FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

SAFETY ASSESSMENT REPORT

APPLICATION A1041 – FOOD DERIVED FROM STEARIDONIC ACID SOYBEAN LINE MON87769

SUMMARY AND CONCLUSIONS

Background

Monsanto Company has developed a genetically modified (GM) soybean, known as MON87769, which produces stearidonic acid (SDA), an omega-3 fatty acid. The modification was achieved through the introduction of genes encoding two enzymes involved in fatty acid metabolism, delta-6 desaturase from the plant *Primula juliae* (PjΔ6D) and delta-15 desaturase from *Neurospora crassa* (NcΔ15D). The genetic modification also results in minor changes in the level of other fatty acids in soybean oil, including linoleic acid and gamma linolenic acid. Conventional soybean lacks a delta-6 desaturase gene, therefore SDA is normally not found in soybean products.

Delta-6 desaturase activity is required to convert alpha linolenic acid (ALA) to SDA in the omega-3 fatty acid biosynthetic pathway. Expression of the introduced delta-6 desaturase gene (*Pj.D6D)* also results in the conversion of linoleic acid (LA) to gamma linolenic acid (GLA), in the omega-6 fatty acid pathway. In order to enhance production of SDA in the soybean, the delta-15 desaturase gene (*Nc.fad3*) was also introduced to the plant. The delta-15 desaturase activity catalyses the conversion of (i) LA to ALA, thereby increasing the pool of ALA available for conversion to SDA, and (ii) GLA to SDA. The combined effect of the two introduced enzymes is that SDA is produced in seeds to a level of approximately 26% of total fatty acids.

The Applicant has not applied to the Office of the Gene Technology Regulator (OGTR), or the Environmental Risk Management Authority (ERMA) for a licence to grow MON87769 soybean in Australia or New Zealand. Rather, it is anticipated that it would be an identity preserved, low acreage crop cultivated in typical soybean growing regions in the United States. If approved, foods derived from soybean MON87769 would only enter the food supply in Australia and New Zealand through imported products.

History of Use

Soybean (*Glycine max* (L.) Merr) is grown as a commercial crop in over 35 countries worldwide. Soybean-derived products have a range of food and feed as well as industrial uses and have a long history of safe use for both humans and livestock. Oil accounts for over 90% of the soybean products consumed by humans. The major food product to be derived from MON87769 is refined SDA soybean oil which can partially replace soybean or other oils in a variety of food applications, including baked goods, breakfast cereals, grain products and pastas, sauces, milk products and soups. Refined SDA soybean oil will not be suitable for frying, nor for the manufacture of edible oil spreads, due to its high content of polyunsaturated fatty acids. Soybean meal from MON87769 is similar in composition to other commodity soybean meal and can be used in a traditional manner in foods.

Molecular Characterisation

Comprehensive molecular analyses of MON87769 indicated insertion of the intended gene cassette encoding the two desaturase enzymes at a single genetic locus. The promoters and leader sequences used in the gene constructs are soybean-derived elements, known to be spatially and temporally active only in the developing seed. Zygosity data generated over three generations confirmed stability of the introduced genetic elements and segregation data indicated that the genes are inherited in a predicted manner according to Mendelian principles.
Bioinformatic analysis of theoretical ORFs associated with the junction regions did not indicate any likelihood of unexpected gene products arising from the integration of the new genes. There are no marker genes encoding antibiotic-resistance in soybean MON87769.

Characterisation of Novel Proteins

The two novel proteins expressed in MON87769 are the enzymes PjΔ6D and NcΔ15D. Both are integral membrane proteins that are members of a family of integral membrane fatty acid desaturases found in all eukaryotic organisms.

Evidence of the function of the PjΔ6D and NcΔ15D proteins in MON87769 was provided. MON87769 accumulates SDA as well as GLA, fatty acids that are novel to soybean seed. *In vitro* assays with crude extracts of MON87769 immature seed and radiolabelled fatty acid precursors showed production of labelled SDA as the unique product. Expression of the PjΔ6D and NcΔ15D proteins in yeast showed the production of SDA and GLA when precursor fatty acids were provided exogenously in the medium.

The PjΔ6D protein is 446 amino acids in length, has a predicted molecular mass of 51 kDa and is identical to the native protein produced in Primrose. The protein is characterised by multiple membrane spanning regions and an amino terminal cytochrome b5 domain carrying heme-binding motifs, similar to other Δ6 desaturases. Mutations at these conserved sites are known to abolish enzyme activity.

The NcΔ15D protein is 429 amino acids in length, has a predicted molecular mass of 49.2 kDa, and differs from the native microbial protein by a single amino acid at the first position after the start codon, which was introduced for cloning purposes. The NcΔ15D protein also shows multiple membrane-spanning regions and conserved motifs characteristic of other integral membrane desaturases.

Western blots were the most reproducible and accurate method for analysing the expression of PjΔ6D and NcΔ15D proteins in plant tissues. The mean levels of PjΔ6D in immature and mature seed were 100 µg/g and 1.8 µg/g dry weight respectively. The mean levels of NcΔ15D in immature and mature seed were 200 µg/g and 10 µg/g dry weight respectively. The identity and physicochemical properties of the PjΔ6D and NcΔ15D proteins as expressed in MON87769 soybean were confirmed in a range of laboratory-based studies. The proteins conformed in size and amino acid sequence to that expected, and did not exhibit any post-translational modification including glycosylation.

Extensive studies were conducted to examine the potential toxicity and allergenicity of the PjΔ6D and NcΔ15D proteins. Bioinformatic studies confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens. Digestibility studies using simulated gastric and intestinal fluids demonstrated that the proteins would be readily degraded as normal dietary protein. Separate acute oral toxicity studies in mice with the PjΔ6D and NcΔ15D proteins, purified from MON87769 immature seed, also confirmed the absence of toxicity. In addition, documentation of the history of safe consumption of the PjΔ6D and NcΔ15D proteins, or close structural and functional homologues, demonstrated

that these proteins are ubiquitous in foods and are directly consumed as part of a normal human diet. Taken together, there is clear evidence that the PjΔ6D and NcΔ15D proteins are neither toxic nor allergenic in humans.

Compositional Analyses

The genetic modification is intended to result in the production of a soybean oil rich in SDA.

While SDA is between 16% and 36% of total fatty acids, there is a concomitant small increase in GLA in MON87769 soybean oil, to approximately 7% of total fatty acids, compared with conventional varieties. Detailed compositional analyses were conducted on whole seed and food fractions including oil, soybean meal and processed products such as lecithin and protein isolate. Analytes measured were proximates (crude fat/protein, fibre, ash), amino acids, fatty acids, vitamin, isoflavone and anti-nutrient content in MON87769, the non-GM parental soybean and six conventional varieties. A number of significant differences between MON87769 and conventional soybean were reported.

Linoleic acid is reduced in MON87769, outside the reference range for commercial soybean. As LA is a substrate for both the Δ6- and Δ15-desaturase reactions, this is an expected finding. Small differences (less than 4% of total fatty acids) in other fatty acid constituents were noted, however these were anticipated as a result of the intentional perturbation to fatty acid metabolic pathways. A majority of amino acids were significantly different in MON87769 compared with the parental line, however the levels were consistent with the published literature range for conventional soybean. Similarly, reduced levels of three soybean isoflavones in MON87769 were well within the range established for conventional soybean. Overall, except for the accumulation of SDA, the changes in composition in MON87769 are not considered to be of any major nutritional significance. The profile of fatty acids in commonly consumed vegetable oils differs according to the plant source, and in addition to this natural variability, significant changes to certain key constituents have been introduced over time using techniques such as traditional plant breeding. Consequently, the compositional variations observed in MON87769 soybean are within the range of natural variation already present in the diet.

The compositional analyses also show the presence of trace amounts of a novel isomer, *trans* SDA (6c,9c,12c,15t-18:4) in MON87769 soybean oil (0.26% total fatty acids). Another isomer, *trans* ALA (9c,12c,15t-18:3) is present in trace amounts in refined, bleached and deodorised (RBD) soybean oil in the non-GM parent (0.14%), and the levels are slightly elevated in RBD oil produced from MON87769 soybean (0.51%). In total, the levels of *trans* fats in MON87769 oil are well below 1% of total fatty acids. Both of these *trans* isomers are consumed as part of a normal human diet and are readily metabolised by the body into energy. A small increase in their levels is not considered to pose any safety concerns.

Additional allergenicity studies using sera from soybean-allergic individuals found no difference in immunoglobulin binding between soybean MON87769, the non-GM control and 24 commercial soybean varieties. These results demonstrate that the levels of endogenous soybean allergens in MON87769 are comparable to the levels in soybean varieties already on the market.

Nutritional Impact

Four animal studies, two using soybean meal (chickens and rats) and two using soybean oil (rats), indicate that MON87769 supports typical growth and wellbeing in rapidly growing animals and had no adverse effects on reproductive parameters in rats over one-generation. The genetic modification, resulting in the accumulation of SDA and other more minor

changes in fatty acid composition, therefore does not adversely affect the nutritional adequacy of the food in terms of the whole diet.

Conclusion

No potential public health and safety concerns have been identified in the assessment of SDA soybean MON87769. On the basis of the data provided in the present Application, and other available information, food derived from soybean MON87769 is as safe for human consumption as other commercially available soybean varieties.

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LIST OF KEY ABBREVIATIONS

ADF	acid detergent fibre
AI	adequate intake
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
trans ALA; 9c,12c,15t-C18:3	*trans* α-linolenic acid
GLA; 6c,9c,12c-C18:3	gamma-linolenic acid
LA; 9c,12c-C18:2	linoleic acid
ALA; 9c,12c,15c-C18:3	alpha-linolenic acid
DNA	deoxyribonucleic acid
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FSANZ	Food Standards Australia New Zealand
GC	gas chromatography
GM	genetically modified
IgE	immunoglobulin E
ILSI	International Life Sciences Institute
kb	kilo base
kDa	kilo Dalton
LLOQ	lower limit of quantitation
LSM	least squares mean
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
NHANES	National Health and Nutrition Examination Survey (U.S.)
NH&MRC	National Health & Medical Research Council (Australia)
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fibre
NUTTAB	Nutrient Tables (Australian Food Composition Tables)
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
ori	origin of replication
PCR	polymerase chain reaction
mRNA	messenger RNA
RBD SBO	refined, bleached & deodorised soybean oil
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SDA; 6c,9c,12c,15c-18:4	stearidonic acid
trans SDA; 6c,9c,12c,15t-18:4	*trans* stearidonic acid
U.S.	United States of America
UTR	untranslated region
WHO	World Health Organisation

1. INTRODUCTION

Soybean line MON87769 has been genetically modified (GM) to produce seeds containing stearidonic acid (SDA), an omega-3 fatty acid. In humans and other mammals, SDA is an intermediate in the metabolic pathway leading to the production of the long chain omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), normally found in fish and marine oil products derived from fish, krill or marine algae. SDA soybean therefore represents a plant-based source of dietary omega-3 polyunsaturated fatty acids (PUFA).

MON87769 was created from conventional soybean by introducing two genes: a delta-6 (Δ6) desaturase gene from *Primula juliae* and a delta-15 (Δ15) desaturase gene from *Neurospora crassa*. The seed-specific expression of these two desaturase enzymes results in the production of SDA and other more minor changes to fatty acids in soybean seeds.

Refined soybean oil from MON87769 contains 20-30% SDA (% total fatty acids), and 5-8% *gamma*-linolenic acid (GLA), neither of which is present in conventional soybean oil. SDA soybean oil also contains slightly higher levels of *alpha*-linolenic acid (ALA) and palmitic acid as well as lower levels of oleic acid and linoleic acid (LA), compared with conventional soybean oil. The variability in the SDA concentration is attributed to natural variation in growing conditions for the soybean.

Stearidonic acid is a fatty acid with 18 carbons and four double bonds (18:4) (see Table 1). As SDA has fewer double bonds than EPA (20:5) and DHA (22:6), the Applicant claims that SDA soybean oil is more stable to oxidation compared with fish oils. This property may expand the potential formulation options for food manufacturers seeking to use oils rich in omega-3 fatty acids.

It is anticipated that SDA soybean oil could partially replace regular soybean oil or other vegetable oils in a variety of packaged foods such as baked goods, breakfast cereals, grain products and pastas, sauces, milk products and soups. SDA soybean oil may also be used in aquaculture and feed applications as an alternative to fish oil and other omega-3 rich feed components. The seed meal from MON87769 soybean is compositionally similar to other commodity soybean meal and can be used in any conventional food and feed applications.

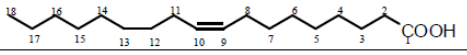
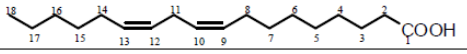
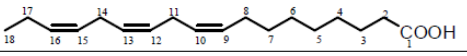
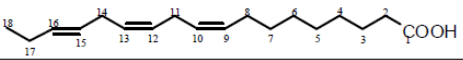
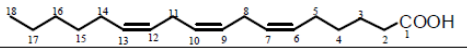
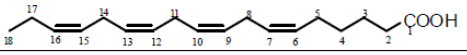
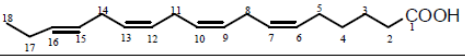
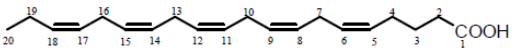
When commercialised, the SDA soybean crop is intended for cultivation and processing in soybean growing regions of northern USA under an identity-preserved (IP) system. Due to its targeted applications, the Applicant anticipates that SDA soybean will be a low acreage crop (<5% of total US soybean acreage) compared with other commercialised soybean lines. This would amount to an area of cultivation of approximately 3-3.5 million acres. There are currently no plans to produce SDA soybean in Australia and/or New Zealand.

2. HISTORY OF USE

2.1 Host Organism

The host organism is a conventional soybean (*Glycine max* (L.) Merr.), belonging to the family Leguminosae.

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	

Soybean is grown as a commercial food and feed crop in over 35 countries worldwide (OECD, 2000) and has a long history of safe use for both humans and livestock. The major producers of soybeans, accounting for 90% of world production, are the U.S., Argentina, Brazil and China. In 2007, soybeans represented 56% of total world oilseed production, and 32% of those soybeans were produced in the U.S. where they provided 71% of the edible consumption of fats and oils (The American Soybean Association, 2008). Australia and New Zealand are net importers of soybean, however Australia grows crops extending from the tropics to temperate regions, mainly in the eastern states and as a rotational crop (James and Rose, 2004).

In many soybean producing countries, GM soybean (mainly with a herbicide tolerant trait) accounts for a significant proportion of the total crop, for example U.S. (91%); Argentina (99%); Brazil (63%); South Africa (87%); Uruguay (99%) (Brookes and Barfoot, 2009).

Typical soybean food products are derived either from whole or cracked soybeans:

- whole soybeans are used to produce soy sprouts, baked soybeans, roasted soybeans and traditional soy foods such as miso, tofu, soy milk and soy sauce.
- cracked soybeans have the hull (seed coat) removed and are then rolled into flakes which undergo solvent extraction to remove the oil. This crude oil is further refined to produce cooking oil, shortening and lecithins as well as being incorporated into a variety of edible and technical/industrial products. The flakes are dried and undergo further processing to form products such as meal (for use in livestock, pet and poultry food), protein concentrate and isolate (for use in both edible and technical/industrial products), and textured flour (for edible uses). The hulls are used in mill feed.

Unprocessed (raw) soybeans are not suitable for food use, and have only limited feed uses, as they contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors (OECD, 2001a). Appropriate heat processing inactivates these compounds.

Soybean oil constitutes approximately 71% of global consumption of edible fats and oils (ASA 2008b), and is the second largest source of vegetable oil worldwide (Soyatech, 2008). Oil from conventional soybeans has five major fatty acid components – palmitic acid (16:0 – 12% of total fatty acid content), stearic acid (18:0 – 4%), oleic acid (18:1 – 23%), linoleic acid (18:2 – 53%) and linolenic acid (18:3 – 8%) (Lee *et al*., 2007).

2.2 Donor Organisms

The donor organism for the PjΔ6D protein, Primula (family *Primulaceae*), is a common garden plant that is used as an ingredient in certain herbal formulations. The whole plant can be utilized for medicinal purposes, including flowers, seeds, leaves and roots. Primrose plants contain significant levels of SDA in the leaves, which is thought to improve the structure and function of chloroplast membranes for growth in their native cold climates. Primrose plants are also used as a food source – young leaves (raw or cooked) are used in soups, and fresh or dried leaves are used as a tea substitute (Facciola 1990).

Reports of contact dermatitis have been associated with several varieties of Primula, however the reaction is triggered by a non-protein allergen, primin, that is secreted from cut leaf and stem trichomes (Horper and Marner 1996).

The donor organism for the Δ15 desaturase protein, *Neurospora crassa* is a ubiquitous fungal organism that has been extensively studied in terms of its biology and genetics. Evidence for its general safety has been reviewed by Perkins and Davis (2000a). The genus *Neurospora* is recognized under the US FDA regulations as GRAS (generally recognised as safe). *N. crassa* is used to manufacture food in a variety of different world regions. It is a major constituent of onchom, a soybean-based pressed cake, which is consumed daily in Indonesia (Matsuo 1997). It is also used in Brazil to process cassava into a fermented beverage (Park *et al*., 1982) and is present in Roquefort cheese prepared by traditional methods in southern France (Perkins and Davis, 2000a). There is no evidence of human health concerns due to oral consumption of *N. crassa*, and it is therefore considered non-pathogenic and non-allergenic (Perkins and Davis, 2000b).

3. MOLECULAR CHARACTERISATION

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects: the transformation method together with a detailed description of the DNA sequences introduced to the host genome; a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation; and the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

Girault, R., Song, Z., Pan, A., Feng, D., Rice, J.F., Tian, Q. and Masucci, J.D. (2009). Amended Report for MSL0021074: Molecular Analysis of Stearidonic Acid Producing Soybean MON87769. Monsanto Study Report MSL0021926 (unpublished).

Silanovich, A. and Girault, R. (2009). Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of the Inserted DNA in Soy MON87769: Assessment of Putative Polypeptides. Monsanto Study Report MSL0021705 (unpublished).

3.1 Method used in the genetic modification to generate MON87769

SDA soybean was developed using *Agrobacterium tumefaciens* strain AB1, carrying plasmid PV-GMPQ1972, to transform embryonic cells of the conventional soybean variety A3525. The plasmid vector utilised a two transfer-DNA (2T-DNA) system. The 2T-DNA system allows for a T-DNA with the traits of interest (T-DNA I) to insert independently from a second T-DNA (T-DNA II) encoding a selectable marker gene, which is used to select transformants that carry both T-DNAs. The Applicant chose this system of transformation in order to effect removal of the marker gene which is no longer required once the plants carrying the genes of interest have been identified. As reported in the literature, this 2T-DNA binary vector approach has been used successfully in 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).

Once selected, the transformed plants were self-pollinated, resulting in segregation of the two independent loci in the progeny. Plants containing only T-DNA I, with the genes of interest, were then maintained, while the plants containing the marker gene (T-DNA II) were discontinued.

Plasmid PV-GMPQ1972 contained the two separate T-DNA constructs, as shown in Figure 1. T-DNA I contained two gene expression cassettes: the *Pj*.D6D and the *Nc*.Fad3 encoding the ∆6- and ∆15-desaturase enzymes respectively. The second T-DNA (T-DNA II) contained a *cp4 epsps* expression cassette, which encodes the CP4 EPSPS protein used as a selectable marker. Expression of CP4 EPSPS in plants confers tolerance to glyphosate.

Agrobacterium-mediated transformation is based on widely published methods. The PV-GMPQ1972 vector contained the left and right border sequences, flanking each of the two T-DNAs. These sequences are necessary for incorporation of the novel DNA into the plant genome.

Meristematic tissue excised from germinated A3525 soybean seed was co-cultured with the *Agrobacterium* containing the vector, and then placed on selection medium containing reagents to inhibit the growth of non-transformed cells and glyphosate, to select transformants. The meristems were then placed on media conducive to shoot and root development, and only rooted plantlets with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

3.2 Breeding of SDA soybean MON87769

The R0 plants generated from the transformation were self-pollinated, and the progeny (R1) plants screened for a single insertion of the T-DNA I cassette. The R1 plants containing T-DNA I (with *Pj.D6D* and *Nc.Fad3* genes) and without T-DNA II (*cp4 epsps* cassette) were advanced. These were again self-pollinated to generate a population of R2 plants, which in turn were repeatedly self-pollinated through to the R6 generation. From this population of transformed plants, the Applicant selected MON87769 as showing the most favourable phenotype and molecular profile. These steps are schematically represented in Figure 2.

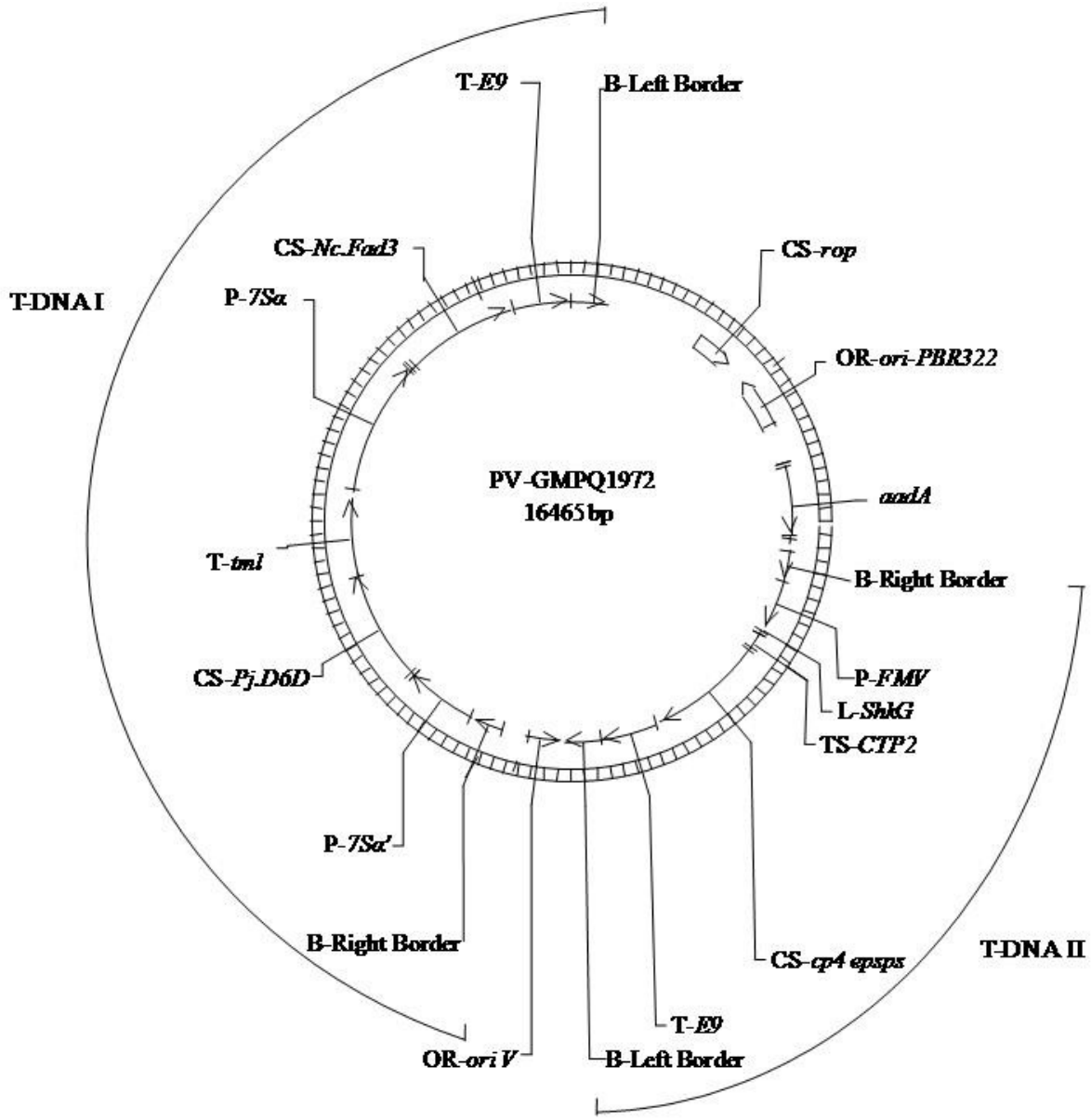


Figure 1: Plasmid PV-GMPQ1972 showing genetic elements comprising T-DNA I and T-DNA II, used to develop SDA soybean MON87769.

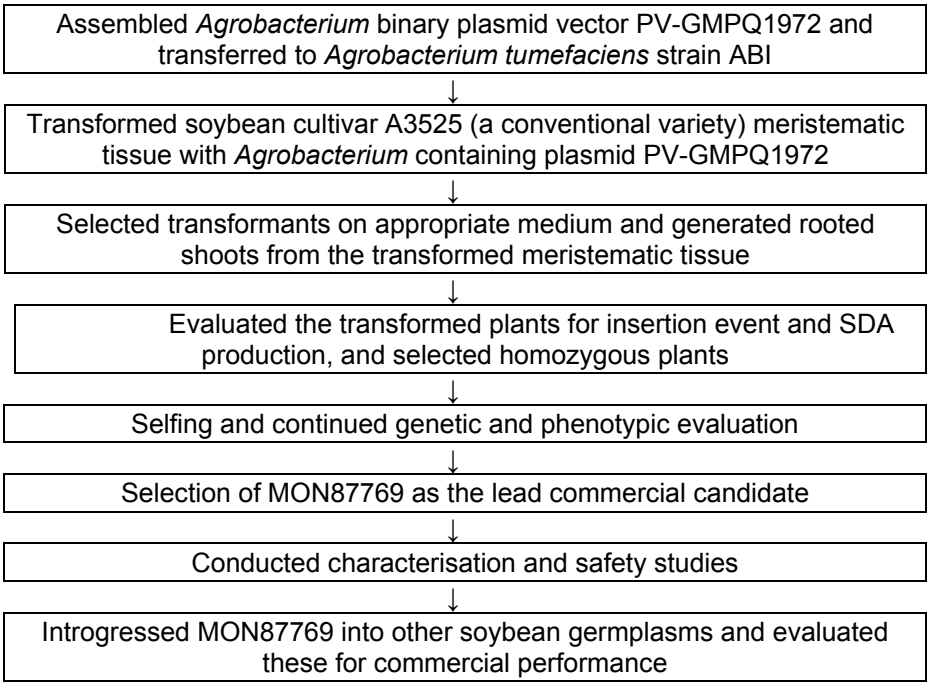


Figure 2: Schematic diagram of the development of SDA soybean MON87769.

3.3 Function and regulation of introduced gene sequences

The plasmid vector PV-GMPQ1972 used in the transformation is approximately 16.5 kb (16465 bp). Information on the key genes of interest and their regulatory elements in T-DNA I (7848 bp) and T-DNA II (3833 bp) is shown in Table 2. Each T-DNA region is delineated by the left and right border sequences (derived from *Agrobacterium*) for incorporation into the plant genome.

Soybean MON87769 contains only T-DNA I (approximately 7.8 kb). Following early event selection, the T-DNA II region containing the *cp4 epsps* gene cassette (glyphosate tolerance trait) was segregated away from T-DNA I by conventional breeding, allowing only those plants expressing the Δ6- and Δ15-desaturases to progress through development. Each genetic element in T-DNA I that was transferred to soybean is discussed below.

Table 2: Description of the key genetic elements contained in the two gene expression cassettes T-DNA I and T-DNA II in plasmid PV-GMPQ1972 (16465 bp)

Genetic element	Nucleotides in Plasmid	Source and Function (Reference)
T-DNA I		
Right Border	9073-9429	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of 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* (soybean) encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle *et al.* 1986) that directs mRNA transcription in soybean seed
Intervening sequence	10322-10337	Sequence used in DNA cloning
CS-*Pj.D6D*	10338-11678	Coding region for the delta-6 desaturase enzyme 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 of *Glycine max* (soybean) encoding the alpha subunit of beta-conglycinin storage protein (Wang and Dubois 2004) that directs mRNA transcription in soybean seed
Intervening sequence	14418-14445	Sequence used in DNA cloning
CS-*Nc.Fad3*	14446-15735	Codon optimised coding sequence for the gene from *Neurospora crassa* encoding delta-15 desaturase enzyme (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	16431-14	Sequence used in DNA cloning
Left Border	15-456	DNA region from *Agrobacterim tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker *et al*. 1983)
T-DNA II		
Right Border	4495-4851	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of T-DNA (Depicker *et al.* 1982)
Intervening sequence	4852-4884	Sequence used in DNA cloning
P-*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-*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-*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 enzyme (Barry *et al.* 1997; Padgette *et al*. 1996)
Intervening sequence	7155-7196	Sequence used in DNA cloning
T-*E9*	7197-7839	3' non-translated region from pea *rbcS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi *et al.* 1984)
Intervening sequence	7840-7886	Sequence used in DNA cloning
Left Border	7887-8328	DNA region from *Agrobacterim tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker *et al*. 1983)

P-Promoter; **CS**-Coding Sequence; **L**-Leader; **TS**-Targeting Sequence; **T-**Transcription terminator and polyadenylation signal sequences.

3.3.1 Promoter elements

The genes of interest are under the regulatory control of the P-*7α'* transcriptional promoter, which is a seed-specific promoter, fused to one of two leader sequences to target the enzymes to intracellular compartments (plastids). The leader sequences are derived from the *Sphas1* and *Sphas2* genes from *Glycine max* (soybean) and encode the alpha prime and alpha subunit of the beta-conglycinin storage protein (alpha'-bcsp) respectively (Doyle et al, 1986; Wang et al. 2004).

3.3.2 Coding sequence Pj.D6D

The *Pj.D6D* gene was isolated from *Primula juliae* (Primrose) and encodes a single polypeptide (PjΔ6D) of 446 amino acids. The PjΔ6D protein is a Δ6-desaturase enzyme which creates a double bond at the 6th position from the carboxyl end of a fatty acid. In MON87769 soybean seed, this enzyme converts ALA (18:3) to SDA (18:4), and also catalyses the conversion of LA (18:2) to GLA (18:3).

3.3.3 Coding sequence Nc.Fad3

The *Nc.Fad3* gene was isolated from *Neurospora crassa* and encodes a single polypeptide (NcΔ15D) of 429 amino acids. This protein is a Δ15-desaturase enzyme which creates a double bond at the 15th position from the carboxyl end of a fatty acid. In MON87769 soybean seed, this enzyme converts LA (18:2) to ALA(18:3), thereby lowering the substrate pool of LA and increasing the flux towards production of SDA. The Δ15-desaturase also catalyses the conversion of GLA (18:3) to SDA (18:4).

3.3.4 Other regulatory elements

The 3' non-translated region of the *tml* gene derived from the octopine-type Ti plasmid of *Agrobacterium tumefaciens* has been fused to the 3' end of the *Pj.D6D* coding sequence. Similarly, the 3' non-translated region of the ribulose 1,5 bisphosphate carboxylase small subunit (*rbcS2*) gene (T-*E9*) from pea (*Pisum sativum*) has been fused to the 3' end of the *Nc.Fad3* coding sequence. These 3' terminal elements direct termination of transcription and polyadenylation of the mRNA of the introduced genes.

3.3.5 Function of the introduced genes

Plant oils are primarily composed of triacylglycerols (TAGs) that in turn comprise 3 fatty acid chains usually 16 or 18 carbons long (Durrett *et al*., 2008). The synthesis of these fatty acids in plants occurs in plastids and essentially results in the formation of 16:0, 18:0 and 18:1 fatty acids; odd-chained and other even-chained fatty acids are produced to a lesser degree. These fatty acids are esterified to acyl carrier protein (ACP). Thioesterases then release them from ACP so that they may be exported to the endoplasmic reticulum for desaturation (addition of double bonds) by fatty acid desaturases (FADs) and assembly into TAGs (see Figure 3). FADs are also found in most animals and in some eubacteria.

The synthesis of polyunsaturated fatty acids in developing oilseeds is catalysed by two membrane-associated FADs that sequentially add a second and third double bond to oleic acid (Kinney, 1999). The second double bond, converting oleic acid (18:1) to linoleic acid (18:2), is added at the ω-6 position by ω-6 (Δ12) desaturase, encoded by the *fad2* gene (Heppard *et al*., 1996; Okuley *et al*., 1994). The third double bond, converting linoleic acid to linolenic acid (18:3), is added at the ω-3 (Δ15) position by an ω-3 desaturase, encoded by the *fad3* gene (Yadav *et al*., 1993).

The purpose of introducing the two desaturase enzymes into soybean is to bring about specific changes in the biosynthesis of fatty acids in the seeds to produce SDA (18:4).

Conventional soybean lacks a Δ6 desaturase, which is necessary to convert α-linolenic acid (ALA) into SDA. However, Δ6 desaturase can also convert linoleic acid into γ-linolenic acid (GLA), and so the addition of the Δ15-desaturase drives the production of more SDA (i) directly from GLA, and (ii) via ALA, while lowering the pool of LA. Addition of both a Δ15-desaturase and a Δ6-desaturase in combination allows increased production and accumulation of SDA in MON87769. The biosynthetic pathway for production of SDA in MON 87760 soybean is depicted in Figure 4.

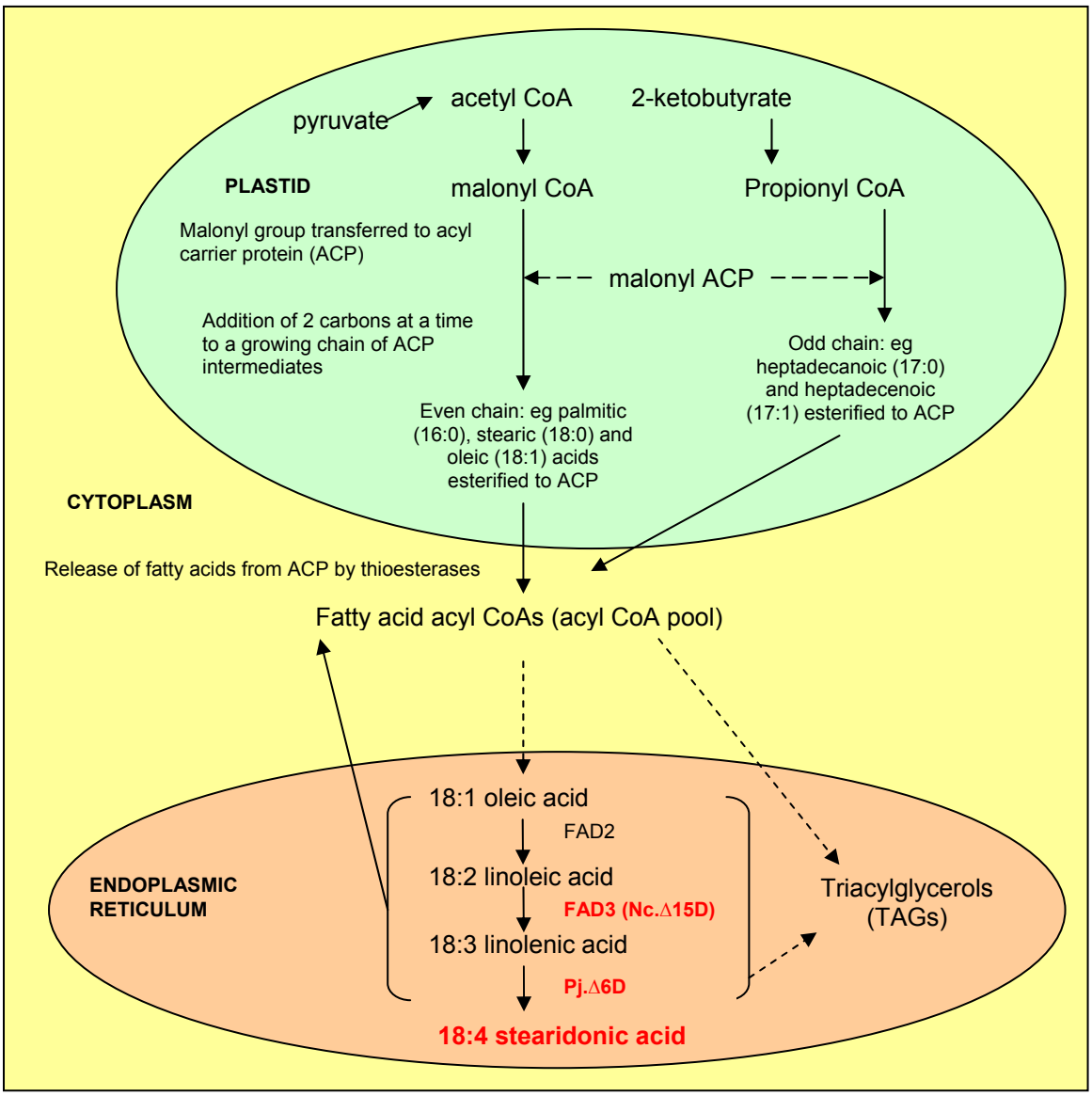


Figure 3: *A simplified schematic summary of the synthesis of fatty acids in the seeds of SDA soybean MON87769 plants (adapted from Kinney,1999 and Durrett et al. 2008).*

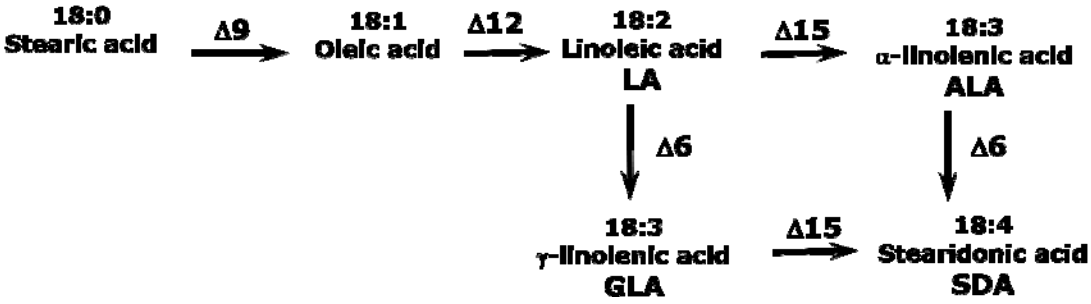


Figure 4: Part of the Fatty Acid Biosynthetic Pathway in Plants showing the function of the ∆6- and ∆15-desaturases leading to the production of SDA in soybean MON87769.

3.4 Characterisation of the genes in the plant

Molecular characterisation studies were carried out on soybean MON87769 to determine the nature of the modification to the soybean genome. The evaluation of T-DNA I insert copy number, insert integrity and the presence or absence of plasmid backbone sequences and T-DNA II was done by Southern blot analysis and sequence analysis. Other techniques such as PCR and DNA sequencing were performed to determine the organisation of genetic elements in the inserted DNA, to characterise the junction regions and examine the flanking genomic DNA.

Leaf tissue from individual seedlings from multiple generations was used in these analyses. Tissue from the R4 generation was used in Southern blots. Generations R3, R4, R5[a], R6[b] and R6[c] were used to investigate the stability of the insert and confirm the absence of T-DNA II and plasmid backbone sequences across generations of MON87769 (see Figure 5).

Tissue from conventional soybean with the same genetic background as MON87769 was used as the control material. Plasmid PV-GMPQ1972 was used as the reference material in the characterisation studies, as the positive hybridisation control in Southern analyses and for generating a suite of DNA template probes.

All control and test leaf material used in the characterisation studies was verified by event-specific PCR prior to use in the analyses.

3.4.1 Transgene copy number and insertion integrity

Genomic DNA from MON87769 and the control soybean line was digested with a variety of restriction endonucleases for use in Southern blot analyses. Using specific restriction enzyme combinations, the number of restriction fragments detected with radiolabelled probes indicates the presence/absence of genetic elements in MON87769 soybean and the relative number of inserts present in the genome. In all, 18 probes were used to examine the Southern blots. Twelve probes were homologous to specific regions which covered the entire length of plasmid PV-GMPQ1972, as described in Table 2. Six more probes were generated to test in detail for elements specific to T-DNA I containing the two desaturase genes. The probes ranged in length from approximately 0.7 kb to 2.0 kb.

Several of the genetic elements used in T-DNA I were derived from soybean. Equivalent restriction digests of conventional soybean were performed in order to determine the background hybridisation signal due to the detection of endogenous sequences.

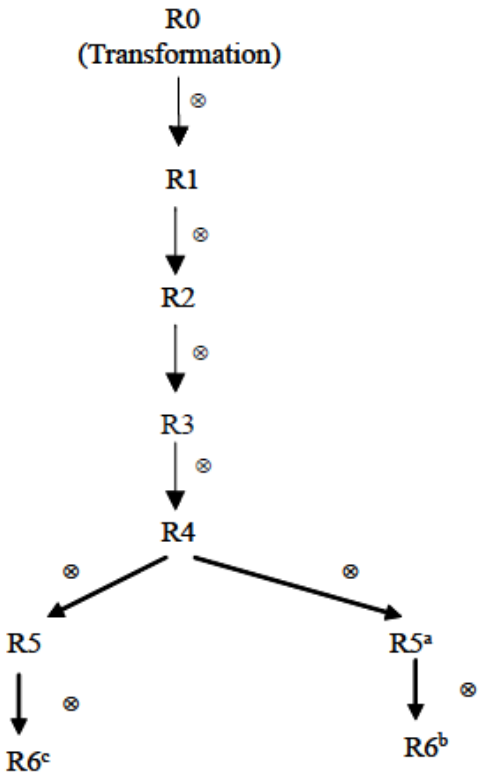


Figure 5: Breeding History of MON87769 showing derivation of generations used for the molecular characterisation analyses (see Sections 3.4 and 3.5).

The signals corresponding to endogenous soybean DNA sequences were detected, as expected, across all tested samples including the non-GM, conventional soybean, and served as positive hybridisation controls in a number of experiments.

On using a restriction enzyme that does not cleave within T-DNA I and overlapping T-DNA probes, a single unique band of approximately 10 kb was detected, in addition to the background hybridisation signals produced by endogenous soybean sequences. This result suggested the presence of T-DNA I at a single locus in the MON87769 genome. A number of separate experiments using other combinations of restriction enzymes produced results that were consistent with this conclusion.

3.4.2 Plasmid backbone DNA analysis

Southern blot analysis was also used to determine whether DNA sequences derived from the backbone of the transformation plasmid (ie. the regions outside of the T-DNAs) could be detected in MON87769. These regions included two bacterial origins of replication (*ori V* and *ori-p*BR322) and the antibiotic resistance gene, *aadA*[1], used only in the construction of the plasmid prior to transformation. Using probes specific for these regions, no detectable hybridisation signal was obtained.

[1] The *aadA* includes a bacterial promoter and coding sequence for an enzyme that confers resistance to spectinomycin and streptomycin and is utilised in laboratory cloning and selection steps only.

This result indicates that MON87769 does not contain any of the backbone sequences from the transformation vector PV-GMPQ1972. There are no antibiotic-resistance marker genes in soybean MON87769.

Table 3: Description of probes used in Southern analyses to characterise the inserted DNA in MON87769 soybean.

Probe	DNA region in PV-GMPQ1972
1	Plasmid backbone 1
2	Plasmid backbone 2
3	Plasmid backbone 3
4	T-DNA II probe 1
5	T-DNA II probe 2
6	Plasmid backbone 4
7	RB / P-*7Sα*'
8	Coding sequence – *Pj.D6D*
9	T - *tml*
10	P – *7Sα*
11	Coding sequence – *Nc.Fad3*
12	T – *E9*/ LB

3.4.3 Analysis for T-DNA II sequence

After the initial transformation and screening for transgenic plants, the T-DNA II sequence was no longer required. This sequence was subsequently segregated away from MON87769, through a number of traditional breeding steps. Southern blot analysis was used to confirm the absence of the T-DNA II region in leaf tissue obtained from plants of generations R3 through to R6 (see Figure 5), using probes 4 and 5 (Table 2). Conventional soybean DNA was used as a negative control. As expected, when examined with a number of overlapping T-DNA II-specific probes, no detectable hybridisation bands were observed, demonstrating that no endogenous soybean sequences produced a positive signal. Plasmid PV-GMPQ1972 and conventional soybean DNA spiked with T-DNA II sequences were used as positive hybridisation controls.

The overlapping T-DNA II probes spanned the Right border sequence, the *E9* 3' non-translated region sequence and the Left border sequence also present in T-DNA I. Due to these sequences being common to both T-DNAs, a characteristic pattern of bands was expected from restriction digests of MON87769 DNA. Southern blot analysis produced only a single band of predicted length (1.6 kb) representing the *E9* 3' non-translated region and Left border sequence.

In another experiment, a band of approximately 6.8 kb was expected to indicate the presence of the Right border sequence. On examination of the Southern blot, this band was not visible and no additional fragments were observed. Further hybridisation experiments also failed to detect the Right border sequence in MON87769 soybean. Subsequent DNA sequence analysis (see Section 3.4.4) revealed only 43 bp of the Right border sequence present in the MON87769 insert. As only a single band representing the Left border sequence was visible, this series of hybridisation experiments showed that T-DNA II elements could not be detected in MON87769 soybean.

Based on the results of multiple Southern blot analyses, it was therefore determined that:

- a single, intact copy of T-DNA I is present in MON87769 soybean
- T-DNA II was not detectable in MON87769 soybean

- backbone sequences from PV-GMPQ1972 outside of the two T-DNA regions are not detectable in MON87769 soybean

3.4.4 Organisation of the genetic elements in MON87769

The organisation of the genetic elements comprising the insert in MON87769 was investigated by PCR and DNA sequence analyses. Five overlapping regions of DNA spanning the entire length of the insert and genomic DNA flanking the insert were amplified using PCR (see Figure 6). As expected, the control without template, and the reactions with conventional soybean as template, did not generate PCR products with any of the primer sets. The reactions with MON87769 genomic DNA produced three internal PCR products of equal size to those obtained using the plasmid control as template. The amplification of fragments of the predicted length established the positions of the PCR products relative to the insert, and confirmed the arrangement and linkage of elements in MON87769 genomic DNA.

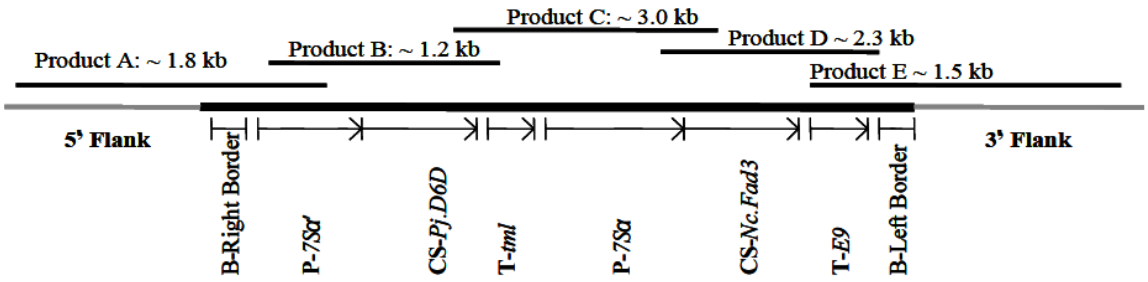


Figure 6: Diagram of the insert in MON87769 showing the expected product size for each amplicon obtained in PCR analysis.

The amplified PCR products were then subjected to routine DNA sequence analysis to further confirm the nature and organisation of genetic elements in the insert. A consensus sequence, representing the insert and a region of flanking genomic soybean DNA at the 5' and 3' ends of the insert, was obtained. The consensus sequence was generated by compiling the data from numerous sequencing reactions.

Based on these data, the length of the insert in MON87769 is known to be 7367 bp, which corresponds to the T-DNA I region in the plasmid between the Right and Left borders. Loss of some border sequence is expected at the site of insertion. At the 5' end of the insert, 933 bp of adjacent genomic DNA sequence was obtained; 831 bp of adjacent sequence was obtained at the 3' end. The sequence information confirmed the organisation of the genetic elements integrated into the soybean.

The 5' and 3' genomic border regions were verified to be of soybean origin by PCR amplification and DNA sequencing of these regions from both MON87769 soybean and control soybean samples. The PCR primer set hybridised to genomic DNA sequence upstream and downstream of the insert. The product obtained from the PCR amplification of the conventional soybean sample was approximately 600 bp. As expected however, a PCR product was not obtained in the MON87769 sample because the reaction conditions would not support the generation of products of the size of the insert (approximately 7400 bp).

After sequencing the 600 bp fragment generated from conventional soybean, several changes were noted when compared with the sequence of the upstream and downstream regions flanking the insert in MON87769 soybean. A deletion of 9 bp, and two insertions of 17 and 8 bp had occurred across the insertion site.

A region of 333 bp at the 5' end and 146 bp at the 3' end of the insert in MON87769 were found to be identical to conventional soybean sequence.

3.4.6 Conclusion about gene characterisation

Comprehensive Southern blot analyses and sequence data indicate that one intact copy of T-DNA I, derived from transformation plasmid PV-GMPQ1972, remains in MON87769 soybean, at a single insertion site in the genome. This means that MON87769 contains one copy each of the *Pj.D6D* and *Nc.Fad3* expression cassettes. T-DNA II and plasmid backbone sequences were not detected in MON87769. Characterisation of the regions flanking the insertion site confirmed these to be native soybean sequence.

3.5 Open Reading Frame analysis

The nature of the process by which genetic material is introduced into an organism may result in unintended effects that include the creation of new open reading frames (ORFs) in the genome of the organism. In theory, these could lead to the production of novel proteins. The safety assessment therefore includes a bioinformatic analysis of any potential ORFs to determine whether there would be any significant homology to other proteins, particularly those in toxin and allergen databases. ORF analysis is entirely theoretical in nature because without essential regulatory elements such as promoters, transcription and translation of these regions is extremely unlikely to occur.

3.5.1 ORF analysis of junction regions

A bioinformatics approach was used to analyse DNA regions spanning the 5' and 3' junctions of the insert in MON87769 for putative polypeptides. The sequence data encompassing the 5' and 3' genomic border sequences were screened for translational 'stop' codons (TGA, TAG, TAA) and the presence of ORFs originating or terminating within the MON87769 insert in all six reading frames. The search focussed on identifying sequences encoding a theoretical peptide of eight amino acids or greater in length (identity over a window of eight contiguous amino acids typically represents the minimum length for an immunologically relevant epitope).

This process revealed five such sequences spanning the 5' junction, and six sequences spanning the 3' junction. These were used as theoretical search sequences for comparisons against the AD8 (2009), TOXIN6 (2009) and PROTEIN (2009) public databases using the FASTA sequence alignment tool. In addition, the putative ORFs were analysed for match proteins in the AD_2009 over eight contiguous amino acids.

The results of these *in silico* sequence alignments showed no structurally relevant similarity with any known toxins or allergens. The results from the alignments of a sliding window of eight amino acids against the protein database similarly demonstrated a lack of immunologically relevant sequence matches. The bioinformatic analyses provide reassurance that, even in the unlikely event of aberrant transcription and translation, a resulting product would not raise a food safety concern.

3.5.2 ORF analysis of introduced coding regions

On the grounds that rare instances of mutations in the coding sequence of genes can occur, with the possibility of producing a novel protein, *in silico* investigation of the *Pj.D6D* and *Nc.Fad3* coding sequences was conducted. Bioinformatic analyses of the entire T-DNA I sequence present in MON87769 soybean were performed by comparison with the same set of public DNA and protein sequence databases as in the previous analysis. Putative peptides in all possible reading frames were deduced from the gene coding sequence.

The results demonstrated that putative proteins derived from alternative reading frames of the *Pj.D6D* and *Nc.Fad3* coding regions, and other sequences contained in the inserted DNA, do not show significant alignment with known toxins, allergens or proteins with adverse biological activity.

3.6 Stability of the genetic change

Four successive generations, including two separate R6 lines, were analysed using Southern blotting to investigate the stability of the insert in MON87769 soybean. DNA was extracted from leaf tissue from generations R3, R4, R5[a], R6[b] and R6[c] for this set of experiments (refer to Figure 5). Blots were examined with six overlapping T-DNA I probes spanning the entire inserted DNA sequence. A number of controls were used in these experiments including conventional soybean spiked with PV-GMPQ1972 or probe template, and conventional soybean alone. All insert-containing samples yielded consistent results across the generations. The array of probes confirmed the presence of T-DNA I gene sequences in all generations tested, and were unable to detect sequences representative of T-DNA II or DNA elements derived from the transforming plasmid.

3.6.1 Segregation analysis

Southern blot analysis demonstrated the segregation of T-DNA I in MON87769 soybean and showed that the introduced DNA was stably integrated into the soybean genome and maintained across successive generations. To confirm genotypic stability, a chi-square statistical test was used to analyse whether there was any difference between the observed segregation ratio and the ratio expected from inheritance of the introduced genes according to Mendelian principles. The chi-square analysis was performed on the zygosity data generated for the T-*tml* 3' genetic element over three successive generations. The breeding path for these segregation data is outlined in Figure 7.

As outlined in the diagram, the R0 plant was self-pollinated to produce R1 seed, which would be expected to segregate 1:2:1 for the insert (1 homozygous:2 hemizygous:1 null segregant). A homozygous R1 selected plant was identified from the segregating population. The selected R1 plant was self-pollinated to produce R2 seed, which was expected to be homozygous for the introduced trait. Subsequent generations were confirmed as homozygous for T-DNA I.

Homozygous MON87769 plants of the R4 generation were crossed to a conventional soybean variety to produce hemizygous F1 seed. F1 plants were self-pollinated to produce F2 seed. Plants in the F2 generation were expected to segregate 1:2:1 for the insert (1 homozygous:2 hemizygous:1 null segregant). Individual plants were tested for the presence and zygosity of the insert. Positive hemizygous plants in the F2 generation were self-pollinated to produce F3 seed. The process of testing and self-crossing was repeated to produce the F3 and F4 generations, and the data used in a chi-square analysis.

The chi-square value obtained for the F2, F3 and F4 generations indicated no significant difference between the observed and expected segregation ratios. These results are consistent with the molecular characterisation information which indicated a single insertion site in MON87769, and a Mendelian pattern of inheritance of the trait.

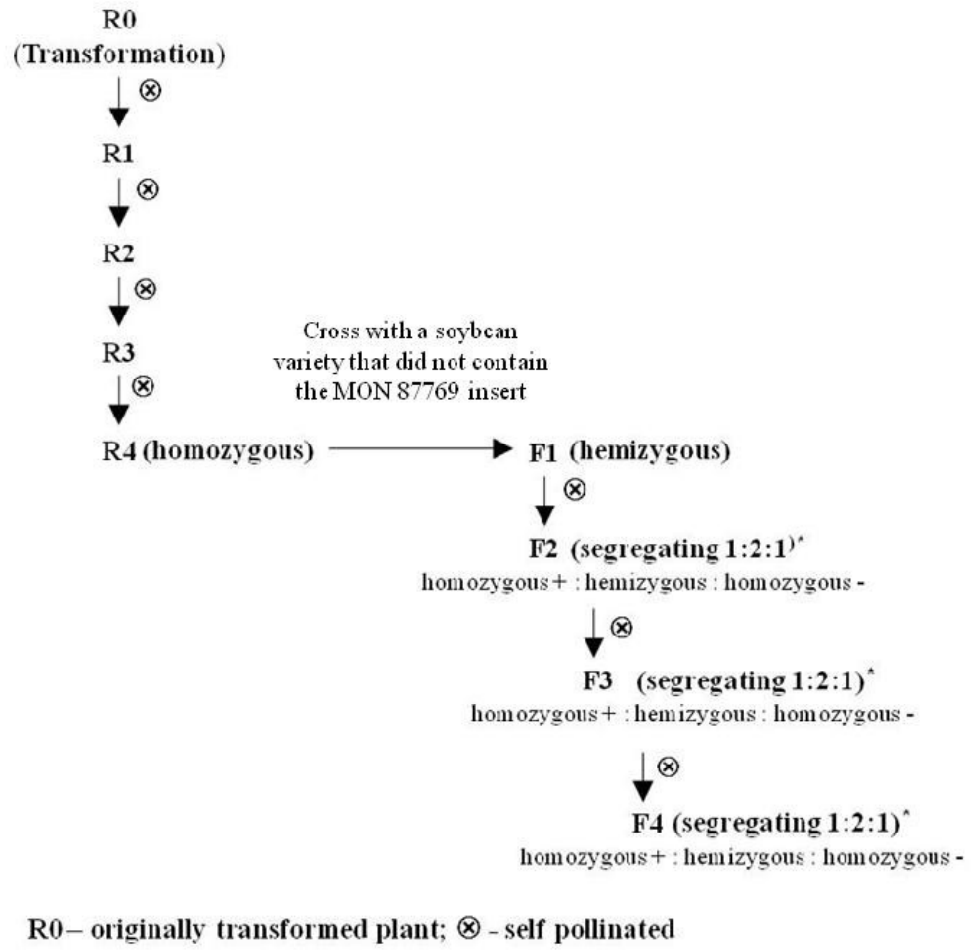


Figure 7: Breeding path for generating segregation data for MON87769 soybean.

3.7 Conclusion about molecular characterisation

Soybean MON87769 contains two complete enzyme coding sequences, and associated regulatory elements, which result in the spatial and temporal expression of the introduced genes only in the developing soybean seed. One gene encodes a Δ15 desaturase from *Neurospora crassa*, NcΔ15D. The other introduced gene sequence encodes a Δ6 desaturase from *Primula juliae*, PjΔ6D. Expression of both genes at the same stage of seed development results in the production of SDA.

Molecular analysis indicated that the introduced genes are linked and inserted at one locus in the soybean genome. A gene conferring tolerance to glyphosate herbicide, *cp4 epsps*, derived from *Agrobacterium* strain CP4, which was originally co-transformed into the soybean, was segregated away from the SDA trait through conventional breeding steps. There are no antibiotic-resistance marker genes in soybean MON87769, and no evidence of backbone sequences derived from the transforming plasmid. Bioinformatic analysis showed that theoretical ORFs would not share homology with known protein toxins or allergens.

Breeding and testing over three generations confirmed the genetic stability of the introduced genetic elements. Segregation data indicated a Mendelian pattern of inheritance, indicative of a single insertion site.

4. CHARACTERISATION OF NOVEL PROTEINS

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, e.g., because they are allergens or anti-nutrients (Delaney *et al*., 2008b). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, quantity and localisation of all novel proteins expressed in the organism, as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Because the expression of novel proteins *in planta* is usually too low to allow purification of sufficient quantities for safety assessment studies, a specific bacterial expression system is generally used to produce larger quantities of individual proteins. It is then necessary to establish the equivalence of the bacterial-produced proteins to the plant-produced proteins through additional characterization analyses. In this case, safety assessment studies were conducted with proteins isolated directly from MON87769 soybean, and therefore equivalence studies were not required.

4.1 Purification of novel proteins in MON87769

MON87769 soybean produces two novel proteins, PjΔ6D and NcΔ15D, both desaturases whose expression is under the control of seed-specific promoters. A range of experimental approaches was used to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity of the two enzymes. The Applicant reported that the expression of desaturase proteins is higher during seed development, therefore immature rather than mature seeds were used for the analyses.

Both PjΔ6D and NcΔ15D are integral membrane proteins. Purification of membrane-bound proteins is known to be technically challenging because they are generally low abundance hydrophobic proteins. Detergents are required to solubilise membrane structure and release proteins from the lipid bilayer while maintaining their solubility. The Applicant experimentally tested a panel of detergents for the ability to release the desaturase proteins from the seed membranes. Using the best performer with protein purification techniques, the desaturase enzymes were obtained in soluble form, allowing for characterisation and further analysis.

4.2 Biochemical function and phenotypic effects of the novel proteins

The PjΔ6D and NcΔ15D proteins are members of a family of integral membrane fatty acid desaturases found in all eukaryotic organisms (plants, animals and fungi) and some prokaryotes. Fatty acid desaturases are non-heme iron-containing enzymes that introduce a double bond with strict specificity between defined carbon atoms of fatty acyl chains. As the double bond is described as *unsaturated*, the enzymes that catalyse the reaction from a single to a double bond are known as *desaturases*.

Unsaturated fatty acids are synthesised *de novo* in all organisms using a common metabolic pathway (Somerville and Browse, 2000; Voet and Voet, 1995). The enzymes involved, including desaturases, are therefore conserved across biological kingdoms. The degree of unsaturation of a fatty acyl chain is a major determinant of the fluidity of biological membranes (Aguilar and Mendoza, 2006).

As noted above in Section 4.1, solubilising integral membrane proteins requires detergents which are likely to disrupt the functional activity of the protein, making study difficult away from an intact membrane system. Although the activity of the desaturases could be demonstrated in crude protein extracts, it was not possible to assay enzyme activity of PjΔ6D and NcΔ15D following their solubilisation and purification from seed membranes. This is presumed to be due to the requirement of the desaturase enzymes for associated electron transfer proteins co-localised in the membrane.

The function of PjΔ6D and NcΔ15D proteins in MON87769 was confirmed in several ways. Firstly, MON87769 produces GLA and accumulates SDA, as expected from the expression and functional integrity of the two introduced desaturase genes (refer to Figure 4). Production of these fatty acids in soybean is novel and therefore a metabolic indicator of PjΔ6D and NcΔ15D activity in the seeds. Secondly, a yeast expression system was developed for *in vivo* studies, as described below. Thirdly, the activity of the introduced desaturases could be demonstrated in crude extracts of immature MON87769 soybean seed when tested *in vitro* with the appropriate substrates, as described in Sections *4.3.2*.

4.3 Characterisation of PjΔ6D

The PjΔ6D protein consists of 446 amino acids with a calculated molecular mass of almost 51 kDa, and a predicted isoelectric point of 8.8. The amino acid sequence of PjΔ6D was analysed for features characteristic of integral membrane desaturases. The active site of integral membrane desaturases consists of three histidine motifs with a total of eight essential histidine residues (Shanklin *et al*. 1994). Three histidine motifs are evident in the deduced amino acid sequence of PjΔ6D, although the first histidine in the third motif has been replaced by glutamine, which also occurs in other Δ6 desaturases. Similar to other Δ6 desaturases (Nakamura and Nara, 2004), PjΔ6D contains the amino-terminal cytochrome b5 domain carrying the heme-binding motifs. The cytochrome b5 domain and glutamine residue are essential for functional Δ6 desaturase activity, as mutations abolish the enzyme activity (Sayanova et al., 2001 and 2000).

4.3.1 In vivo activity studies

The *Pj.D6D* gene was cloned into a yeast expression vector and transformed into *Saccharomyces cerevisiae* for heterologous expression and whole-cell substrate feeding studies. The Applicant identified yeast as a suitable system for the functional evaluation of the desaturases for several reasons: (i) yeast contain an extensive ER membrane system and expresses required enzymes, (ii) expression vectors and cell strains are readily available, and (iii) *S. cerevisiae* is composed of only four fatty acids (palmitic acid, stearic acid, palmitoleic acid and oleic acid).

S. cerevisiae cells containing the *Pj.D6D* expression vector were grown in the absence and presence of exogenous fatty acids. In the absence of exogenous fatty acids, the yeast cells produced two new fatty acids (16:2 c6,9 and 18:2 c6,9), which are both products of Δ6 desaturation of the endogenous monounsaturated fatty acids palmitoleic (16:1) and oleic acid (18:1). Cultures grown in the presence of exogenous oleic acid showed even higher levels of the fatty acid 18:2 c6,9. Both GLA and SDA were produced when an equal mixture of LA and ALA was included in the medium.

4.3.2 PjΔ6D enzyme activity

An intact membrane system containing the desaturase and associated electron transfer proteins are minimal requirements for studying activity *in vitro*. The PjΔ6D protein requires a source of cyt b5 and b5 reductase, as well as substrate lipids.

The Applicant was able to measure enzyme activity specific to the PjΔ6D protein in crude homogenates of very young, fresh MON87769 seed, using green immature seed up to 4 mm. Enzyme activity was measured by the formation of ^{14}C-SDA in the assay mixture when radiolabelled substrate, ^{14}C-ALA-CoA, was incubated with the crude MON87769 homogenate. Detection of SDA in the reaction mixture is indicative of the functional activity of the PjΔ6D protein, since Δ6 desaturase activity is normally lacking in soybean.

4.3.3 Protein identity of PjΔ6D derived from MON87769 soybean

Studies submitted:
Finnessy, J.J., Dong, J., Lee, T.C. and Rice, E.A. (2008). Characterisation of *Primula juliae* Δ6 Desaturase Protein Isolated from Immature Seeds of Soybean MON87769. Monsanto Study Report MSL0021307 (unpublished).

Several analytical techniques were used to determine the identity as well as the physicochemical and functional properties of the plant-derived PjΔ6D protein purified from immature seeds of MON87769 soybean. A total weight of 11 kg of immature soybean seeds (Orion Lot No. 10002214) was used for the purification of PjΔ6D. The identity of the batch of seed material used in these analyses was confirmed by event-specific PCR analysis.

The total protein concentration of the purified PjΔ6D enzyme was determined by amino acid analysis using a method that allows for high sensitivity fluorescent detection of amino acids. The average concentration across five independently prepared dilutions of the PjΔ6D preparation was 0.52 mg/ml.

To determine purity, multiple aliquots of the MON87769-produced PjΔ6D preparation were subjected to SDS-PAGE, followed by Coomassie blue staining and densitometric analysis. The dominant protein band corresponded to a polypeptide of approximately 46 kDa. The average purity of the PjΔ6D preparation was estimated to be 47%. Another band, corresponding to a protein of approximately 7 kDa and constituting 10.6% of the total protein loaded in each lane, was readily visible on the stained gel. Other, mainly lower molecular weight bands were also more faintly visible. Given the level of purity achieved in the preparation, a faint ladder of background bands would be expected on a stained gel. The final concentration of the PjΔ6D protein in the preparation was 0.24 mg/ml.

- N-terminal sequencing of the major 46 kDa protein identified a sequence of 15 amino acids that matched the expected N-terminal sequence of the MON87769-produced PjΔ6D protein. The expected N-terminal amino acid sequence was deduced from the coding region of the full-length *Pj.D6D* gene. The N-terminal amino acids of the second protein band, of approximate molecular weight 7 kDa, were also sequenced for characterisation purposes. The results yielded multiple amino acid identifications during each cycle which indicated the presence of multiple proteins of similar size and abundance. This clearly indicated the presence of lower molecular weight proteins or cleavage products of larger molecular weight proteins in minor amounts in the protein preparation. It was not possible to determine whether any of these proteins were degradation products of the PjΔ6D protein.

- Immunoblot analysis confirmed the identity of PjΔ6D. Following SDS-PAGE, protein bands were electrotransferred to a membrane, which was probed with a goat anti-PjΔ6D antibody. A second antibody (rabbit anti-goat IgG) conjugated with horseradish peroxidase was applied for visual detection of immunoreactive bands. Based on the protein concentration measurements, adjacent lanes were loaded with 4, 6, 8, 10 and 12 ng respectively. An immunoreactive band migrating at approximately 46 kDa was observed in each lane. The intensity of the signal increased with increasing amounts of protein loaded.

- MALDI-TOF mass spectrometry was used to further confirm the identity of the PjΔ6D protein. This method of analysis allows for the matching of a sufficient number of observed mass fragments to expected (theoretical) mass fragments following trypsin digestion of a protein. There were 30 unique protein fragments identified that matched the expected masses of tryptic fragments of the PjΔ6D protein. The identified masses were used to assemble a coverage map that indicated the position of those matched peptide sequences within the protein sequence. A total of 188 of 446 (42.2%) amino acid residues were identified and mapped to the predicted positions within the PjΔ6D sequence. This significant degree of matching confirms the identity of the protein as PjΔ6D.

4.3.4 Glycosylation analysis

Many eukaryotic proteins are glycoproteins that have been post-translationally modified by the addition of carbohydrate moieties (glycans) covalently linked to the polypeptide backbone. Glycosylation that occurs on side chains of asparagine residues is termed N-glycosylation. The addition of N-acetylglucosamine to the β-hydroxyl of either serine or threonine residues is known as O-glycosylation. The carbohydrate component may represent from <1% to >80% of the total molecular weight of glycoprotein. There is one report in the literature of the expression of non-native proteins in transgenic plants leading to aberrant glycosylation, with the potential to lead in turn to altered immunogenicity (see Prescott *et al.,* 2005).

The PjΔ6D protein is an integral membrane protein derived from higher plants (Primrose), and two putative N-glycosylation sites have been identified (Asparagine-X-Serine/Threonine) (Marshall, 1972). The existence of sites however does not imply that the protein is actually glycosylated *in vivo*. To assess whether the PjΔ6D protein, as expressed in MON87769 soybean seeds, is glycosylated, analysis of the protein for the presence of covalently bound carbohydrate moieties was undertaken using a GE Glycoprotein Detection Module, which detects N- and O-linked carbohydrates. Transferrin, a naturally glycosylated mammalian protein, was used as the positive control. No detectable signal was obtained suggesting that PjΔ6D isolated from MON87769 seeds is not glycosylated *in planta*.

4.3.5 Conclusion

Studies on the PjΔ6D desaturase, expressed in MON87769 soybean seeds, indicate that it is identical to the native protein produced in Primula. A range of methods confirmed the identity of PjΔ6D purified from immature MON87769 soybean seeds. The major protein with apparent molecular weight of 46 kDa (i) correlated with the predicted molecular weight, (ii) was recognized by anti-PjΔ6D antibodies, (iii) matched the expected first 15 amino acids at the N-terminus of the protein, and (iv) produced tryptic mass fragments that matched a high percentage of the expected mass fragments of the trypsin-digested PjΔ6D protein. The experimental evidence indicates that PjΔ6D expressed in MON87769 soybean is not a glycosylated protein.

4.4 Characterisation of NcΔ15D

The amino acid sequence of the NcΔ15D desaturase protein is identical to the native protein produced in *Neurospora crassa*, with the exception of a single amino acid change (threonine to alanine) at the first amino acid position after the start codon. This was necessary to facilitate the insertion of the gene into the plant transformation vector. The NcΔ15D protein expressed in MON87769 consists of 429 amino acids with a calculated molecular mass of 49.2 kDa, and a predicted isoelectric point of 7.1. As with the Δ6 desaturase, analysis of the deduced amino acid sequence of NcΔ15D showed three histidine motifs, characteristic of integral membrane desaturases.

Similar to the PjΔ6D protein, the NcΔ15D protein also showed multiple membrane-spanning regions based on the patterns observed from hydropathy plots.

4.4.1 In vivo activity studies

The *Nc.Fad3* gene (encoding NcΔ15D) was cloned into a yeast expression vector and transformed into *Saccharomyces cerevisiae* for studies similar to those described above for the Δ6 desaturase. *S. cerevisiae* cells containing the *Nc.Fad3* expression vector were grown in the absence and 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. This reaction is known only to be catalysed by the NcΔ15D enzyme. All of the 18 or 20 carbon fatty acids were substrates for NcΔ15D catalysed reactions and all were desaturated three carbons from the methyl terminus (omega-3 desaturation).

4.4.2 NcΔ15D enzyme activity in vitro

The Applicant was unable to demonstrate the functional activity of the NcΔ15D protein in the crude homogenate of immature MON87769 soybean seeds. This was due to the inability to distinguish the two 18:3 fatty acid isomers, ALA and GLA, in this system. Moreover, soybean contains an endogenous Δ15 desaturase which means that, to some extent, the Δ15 desaturation reaction occurs naturally in conventional soybean.

4.4.3 Protein identity of NcΔ15D derived from MON87769 soybean

Studies submitted:

Dong, J.G., Lee, T.C., Finnessy, J.J. and Rice, E.A. (2008). Characterisation of *Neurospora crassa* Δ15 Desaturase Isolated from Immature Seeds of Soybean MON87769. Monsanto Study Report MSL0021308 (unpublished).

Several analytical techniques were used to determine the identity as well as the physicochemical and functional properties of the plant-derived NcΔ15D protein purified from immature seeds of MON87769 soybean (Orion Lot No. 10001516). Two batches of seeds with a total weight of 13.5 kg were used for this study. One batch of seeds was harvested from MON87769 soybean grown in Hawaii, while the second batch was obtained from MON87769 plants grown at a Trait Development Site in the US (Wyoming, Illinois). The identity of both batches of seeds was confirmed by event-specific PCR analysis.

To determine the purity and molecular weight of the NcΔ15D preparation, multiple aliquots were subjected to SDS-PAGE, followed by Coomassie blue staining and densitometric analysis. The gel was relatively clear of contaminating bands with the dominant protein band corresponding to a polypeptide of approximately 46 kDa. The average purity of the NcΔ15D preparation was estimated to be 74%.

- Immunoblot analysis was used to confirm the identity of the Nc Δ15D protein in the preparation. Following SDS-PAGE, protein bands were electrotransferred to a membrane, which was probed with a goat anti-NcΔ15D antibody. A second antibody (rabbit anti-goat IgG) conjugated to horseradish peroxidase was applied for visual detection of immunoreactive bands. Based on purity calculations, adjacent lanes were loaded with 2, 4, 6, 8 or 10 ng respectively. A major immunoreactive band migrating at the expected molecular weight of approximately 46 kDa was observed in each lane in a concentration dependent manner. Two minor bands, with molecular weight of approximately 27 kDa and 30 kDa, were also detected by the antibody.

These could be explained by the presence of small amounts of partially degraded NcΔ15D protein in the preparation, or other cross-reacting proteins that co-purified with NcΔ15D.

- N-terminal sequencing of the major 46 kDa immunoreactive protein band (using Edman degradation) identified a sequence of 15 amino acids that exactly matched the expected N-terminal sequence of the NcΔ15D protein, minus the N-terminal methionine. The expected amino acid sequence of the N-terminus of the MON87769-produced NcΔ15D protein was deduced from the coding region of the full-length *Nc.D15D* gene. Removal of the N-terminal methionine during post-translational processing is a common observation and has no significance in terms of protein structure or activity.

- MALDI-TOF mass spectrometry was used to further confirm the identity of the NcΔ15D protein. This method of analysis allows for the matching of a sufficient number of observed mass fragments to expected (theoretical) mass fragments following trypsin digestion of a protein. There were 15 unique protein fragments identified that matched the expected masses of tryptic fragments of the NcΔ15D protein. The identified masses were used to assemble a coverage map that indicated the position of those matched peptide sequences within the protein sequence. A total of 193 of 429 (45%) amino acid residues were identified and mapped to the predicted positions within the NcΔ15D sequence. This degree of matching confirmed the identity of the protein as NcΔ15D.

4.4.4 Glycosylation analysis

The NcΔ15D protein is an integral membrane protein of microbial origin, and contains two putative N-glycosylation sites (Asparagine-X-Serine/Threonine) (Marshall, 1972). The existence of sites however does not imply that the protein is actually glycosylated *in planta*. To assess whether the NcΔ15D protein, as expressed in MON87769 soybean seeds, is glycosylated, analysis of the protein for the presence of covalently bound carbohydrate moieties was undertaken using a GE Glycoprotein Detection Module, which detects N- and O-linked carbohydrate. Transferrin, a naturally glycosylated mammalian protein, was used as the positive control. No detectable signal was obtained suggesting that NcΔ15D isolated from MON87769 seeds is not glycosylated *in vivo*.

4.4.5 Conclusion

The identity of NcΔ15D purified from immature MON87769 soybean seeds was confirmed using a range of methods. The major protein band with apparent molecular weight of 46 kDa, correlated with the expected molecular weight of the Δ15 desaturase enzyme and was recognized by anti-NcΔ15D antibodies on Western blots. The sequence of the first 15 amino acids at the N-terminus of the major protein matched the known sequence for the NcΔ15D protein. The plant purified protein yielded tryptic mass fragments that matched a high percentage of those expected in the trypsin-digested NcΔ15D protein. The experimental evidence also indicated that NcΔ15D expressed in MON87769 soybean is not a glycosylated protein.

4.5 Expression of novel proteins in MON87769

Study submitted:
Zhao, Q. and Silanovich, A. (2008). Assessment of Delta 6 and Delta 15 Desaturase Protein Levels in Tissues from MON87769 Soybean Grown in 2006 U.S. Field Trials. Monsanto Study Report MSL 0021169 (unpublished).

Because the two novel desaturase enzymes in MON87769 are integral membrane proteins,

they could not be detected or measured in extracts of plant tissue using a standard liquid phase assay system, such as ELISA. The technical difficulties arise from the need to use a detergent to extract proteins from the membrane which subsequently interferes with solubilised protein-antibody interactions.

As an alternative to ELISA, optimized Western blot methods were used to quantitate the levels of PjΔ6D and NcΔ15D in tissue samples from MON87769 soybean. The 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 purified protein standard that could be reliably determined. The Limit of Detection (LOD) was defined as the lowest amount of the PjΔ6D and NcΔ15D proteins visually observed on X-ray films. The levels of PjΔ6D and NcΔ15D proteins in various tissues of MON87769 were estimated by densitometric analysis of X-ray films exposed to immunoblots probed with antibodies specific to each protein, and visualized using chemiluminescent detection reagents.

Tissues were collected from soybean plants grown at five separate field sites in North America[2] during the 2006 season. At each site, three replicated plots of MON87769 and the conventional soybean control (A3525) were grown using a randomized complete block field design. Over-season leaf (OSL), forage, root, mature seed and immature seed samples were collected per replicated plot from all field sites, as outlined below.

Twenty sets of the youngest expanded trifoliate leaves were randomly collected from plants of each plot. OSL samples were collected as follows:

Over-season leaf (OSL)	**Plant development stage**	**Days after planting**
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 (above ground) portion of six plants was collected from each plot at each site at the R6 development 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. The R6 stage was reached 81-108 days after planting.

Immature and mature seed were harvested as follows:

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

Goat polyclonal antibodies (IgG) specific to a peptide fragment of either the Δ6 or the Δ15 desaturase proteins were produced and affinity purified for use in the Western blot analyses. The non-transgenic controls used for the reference standards for each tissue type were pooled from all sites. The reference material was purified Δ6- and Δ15-desaturase proteins produced in *E.coli*.

The levels of expression of the PjΔ6D and NcΔ15D proteins in various plant tissues are presented in Tables 3 and 4 respectively. Protein levels for all tissue types were calculated as µg/g plant tissue on a fresh weight (FW) basis. For tissues containing measurable quantities of the PjΔ6D and NcΔ15D proteins, moisture content was also measured in order to calculate the protein levels on a dry weight (DW) basis.

[2] Two sites were located in Iowa, and single sites were in Illinois, Michigan and Ohio.

When applicable, the arithmetic mean and standard deviation (SD) were calculated for each tissue across all sites (n=15 for all tissues). The LOQ for the PjΔ6D desaturase Western blot assay for immature and mature seed was 4.0 µg/g FW and 2.0 µg/g FW respectively; for all other tissues the LOQ was 1.0 µg/g FW. The LOQ for the NcΔ15D desaturase Western blot assay for immature and mature seed was 10.0 µg/g FW and 2.0 µg/g FW respectively. The LOQ for OSL and root samples was 2.0 µg/g FW and forage was 1.0 µg/g.

Table 4: Summary of the levels of PjΔ6D in tissues collected from MON87769 soybean, grown in the US in 2006 season (combined from 5 sites).

Tissue	PjΔ6D Mean µg/g FW (SD)	Range µg/g FW	PjΔ6D Mean µg/g DW[1] (SD)	Range µg/g DW	LOD µg/g FW
OSL-1	< LOD	na	na	na	0.2
OSL-2	< LOD	na	na	na	0.2
OSL-3	< LOD	na	na	na	0.1
OSL-4	< LOD	na	na	na	0.1
Root	< LOD	na	na	na	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 Seed	1.7 (0.86)	0.45-3.0[2]	1.8(0.95)	0.5-3.2	0.4

na- not applicable; LOD- limit of detection
[1] Dry weight values were calculated by dividing the fresh weight values by the dry weight conversion factors obtained from moisture analysis data.
[2] The low end of range was below the LOQ of the Western blot analysis, but above the LOD of 0.4 µg/g FW determined by serially diluting the protein standard to the lowest amount producing 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.

Table 5: Summary of the levels of NcΔ15D in tissues collected from MON87769 soybean grown in the US in 2006 season (combined from 5 sites).

Tissue	NcΔ15D Mean µg/g FW (SD)	Range µg/g FW	NcΔ15D Mean µg/g DW[1] (SD)	Range µg/g DW	LOD µg/g FW
OSL-1	< LOD	na	na	na	0.5
OSL-2	< LOD	na	na	na	1.0
OSL-3	< LOD	na	na	na	0.5
OSL-4	< LOD	na	na	na	1.0
Root	< LOD	na	na	na	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[2]	10(6.5)	4.8-25	2.0

na- not applicable; LOD- limit of detection
[1] Dry weight values were calculated by dividing the fresh weight values by the dry weight conversion factors obtained from moisture analysis data.

Due to the use of seed specific promoters, the levels of expression of the novel proteins in MON87769 soybean were expected to be enhanced in seeds. Both desaturase proteins were found at the highest levels in immature and mature seed, and at low levels in forage, which usually contains a small amount of immature seed.

The mean PjΔ6D protein levels across all sites for immature seed, mature harvested seed and forage were 100, 1.8 and 16 µg/g of plant tissue on a dry weight basis, respectively. The mean NcΔ15D protein levels across all sites for immature seed, mature seed and forage were 200, 10 and 14 µg/g of plant tissue on a dry weight basis, respectively.

Neither PjΔ6D nor NcΔ15D were detected in tissue samples harvested from the conventional control soybean.

4.6 Potential toxicity and allergenicity of the novel proteins in MON87769

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein.

The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate the need for further investigations.

4.6.1 History of human consumption

PjΔ6D

Desaturases are ubiquitous in nature, being present in many commonly eaten plants, yeast and animals. The donor organism of the Δ6 desaturase enzyme is *P. juliae*, a member of a large genus of plants commonly known as Primrose. While plants in this genus are not commonly consumed as food, the Δ6 desaturase enzyme from *P. juliae* 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. The enzyme also shares high identity with Δ8 desaturase from *Brassica napus* (canola), which is a major food crop worldwide.

The PjΔ6D protein shares amino acid sequence homology with a variety of proteins in the subfamily of desaturases (structural and catalytic similarities) that are ubiquitous in the diet of humans and other animals. There is strong conservation of the three histidine box motifs required for binding two iron atoms at the catalytic centre of integral membrane desaturases (Hashimoto *et al.* 2008). Homologues of PjΔ6D are found across a broad spectrum of organisms with a safe history of consumption as food including plants, fungi and vertebrates, as well as cyanobacteria.

Desaturases that are most similar to PjΔ6D are in the plant species *Echium plantagineum* (echium) and *Borago officinalis* (borage). Both of these species have been used to produce oils for human consumption. Cold-pressed oils from these plant species, which are relatively high in GLA and/or SDA, are used as dietary supplements. Evening primrose oil also contains high levels of GLA due to the presence of a Δ6 desaturase enzyme. The PjΔ6D protein in MON87769 shares approximately 57% amino acid identity with Δ8 desaturase from *Brassica napas* (canola), and approximately 25% identity to the Δ6 desaturase from two widely consumed fresh water fish species, *Oncorhynchus mykiss* (rainbow trout) and *Cyprinus carpio* (common carp).

NcΔ15D

The NcΔ15D protein is similar to several proteins that are ubiquitous in the human diet and commonly consumed in many foods derived from plant and fungal sources.

The enzyme is in the subfamily of "omega" desaturases, including Δ12 and Δ15 desaturases, which are required for the synthesis of 18:2 or 18:3 fatty acids. Virtually all plant species contain Δ12 and Δ15 desaturases, which are involved in the synthesis of LA and ALA from oleic acid. Vertebrate animals lack these desaturases and therefore must obtain LA and ALA from their diet. All Δ12 and Δ15 desaturase proteins share nearly identical hydrophobicity profiles and show strong conservation of three histidine box motifs required for binding two iron atoms at the catalytic centre (Hashimoto *et al*. 2008). The highly conserved histidine motifs in these proteins suggest that they share a common overall membrane topology.

Based on its amino acid sequence, the NcΔ15D protein shares varying degrees of identity with other desaturase proteins from a variety of vegetable crops, including broccoli, cabbage and cauliflower (family *Brassicaceae*), and oil crops such as canola. Significant identity also exists between the NcΔ15D protein and Δ12 or Δ15 desaturases found in fruit species such as *M. domestica* (apple), *L. esculatum* (tomato), *M. charantia* (pear), *P. americana* (plum) and *O. europaea* (olive). The NcΔ15D protein in MON87769 shares approximately 37% sequence identity to Δ15 desaturase from *Mortierella alpina*, a fungus which is currently used for the commercial production of arachidonic acid used in the fortification of infant foods[3].

4.6.2 Similarities with known protein toxins and allergens

The potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on: the source of the novel protein; any significant amino acid sequence similarity between the novel protein and known allergens; the structural properties of the novel protein, including susceptibility to digestion; and specific serum screening if the novel protein is derived from a source known to be allergenic, or has amino acid sequence similarity with a known allergen. In some cases, such as where the novel protein has sequence similarity to a known allergen, additional *in vitro* and *in vivo* immunological testing may be warranted. Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

Study submitted:
Silanovich, A. and Tu, H.(2009). Updated Bioinformatics Evaluation of Δ6 and Δ15 Desaturases Utilizing the AD_2009, TOX_2009 and PRT_2009 Databases. Monsanto Company Study Number: RAR-09-520. (unpublished)

Bioinformatic analyses are useful for assessing whether introduced proteins share any amino acid sequence similarity, and therefore potential higher structural similarity, with known protein toxins, allergens, or other biologically active proteins that may have an adverse effect on human health. Potential similarities of the PjΔ6D and NcΔ15D proteins with sequences in several protein databases were evaluated using the FASTA sequence alignment tool. In addition to the FASTA comparisons of each query sequence to allergens, an eight amino acid sliding window search was performed.

The bioinformatic analyses used the AD_2009, TOX_2009 and PRT_2009 databases. The allergen, gliadin and glutenin sequence database (AD_2009) was obtained from the Food Allergy Research and Resource Program Database (FARRP, 2009)[4], and contains 1,386 sequences. The PRT_2009 database was compiled from GenBank (National Centre for Biotechnology Information), and contains 14,717,352 protein sequences.

[3] EFSA has evaluated *M. alpina* as a safe source of arachidonic acid-rich oil for infant formula use. See: http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902043916.htm
[4] Located at http://www.allergenonline.com

The TOX_2009 database is a subset of 7,651 sequences derived from the larger PRT_2009 database filtered to remove non-toxin proteins.

A parameter known as the E-score (expectation score) statistically represents the probability that a particular sequence alignment is due to random chance. Comparisons between highly homologous proteins yield E-scores approaching zero, indicating the very low probability that such matches would occur by chance. Conversely, a larger E-score indicates a lower degree of similarity.

The results of the bioinformatic analysis for similarity with known toxins did not reveal any alignments with an E-score below 1.0, demonstrating that no structurally relevant similarity exists between the PjΔ6D or the NcΔ15D proteins and any known toxin in the database. The analysis of similarity with proteins in the allergen database revealed no significant homology. In addition, there were no immunologically relevant identities of eight contiguous amino acids detected when the PjΔ6D the NcΔ15D protein sequences were compared to the AD_2009 sequences using a sliding window search.

4.6.3 In vitro digestibility

One characteristic of many known protein allergens is the ability of the protein to withstand proteolytic digestion by enzymes present in the gastrointestinal tract. Presentation of intact protein allergens or their fragments to the intestinal immune system can lead to immune-mediated food allergy in particularly susceptible individuals. Complete enzymatic digestion of an ingested protein involves exposure firstly to pepsin in the acidic conditions of the stomach, followed by exposure to pancreatic enzymes in the duodenum. In the body, pepsin-mediated digestion greatly reduces the possibility of intact protein or protein fragments reaching the absorptive surface of the small intestine.

As a consequence of the correlation between resistance to enzyme proteolysis and potential allergenicity, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. *In vitro* digestibility studies utilise simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), which contain the appropriate digestive enzymes. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an immune response.

Studies using only SIF are limited because ordinarily an ingested protein is first exposed to pepsin-mediated hydrolysis in the acidic environment of the stomach, before being exposed to further digestion in the small intestine. In instances where transient stability of a protein is observed in laboratory studies with SGF, digestibility can be further investigated with sequential exposure to SIF. The results of *in vitro* digestibility studies do not provide unequivocal evidence of a lack of toxicity or allergenicity, but rather contribute to a weight of evidence leading to that conclusion.

Study submitted:
Kapadia, S. A., Bhakta, T., Lee, T.C. and Rice, E.A. (2009). Assessment of the *in vitro* Digestibility of the Primula juliae Desaturase Protein (PjΔ6D) in Simulated Gastric and Simulated Intestinal Fluids. Monsanto Company, MSL: 0021428 (unpublished)

This study examined the *in vitro* digestibility of PjΔ6D protein, purified from immature MON87769 soybean seeds, in SGF, SIF, and in a sequential digestion where the protein was first exposed to SGF followed by exposure to SIF. SGF contained pepsin in a buffer adjusted to an acidic pH 1-2. SIF contained pancreatin, a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The assay protocols followed the standardised digestion protocol published in Thomas *et al*., 2004.

SGF assay

Digestibility of the protein in SGF was measured by incubating samples at 37º for selected times (0.5, 2, 5, 10, 20, 30 and 60 minutes) and subjecting the reaction mixes to SDS-PAGE. Protein digests were visualised by Coomassie staining of the resulting gel, and were also analysed by Western blots using a PjΔ6D specific antibody.

Visual examination of the stained gel showed that the full-length PjΔ6D protein was rapidly hydrolysed in SGF, with over 99% of the protein digested within 30 seconds. Several lower molecular weight fragments (at or below approximately 5 kDa) were faintly observed in samples between 30 seconds and 60 minutes.

Further analysis was conducted on the protein fragments of molecular weight <5 kDa observed on the Coomassie stained gel. These fragments were not detectable with the PjΔ6D specific antibody used in Western blots (polyclonal goat anti-PjΔ6D sera). N-terminal sequencing of some of these fragments showed no match to the predicted amino acid sequence of the PjΔ6D protein. Given the level of purity of the plant-purified PjΔ6D protein (47%), these fragments are most likely to correspond to soybean proteins which co-purified with the PjΔ6D protein.

Western blot analysis of the SGF digests demonstrated that the PjΔ6D protein was digested below the LOD (estimated at 0.5 ng in this assay system) within 30 seconds of incubation with SGF. Calculations based on the LOD show that this was equivalent to the digestion of over 96% of the PjΔ6D protein within 30 seconds of exposure to SGF. On the Western blot, a single immunoreactive band corresponding to approximately 10 kDa molecular weight was observed at 30 seconds, however this band had disappeared at the next time point (2 minutes) indicating rapid digestion of the PjΔ6D fragment. No immunoreactive bands were detected in the remaining samples corresponding to incubation times of 2 minutes to 60 minutes.

Sequential SGF and SIF assay

In a separate experiment, the PjΔ6D protein preparation was exposed to digestion with SGF (pepsin) and SIF (pancreatin) sequentially. After digestion in SGF for 2 minutes, the reaction mixture was quenched and then exposed to SIF for additional times (0.5, 2, 5, 10, 30, 60 and 120 minutes). As before, the reaction mixes were run on SDS-PAGE for visual analysis and analysed in Western blots.

After 2 minutes in SGF alone, there was no visible full-length PjΔ6D protein and the low molecular weight bands (<5 kDa) were again faintly visible. However, these fragments were no longer visible after 2 minutes exposure to SIF. As observed in the previous experiment with SGF alone, the results of the Western blot analysis showed no immunoreactive protein fragments at the 2 minute timepoint, and therefore no further digestion with SIF was detected.

SIF assay

In vitro digestibility of the PjΔ6D protein in SIF alone was evaluated by Western blots. Incubation times were 5, 15, 30 minutes, and 1, 2, 4, 8 and 24 hours. This analysis demonstrated that a band corresponding to the full-length PjΔ6D protein was digested below the LOD within 5 minutes of incubation in SIF. No proteolytic fragments of the PjΔ6D protein were detected in any of the digestion samples. These data indicate that the PjΔ6D protein degrades rapidly when exposed to pancreatin at neutral pH.

Study submitted:
Kapadia, S. A., Lee, T.C. and Rice, E.A. (2008). Assessment of the *in vitro* Digestibility of the *Neurospora crassa* Δ15 Desaturase Protein (NcΔ15D) in Simulated Gastric and Simulated Intestinal Fluids. Monsanto Company, MSL: 0021427 (unpublished)

This study examined the *in vitro* digestibility of NcΔ15D protein, purified from immature MON87769 soybean seeds, in SGF, SIF, and in a sequential digestion where the protein was first exposed to SGF followed by exposure to SIF (as above). The assay protocols followed the standardised digestion protocol published in Thomas *et al*., 2004. Digestibility was analysed using SDS-PAGE and Western blot methods.

SGF assay

Approximately 0.8 µg (total protein) of the NcΔ15D protein preparation was incubated with SGF at 37º for selected times (0.5, 2, 5, 10, 20, 30 and 60 minutes). The reaction mixes were analysed by SDS-PAGE and digestion products were visualised by Coomassie staining of the gel. Visual examination of the stained gel showed that the full-length NcΔ15D protein was rapidly hydrolysed in SGF, with over 97% of the protein digested within 30 seconds. Fragments of approximately 17 kDa and 12 kDa were faintly observed up to 5 minutes and 10 minutes respectively. Other lower molecular weight fragments (< 5 kDa) were only faintly observed up to 60 minutes.

Analysis of the SGF digests demonstrated that more than 96% of the NcΔ15D protein was digested within 30 seconds following exposure to SGF. The two fragments of apparent molecular weight approximately 17 kDa and 12 kDa were also visible on the Western blot, up to reaction times of 5 and 10 minutes respectively, indicating that these fragments represented digestion products of the NcΔ15D protein. The smaller size fragments were however not observed on the Western blot, indicating that either the NcΔ15D specific antibodies do not detect these fragments, or that the fragments were not derived from the NcΔ15D protein. No other immunoreactive digestion products were detected.

N-terminal sequencing was carried out on the low molecular weight fragments not recognised by the anti-NcΔ15D antibody used in the Western blots. The sequence obtained for the approximately 5 kDa fragment did not match the expected sequence of the NcΔ15D protein, and its identity was not established. Given the level of purity of the NcΔ15D protein, it is likely that this fragment represents a soybean protein that co-purified with the NcΔ15D protein. The sequence of the fragment of approximately 4 kDa matched the expected sequence of the NcΔ15D protein to a region starting at amino acid 376 of the NcΔ15D protein.

Sequential SGF and SIF assay

To further evaluate the digestibility of the NcΔ15D protein, aliquots of the protein preparation were exposed to sequential digestion in SGF and SIF. After digestion in SGF for 2 minutes, the reaction mixture was quenched and then exposed to SIF for additional times (0.5, 2, 5, 10, 30, 60 and 120 minutes). As before, the reaction products were visually analysed after SDS-PAGE and in Western blots.

As apparent in the previous experiment with SGF alone, after 2 minutes there was no visible full-length NcΔ15D protein and only low molecular weight bands (<5 kDa) were faintly visible. These fragments were no longer visible after 5 minutes exposure to SIF. On the corresponding Western blot, the two digestion fragments of the NcΔ15D protein (molecular weight approximately 17 kDa and 12 kDa) were detected at T0 but were not detected at the first timepoint following addition of SIF (30 seconds).

No other immunoreactive bands were detected at any of the digestion timepoints thereafter, indicating that the NcΔ15D protein was rapidly degraded following the addition of SIF. As before, the low molecular weight fragments were not detectable on the Western blot in any samples containing SGF alone, or SGF and SIF.

SIF assay

In vitro digestibility of the NcΔ15D protein in SIF alone was evaluated by Western blots. Incubation times were 5, 15, 30 minutes, and 1, 2, 4, 8 and 24 hours. This analysis demonstrated that a band corresponding to the full-length NcΔ15D protein was digested below the LOD within 5 minutes of incubation in SIF. As 5 minutes was the first timepoint in this experiment, no proteolytic fragments of the NcΔ15D protein were detected in any of the digestion samples. These data indicate that the NcΔ15D protein degrades rapidly when exposed to pancreatin at neutral pH.

4.6.4 Acute oral toxicity

The safety of the PjΔ6D and NcΔ15D proteins purified from seed of MON87769 soybean plants was further evaluated in an acute oral toxicity study, in which a single dose of the test proteins was administered to mice.

Study submitted:
An Acute Toxicity Study of Delta 6 Desaturase and Delta 15 Desaturase Proteins Administered by the Oral (Gavage) Route to Mice. Charles River Laboratories, Ohio, USA. Study Director: J.W. Smedley, completed October 2008. Sponsor: Monsanto Company, Study No. CRO-2007-324
Analytical sub-report:
Dong, J.G., Lee, T.C. and Rice, E.A. (2008). Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Soybean MON87769-Produced *Primula juliae* Delta-6 Desaturase and *Neurospora crassa* Delta-15 Desaturase Proteins. MSL: 0021314; Study No. CRO-2007-324

The test materials were thoroughly characterised prior to the study. Purification of integral membrane proteins requires removal of the membranes and replacement of the lipids surrounding the hydrophobic (membrane-spanning) regions of the proteins with the appropriate detergent, to keep the protein solubilised. To use the test proteins in the acute mouse gavage study, the level of the detergent was reduced to a minimum necessary to keep the protein in solution. After buffer exchange, the protein was concentrated to the highest level attainable. The dosage administered to the mice during the study was the highest practical dose based on the physical properties of the proteins and their low expression levels in mature seed.

Test articles	PjΔ6D from MON87769 soybean seed (ID No. 10001532), diluted solution (Test Dosing Solution 1, TDS1) and NcΔ15D from MON87769 soybean seed (ID No. 10001516), diluted solution (Test Dosing Solution 2, TDS2)
Test Species	CD -1 mice (males approximately 8 weeks of age with body weights ranging from 28.5 to 33.8 grams; females approximately 10 weeks of age with body weights ranging from 23.1 to 29.5 grams)
Control	Bovine serum albumin, solubilised (Control Dosing Solution, CDS)

The target dose levels were selected to be at least 100 times the conservative estimates of human exposure to the test proteins, PjΔ6D and NcΔ15D. The treatment articles and control material were administered once by oral gavage in the same dose volume, 33.3 ml dosing solution per kg body weight. The overall experimental design was as follows:

Group No.	No. of Animals		Treatment	Analytically-Determined Dose Level (mg protein/kg body weight)
	Male	Female		
1	10	10	Bovine Serum Albumin (BSA)	44.0
2	10	10	Delta 6 Desaturase	4.66
3	10	10	Delta 15 Desaturase	37.3

Mice were observed twice daily for general health/mortality/moribundity. Detailed clinical observations were performed twice on Day 0 (post dose) and once daily thereafter for 14 days. Individual body weights were recorded on Day 0 (prior to fasting), Day 0 (prior to dosing), and on Days 7 and 14. Food consumption measurements were recorded on Days 0, 7 and 14. At the end of the study all animals were killed and examined post mortem for organ or tissue damage or dysfunction.

All mice survived through the duration of the study. No clinical signs of toxicity could be related to the test proteins during the course of the study. There were no test article-related effects on body weight during the study, with the mean weight gain being comparable throughout all groups. Food consumption was also comparable for all groups. At necropsy on day 14, no gross lesions were present that could be related to the test articles.

Under the conditions of this study, there were no observed adverse effects from the administration of the PjΔ6D and NcΔ15D proteins as a single oral dose to mice. These results support the conclusion that the PjΔ6D and NcΔ15D proteins expressed in MON87769 soybean seed are not acutely toxic.

4.6 Conclusion from characterisation of novel proteins

Two novel proteins are expressed in the seed of soybean MON87769. The PjΔ6D protein is identical to the native enzyme produced by *Primula juliae* and consists of 446 amino acids with a predicted molecular mass of approximately 51 kDa. The NcΔ15D protein is identical to the native enzyme produced in *Neurospora crassa* and consists of 429 amino acids with a predicted molecular mass of approximately 49 kDa. Both proteins are integral membrane desaturases and are characterised by multiple hydrophobic membrane-spanning regions and the presence of three highly conserved histidine-rich motifs at their catalytic centre.

Protein expression studies on major plant tissues showed that PjΔ6D and NcΔ15D are detected in immature and mature soybean seed. The mean levels for immature and mature seed for the PjΔ6D protein were 100 and 1.8 µg/g dry weight respectively, and for the NcΔ15D protein were 200 and 10 µg/g dry weight respectively. As the mean level of total protein (dry weight) in mature MON87769 seed is approximately 42%, the PjΔ6D and NcΔ15D proteins correspond to approximately 0.0004% and 0.0024% respectively, which represents negligible exposure to the proteins through the consumption of whole soybean or protein-containing fractions. The proteins were also detected at low levels in forage (used as feed for livestock) due to the presence of small amounts of immature seed.

Extensive studies were done to confirm the identity and physicochemical and functional properties of the desaturase proteins purified directly from MON87769 soybean seeds. The proteins conformed in size and amino acid sequence to that expected, and demonstrated the predicted enzymatic activity. There was no indication that either of the desaturases is glycosylated in MON87769. Bioinformatic studies with the PjΔ6D and NcΔ15D protein sequence confirmed the absence of any biologically significant amino acid sequence

similarity to known protein toxins or allergens. Digestibility studies demonstrated that the proteins would be rapidly degraded in the gastrointestinal tract, similar to other dietary proteins. Separate oral toxicity studies in mice confirmed the absence of acute toxicity of the PjΔ6D and NcΔ15D proteins. Furthermore, humans are likely to have been exposed to both of these proteins in the diet, or to proteins with similar structure and function. Taken together, the studies investigating the safety of the novel desaturase enzymes, PjΔ6D and NcΔ15D, in MON87769 soybean do not indicate any potential toxicity or allergenicity in humans.

5. COMPOSITIONAL ANALYSES

Where there has been a deliberate change in the composition of food brought about by the genetic modification, compositional analyses are primarily important for evaluating the intended effect. These analyses are also important to determine if any unexpected changes in composition have occurred to the food, and to establish its nutritional adequacy.

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical and not necessarily of value to a safety assessment. The aim is to analyse those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxicity and level may be significant to health (eg. solanine in potatoes).

5.1 Key components

For soybean there are a number of components that are considered to be important for compositional analysis (EuropaBio, 2003; OECD, 2001a). As a minimum, the key nutrients of soybean seed appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, mineral and vitamin levels may be considered. International guidance also suggests that levels of the key anti-nutrients phytic acid, trypsin inhibitors, lectins, isoflavones and stachyose and raffinose should be determined for new varieties of soybean. The reasons for focussing on these particular anti-nutrients are:

- phytic acid causes chelation of mineral nutrients (including calcium, magnesium, potassium, iron and zinc) thereby making them unavailable to monogastric animals, including humans
- trypsin inhibitors interfere with digestion of protein; lectins are proteins that bind to carbohydrate-containing molecules. Both trypsin inhibitors and lectins can inhibit animal growth. The activity of trypsin inhibitors and lectins is heat-labile, which means that they are inactivated during processing of soybean protein products and soybean meal, so that the final edible product should contain minimal levels of these anti-nutrients.
- isoflavones are reported to have biochemical activity including estrogenic, anti-estrogenic and hypocholesterolaemic effects which have been implicated in adversely affecting animal reproduction. Major isoflavones in soybeans include daidzein, genistein, glycitein and coumestrol.

- stachyose and raffinose are low molecular weight carbohydrates (oligosaccharides) that are incompletely digested in humans. Depending on the degree of cooking, when ingested, they are associated with production of intestinal gas.

5.2 Study design

Studies submitted:
Drury, S.M., Riordan, S.G., Miller K.D. and Sorbet, R. (2008) Compositional Analyses of Forage and Seed Collected from Stearidonic Acid-Containing Soybeans, MON87769, Grown in the United States during 2006. Monsanto Company, Covance Laboratories, Certus International Inc.. MSL: 0020866, Covance Study No. 6103-693 (unpublished).

The test (MON87769), control (A3525), and 10 commercially available soybean varieties were grown under similar conditions at five replicated field sites across the US[5] during the 2006 growing season. The commercial soybean varieties were included as references to provide data for the development of a 99% tolerance interval for each analysed component. Soybean A3525 was the original transformed line and therefore represented the isogenic control line for the purposes of the comparative analyses. The identity of the test, control and reference lines was verified by event-specific PCR analysis and confirmed by the Study Director via chain-of-custody documentation prior to their use in the study.

Seeds of the test and control soybean lines were planted in a randomised complete block design, three replicated plots at each of the five sites. Three of the ten commercial soybean varieties were grown at each of the five sites (each as a single replicate), amounting to a total of 15 reference values in the study. This design meant that several of the reference lines were grown at multiple sites.

Seed and forage from MON87769 and the conventional control plants were harvested from all replicated plots and analysed for composition. Forage was collected at the R6 plant growth stage, and seed harvested at physiological maturity. The reference varieties were planted, harvested, processed and analysed using the same methods as used for soybean MON87769 and the control line. Any statistically significant differences between soybean MON87769 and the control could also be compared to the reference range to assess whether the differences were likely to be biologically meaningful.

Data from the commercial varieties were used to calculate population tolerance intervals for each compositional component. Tolerance intervals are expected to contain, with 95% confidence, 99% of the values contained in the population of commercial lines. The population tolerance interval, together with the combined range of values for each analyte available from the published literature (ILSI, 2006; Kim *et al*., 2005; OECD, 2001a; Taylor *et al*., 1999), were used to interpret the compositional data for soybean MON87769. Any mean value for a MON87769 analyte that fell within the tolerance interval and/or the combined literature range was considered to be within the normal variability of commercial soybean cultivars, even if the mean value was statistically different from the control. In assessing the significance of any difference between the mean analyte value for soybean MON87769 and the control, a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

[5] The five US sites were: Site 1A-1 Richmond, IA; Site 1A-2 Bagley, IA; Site IL, Carlyle, IL; Site MI, Conklin, MI; Site OH, New Holland, OH.

For those comparisons in which the soybean MON87769 test result was statistically different from the control, the test mean was compared to the 99% tolerance interval derived from the commercial varieties. This interval is expected to contain, with 95% confidence, 99% of the values obtained from the population of commercial reference varieties.

5.3 Analyses of key components

Soybean MON87769 produces SDA , an omega-3 fatty acid, through the introduction of genes encoding the Δ6- and Δ-15 desaturase enzymes. Because of the use of seed-specific promoters, SDA is produced only in the seeds of MON87769 soybean plants. Compositional analyses of the seed samples included proximates (protein, fat, ash, moisture, and carbohydrate by calculation), acid detergent fibre (ADF), neutral detergent fibre (NDF), fatty acids (C8-C24), amino acids, isoflavones (daidzein, glycitein and genistein), vitamin E and anti-nutrients (stachyose, raffinose, phytic acid and trypsin inhibitor).

The compositional components for the test and control samples were statistically analysed using a mixed model analysis of variance. The data from the five replicated sites were analysed separately and as a combined data set. Overall, 75 different analytical components were measured, however for 26 of these analytes, some values were below the limit of quantitation (LOQ) of the assay system. Where more than 50% of the values were below the LOQ, the analyte results were not included in the statistical analysis. This applied to a number of fatty acids as detailed in Section *5.3.2*. Due to the intended change in the fatty acid profile, these were presented separately from the other components.

The results of a similar compositional analysis of forage samples were provided in this Application. However, the focus of this assessment is necessarily on the food uses of soybean and therefore the forage data are not presented in this report.

5.3.1 Proximates and fibre

The combined site results of the proximate and fibre analysis of soybean MON87769 and the control line are shown in Table 5, along with the results for the commercial reference lines. Statistically significant differences between MON87769 and the conventional control ($p < 0.05$) were observed for protein and carbohydrate (calculated), but the mean value for MON87769 soybean for each of these analytes was within the statistical tolerance intervals for commercial soybean cultivars and the ranges reported in the literature (ILSI 2006; Taylor *et al*., 1999). No other statistically significant differences were observed in proximates or fibre analysis.

Table 6: Combined Site Results of Proximates (Protein, Fat, Ash, Moisture and Carbohydrates) and Fibre (Acid Detergent Fibre and Neutral Detergent Fibre) Analysis in Soybean Seed – Statistical Summary

Analyte (Units)	MON87769 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[3]	Commercial reference range (99% tolerance interval[2])
Protein (% DW)	41.92 (0.27) [40.92 – 43.36]	39.75 (0.27) [38.22 – 41.58]	<0.001	37.52 – 42.37 (33.37, 46.00)
Total Fat (% DW)	15.91 (1.05) [12.95 – 19.03]	15.94 (1.05) [12.73 – 8.80]	0.955	13.99 – 20.56 (11.04, 25.03)
Ash (% DW)	5.72 (0.092) [5.23 – 6.17]	5.63 (0.092) [5.24 – 6.07]	0.106	5.59 – 6.20 (5.16, 6.64)
Moisture (%FW)	7.47 (0.17) [6.71 – 8.21]	7.41 (0.17) [6.84 – 8.11]	0.572	6.68 – 8.16 (5.23, 9.56)
ADF (%DW)	16.77 (0.42) [14.38 – 18.31]	16.90 (0.42) [13.80 – 18.15]	0.794	14.57 – 18.85 (10.36, 22.77)
NDF (%DW)	16.84 (0.38) [15.06 – 19.15]	17.18 (0.38) [14.43 – 19.37]	0.411	15.03 – 18.92 (10.91, 22.59)
Carbohydrate (%DW)	36.45 (0.99) [33.23 – 39.93]	38.68 (0.99) [35.30 – 42.60]	<0.001	33.50 – 40.22 (26.76, 45.99)

[1] DW – dry weight; FW – fresh weight; S.E. – standard error;
[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
[3] Probability values <0.05 are statistically significant.

5.3.2 Fatty acids in MON87769 soybean

Major changes in fatty acid composition of MON87769 seed are intended as a result of the genetic modification. The mean values for the fatty acid components novel to MON87769 soybean seed are presented in Table 6. The levels of SDA (18:4) in harvested seed ranged from 16.83 to 33.92% of total fatty acids, with a mean level of 26.13%. Due also to the Δ6 desaturase activity, the levels of GLA (18:3) were expected to increase while LA (18:2) levels were expected to decrease. The levels of GLA in MON87769 seed ranged from 6.07 to 8.03% of total fatty acids (mean 7.09%).

Lower levels of two other fatty acids, trans-SDA (mean of 0.18%, range 0.06 – 0.26% of total fatty acids) and trans-ALA (mean of 0.44%, range 0.38 – 0.48% of total fatty acids) were also observed. The formation of trans-ALA and trans-SDA is due to the known spontaneous trans-isomerisation of unsaturated fatty acids. The rate of trans-isomerisation reportedly increases with an increasing degree of unsaturation (Chardigny *et al*. 1996). As SDA and ALA together represent approximately 35 – 40% of total fatty acids in MON87769, trans-ALA and trans-SDA are expected to be present. Conventional soybean seed does not contain detectable levels of SDA, GLA or the two trans-isomers.

Table 7: Combined-Site Summary of SDA, GLA, Trans-SDA and Trans-ALA Levels in harvested MON87769 soybean 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.

Overall, 34 different fatty acid analytes were measured in the compositional analysis of MON87769 and control seed (A3525), ranging from caprylic acid (C8:0) through to docosahexanenoic acid (C22:6), however for a majority of these analytes, the values were less than 0.02% of the seed. In addition to the results presented in Table 6, a statistical comparison between MON87769 soybean and the control was possible for eight other fatty acids present in seed (Table 7). The levels of six of these fatty acids were significantly different in MON87769 compared with the control in the combined-site analysis and in more than one individual-site analysis. These findings were not unexpected as a result of the changing flux of intermediates in fatty acid metabolic pathways in MON87769 soybean seed.

In summary, the fatty acid analyses of harvested MON87769 seed showed:

- Oleic acid, LA and ALA were all significantly ($p < 0.05$) different from the conventional control in the combined-site analysis and in all five individual-site analyses. Since these fatty acids are directly involved in the pathway to SDA, their concentrations are interdependent with that of other fatty acids.
- Arachidic acid was significantly different from the control in the combined-site analysis and in four individual-site analyses.
- Palmitic acid and behenic acid values were significantly different in the combined-site analysis and in two individual-site analyses.

Except for LA, the differences in fatty acid levels were relatively small in magnitude (<4% of total fatty acids) and/or the mean values and ranges in MON87769 seed were within the 99% tolerance interval for the population of conventional reference varieties.

The levels of LA in MON87769 were significantly lower than in the control line as evident in the combined-sites analysis and in all individual-site analyses. Given that LA is a substrate for both the Δ6- and Δ15-desaturase catalysed reactions, levels would be expected to be significantly depleted in MON87769 soybean, compared with conventional soybean.

Table 8: Comparison of Fatty Acids in Soybean Seed – Combined Site Results

Fatty Acid (% total FA)	MON87769 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[1]	Commercial reference range (99% tolerance interval[2])
Palmitic acid (C16:0)	12.06 (0.13) [11.53 – 12.54]	11.77 (0.13) [11.14 – 12.08]	<0.001	9.88 – 12.33 (7.28, 14.20)
Stearic acid (C18:0)	4.19 (0.10) [3.73 – 4.53]	4.15 (0.10) [3.85 – 4.44]	0.245	3.68 – 4.89 (2.87, 5.85)
Oleic acid (C18:1 Cis)	15.18 (0.95) [12.66 – 18.80]	19.19 (0.95) [17.24 – 21.17]	0.001	16.70 – 23.16 (12.56, 27.98)
Linoleic acid (C18:2 9c,12c)	22.78 (1.64) [16.46 – 30.81]	54.93 (1.64) [54.05 – 56.04]	<0.001	53.36 – 57.39 (50.46, 59.96)
A-Linolenic acid (C18:3)	11.18 (0.46) [10.20 – 11.80]	9.20 (0.46) [7.42 – 10.66]	0.016	6.95 – 10.58 (3.72, 13.46)
Arachidic acid (C20:0)	0.34 (0.009) [0.31 – 0.37]	0.31 (0.009) [0.28 – 0.34]	<0.001	0.27 – 0.36 (0.20, 0.45)
Eicosenoic acid (C20:1)	0.14 (0.023) [0.075 – 0.20]	0.13 (0.023) [0.069 – 0.19]	0.282	0.071 – 0.19 (0, 0.31)
Behenic acid (C22:0)	0.29 (0.007) [0.26 – 0.31]	0.32 (0.007) [0.28 – 0.37]	0.023	0.30 – 0.41 (0.22, 0.49)

[1] Probability values <0.05 are statistically significant.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Significance of compositional differences

The fatty acid composition of conventional soybean oil varies widely depending on agricultural and environmental factors and the geographical location in which the soybean crop is grown (Gunstone *et al*.). The consumption of traditional varieties of soybean with very different compositions from one another has occurred over time and does not raise food safety concerns. Except for the production of SDA, the composition of oil derived from MON87769 soybean is not unusual for a typical vegetable oil, despite other more minor compositional changes.

To demonstrate the degree of natural variability, the data in Table 9 show that the levels of ALA and LA in vegetable oils vary widely according to the source. In particular, the level of LA in MON87769 soybean is comparable to that in conventional canola oil, as well as being similar to, or lower than, the levels in oil derived from almond, avocado and flaxseed. Moreover, the decrease in LA in MON87769 to approximately 20% of total fatty acids is smaller than the decrease found in approved High Oleic acid soybean varieties, where LA levels decreased to approximately 4% of total fatty acids. In this case, conservative dietary modelling scenarios showed that significantly lower levels of LA in soybean oil would have minimal nutritional impact on the whole diet (FSANZ Application A1018 – Supporting Document 1). The consumption of vegetable oils with variable fatty acid profiles therefore does not pose any safety concerns.

Table 9: Indicative linoleic acid (LA) and α-linolenic acid (ALA) content (% total oil) in a number of commercially available refined vegetable oils.

Source of oil	α-Linolenic acid (% total fat)	Linoleic acid (% total fat)	Reference
Almond (natural level)	0	17.4	Cordain (2002)
Avocado (natural level)	0.96	12.53	Cordain (2002)
Canola	9.3	20.3	Dow AgroSciences (2009)
Corn	1.16	53.52	Oilseeds International, Ltd (2002)
Cottonseed	0.20	51.5	Cordain (2002)
Flaxseed	53.3	12.7	University of Florida (2003)
Peanut	0	32	Oilseeds International, Ltd (2002)
Olive (natural level)	0.79	9.21	Cordain (2002)
Soybean	6.8	51.0	Cordain (2002)

Fatty acid metabolism

Fatty acid oxidation provides the major energy source for many tissues in the body except brain. The degradation of fatty acids in humans and animals occurs in the mitochondria of cells in a cyclic process called β-oxidation. In this process, two carbon units are cleaved from the carboxy-terminus as acetyl-CoA subunits. Acetyl-CoA units are able to directly enter the tricarboxylic acid (TCA, or Krebs, cycle) to generate usable energy. The *cis* double bonds in polyunsaturated fatty acids such as LA (C18:2cΔ9, 12), however require the activity of two additional enzymes, enoyl-CoA isomerase and 2,4-dienoyl-CoA-reductase, to undergo complete oxidation. The isomerase converts a *cis* double bond to *trans*, and following several further reactions (hydration and dehydrogenation), the intermediate can then undergo the remaining cycles of β-oxidation to the final two carbon product. The two auxiliary enzymes allow all of the even-chain polyunsaturated fatty acids to be similarly degraded, with a slight reduction in overall energy yield. It should be noted however that metabolic pathways for oxidising *trans* unsaturated fatty acids present in the diet are not fully resolved.

5.3.3 Amino acids

Levels of 18 amino acids were measured in seed from soybean MON87769 and the conventional control soybean. Results of the combined site analysis (Table 10) show a statistically significant difference between SDA soybean and its conventional soybean control in all but one of these measurements (tryptophan).

Statistically significant differences were observed in five amino acids (proline, arginine, cystine, glycine and phenylalanine) in more than one individual test site analysis, as well as the combined site analysis, as follows

(i) Proline levels were significantly different in three of the five individual-site analyses, and
(ii) Arginine, cystine, glycine and phenylalanine were significantly different in two of the five individual-site analyses.

The levels of aspartate, glutamate, histidine, leucine, isoleucine, lysine and valine were significantly different between SDA soybean and the control in the combined-site and one individual-site analyses. For the remaining amino acids (alanine, methionine, serine, threonine and tyrosine), the differences were found to be statistically significant only in the combined-site analysis.

The data presented in Table 11 show that for each significantly different analyte, the magnitude of the difference between MON87769 soybean and the conventional control was small. More importantly, from a food safety perspective, all but one of the amino acid analytes that were statistically significantly different were within the range obtained for the commercial reference varieties grown at the same time. For all amino acids, the mean and range observed in MON87769 harvested seed were within the 99% tolerance interval for the population of conventional reference varieties.

Overall, the proximate analysis also showed a small difference in the protein levels in MON87769 soybean compared with the control (see Table 6). Although this difference was statistically significant, the magnitude of the difference was small, and the mean level and range in the combined-site analysis fell well within the commercial reference range, within the 99% tolerance interval for the population of conventional reference varieties and within the range of values obtained from the published literature and/or the ILSI Crop Composition Database. Based on these findings, the small differences in amino acids and protein observed in MON87769 soybean seed compared with its conventional counterpart are not considered to be biologically significant.

5.3.4 Isoflavones

The levels of the three basic categories of isoflavones in soybean seed, namely daidzein, genistein and glycitein were measured in harvested seed from MON87769, control A3525 and commercial soybean varieties (OECD, 2001). The results of the combined site analysis are presented in Table 12.

The mean dry weight concentrations of genistein, daidzein and glycitein in seed of MON87769 soybean were significantly ($p < 0.05$) decreased compared to the levels in the conventional control (A3525). As evident from the commercial reference range and the 99% tolerance intervals, the natural variation in the levels of these analytes in conventional soybean varieties is quite broad. It is well-documented that soybean isoflavone levels are greatly influenced by many factors including environmental conditions, variety and agronomic practices (Messina, 2001; Nelson et al. 2001). The mean levels of daidzein, genistein and glycitein in MON87769 soybean are within the 99% tolerance intervals established for conventional soybean varieties grown at the same test sites as MON87769 and the near isogenic control. Furthermore, the levels of isoflavones in MON87769 soybean are within the broad literature range and the ILSI Crop Composition Database range, and therefore do not raise a safety issue.

Table 12: Isoflavone Content of Soybean Seed – Combined Site Analysis

Analyte (µg/g DW)	MON87769 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[1]	Commercial reference range (99% tolerance interval[2])
Daidzein	1187.81 (188.32) [957.23 – 1838.91]	1807.36 (188.32) [1380.05 – 2775.08]	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]	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]	0.004	74.87 – 189.64 (0, 243.40)

[1] Probability values <0.05 are statistically significant.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 11: Comparison of Amino Acids in Soybean Seed – Combined Site Analysis

Amino Acid (% DW)	MON87769 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[1]	Commercial reference range (99% tolerance interval[2])
Alanine	1.78 (0.0084) [1.76 – 1.84]	1.74 (0.0084) [1.68 – 1.81]	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]	<0.001	2.61 – 3.15 (2.13, 3.62)
Aspartic acid	4.54 (0.035) [4.21 – 4.60]	4.36 (0.035) [4.21 – 4.60]	0.007	4.01 – 4.71 (3.45, 5.29)
Cystine	0.62 (0.0098) [0.56 – 0.64]	0.60 (0.0098) [0.56 – 0.64]	<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]	<0.001	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]	0.003	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]	<0.001	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]	<0.001	1.62 – 2.00 (1.34, 2.28)
Leucine	3.19 (0.017) [3.13 – 3.32]	3.09 (0.017) [3.01 – 3.19]	<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]	<0.001	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]	0.038	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]	0.002	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]	<0.001	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]	0.043	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]	0.035	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]	0.069	0.43 – 0.52 (0.35, 0.59)
Tyrosine	1.40 (0.016) [1.27 – 1.50]	1.34 (0.016) [1.26 – 1.44]	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]	<0.001	1.70 – 2.11 (1.42, 2.41)

[1] Probability values <0.05 are statistically significant.

[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

5.3.5 Anti-nutrients

Trypsin inhibitors are naturally-occurring substances that interfere with the digestion of proteins, resulting in decreased animal growth (Liener, 1994). Lectins also occur naturally in soybean, and can be toxic to animals if the soybean is consumed raw. Both lectins and trypsin inhibitors are heat-labile and should be inactivated by the processing of soybean seeds into protein or seed meal products. Provided that soybean has been adequately processed, there are minimal residual levels of these antinutrients remaining in the edible soybean fractions. Stachyose and raffinose are low molecular weight carbohydrates present in raw soybean. Cooking of soybean significantly reduces the intestinal effects caused by the fermentation of these substances by bacteria in the gut.

The levels of these key antinutrients were measured in harvested seed from MON87769 soybean and the conventional control A3525 (Table 13). There were no significant differences in the levels of antinutrients in MON87769 soybean seed when compared with the conventional control soybean. In addition, all values were within the reference range observed for a number of conventional soybean varieties.

Table 13: Antinutrients in Soybean Seed – Combined Site Results

Antinutrient (Units)	MON87769 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[1]	Commercial reference range (99% tolerance interval[2])
Lectins (H.U.[3] / mg DW)	3.55 (0.80) [0.55 – 8.07]	3.73 (0.80) [0.71 – 11.32]	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]	0.357	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]	0.057	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]	0.259	2.23 – 3.29 (1.61, 4.05)
Trypsin Inhibitor (T.I.U. / mg DW)	33.81 (2.81) [24.30 – 54.80]	31.10 (2.81) [21.34 – 41.69]	0.190	24.29 – 46.29 (8.09, 57.27)

[1] Probability values <0.05 are statistically significant.
[2] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.
[3] H.U. = haemagglutinating units.

5.3.6 Vitamins

There was no statistically significant difference in the level of vitamin E in harvested seed from MON87769 compared to the control soybean (data from combined-site analysis). All values fell within the range observed for a number of commercial soybean varieties grown concurrently at the test sites.

	MON87769 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value	Commercial reference range
Vitamin E (mg / 100g DW seed)	1.56 (0.26) [0.86 – 2.54]	1.43 (0.26) [0.70 – 2.22]	0.271	0.27 – 2.93

5.3.7 Summary of seed compositional analyses

A range of statistically significant compositional differences between MON87769 soybean seed and the conventional control were evident from the results of the combined site analysis.
As intended from the genetic modification which introduced deliberate changes in the fatty acid composition of the oil, SDA constitutes approximately 26% of the fatty acids in MON87769 soybean seed, whereas SDA is not detected in conventional soybean seed. The oil from MON87769 also contains increased levels of trans ALA and very low amounts of a novel isomer, trans SDA. Differences in other fatty acids were also observed, however the levels were generally within the range corresponding to commercial soybean varieties. Lowered levels of LA and ALA are also due to the altered fatty acid metabolic pathways, but are compatible with the levels of these fatty acids in other commonly consumed vegetable oils and therefore do not represent a food safety concern.

In the proximate analysis, protein was significantly increased and carbohydrates were significantly decreased in MON87769 soybean seed, however both were within the range of natural variation for commercial soybean varieties. The levels of all amino acids were only slightly increased in MON87769 soybean seed compared with the control, although the difference was statistically significant in all cases except for tryptophan. Isoflavones were significantly decreased compared to the control but were within the commercial reference range. There were no significant differences in the levels of vitamin E and antinutrients measured in MON87769 soybean and the control.

5.4 Composition of processed soybean fractions

Seed samples from MON87769, A3525 (isogenic control), and eight conventional soybean varieties were harvested from two additional field trial sites in the U.S. during the 2006 growing season. The seed was 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 food fractions were analysed according to the principles outlined in the OECD Consensus Document for soybean composition (OECD, 2001). The DT fraction was analysed for proximates (moisture, protein, fat and ash), ADF, NDF, amino acids, trypsin inhibitors and phytic acid. The RBD oil was analysed for fatty acids (C8-C24) and vitamin E (α-tocopherol). Protein isolate was analysed for amino acids and moisture. The crude lecithin fraction was analysed for fatty acids (C8-C24) and phosphatides (α-phosphatidic acid, α-phosphatidylcholine, α-phosphatidylethanolamine and α-phosphatidylinositol). A comparison of the results of the compositional analysis of fractions obtained from MON87769 soybean and conventional soybean showed a number of statistically significant differences, as summarised below.

In soybean meal, four amino acids (aspartic acid, glutamic acid, histidine and tryptophan) as well as ADF and carbohydrates values were significantly different ($p<0.05$). The magnitude of the differences in each case was small, and the mean and range of values for each of these analytes in MON87769 were within the 99% tolerance interval for the population of conventional soybean varieties. Defatted toasted soybean meal typically contains a small amount of oil after processing. Analysis indicated that DT soybean meal from MON87769 seed contained 1.17% oil, which is consistent with average oil content for soybean meal. Given the low levels of oil present in the seed meal, the altered fatty acid profile in oil from MON87769 would have negligible impact on the overall composition of the seed meal.

Soybean protein isolate was prepared by removing all non-protein material from defatted soybean flour. Following analysis for amino acids and moisture, only the level of leucine was significantly different in MON87769, compared with the conventional control. The mean and range of values were comparable to those of commercial soybean varieties.

Fatty acids (C8-C24) and vitamin E levels were analysed in RBD oil samples from MON87769, A3525 and eight conventional soybean reference varieties. Four additional fatty acids (SDA, GLA, trans-SDA, trans-ALA), not detected in conventional soybean seed, were measured in all RBD oil preparations.
The results of these specific analyses are reported in Table 14 (expressed as % total fatty acids).

Overall, the levels of these constituents in RBD soybean oil from MON87769 were consistent with the results from the analysis of harvested seed samples (refer Tables 6 and 7), except for one additional observation. Although *trans*-ALA was not detected in conventional soybean seed, it was observed in low amounts (mean of 0.14% of total fatty acids) in RBD oil prepared from the conventional soybean seed A3525. This is attributed to the effects of processing of the oil from the seed.

As would be expected due to the higher levels of ALA, *trans*-ALA in RBD oil from MON87769 is also increased relative to the conventional control. Differences in some other fatty acids were also found (palmitic, stearic and lignoceric acids), although these were not considered to be of nutritional significance, as the difference in each case was small. In addition, RBD oil from MON87769 contained approximately 20% higher levels of vitamin E compared to that from the conventional control.

Finally, the fatty acid and phosphatide levels in crude lecithin from MON87769 were comparable to the levels in the conventional soybean control. As might be expected from the introduced trait, there were detectable levels of SDA, GLA, and the two *trans* isomers in crude lecithin from MON87769, however the absolute amounts were low.

Table 14: Summary of levels of SDA, GLA, *trans*-ALA and *trans*-SDA in RBD soybean oil from MON87769 and the Control – data from the Combined-site Analysis (% total fatty acids)

Fatty Acid	MON87769 Mean (S.E.)	MON87769 Range	A3525 Control Mean	p-value
18:4 Stearidonic Acid (SDA)	22.62 (3.08)	[16.88 – 28.35]	ND[1]	NA[2]
18:3 γ-Linolenic Acid (GLA)	6.68 (0.26)	[6.19 – 7.19]	ND	NA
18:4 6c,9c,12c,15t (Trans-SDA)	0.26 (0.052)	[0.17 – 0.39]	ND	NA
18:3 9c,12c,15t (Trans-ALA)	0.51	[0.47 – 0.54]	0.14	0.035

[1] ND= not detected
[2] NA=not applicable

5.5 Allergenicity assessment

Soybean is one of eight major allergenic foods that together are responsible for over 90% of all verifiable food allergies (FAO, 1995). Soybean is somewhat less allergenic than other foods in this group, and rarely causes severe, life-threatening reactions (Cordle, 2004). Moreover, allergy to soybean is more prevalent in children than adults, and may be considered a transient allergy of infancy/childhood (Sicherer *et al.* 2000). Due to its inherent allergenicity, further testing of MON87769 soybean was conducted to determine whether the introduction of the genes and production of the PjΔ6D and NcΔ15D proteins had any unintended effect on allergenicity, relative to conventional non-GM soybean.

Study submitted:
Rice, E.A., Nemeth, M.A. and Bannon, G.A. (2007). Assessment of Human IgE Binding to SDA Soy, Control, and Reference Soy Extracts. MSL: 20553[6] (unpublished)

This study determined the IgE *in vitro* binding levels of serum obtained from clinically documented soybean allergic patients, to protein extracts prepared from MON87769 soybean seed, and two other SDA-producing transgenic soybean lines (not under assessment). The identity of the soybean extracts was verified by event-specific PCR. Protein extracts from 24 commercial soybean varieties were also tested to establish a reference range of IgE binding. The commercially available soybean varieties included both conventional and GM lines already on the market and used for human consumption.

[6] Testing site: Prof. Dr. Stefan Vieths, Paul-Erlich-Institut, Division of Allergology, Langen, Germany.

The level of IgE binding provided an estimate of the amount of endogenous soybean allergens present in the seeds.

Serum samples from 16 soy-allergic patients and 6 non-allergic individuals were used to assess the range of IgE binding to each soybean extract. Only soy allergic patients with a documented history of anaphylactic reactions to soybean and a Double-Blind Placebo Controlled Food Challenge (DBPCFC) were included.

The protein extracts were prepared from the ground seed of the SDA soybean lines, non-GM control (A3525), and the commercial soybean varieties for analysis of IgE binding by a validated ELISA method. Determining the levels of direct IgE binding using an enzyme-linked ELISA has been shown to be an appropriate method for this analysis (Sten *et a*l. 2004; Thomas *et al*. 2005). Each soybean extract (at 10 µg total seed protein/ml) was tested in triplicate. Soy-specific IgE was quantified from a soy-specific IgE standard curve. Detection of soy-specific IgE was achieved by using biotin conjugated anti-human IgE polyclonal antibody from goat.

To compare levels of IgE binding, all ELISA values were subjected to a statistical data evaluation. The values obtained for the reference soybean extracts were used to calculate a 99% tolerance interval for each serum. The IgE binding levels obtained for the protein extract from MON87769 soybean were compared to the calculated tolerance intervals. The results showed that IgE values for the MON87769 soybean and A3525 control were within the established tolerance intervals obtained for each serum. None of the tested soybean varieties showed IgE binding to sera from non-allergic individuals. This study confirms that the levels of endogenous allergens in MON87769 soybean are comparable to those in soybean varieties currently available for human food uses.

5.6 Conclusion from compositional studies

Detailed compositional analyses were done to establish the nutritional adequacy of seed-derived products from MON87769 soybean, and to characterise the intended as well as any unintended compositional changes. Analyses were done of proximate (crude fat/protein, fibre, ash), amino acid, fatty acid, vitamin, isoflavone and anti-nutrient content. The levels of these analytes were compared to those in the conventional parental line A3525 and to 10 non-GM commercially available soybean varieties grown concurrently at the test sites. These analyses demonstrated that the fatty acid profile in MON87769 soybean seed is significantly changed from that of the parental line, and conventional soybeans.

The mean SDA content in MON87769 soybean oil is approximately 26% of fatty acids. Concomitantly, mean levels of linoleic acid decreased from 54.9% to 22.8%. There was also a small increase in the mean levels of α-linolenic acid in MON87769 soybean, from 9.2% to 11.2% of fatty acids. The level of LA, while significantly lower than that in the control, is nonetheless within the normal range found in soybeans, and is a much smaller change than that observed in High Oleic Acid soybean 305423, previously assessed by FSANZ (Application A1018) and considered safe for human consumption. The differences in levels of the three fatty acids are attributable to the two components of the genetic modification, ie the co-expression of the Δ6- and Δ15-desaturase enzymes in the seed, leading directly to the production of SDA, a small increase in ALA, and a decrease in the pool of LA.

Conventional soybean oil does not normally contain stearidonic acid, nor the *trans* isomer, although the levels of the latter are acceptably low (<0.5% of total fatty acids). SDA is readily metabolised in the body through normal fatty acid degradative pathways. The consumption of SDA is part of a normal human diet, and the presence of SDA in soybean oil does not raise any food safety concerns.

While minor increases in the levels of most amino acids and total protein were observed, the changes are within the range of values observed for commercial soybean varieties and are therefore consistent with the range of natural variation currently in the food supply. The compositional data are consistent with the conclusion that there are no changes in key constituents in MON87769 seed that represent a safety concern, when compared to commercial soybean varieties or other commonly consumed vegetable oils.

6. NUTRITIONAL IMPACT

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence indicates that feeding studies using target livestock species will add little to the safety assessment (OECD, 2003; FSANZ, 2007; EFSA, 2008). However, if the compositional analysis indicates biologically significant changes in the levels of certain nutrients in the GM food, additional nutritional assessment may assist to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. The assessment should include consideration of the bioavailability of the modified nutrient.

In the case of MON87769, there are significant changes in the fatty acid profile of soybean oil, specifically the production of SDA (C18:4) and a moderate decrease in the level of LA (C18:2), as well as minor changes (<4%) in other key fatty acids. Although the composition of the oil has changed, all components of MON87769 soybean oil are typical constituents of the human diet.

In 2008, the Codex Alimentarius Commission adopted a number of annexes to its Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (Codex Plant Guideline, adopted in 2003). The purpose of one of these, Annex 2: Food Safety Assessment of Foods Derived from Recombinant-DNA Plants Modified for Nutritional or Health Benefits, was to provide general guidance on additional factors to be taken into account in the safety assessment of a GM food, modified for a nutritional or health benefit (CAC/GL 45-2003).

The Codex Plant Guideline states that "*When the modification results in a food product, such as vegetable oil, with a composition that is significantly different from its conventional counterpart, it may be appropriate to use additional conventional foods or food components (i.e. foods or food components whose nutritional composition is closer to that of the food derived from recombinant-DNA plant) as appropriate comparators to assess the nutritional impact of the food.*" FSANZ has conducted a separate nutrition assessment of MON87769 soybean to determine whether the changes in the composition of the oil would have a significant impact on the diet (see **Supporting Document 2**).

6.1 Feeding studies

The Applicant has conducted several feeding studies in animals to support the safety and nutritional adequacy of food derived from soybean MON87769. Two studies – a 90-day in rats and a 42-day broiler study – used soybean meal produced from MON87769 soybean and the conventional control.

A 28-day gavage study and a 90-day one generation reproduction feeding study in rats used SDA soybean oil produced from MON87769. Details of the latter studies in rats were published in 2008 (Hammond *et al*. 2008).

6.1.1 Studies in rats with soybean oil

Published study:

Hammond, B.G., Lemen, J.K., Ahmed, G., Miller, K.D. Kirkpatrick, J. and Fleeman, T. (2008). Safety assessment of SDA soybean oil: Results of a 28-day gavage study and a 90-day/one generation reproduction feeding study in rats. *Regulatory Toxicology and Pharmacology*, 52, 311-323.

Gavage study

Four groups of CrlCD Sprague-Dawley rats (10 rats/sex) were administered soybean oil orally by gavage for 28 consecutive days. A diet control group (Group 1) was not gavaged with oil. The treatment groups each received the same dosage of oil (3 ml/kg bw/day), made up as follows:
Group 2 – 3.0 ml control soybean oil;
Group 3 – 0.3 ml SDA soybean oil mixed with 2.7 ml control soybean oil;
Group 4 – 1.0 ml SDA soybean oil mixed with 2.0 ml control soybean oil;
Group 5 – 3.0 ml SDA soybean oil.

All animals were observed twice daily for clinical signs; individual body weights and food consumption were recorded weekly. A detailed physical examination was performed pre-treatment, at weekly intervals during the treatment period, and on the day of necropsy. Blood and urine samples were collected for clinical pathology evaluations (haematology, serum chemistry, urinalysis) from all animals immediately prior to the end of treatment. At necropsy, animals were subjected to gross examination and approximately 40 tissues were collected from each animal. Organ weights were measured; organs from the high dose SDA and control rats only (both males and females) were examined microscopically.
No adverse clinical signs were observed over the course of the study, and all animals survived to the scheduled necropsy. There were no treatment-related effects on body weights or food consumption across any groups, nor any adverse findings in clinical pathology parameters. At necropsy, there were no adverse macroscopic findings, no changes in organ weights, and no histological findings that could be related to the treatment. Overall, the comparison between the control and the SDA treated groups showed no adverse effects related to the oral administration of SDA soybean oil, even at the highest dose tested, equivalent to >600 mg SDA/kg bw/day.

Reproduction feeding study

Groups of male (n=25) and female (n=45) rats were administered control soybean oil, SDA soybean oil or menhaden oil in the diet. Males were subjected to the diet treatment for a minimum 70 days prior to mating, until the conclusion of the study. The females were divided into two groups as follows:
Group 1 – toxicology females (20/group), not mated, subjected to dietary treatment for a minimum of 90 days until the conclusion of the study; and
Group 2 – mated females (25/group), in the reproduction phase of the study, subjected to dietary treatment for a minimum of 70 days prior to mating and continuing through gestation and lactation. Dams and their progeny were sacrificed at weaning on postnatal day 21.

The behaviour and health of the animals were monitored at regular intervals, and various parameters including body weights and food consumption measured. In addition, clinical parameters were evaluated in randomly selected males and non-mated females (10/group) at selected times. All progeny were observed daily for clinical signs and general well-being; a detailed physical examination and body weights were recorded at specified times. At the conclusion of the study, a full complement of tissues was harvested from all adult animals.

There were no treatment-related adverse effects on any of the fertility and reproductive performance parameters assessed in this study. The reproductive performance of male and female rats was not different between the control and SDA groups. Similarly, there were no differences between control and SDA-treated groups in the progeny; all findings were within age-related normal limits for the pups and were similar across all groups.

Discussion and Conclusions

The rat was considered a suitable model to use to investigate potential dietary effects in humans following exposure to oils containing high amounts of certain fatty acids such as SDA. As noted by the authors, it has been demonstrated (in feeding studies with ALA and SDA) that the rat is biochemically equipped to convert SDA to EPA. The 28-day gavage study and the 90-day/one generation reproduction feeding study found no treatment-related adverse effects from the oral administration of SDA soybean oil (containing 20-26% SDA), at the highest amount tested. These results equate to a daily SDA dose of >600 mg/kg bw (28-day gavage study) and >1000 mg/kg bw in the feeding/reproduction study.

6.1.2 90-day study in rats with processed soybean meal

Study submitted:
A 90-day Feeding Study in Rats with Processed Meal from MON87769 Soybeans. Study Director: J.B. Kirkpatrick. Study Number WIL-50333. Study Completed: December, 2008. Performing laboratories: WIL Research Laboratories, Covance Laboratories, Midwest ToxPath Sciences Inc. Monsanto Study Report 0021746.

The study included three groups of Sprague-Dawley (Crl:CD®[SD]) rats, with each group consisting of 20 males and 20 females. Group 1 received a diet containing conventional control (A3525) soybean meal formulated into the diet to 15% (w/w). Groups 2 and 3 received a test diet containing MON87769 soybean meal incorporated into the diet to 5 and 15% (w/w) respectively.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly for the duration of the 13-week study. On the day of scheduled necropsy, clinical pathology evaluations (haematology, coagulation, serum chemistry and urinalysis) were performed on 10 rats/sex/group. All animals were subjected to macroscopic examination at necropsy and selected organs weighed. Selected tissues were examined microscopically from all animals fed the A3525 control diet (15%) and the 15% MON87769 soybean meal diet.

With one exception, all rats survived the course of the study. The single mortality was considered to be incidental to the diet treatment (5% MON87769 diet), and there were no adverse clinical signs observed in any groups. Body-weights, food consumption and organ weights were comparable across all groups.
There were no test-diet related adverse effects on clinical pathology parameters, and no macroscopic or microscopic findings. The conclusion from this study was that the incorporation of MON87769 soybean meal into the diet of rats up to a concentration of 15% (w/w) for at least 90 days had no adverse effect on the growth and health of rats.

6.1.3 Broiler feeding study

Study submitted:
Comparison of Broiler Performance and Carcass Parameters When Fed Diets Containing Soybean Meal Produced from MON87769, Control, or Reference Soybeans. Study Director: S.W. Davis. Performing laboratories: Colorado Quality Research, Inc; Global Poultry Consulting, Inc; University of Missouri Experiment Station Chemical Laboratories; Monsanto Company, Quality Assurance Unit. Monsanto Study No: 07-01-83-40

This study compared the nutritional performance of soybean meal prepared from MON87769, control A3525 and conventional reference soybean varieties when incorporated into the diet of rapidly-growing broiler chickens (Ross x Ross 308) for approximately 42 days. The test, control and reference soybean meal was produced from soybeans grown at two sites in the U.S. and processed to yield oil and meal. The reference articles were six different non-GM soybean varieties. Characterisation, including verification of identity of the samples prior to processing by PCR analysis, and nutrient/anti-nutrient, mycotoxin and pesticide analyses of the soybean meal was carried out before use in the diets.

Equal numbers of healthy one day old male and female chicks were assigned to one of the eight different dietary treatments (100 birds per treatment). Diets were formulated to maximise the amount of soybean meal included, while meeting dietary specifications for starter and grower/finisher diets. The diets consisted of either the test, control or reference soybean meals, mixed with corn grain and corn gluten meal, and were formulated to be isocaloric and contain approximately the same amount of soybean meal. Feed and drinking water were provided *ad libitum*. Starter diet was provided from Day 0-21 and the grower/finisher diet was provided for the remainder of the feeding study.

Birds were observed at least twice daily for overall condition and health. Body weights were measured at the beginning and end of the study. Feed intake was determined for Days 21 and 42, and the average and adjusted feed:gain calculated. Male birds were processed on Day 43 and the females were processed on Day 44 to collect a set of performance parameters and carcass yield data for individual birds as follows: live weight, fat pad weight, chilled weight, breast meat weight (skinless, boneless), wings (bone in, skin on), thighs (bone in, skin on) and legs (bone in, skin on).

Each measurement to be statistically analysed was processed by two different procedures. The basic method was a two-factor analysis of variance under a randomised complete block structure. The two factors were diet and sex of the birds. Differences between means were considered significant at $P< 0.15$. The second analysis was a comparison of the test diet with the population of control and reference diets of which the seven conventional diets (A3525 and six commercial reference diets) were a sample. This required a mixed linear model analysis with an additional variance component for random between-diet effects. Analyses were averaged over sex, unless there was a significant diet-by-sex interaction, which resulted in analyses by sex of the birds.

The mortality rate of birds during the period 7-42 days was an average of 3.3% (range 2-8%) across all treatment groups. The mortality from day 7-42 was 2.0% for birds receiving diets containing soybean meal produced from MON87769 soybean. The growth performance of broilers fed diets containing MON87769 was not different ($P\geq 0.05$) than that of broilers fed diets with control soybean meal produced from conventional soybeans of similar genetic background, and of the population of birds fed conventional reference soybean meal. Small, but statistically significant, differences in some carcass yield measurements (fat pad weight / wing weight) between broilers fed diets containing MON87769 and those fed conventional control soybean meal were noted, but were not considered to be biologically meaningful.

Measurement of fat, moisture and protein content of skinless breast and thigh meat samples collected during bird processing showed no differences among dietary treatments. Overall, the diet containing MON87769 soybean meal was as wholesome as the diets formulated with conventional control or reference soybean meal in terms of the ability to support growth and normal development in the birds, and produced commercial quality outcome measures comparable to the conventional diets. Based on these results, it was concluded that soybean meal derived from MON87769 is nutritionally equivalent to that from the non-GM control soybean with a comparable genetic background, and other conventional soybean varieties.

6.2 Conclusion

Several animal feeding studies were conducted, including a gavage study and one-generation feeding study in rats with soybean oil, a 90-day rat study with processed soybean meal, and a nutritional study in chickens with soybean meal. These studies clearly indicate no adverse effects from the oral administration of oil and processed meal from MON87769 soybean, and demonstrate that MON87769 soybean meal is nutritionally equivalent to conventional soybean meal in terms of supporting typical growth and commercial production of broiler chickens.

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