 is intended for commercialisation.

PART 3 REGULATORY / LEGISLATIVE IMPLICATIONS

3.1 Other Approvals

a) Relevant Overseas Approvals

Monsanto has submitted a food and feed safety and nutritional assessment summary for MON 87769 to the United States Food and Drug Administration (FDA) and has also requested a Determination of Nonregulated Status for MON 87769 from the United States Department of Agriculture-Animal and Plant Health and Inspection Service in 2009. Similarly, food, feed and environmental submissions have or will be made to CFIA, and Health Canada, and an import submission for food and feed use of MON 87769 to the European Food Safety Authority (EFSA).

Regulatory submissions have been or will be made to countries that import significant soybean or food and feed products derived from U.S. soybean and have functional regulatory review processes in place. These include submissions to a number of additional governmental regulatory agencies including, but not limited to, Ministry of Agriculture (MOA) of China, Ministry of Health, Labor and Welfare (MHLW) and Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan, and the Korean Food and Drug Administration (KFDA) and the Rural Development Administration (RDA). As appropriate, notifications will be made to countries that import significant quantities of U.S. soybean and soybean products and do not have a formal regulatory review process for biotechnology-derived crops.

b) Approval Refusal

No application has been rejected or withdrawn by any regulatory body.

3.2 Regulatory Impact Statement

As described previously, soybean is one of the most agriculturally important crops in the world. This application – if approved – will ensure imports of food derived from MON 87769 soybean comply with the *Australian New Zealand Food Standards Code*. This will ensure that there is no potential for trade disruption on regulatory grounds.

PART 4 STATUTORY DECLARATION – AUSTRALIA

I, Amanda Forster, declare that the information provided in this application fully sets out the matters required and that the same are true to the best of my knowledge and belief, and that no information has been withheld that might prejudice this application.

Signature: _____

Declared before me ……

This …….. day of …….. 2009.

PART 5 REFERENCES

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