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

Supporting Document 1

APPLICATION A1049 – FOOD DERIVED FROM HERBICIDE-TOLERANT, HIGH OLEIC ACID SOYBEAN LINE MON87705

SAFETY ASSESSMENT REPORT

SUMMARY AND CONCLUSIONS

Background

Soybean line MON87705 has been genetically modified (GM) to increase levels of oleic acid, a monounsaturated fatty acid, and to decrease levels of linoleic acid, a polyunsaturated fatty acid (PUFA), in soybean oil to improve its suitability for food and industrial uses. At the same time, a slight reduction in saturated fats was achieved. The changes in the fatty acid profile are due to the down-regulation of two key enzymes, FATB and FAD2, involved in the soybean seed fatty acid biosynthetic pathway. This was achieved through the use of RNA-based suppression of two endogenous soybean genes, *fatb1a* and *fad2-1a*. Soybean MON87705 also contains the 5-enolpyruvylshikimate-3-phophate synthase gene derived from *Agrobacterium sp.* strain CP4. This gene is referred to as *cp4 epsps*, and confers tolerance to the herbicide glyphosate.

The oxidative stability of vegetable oils is greatly influenced by the proportion of monounsaturated to polyunsaturated fatty acids. Conventional soybean oil typically contains 60-65% PUFA (mostly in the form of linoleic acid) which are susceptible to oxidation and degradation at high temperatures. Soybean oil from MON87705 is anticipated to have enhanced oxidative stability relative to conventional soybean oil, due to the increase in monounsaturated fatty acid and decrease in PUFA. High oleic acid soybean oils are estimated to have oxidative stability around 17 times greater than conventional soybean oil (Frankel, 2005), providing a more stable oil for use in food formulations.

This safety assessment report addresses only food safety and nutritional issues. It does not address: environmental risks related to the environmental release of GM plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants; or the safety of food derived from the non-GM (conventional) plant.

History of Use

Soybean (*Glycine max* L. Merr.) has a long history of safe use as food for humans and feed for livestock, as well as a range of industrial uses. Soybean oil constitutes approximately 71% of global consumption of fats and oils (ASA, 2008), and is the second largest source of vegetable oil worldwide (Soyatech, 2009).

Soybean MON87705 is intended primarily for use as a broad acre field soybean and not for vegetable or garden use. After extraction of the nutritionally altered soybean oil from MON87705, the remaining processed fractions would likely be blended with those derived from other commercial soybean varieties before entering the food supply in a range of processed products.

Molecular Characterisation

Comprehensive molecular characterisation of MON87705 indicated that a functional two-gene suppression cassette and intact *cp4 epsps* gene co-integrated into the soybean genome at a single insertion site. The complete *FAD2-1A/FATB1-A* suppression cassette, under the control of a seed specific promoter, expresses an RNA that contains an inverted repeat of the soybean *fad2-1a* and *fatb1-a* gene segments. The RNA transcript forms a double-stranded RNA structure (hairpin) that suppresses endogenous *FATB* and *FAD2* RNA levels via RNA interference (RNAi) mechanisms, subsequently leading to the desired fatty acid phenotype. Expression of the *cp4 epsps* gene in chloroplasts confers tolerance to glyphosate containing herbicides.
Some genomic rearrangements were observed at the insertion site however bioinformatic analysis of possible open reading frames (ORFs) formed as a result of these rearrangements did not indicate any likelihood of unexpected gene products with homology to known toxins or allergens. The genetic elements introduced into MON87705 were demonstrated to be stably integrated and segregation data are consistent with a Mendelian pattern of inheritance for a single genetic locus.

Characterisation of Novel Protein

Soybean MON87705 expresses one novel protein, CP4 EPSPS, which is expressed in leaves, roots, forage and seeds. The average CP4 EPSPS content in seeds of MON87705 soybean is 110 µg/g dry weight.

The safety of CP4 EPSPS has been assessed several times as a novel protein in glyphosate tolerant crops. Based on a dossier of studies and a short history of use in GM crops, the CP4 EPSPS protein has been confirmed to lack any biologically significant sequence similarity to known protein toxins or allergens, and is readily degraded in conditions that simulate normal digestion. The evidence supports the conclusion that the CP4 EPSPS protein is neither toxic nor allergenic in humans.

Compositional Analyses

Detailed compositional analyses of seeds harvested from MON87705 soybean, the isogenic control and a number of other commercial soybean varieties grown at different field sites, confirm that MON87705 contains the same five major fatty acids as found in conventional soybean, but in different proportions. Oleic acid is significantly increased from approximately 23% in conventional soybean to around 76% of total fatty acids in MON87705 soybean. The levels of linoleic acid are significantly decreased from 53% total fatty acids to approximately 10% in MON87705. In addition the saturated fatty acids, palmitic and stearic acids, comprise 6% of fatty acids in MON87705 compared with 15% in conventional soybean. Smaller differences in other fatty acids were noted although the absolute levels were within the range established for commercial reference soybean varieties, or reported in the published literature.

Except for the intended changes to four major fatty acids, the composition of MON87705 seed is comparable to that of the conventional soybean control. No significant differences between MON87705 and conventional soybean were observed from analysis of proximates, fibre, amino acids, key nutrients and anti-nutrients present in soybean. Comparisons of the

composition of processed soybean fractions: refined, bleached and deodorised soybean oil, protein isolate and lecithin from MON87705 and conventional soybean varieties, confirmed the intended changes in the fatty acid profile of MON87705 soybean oil and did not reveal other differences of any biological or nutritional significance.

Nutritional Impact

Dietary exposure assessments of the key fatty acids in MON87705 using consumption data from the United Kingdom and the United States indicated that substitution of MON87705 soybean oil for conventional soybean oil would result in higher intakes of the monounsaturated fatty acid, oleic acid and marginally lower intakes of saturated fats normally found in vegetable oils.

Two feeding studies using diets containing soybean meal prepared from MON87705, the conventional control and other conventional soybean varieties were conducted to compare the nutritional performance of MON87705 soybean with conventional varieties.
A 90-day feeding study in rats and a 42-day study in broiler chickens both demonstrated that there were no differences between a diet containing MON87705 soybean meal and diets containing conventional soybean meal in terms of the ability of the diet to provide adequate nutrition to animals to support typical growth and wellbeing.

Conclusion

No potential public health and safety concerns have been identified in the assessment of high oleic acid soybean MON87705. On the basis of the data provided in the present application, and other available information, food derived from soybean MON87705 is considered as safe for human consumption as food derived from conventional soybean varieties.

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1 INTRODUCTION

Soybean line MON87705 has been genetically modified (GM) to produce an altered fatty acid profile and for tolerance to glyphosate. The intended nutritional change, to increase the level of oleic acid (C18:1) in soybean oil, was brought about by silencing two endogenous genes involved in fatty acid biosynthesis in soybean plants. The net effect of the modification also results in decreased levels of linoleic acid (C18:2), and saturated fatty acids palmitic acid (C16:0) and stearic acid (C18:0), relative to conventional soybean oil. Due to the higher oleic acid levels, soybean oil from MON87705 is suitable for a greater range of food applications compared with conventional soybean oil.

The glyphosate tolerance trait introduced into MON87705 plants has been described previously by FSANZ. Tolerance to glyphosate herbicides is conferred by expression of *cp4 epsps*, a gene derived from the comMONsoil bacterium, *Agrobacterium sp.* strain CP4.

2 HISTORY OF USE

2.1 Donor organism

The donor for the only novel gene present in MON87705 soybean (*cp4 epsps*) is *Agrobacterium tumefaciens,* a Gram-negative, non-spore forming, rod-shaped bacterium commonly found in soil. It is closely related to other soil bacteria involved in nitrogen fixation by certain plants. As such, *Agrobacterium* and related nitrogen fixing bacteria are commonly found in association with plants, especially plant roots. Many strains of *Agrobacterium* naturally contain a plasmid (the Ti plasmid) which has the ability to enter plant cells and insert a portion of the plasmid into plant chromosomes. The result of this is the forced production by the plant of specialised amino acids (called opines) that the bacterium uses as a nitrogen source. As such, *Agrobacterium* is a plant pathogen that causes root deformation in several crop species. However, techniques have been developed that allow use of *Agrobacterium* sp to transform a broad range of plant species without causing adverse effects in the host plant.

As *Agrobacterium* species are commonly found in soil and in association with plants, it is likely that humans have had long exposure to them. They are not known to be pathogenic to humans or animals and are not commonly allergenic (FAO/WHO, 1991). The *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 has been used widely to confer glyphosate-tolerance to GM crop plants, and is not associated with any safety concerns.

2.2 Host organism

Soybean (*Glycine max* (L.) Merr.) is grown as a commercial crop in over 35 countries worldwide and has a long history of safe use as human food and feed for livestock. The major producers of soybeans, accounting for 90% of world production, are the United States, Argentina, Brazil and China. In 2007, soybeans represented 56% of total world oilseed production, and 32% of those soybeans were produced in the United States (The American Soybean Association, 2008). In many soybean producing countries, GM soybean (mainly with a herbicide tolerant trait) accounts for a significant proportion of the total soybean grown e.g. United States (91%); Argentina (99%); Brazil (63%); South Africa (87%); Uruguay (99%) (Brookes and Barfoot, 2009).

Australia, while a net importer of soybean, grows conventional crops in latitudes extending from the tropics (16^{o} S) to temperate regions (37^{o} S), mainly in the eastern states and as a rotational crop (James and Rose, 2004). The seed is used mainly to produce meal for use in animal feed (Grey, 2006).

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. 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, 2001). Appropriate heat processing inactivates these compounds.

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:
A. Skipworth, K.R. Lawray, Q. Tian, J.D. Masucci. (2009). Amended report for MSL0022130: Molecular analysis of soybean MON87705. Monsanto study report MSL0022384

H. Tu and A. Silvanovich. (2009). Bioinformatics evaluation of DNA sequences flanking the 5'and 3' junctions of the inserted DNA in MON87705: Assessment of putative polypeptides. Monsanto study report MSL 0021929

A. Silvanovich and H. Tu. (2009). Additional bioinformatic evaluation of DNA sequences flanking the 5' junction of the inserted DNA in MON87705: assessment of putative polypeptides. Monsanto study report MSL 0022346

H. Tu and A. Silvanovich. (2010). Updated bioinformatics evaluation of the DNA sequences flanking the insertion site in MON87705: BLASTn and BLASTx analyses. Monsanto laboratory project study number RAR-10-071

H. Tu and A. Silvanovich. (2010). Updated bioinformatics evaluation of the transfer DNA in MON87705 utilizing the AD_2010, TOX_2-10, and PRT_2010 databases. Monsanto laboratory project study number RAR-10-070

3.1 Method used in the genetic modification

Conventional soybean cultivar A3525 was transformed with the plasmid PV-GMPQ/HT4404 (see Figure 1), using standard *Agrobacterium*-mediated transformation methods (Martinell *et al.*, 2002). The vector consisted of two independent T-DNAs, each flanked by the Left and Right border sequences from *Agrobacterium*.
Meristem tissues were excised from the embryos of germinated A3525 seed, and co-cultured with *Agrobacterium tumefaciens*, carrying PV-GMPQ/HT4404 (see Figure 1). After co-culturing, the meristem tissues were placed on glyphosate-containing medium to select for transformed tissues. The selected tissues were then cultured on medium conducive to shoot and root development, and rooted plants with normal phenotypic characteristics were transferred to soil for further assessment.

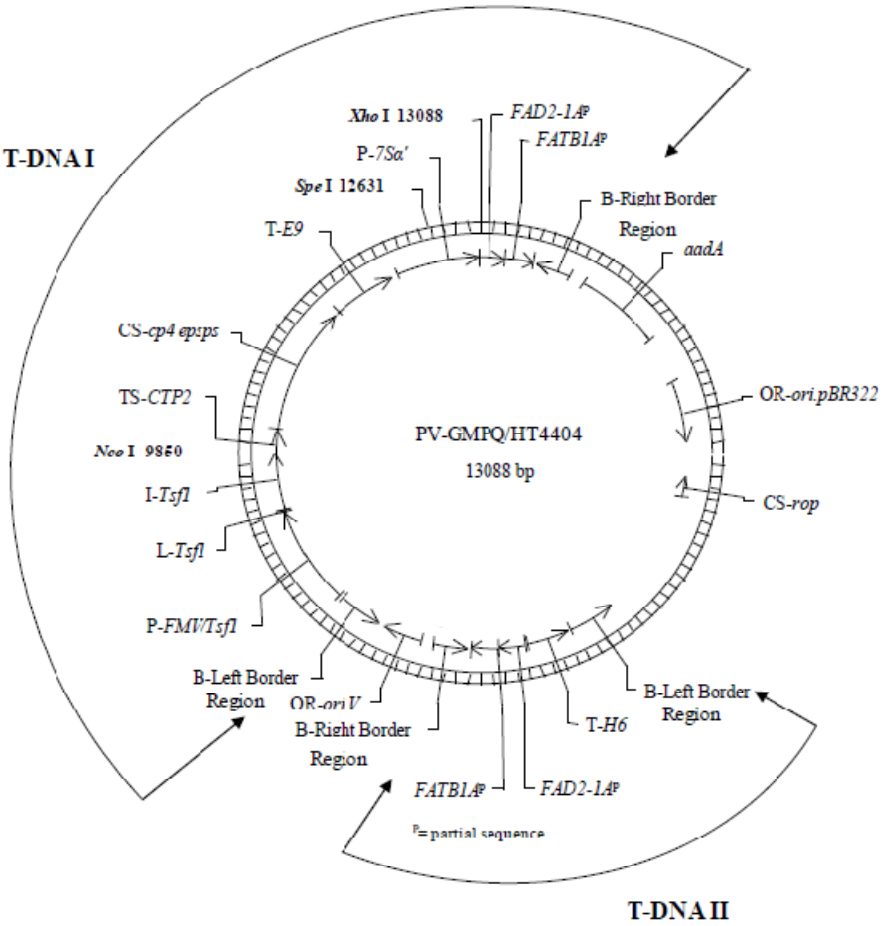

Figure 1: Map of plasmid PV-GMPQ/HT4404 showing two independent T-DNAs, labelled T-DNA I and T-DNA II (see Section 3.2).

These R0 plants were self-pollinated to create the R1 generation. R1 plants were screened for homozygosity of the inserted *cp4 epsps* gene. Only those homozygous plants that also produced the desired fatty acid composition were advanced for development and further characterisation. MON87705 was selected based on its desired phenotypic and agronomic characteristics, and molecular profile, as described in Figure 2.

3.2 Function and regulation of introduced gene sequences

Information on the genetic elements present in the transforming plasmid PV-GMPQ/HT4404 is provided in Table 1. The vector contained two independent T-DNAs, as described in the following sections. T-DNA I included a *cp4 epsps* expression cassette and a partial gene suppression cassette. T-DNA II contained another partial gene suppression cassette. The suppression cassette comprised the sequences and regulatory elements necessary for the RNA-based suppression of the endogenous *FAD2* and *FATB* genes.

The FATB enzymes, localised in plastids, are acyl-acyl carrier protein (ACP) thioesterases, which hydrolyse saturated fatty acids from the ACP-fatty acid moiety. In MON87705 soybean, suppression of *FATB* results in a decrease in transport of saturated fatty acids out of the plastid, thus increasing their availability for other reactions, specifically conversion to oleic acid via a desaturation reaction.

The FAD2 enzymes are delta-12 fatty acid desaturases, catalysing the conversion of oleic acid (C18:1) to linoleic acid (C18:2). Suppression of FAD2 in MON87705 soybean causes

reduced desaturation of oleic acid to linoleic acid, resulting in more oleic acid being available for transport from the endoplasmic reticulum to the oil body.

The combined effect of the RNA-based suppression of the two genes in MON87705 is that soybean oil contains (i) reduced levels of saturated fatty acids (C16:0 palmitic acid and C18:0 stearic acid), (ii) increased levels of monounsaturated oleic acid, and (iii) decreased levels of linoleic acid, compared with conventional soybean oil.

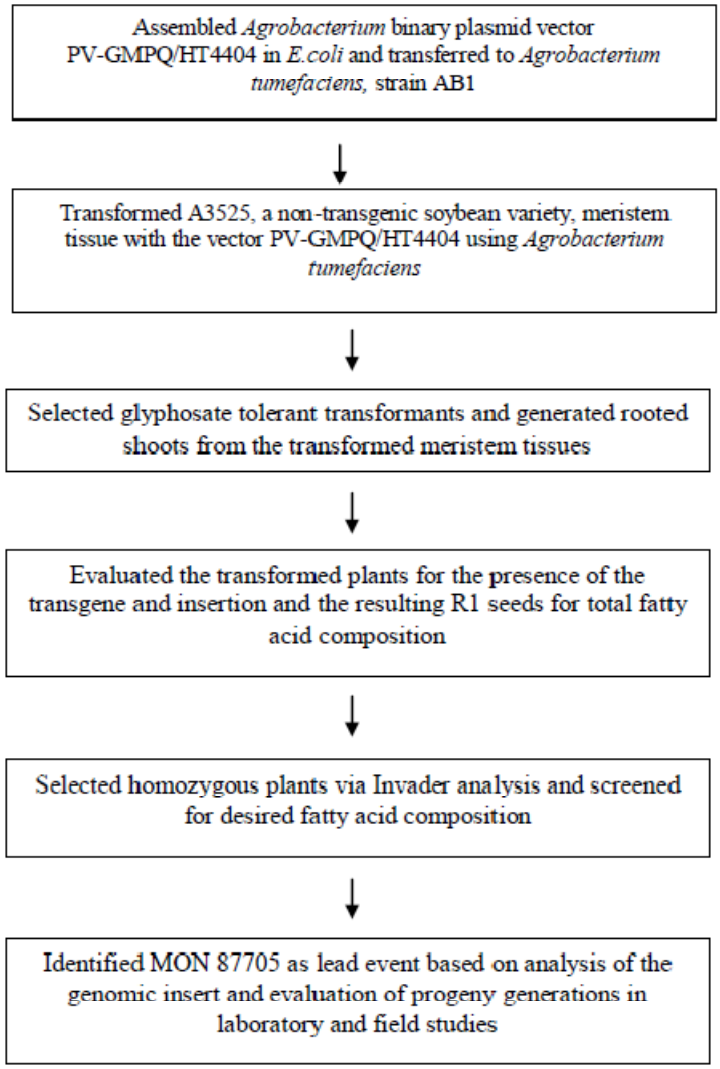

Figure 2: Summary of the development of soybean MON87705.

3.2.1 The FAD2-1A/FATB1-A suppression cassettes

The partial suppression sequences were derived from the *fad2-1a* and *fatb1-a* genes from soybean. T-DNA I contains sequences corresponding to the sense strand of a portion of the soybean *FAD2-1A* intron, and a portion of the *FATB1-A* 5' UTR (untranslated region), including the plastid targeting sequence.

Table 1: Description of the genetic elements contained in the vector PV-GMPQ/HT4404

Genetic element	Location in Plasmid	Source and Function (Reference)
T-DNA I		
Left Border	7657-8098	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of T-DNA (Barker *et al*., 1983)
Intervening sequence	8099-8134	Sequence used in DNA cloning
P-*FMV/Tsfl*	8135-9174	Chimeric promoter consisting of enhancer sequences from the promoter of the Figwort Mosaic Virus 35S RNA (Richins *et al*., 1987) combined with the promoter from the *Tsfl* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1 alpha (Axelos *et al*., 1989)
L-*Tsfl*	9175-9220	5' untranslated leader (exon 1) from the *Tsfl* gene of *Arabidopsis thaliana* - encodes elongation factor EF-1 alpha (Axelos *et al*., 1989)
I-*Tsfl*	9221-9842	Intron with flanking exon sequence from the *Tsfl* gene of of *Arabidopsis thaliana* that encodes elongation factor EF-1 alpha (Axelos *et al*., 1989)
Intervening sequence	9843-9851	Sequence used in DNA cloning
TS-*CTP2*	9852-10079	Targeting sequence from the *ShkG* gene encoding the transit peptide region of *Arabidopsis thaliana* EPSPS (Klee *et al*., 1987) that directs transport of CP4 EPSPS to the chloroplast.
CS-*cp4 epsps*	10080-11447	Codon modified coding sequence of th3e *aroA* gene from *Agrobacterium sp.* strain CP4, encoding the CP4 EPSPS protein (Barry *et al*., 1997; Padgette *et al*., 1996)
Intervening sequence	11448-11505	Sequence used in DNA cloning
T-*E9*	11506-12148	3' untranslated region of the pea *RbsS2* gene which functions to direct polyadenylation of the mRNA (Coruzzi *et al*., 1984)
Intervening sequence	12149-12236	Sequence used in DNA cloning
P-*7Sα'*	12237-13077	Promoter and leader from the *SphasI* gene of *Glycine max* encoding beta-conglycinin storage protein (alpha'-bcsp) (Doyle *et al*., 1986) that directs transcription in seed
Intervening sequence	13078-11	Sequence used in DNA cloning
***FAD2-1A*^{P}**	12-277	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti *et al*., 2003) which forms part of the suppression cassette
***FATB1-A*^{P}**	278-578	Partial sequence from the 5' untranslated region and the plastid targeting sequence from the *Glycine max FATB1-A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti *et al.,* 2003) which forms part of the suppression cassette.
Intervening sequence	579-616	Sequence used in DNA cloning
Right Border	617-973	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Zambryski *et al*., 1982)
T-DNA II		
Left Border	5128-5569	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al*., 1983)
T-*H6*	5668-6103	3'UTR sequence of the *H6* gene from *Gossypium barbadense* encoding a fibre protein involved in secondary cell wall assembly (John and Keller, 1995)
Intervening sequence	6104-6115	Sequence used in DNA cloning
***FAD2-1A*^{P}**	6116-6381	Partial sequence from intron #1 of the *Glycine max FAD2-1A* gene that encodes the delta-12 desaturase (Fillatti *et al.,* 2003) which forms part of the suppression cassette
***FATB1-A*^{P}**	6382-6682	Partial sequence from the 5' untranslated region and the plastid targeting sequence from the *Glycine max FATB1-A* gene that encodes the palmitoyl acyl carrier protein thioesterase (Fillatti *et al.,* 2003) which forms part of the suppression cassette.
Intervening sequence	6683-6693	Sequence used in DNA cloning
Right Border	6694-7024	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Zambryski *et al*., 1982)

T-DNA II contains the same partial gene segments, but the sequence encodes the corresponding antisense strand, followed by the *H6* 3' untranslated sequence to terminate transcription (John and Keller, 1995).

Homologous recombination between the border sequences of adjacent T-DNAs I and II, leads to the formation in the soybean genome of a single segment of inserted DNA corresponding to the intact *cp4epsps* expression cassette (see below), and a functional RNAi-mediated *fad2-1a/fatb1-a* suppression cassette. Transcription of the suppression cassette leads to production of RNA which forms a double-stranded hairpin structure due to the presence of the complementary partial gene sequences. This double-stranded RNA (dsRNA) structure specifically interferes with expression of the endogenous *fad2-1a* and *fatb1-a* genes via RNA interference mechanisms (Siomi and Siomi, 2009), thus reducing the amount of FAD2-1A and FATB1-A proteins produced in the cell (see Figure 3).

Transcription of these sequences is under the regulation of the seed specific promoter *7Sα'* from soybean itself, which drives expression in immature seeds (Doyle *et al*., 1986). The use of a tissue specific promoter limits the phenotype (modification to oil composition) to the seeds of the plant.

3.2.2 The cp4epsps expression cassette

MON87705 soybean contains a functional *cp4epsps* expression cassette comprising the *epsps* gene sequence derived from *Agrobacterium* sp. strain CP4. The *cp4epsps* gene is under the regulation of the FMV/*Tsf1* promoter, a chimeric promoter consisting of enhancer sequences from the 35S promoter of the Figwort Mosaic Virus (FMV; Richens *et al*., 1987) combined with the promoter from the *Tsf1* gene of *Arabidopsis thaliana*, which encodes the elongation factor EL-1 alpha (Axelos *et al*., 1989). The cassette also contains sequences corresponding to the 5' untranslated region of the *Tsf1* gene, and CTP2 (chloroplast transit peptide) from *Arabidopsis* which targets the CP4EPSPS protein to the chloroplast (see Table 1).

Expression of the CP4 EPSPS protein in MON87705 confers tolerance to glyphosate-containing herbicides. The CP4 EPSPS protein in MON87705 is the same as that expressed in several glyphosate-tolerant crops previously assessed and approved by FSANZ, such as soybean (Applications A338 and A592), corn (Application A416), canola (Application A363), sugarbeet (Applications A378 and A525) and cotton (Applications A355 and A553).

3.3 Characterisation of the genes in the plant

Evaluation of insert copy number, insert integrity and the presence or absence of plasmid backbone sequences was achieved using Southern blot analysis and DNA sequence analysis of plant material from the R3 generation (see Figure 4) of soybean MON87705. The Southern analysis used multiple probes (see

Table **2: Probes used in Southern hybridisation analyses.**
) corresponding to specific segments of the entire transforming plasmid PV-GMPQ/HT4404 to characterise the nature of the insert in MON87705. These experiments can determine whether the entire PV-GMPQ/HT4404 was incorporated into the genome, the T-DNAs are present at more than one insertion site, or whether incorporation of more than one copy of the T-DNAs within the one site has occurred. The parent line, A3525, was used as a control.

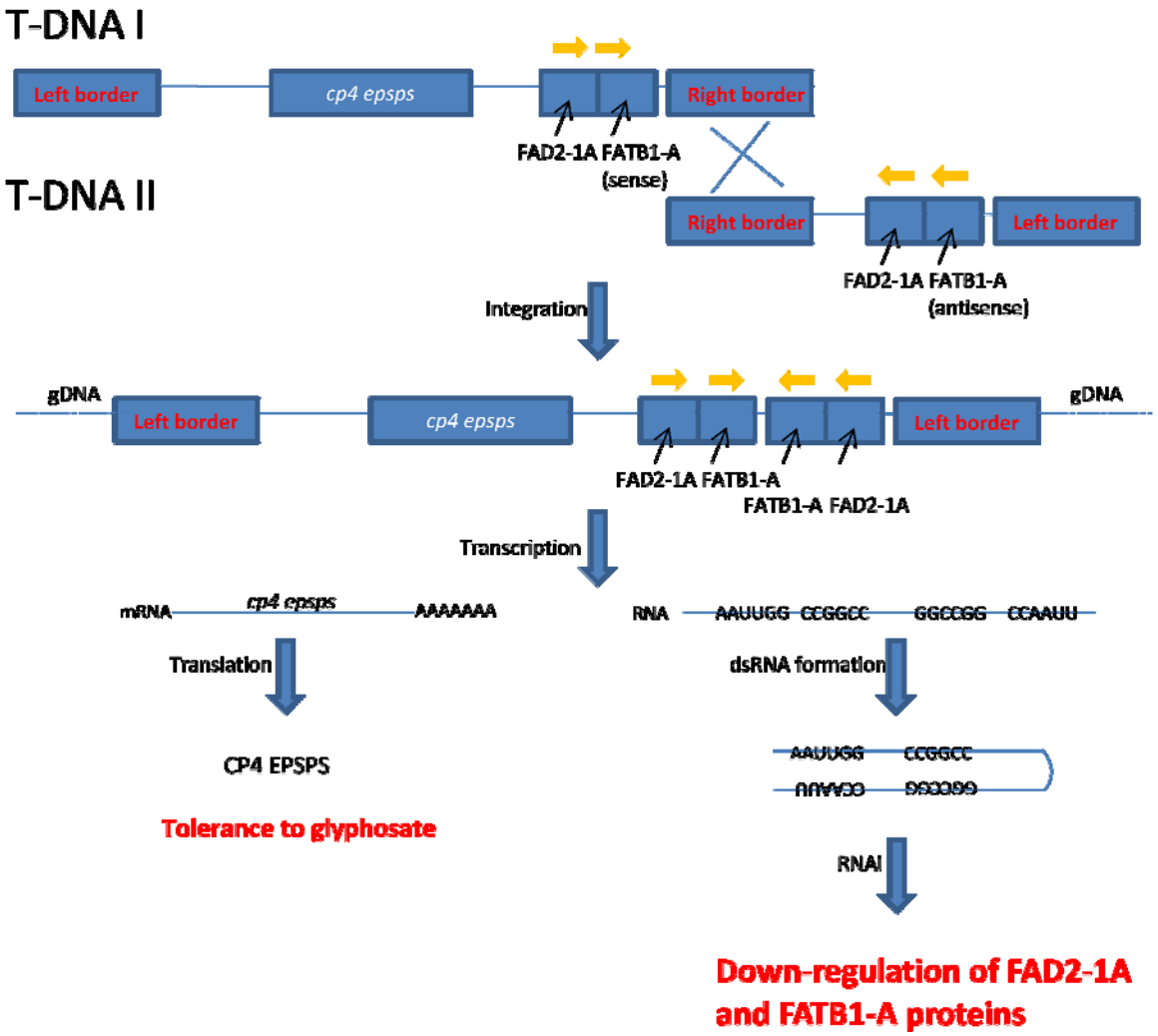

Figure 3: Schematic diagram showing the mechanism of gene suppression resulting from the co-integration of T-DNA I (containing the cp4 epsps cassette and the sense-strand partial suppression cassette) and T-DNA II (containing the antisense-strand partial suppression cassette) into the soybean genome (gDNA).

Table 2: Probes used in Southern hybridisation analyses.

Probe	DNA Probe	Element Sequence Spanned by DNA Probe
1	T-DNA I Probe 1A	Left Border + P-*FMV/Tsf1* + L-*Tsf1* + I-*Tsf1* (portion)
2	T-DNA I Probe 2B	I-*Tsf1* (portion) + TS-*CTP2* (portion)
3	T-DNA I Probe 3C	TS-*CTP2* (portion) + CS-*cp4 epsps* (portion)
4	T-DNA I Probe 4D	CS-*cp4 epsps* (portion) + T-*E9* + P-*7Sα'* + *FAD2-1A* (portion)
5	T-DNA I Probe 5E	*FAD2-1A* (portion) + *FATB1A* + Right Border
6	T-DNA II Probe 1A	T-*H6* + *FAD2-1A* + *FATB1A*
7	Backbone Probe 1	Backbone sequence
8	Backbone Probe 2	Backbone sequence
9	Backbone Probe 3	Backbone sequence
10	Backbone Probe 4	Backbone sequence

3.3.1 Analysis for transgene copy number and plasmid backbone

Genomic DNA was extracted from leaf tissue harvested from two seedlings of the R3 generation and digested with a suite of restriction endonucleases for use in these experiments. As indicated in Table 2, probes 1-6 corresponded to sequences within the two T-DNAs, whereas probes 7-10 corresponded to backbone sequences derived from the transforming plasmid. Using appropriate radiolabelled probes, the Southern blots demonstrated that MON87705 contains sequences derived from T-DNA I and T-DNA II integrated at a single insertion site in the genome.

None of the plasmid probes hybridised with DNA from either MON87705 or control (conventional) soybean, while control DNA spiked with a known quantity of the plasmid was readily detected. These data indicate that the plasmid backbone is not present in MON87705.

3.3.2 Antibiotic resistance marker genes

No bacterial marker genes encoding antibiotic resistance are present in soybean MON87705. Molecular data, including several Southern blots, demonstrated that plasmid backbone DNA was not integrated into the soybean genome during transformation, and the T-DNAs do not contain genes encoding antibiotic resistance.

3.3.3 Insertion site analysis

Sequencing of the insert and flanking soybean DNA confirms the organisation of the genetic elements within the inserted DNA, determines the 5' and 3' insert-to-plant junction regions and confirms that the sequences flanking the ends of the insert in MON87705 are native to the soybean genome. The precise organisation of the genetic elements comprising the insert in MON87705 was investigated by sequencing at least six overlapping PCR products and assembly of the data into a consensus sequence.

The inserted DNA in MON87705 is 7251 bp in length and matches the sequence of the T-DNA regions of the transforming plasmid PV-GMPQ/HT4404. The sequence analysis showed a truncation of 30 bp at the 3' end of the *FATB1-A* antisense segment. The small truncation apparently does not have an impact on the RNAi mechanism of reducing levels of *FATB1-A* transcription, resulting in the intended fatty acid phenotype.

Comparison with genomic sequence of the same region in the control soybean (A3525) verified that the 5' and 3' flanking regions adjacent to the combined T-DNA insertion site in MON87705 are native soybean sequence. However, the sequence comparison indicated a deletion of 36 bp and insertion of a 2374 bp segment, just 5' to the insertion site in MON87705. The additional 2374 bp (containing a single base pair change from wildtype) is duplicated genomic soybean sequence derived from the region 3' to the site of T-DNA insertion. These molecular rearrangements are most likely the result of DNA repair mechanisms in the plant during the *Agrobacterium*-mediated transformation process.

3.4 Open Reading Frame analysis

The nature of the process by which genetic material is introduced into an organism may result in unintended effects that could 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 or peptides. A bioinformatic analysis of any new ORFs created by the transformation can be used to determine whether any putative peptides have homology to known protein toxins or allergens.

In this analysis, an ORF was defined as any sequence between two translational 'stop' codons that would encode a peptide of at least eight amino acids.

The introduced T-DNA sequence as well as the 5' and 3' insert-to-plant junction regions were analysed in all six reading frames for the presence of ORFs, and the translated sequences were subsequently compared to sequence information in the following three databases, using the FASTA sequence comparison algorithm:

(i) AD_2010 database – contains allergen, gliadin and glutenin sequences and was assembled from sequences found on the FARRP allergen database. This database contains 1,471 sequences;
(ii) PRT_2010 database – represents all currently known publicly available protein sequences and consisted of GenBank release 175.0 (December 15 2009). It contains at least 17,815,538 sequences;
(iii) TOX_2010 database – assembled from the above PRT_2010 database. Protein sequences were selected using a keyword and filtered to remove likely non-toxin proteins. The TOX_2010 database contains 8,448 sequences.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences (ORFs) and protein sequences in the databases. The extent of any structural relatedness was evaluated by detailed visual inspection, the calculated percent identity and the *E*-score. In addition to structural similarity, each putative peptide was screened for short matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope.

3.4.1 ORF analysis of junction regions

Six possible ORFs of varying lengths were identified at both the 5' junction and 3' junction regions in MON87705. The results of the bioinformatic alignment searches demonstrated no structurally relevant similarity between the putative peptide sequences with known toxins, allergens or other biologically active proteins. Furthermore, no short (eight amino acids) matches were shared between any of the putative peptides and protein sequences in the allergen database.

3.4.2 ORF analysis of introduced coding regions

The results of the search comparisons showed no relevant structural similarity to known allergens or toxins for any of the putative peptides identified from the entire inserted DNA sequence in MON87705. As expected, the FASTA search of the PRT_2010 database showed significant alignment with patent sequences described as 5-enolpyruvylshikimte-3-phosphate synthase, or CP4 EPSPS. Alignment was also found with an acyl-ACP thioesterase, which would be expected due to the segments of the soybean FATB1A coding sequence on the forward and reverse complement strands. Based on the nature of the T-DNA insert, these two alignments reflect expected conserved sequence and do not indicate the potential for adverse biological activity.

3.5 Stability of genetic changes

The breeding steps involved in the development of MON87705 are summarised in Figure 3. The original transformant (R0 generation) was self-pollinated to produce R1. The process of self-pollination was used to produce the next three generations (R2, R3, R4) leading to the selection of MON87705 for commercial development.

This line exhibited the intended phenotype (changes in fatty acid composition) associated with a single, intact DNA insert. Plant material from the R3 generation was used for the detailed molecular characterisation studies described above. In addition, R5 seed was used for compositional analysis, and R5 plants were used for CP4 EPSPS protein expression

analyses in multiple tissues. Compositional analyses of processed soybean fractions were carried out on plant material from the R6 generation. Generations R5 and R6 were used for analysing the stability of the insert.
In order to determine if the inserted DNA (*cp4 epsps/fad2-1a/fatb1-a*) is stable across multiple generations, Southern blot fingerprint analysis was applied to four successive generations (R3 – R6) of MON87705. Genomic DNA from leaf tissue from each generation was examined using two radiolabelled probes (Table 2, probes one and six). This fingerprint strategy consisted of two border segments that assessed not only the stability of the inserted DNA, but also the stability of soybean genomic DNA directly adjacent to the insert. If the insert was unstable, this would be evident in the detection of additional bands, the loss of bands, or a change in the size of bands visualised on the blots. The banding pattern obtained in the fingerprint analysis was identical for all four generations, indicating that the inserted DNA in MON87705 is stable across multiple generations.

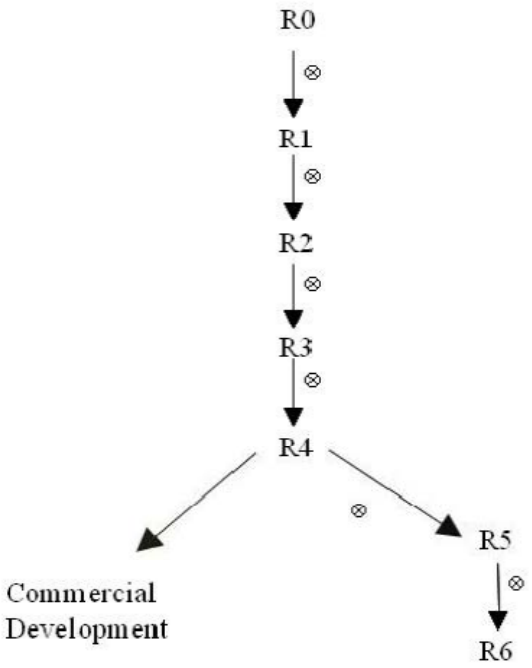

Figure 4: Breeding history of MON87705. All generations were self pollinated.

3.5.1 Segregation analysis

A segregation analysis was undertaken to test if the *cp4 epsps/fad2-1a/fatb1-a* locus segregates according to Mendelian principles. The R4 generation was crossed with a conventional soybean variety not containing the *H6 3' UTR* (see Figure 5). The F1 generation from this cross was self-fertilised four times, producing F2, F3, F4 and F5 generations. The R1-R4 and F1-F5 generations were examined to establish whether the *cp4 epsps/fad2-1a/fatb1-a* locus segregated in the expected ratios. For homozygous generations R1-R4, the expected ratio was 1:0, whereas the F1-F5 generations were expected to generate a ratio of 1:2:1 (homozygous positive: hemizygous positive: homozygous negative). To determine whether the observed segregation ratios deviated significantly from the expected segregation ratios, a Chi-square (χ^2) analysis was undertaken.

All plants in generations R1-R4 showed the expected segregation ratio. The observed ratios in the F2 generation did not differ significantly from the expected ratios. However, as some plants in the F3 generation were unable to be analysed (resulting in a small sample size), larger numbers of plants from the F4 and F5 generations were analysed. These generations also did not differ significantly from the expected ratios.

Taken together, the molecular data (PCR, Southern analysis) and the segregation data indicate that the novel DNA introduced into MON87705 is stable. No recombination was observed in multiple plants over multiple generations.

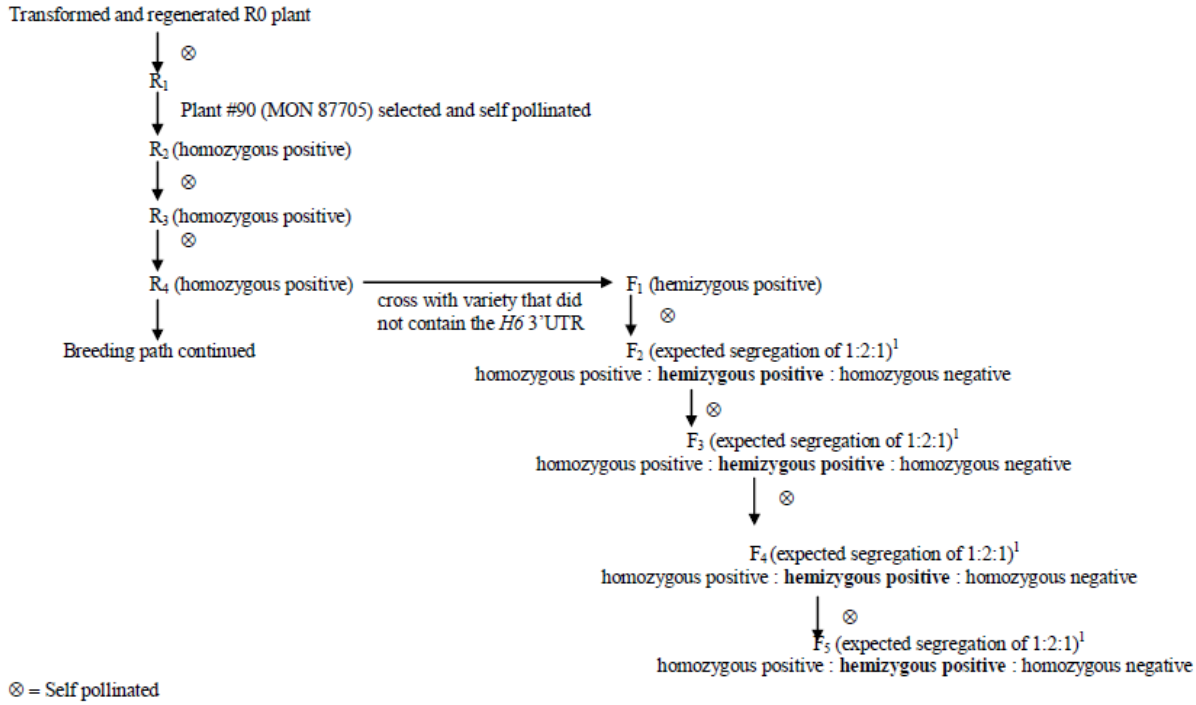

Figure 5: Breeding strategy for the generation of segregation data for MON87705 soybean

3.6 Conclusion about molecular characterisation

Soybean MON87705 contains one introduced coding sequence and two non-coding partial gene sequences with associated regulatory elements inserted at a single locus. The *cp4 epsps* gene is derived from *Agrobacterium sp.* strain CP4 and confers resistance to glyphosate. The partial gene sequences correspond to intron #1 from the *fad2-1a* gene and the 5' untranslated region of the *fatb1-a* gene, both from soybean. Transcription of these two sequences leads to RNA-mediated suppression of the corresponding endogenous soybean genes encoding the FAD2-1A and FATB1-A proteins, both involved with fatty acid metabolism. The consequence of this suppression is an increase in oleic acid and a decrease in the levels of linoleic acid and saturated fatty acids in soybean oil.

Comprehensive molecular analyses of soybean MON87705 indicate that T-DNA I and T-DNA II integrated into the soybean genome as a single functional genetic insert at one locus. Despite some molecular rearrangement at the site of insertion, bioinformatic analysis of putative ORFs at the junction regions, and the complete insert itself, showed no structural homology with known toxins or allergens. The stability of the introduced genetic elements over at least four generations was confirmed at the molecular level and by phenotype, and segregation data demonstrated a Mendelian pattern of inheritance, indicative of a single insertion site.

3.7 Mode of action and evidence of suppression of FATB and FAD2 genes

As outlined in Section 3.2, the altered fatty acid profile in MON87705 soybean oil is achieved through suppression of the levels of endogenous soybean enzymes *FATB* (acyl-acyl carrier

protein thioesterases) and *FAD2* (delta-12 desaturases) via an RNA-mediated gene suppression mechanism (see Figure 3). As the mode of action of gene suppression is mediated through RNA transcripts, no novel proteins are produced from the partial gene segments in each of the T-DNAs, which together form the functional suppression cassette.

3.7.1 Northern blot analysis of MON87705 soybean seed

Northern blot analysis was used to examine the RNA levels of the endogenous *FAD2-1A* and *FATB1-A* genes in MON87705 soybean, compared to that in the conventional control soybean (A3525). Messenger (poly A rich) RNA isolated from immature seed of MON87705 plants at the R5/6 stage of growth was examined with probes to detect *FAD2-1A* and *FATB1-A* transcripts.

The four replicates of the conventional soybean control (immature seeds) produced a strong hybridisation signal at approximately 1.5 kb, which is the expected size of the *FAD2-1A* transcript. In contrast, the four replicates of MON87705 immature seeds produced a very faint hybridisation signal at approximately 1.5 kb, indicating a greatly reduced level of RNA relative to the conventional control. A separate experiment to detect RNA corresponding to the *FATB1-A* gene in conventional soybean produced a strong signal at the expected size of approximately 1.8 kb, whereas the signal produced from the MON87705 samples was significantly reduced. The hybridisation signals for the internal positive control (actin transcript) from conventional soybean and MON87705 immature seeds showed similar intensities, indicating that the amounts of RNA loading, RNA quality and hybridisation were similar for both sets of samples in these experiments. These data confirm a reduction in the levels of detectable *FAD2-1A* and *FATB1-A* RNAs in MON87705 soybean compared to conventional soybean.

4 CHARACTERISATION OF NOVEL PROTEINS

In considering the safety of novel proteins, it is important to consider that human diets normally include a large and extremely diverse range of proteins that are typically consumed without causing any adverse effects. Only a small fraction of dietary proteins have the potential to impair health, for example because they are allergens or anti-nutrients (Delaney *et al*., 2008). As proteins perform a wide variety of biological functions, a safety assessment must include consideration of the physicochemical and biochemical properties of novel proteins, including potential toxic, anti-nutritional and allergenic effects.

To effectively identify any potential dietary hazard, knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the GM organism, as well as a detailed understanding of the protein's biochemical function and phenotypic effects is required. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

4.1 Description and function of the novel protein

The single novel protein produced in soybean line MON87705 is 5-enolpyruvylshikimate-3-phosphate synthase, which is derived from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). EPSPS is an essential enzyme involved in the biosynthesis of aromatic amino acids via the shikimate metabolic pathway. This metabolic pathway is present in all plants (including soybean), bacteria and fungi. As EPSPS proteins are found widely in nature, they are typically found in foods derived from plants and microbes.

Glyphosate, the active ingredient in the herbicide Roundup®, specifically binds to the EPSPS enzyme, thereby blocking normal protein synthesis in plant cells, ultimately leading

to the death of the plant. The CP4 EPSPS enzyme is however significantly less sensitive to glyphosate compared with the endogenous soybean EPSPS. Plants expressing CP4 EPSPS can therefore continue to function in the presence of the herbicide, conferring glyphosate tolerance to the whole plant.
Based on the *cp4 epsps* gene sequence in MON87705 soybean, the CP4 EPSPS protein consists of a single polypeptide of 455 amino acids with a calculated molecular weight of 47.6 kDa. The CP4 EPSPS protein expressed in MON87705 is identical to the CP4 EPSPS proteins in other glyphosate-tolerant crops assessed by FSANZ. Previous safety assessments of other glyphosate tolerant crops[1] already approved in the Code have established the safety of the CP4 EPSPS enzyme in terms of the diet, and the protein is now considered to have a history of safe human consumption. For the purposes of assessing the safety of MON87705 soybean, the weight of evidence should therefore be on establishing the equivalence of the CP4 EPSPS protein as expressed in MON87705 to reference CP4 EPSPS proteins previously determined to raise no safety concerns. In addition, the Applicant has updated a bioinformatic analysis of the CP4 EPSPS protein using the most recent protein databases. Previous studies examining potential toxicity and allergenicity have been cited as references.

4.2 Equivalence studies

Study submitted:
C. Wang, S.A. Kapaida, L.A. Burzio and E.A. Rice (2009). Characterization of the CP4 EPSPS protein purified from the seed of MON87705 and comparison of the physicochemical and functional properties of the plant-produced and *E. coli*-produced CP4 EPSPS proteins. Monsanto Study Report MSL0021863

As commonly occurs in GM crops, the amount of CP4 EPSPS protein that could be purified directly from MON87705 soybean seed was insufficient to carry out the numerous safety evaluations required for assessment. Instead, larger quantities of the CP4 EPSPS protein were produced in the laboratory using a bacterial expression system in *E. coli*. Using a small amount of the protein isolated from harvested MON87705 seed for comparison, studies were conducted to confirm its identity and demonstrate that the microbially-produced protein is equivalent to that produced in the GM soybean plants.

A panel of analytical techniques allowed characterisation of the proteins from the two sources, including: N-terminal sequence analysis, MALDI-TOF mass spectrometry, SDS-PAGE, Western blots, enzyme activity assays and glycosylation analysis.

4.2.1 Molecular weight and purity analysis

The molecular weight of the MON87705-produced and *E. coli*-derived CP4 EPSPS protein preparations was estimated on SDS-PAGE. The proteins co-migrated at an apparent molecular weight of approximately 43.8 kDa, which differs only slightly from the estimated molecular weight of 44.6 kDa (based on amino acid sequence).In addition, the purity of the two enzyme preparations was determined using a densitometer with analytical quantification software, and found to be equivalent.

4.2.2 Immunoreactivity

The identity and immunoreactivity of the CP4 EPSPS protein produced in MON87705 seed and in the bacterial expression system were investigated by Western blot analysis. The

[1] CP4EPSPS is expressed in glyphosate-tolerant soybean [40-3-2, MON89788], glyphosate-tolerant corn [NK603], glyphosate-tolerant canola [GT73], glyphosate-tolerant sugarbeet [H7-1], glyphosate-tolerant cotton [MON88913].

proteins from both sources were detected by goat anti-CP4 EPSPS antibody in a concentration dependent manner. The observed immunoreactivities of the plant- and microbially-produced proteins were similar based on densitometric analysis of the Western blots.

4.2.3 N-terminal sequence analysis

Amino-terminal sequencing of the first 10 amino acids of the plant-produced CP4 EPSPS protein showed an exact match with the expected sequence, except for the N-terminal methionine, which is routinely cleaved from nascent proteins by methionine aminopeptidase (Polevoda and Sherman, 2000).

4.2.4 MALDI-TOF analysis

Trypsinised MON87705-derived CP4 EPSPS protein was subjected to matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). Thirty unique peptide fragments were identified that corresponded to the expected masses of CP4 EPSPS trypsin-digested peptides. The identified peptides were used to assemble a coverage map corresponding to approximately 80% (362 of the 455 amino acids) of the total protein. This analysis further confirmed the identity of the CP4 EPSPS protein produced in MON87705 soybean.

4.2.5 Functional activity

The functional activity of both the MON87705- and *E.coli*-produced CP4 EPSPS proteins was measured using a phosphate release assay. The assay measures the release catalysed by the CP4 EPSPS enzyme of inorganic phosphate from the substrate phosphoenolpyruvate (PEP). In this assay, protein specific activity is expressed as units per milligram of protein (U/mg), where a unit is defined as one µmole of inorganic phosphate released from PEP per minute at 25ºC. The results indicated a specific activity of 4.10 U/mg for the MON87705 produced enzyme, and 4.38 U/mg for the bacterially-produced reference CP4 EPSPS enzyme. These results demonstrate that the CP4 EPSPS proteins from both sources have equivalent functional activities.

4.2.5 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; 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. In contrast, the non-virulent *E. coli* strains used in the laboratory for cloning and expression of novel proteins lack the necessary biochemical machinery for protein glycosylation.
To investigate whether CP4 EPSPS produced in MON87705 is glycosylated, the purified protein sample was analysed using a commercially available glycoprotein staining kit. The bacterially-produced CP4 EPSPS protein was used as the negative control in this test. As faint bands were observed for both the MON87705-derived and the *E.coli*-derived CP4 EPSPS, the results did not indicate glycosylation of the plant-produced protein. In addition, carbohydrate moieties attached to the plant-produced protein would be likely to alter the electrophoretic mobility compared with the mobility of the microbially-produced form. As this also was not observed, the evidence indicated that CP4 EPSPS is not glycosylated in MON87705 soybean. The mass spectronomy data, in which all identified peptide masses

matched the expected non-modified masses, further supported the conclusion that CP4 EPSPS expressed in MON87705 soybean is not glycosylated.

4.2.6 Conclusion

Multiple analyses confirmed the identity of the CP4 EPSPS produced in MON87705 soybean, and established the equivalence of the plant-produced protein to the bacterially-produced CP4 EPSPS reference protein. The CP4 EPSPS reference protein produced in *E. coli* is therefore a valid surrogate for use in further safety evaluations of the novel protein.

4.3 CP4 EPSPS protein expression analysis

Study submitted:
T. Geng and K. Niemeyer (2009). Assessment of CP4 EPSPS levels in leaf, seed, root and forage tissues from MON87705 soybean grown in 2007/2008 Chile field trials. Monsanto study report MSL0021832

The levels of CP4 EPSPS protein in a number of plant tissues were measured using a validated enzyme linked immunosorbent assay (ELISA). Various tissues of MON87705 were collected during the 2007/2008 growing season from five separate field sites in Chile, considered to be representative of typical soybean growing regions suitable for commercial production.

The conventional control (A3525) was grown at the same locations and at the same time as MON87705. Over-season leaf (OSL), root, forage and mature seed from MON87705 and control soybean were collected and analysed. Leaf samples (OSL 1-4) corresponded to four stages of growth respectively: V3-V4, V6-V8, V10-V12 and V14-V16. Forage and root samples were collected at approximately R6 stage. Seed was collected at the R8 stage.

The results indicate that CP4 EPSPS is expressed in all MON87705 tissues, with the highest levels found in the leaves of the plant (Table 3). The average amount of CP4 EPSPS protein in mature seeds of soybean MON87705 was 110 µg/g dry weight (dw), with a range of 40-210 µg/g. The mean % dw of total protein in seed harvested from MON87705 was 35.5% (353,000 µg/g). The CP4 EPSPS protein therefore represents 0.031% of total seed protein. As expected, the levels of the CP4 EPSPS protein in the conventional control soybean (A3525) were below the assay limit of detection (LOD) for all tissue types.

Table 3: Mean CP4 EPSPS Protein Levels in Plant Tissues from MON87705 Soybean grown in Chile Field Trials in 2007/2008.

Tissue Type[1]	CP4 EPSPS protein µg/g fw (SD)[2,4]	Range[3,4] (µg/g fw)	CP4 EPSPS protein µg/g dw (SD)[2,5]	Range (µg/g dw)[3,5]	LOQ/LOD (µg/g fw)[4]
OSL-1	36 (14)	16-65	200 (72)	84-340	0.57/0.26
OSL-2	110 (51)	60-230	530 (230)	290-1000	0.57/0.26
OSL-3	51 (21)	11-84	220 (94)	47-350	0.57/0.26
OSL-4	51 (21)	27-94	210 (92)	110-410	0.57/0.26
Forage	32 (5.3)	22-40	120 (24)	77-160	0.57/0.10
Root	24 (6.4)	14-34	77 (24)	41-120	0.57/0.11
Mature Seed	100 (39)	35-190	110 (44)	40-210	0.34/0.26

[1]The OSL-1, OSL-2, OSL-3, OSL-4 samples were collected approximately at V3 – V4, V6-8, V10-V12; and V14-V16 stages, respectively. The forage and root were collected approximately at R6 stage, and the mature seed was collected at R8 stage.
[2]The means and standard deviations were calculated for each tissue type across all sites (n=15 for all tissues, except OSL-2 where n=12 and OSL-3 where n=19).
[3]Minimum and maximum values were determined for each tissue type across all sites.
[4]Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fw) basis.
[5]Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a dry weight (dw) basis. The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factors obtained from moisture analysis data.

4.4 Bioinformatics review of CP4 EPSPS

Study submitted:
H. Tu and A. Silvanovich. (2010). Bioinformatics Evaluation of the CP4 EPSPS Protein Utilizing the AD_2010, TOX_2010, and PRT_2010 Databases. Monsanto study report MSL0022522

Bioinformatic analyses determine whether introduced proteins share any amino acid sequence similarity with known allergens or protein toxins. An *in silico* evaluation of the CP4 EPSPS protein sequence has been conducted previously on several occasions for the assessment of other glyphosate-tolerant crops. All earlier reports have concluded that the CP4 EPSPS protein was not similar to known allergens, toxins, or other biologically active proteins that may adversely affect human or animal health. However, as new protein sequences are added to publicly available databases on an ongoing basis, the Applicant has reviewed the bioinformatic evaluation of CP4 EPSPS using updated sequence information.

In order to determine if the CP4 EPSPS protein shares significant sequence similarity to new sequences contained in updated allergen, toxin or protein databases, the CP4 EPSPS protein sequence was used as a query for a FASTA and Sliding Window search of the AD_2010 database, and a FASTA search of the TOX_2010 and PRT_2010 databases. The revised allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP[2] (2010) and was used as provided. The AD_2010 database is published and contains 1,471 sequences.

[2] Food Allergy Research and Resource Program (FARRP, University of Nebraska, US)

The new toxin (TOX_2010) and protein (PRT_2010) sequence databases were assembled for the bioinformatic analysis. The PRT_2010 database contains 17,815,538 sequences and was reformatted from the GenBank protein database, release 175.0 (December 15, 2009), which was downloaded from NCBI. The toxin database is a subset of sequences derived from the PRT_2010 database. It contains 8,448 sequences selected by using a keyword search and filtered to remove likely non-toxin proteins. A complete description of the AD_2010, PRT_2010, and TOX_2010 databases can be found in Monsanto Technical Report MSL0022498 (Tu and Silvanovich, 2010).

Using CP4 EPSPS as the query sequence to search the AD_2010 database, no alignment met or exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2003). Furthermore, no eight contiguous amino acid identities were detected when the CP4 EPSPS protein sequence was compared to the AD_2010 sequence database. These results indicate that the CP4 EPSPS, when compared with more recent databases, fails to show any significant sequence homology with known allergens.

Similarly, the bioinformatic search of the TOX_2010 database, failed to show any meaningful alignment of the CP4 EPSPS with known toxins. As expected, a 100.00% identity match was found with CP4 EPSPS in the PRT_2010 database. These results confirm previous reports that there are no biologically relevant sequence similarities observed between the CP4 EPSPS protein and allergen, toxin, or other biologically active proteins.

4.5 Summary and conclusion from characterisation of novel protein

The one novel protein expressed in MON87705 soybean is CP4 EPSPS, an enzyme derived from the comMONsoil organism *Agrobacterium* sp. strain CP4. The CP4 EPSPS enzyme has a lower affinity for glyphosate, allowing protein synthesis to continue in the plant in the presence of the herbicide. Extensive studies confirmed the identity and physicochemical and functional properties of the CP4 EPSPS protein expressed in MON87705 soybean, and demonstrated that it is identical to previously assessed CP4 EPSPS proteins used to confer glyphosate-tolerance in other GM crops already approved in the Code. Expression of CP4 EPSPS is relatively low in MON87705 soybean seed, constituting around 0.03% of total protein.

A revised bioinformatic analysis of the CP4 EPSPS sequence using updated protein, allergen and toxin databases confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens. These data, in addition to the conclusions drawn from previous assessments and a limited history of safe use in other GM crops, now provide further evidence to show that the CP4 EPSPS protein is not toxic, nor likely to be allergenic 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 targeted. 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 can 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, 2001). 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 or processing, when ingested they can be associated with production of intestinal gas.

5.2 Study design

Study submitted:
D.R. Lundry, S.G. Riordan, K.D. Miller and R. Sorbet. (2010).Composition Analyses of Forage and Seed Collected from MON87705 Produced in Chile during the 2007-2008 Growing Season. Monsanto study report MSL0021756

The test (MON87705), control (A3525), and 19 commercial soybean varieties (conventional) were grown under typical agricultural conditions in Chile during the 2007/2008 season. Seed and forage were collected from the test, control plants and four reference varieties, grown at the same time in replicated plots at each of five separate field trial sites[3]. Forage was collected at the R6 plant growth stage, and soybean seed was harvested at physiological maturity.

Compositional analysis included proximates (ash, fat, moisture and protein), carbohydrates (by calculation), acid detergent fibre (ADF), neutral detergent fibre (NDF). Seed samples were further analysed for levels of amino acids (18), fatty acids (26, C8-C24), trypsin inhibitors, phytic acid, lectin, isoflavones (daidzein, glycitein and genistein), vitamin E (α-tocopherol), raffinose and stachyose.

The conventional reference varieties establish a range of natural variability for each of the nutrient analytes measured. Data from the commercial cultivars 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 grown concurrently to the test and control lines. The compositional data

[3] Due to the slower maturity of one reference variety, all replicates of this variety at one site were damaged by an early frost and were excluded from the study.

were analysed using a mixed model analysis of variance. The five replicated sites were analysed both individually and combined. This analysis included reproducibility across individual sites and the magnitude of differences.
For those comparisons in which the soybean MON87705 test result was statistically different from the control, the test mean was compared with the 99% tolerance interval derived from the commercial cultivars. A value that did not lie within the 99% tolerance interval, was also compared with values in the published literature.

5.3 Analyses of key components

The focus of this assessment is necessarily on the food uses of soybean, therefore only compositional data for harvested soybean seeds are presented.

5.3.1 Proximates and fibre

The proximates and fibre analysis of soybean MON87705 seed and the conventional control are shown in Table 2. In the combined site analysis, a statistically significant difference was observed in total fat content between soybean MON87705 (18.29%) and the parental control (19.33%, p-value <0.001). The mean value for soybean MON87705 was within the statistical tolerance intervals for commercial soybean cultivars (16.91-23.48%). No statistically significant differences were observed between soybean MON87705 and the control mean values for ADF, NDF, ash, carbohydrates, moisture or protein.

5.3.2 Amino acids

The levels of 18 amino acids were measured in seed harvested from MON87705 and the parental control. Since asparagine is converted to aspartic acid, and glutamine is converted to glutamic acid during analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine.

While there were statistically significant differences in some amino acids between MON87705 and the parental control, these occurred randomly at individual sites only. In the combined site analysis, although statistically significant differences between MON87705 and the control soybean were found for two amino acids (arginine and cystine), the absolute difference in each case was negligible (≤ 0.1%) (see Table 7). In addition, all values were within the relevant commercial tolerance intervals for conventional soybean varieties.

Table 2: Combined-site Results of Proximates and Fibre Analysis in Soybean Seed – Statistical Summary

Component (Units)[1]	MON87705 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Proximate (% DW, unless noted)						
Ash	6.06 (0.13) [5.46-6.54]	6.13 (0.13) [5.48 - 6.55]	-0.072 (0.081) [-0.56 - 0.26]	-0.30, 0.15	0.421	(5.64 - 6.82) [5.26, 7.17]
Carbohydrates	40.35 (0.86) [36.69-43.52]	39.93 (0.86) [37.46 - 42.96]	0.42 (0.37) [-1.08 - 2.00]	-0.59, 1.43	0.317	(32.79 - 42.29) [30.78, 45.86]
Moisture (%FW)	10.76 (0.37) [8.96-12.30]	11.56 (0.37) [10.20 - 12.70]	-0.80 (0.44) [-3.40 - 0.90]	-2.02, 0.42	0.141	(6.89 - 12.50) [5.51, 13.37]
Protein	35.32 (0.99) [31.48-38.59]	34.66 (0.99) [30.71 - 37.40]	0.66 (0.36) [-0.94 - 3.08]	-0.33, 1.65	0.141	(29.51 - 40.25) [26.12, 43.51]
Total Fat	18.29 (0.39) [16.55-19.50]	19.33 (0.39) [17.63 - 20.32]	-1.04 (0.16) [-2.16 - -0.20]	-1.39, -0.69	**<0.001**	(16.91 - 23.48) [15.35, 25.95]
Fibre (%DW)						
Acid detergent fibre	17.14 (0.54) [15.71 - 19.31]	16.14 (0.54) [13.36 - 18.02]	1.00 (0.74) [-1.84 - 4.03]	-1.06, 3.05	0.249	(12.46 - 21.25) [12.71, 19.29]
Neutral detergent fibre	18.44 (0.85) [13.41 - 22.18]	17.83 (0.86) [14.61 - 21.09]	0.60 (1.03) [-4.24 - 4.33]	-2.25, 3.46	0.590	(12.25 - 20.89) [12.07, 21.51]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.
Statistically significant differences between MON87705 and the control are in **bold** (P<0.05).

Table 3: Amino acids in MON87705 and Control Soybean Seed: Combined-site analysis

Amino Acid (%DW)[1]	MON87705 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[2]	Commercial Range [99% Tolerance Interval[3]]
Alanine	1.59 (0.033) [1.49 - 1.70]	1.57 (0.033) [1.43 - 1.66]	0.243	1.34 - 1.78 [1.25, 1.92]
Arginine	2.78 (0.11) [2.43 - 3.16]	2.68 (0.11) [2.31 - 2.99]	**0.048**	2.15 - 3.23 [1.81, 3.62]
Aspartic acid	4.08 (0.13) [3.67 - 4.49]	4.00 (0.13) [3.51 - 4.43]	0.279	3.37 - 4.76 [3.02, 5.11]
Cystine	0.61 (0.0075) [0.57 - 0.64]	0.59 (0.0076) [0.55 - 0.63]	**0.043**	0.53 - 0.64 [0.49, 0.69]
Glutamic acid	6.46 (0.24) [5.72 - 7.19]	6.32 (0.24) [5.42 - 7.09	0.300	5.14 - 7.73 [4.42, 8.48]
Glycine	1.59 (0.039) [1.47 - 1.74]	1.56 (0.039) [1.41 - 1.67]	0.265	1.30 - 1.79 [1.19, 1.95]
Histidine	0.96 (0.023) [0.88 - 1.04]	0.94 (0.023) [0.84 - 1.01]	0.065	0.79 - 1.07 [0.74, 1.16]
Isoleucine	1.71 (0.050) [1.56 - 1.88]	1.67 (0.051) [1.45 - 1.86]	0.344	1.37 - 2.00 [1.23, 2.15]
Leucine	2.73 (0.083) [2.47 - 3.01]	2.69 (0.083) [2.37 - 2.94]	0.325	2.26 - 3.14 [2.06, 3.41]
Lysine	2.39 (0.053) [2.19 - 2.55]	2.33 (0.053) [2.10 - 2.51]	0.080	2.00 - 2.63 [1.87, 2.81]
Methionine	0.55 (0.0088) [0.51 - 0.58]	0.53 (0.0089) [0.49 - 0.57]	0.141	0.46 - 0.59 [0.43, 0.63]
Phenylalanine	1.82 (0.056) [1.64 - 2.00]	1.80 (0.056) [1.58 - 1.99]	0.523	1.50 - 2.11 [1.35, 2.31]
Proline	1.77 (0.057) [1.59 - 1.95]	1.72 (0.057) [1.52 - 1.90]	0.145	1.43 - 2.03 [1.29, 2.21]
Serine	1.75 (0.059) [1.49 - 1.98]	1.77 (0.059) [1.45 - 1.94]	0.655	1.55 - 2.05 [1.44, 2.15]
Threonine	1.33 (0.030) [1.20 - 1.45]	1.33 (0.030) [1.18 - 1.47]	0.867	1.19 - 1.48 [1.12, 1.53]
Tryptophan	0.42 (0.011) [0.37 - 0.46]	0.41 (0.012) [0.35 - 0.44]	0.831	0.33 - 0.48 [0.30, 0.50]
Tyrosine	1.25 (0.029) [1.17 - 1.33]	1.22 (0.029) [1.10 - 1.32]	0.124	1.07 - 1.39 [0.99, 1.49]
Valine	1.83 (0.052) [1.69 - 2.02]	1.77 (0.053) [1.55 - 1.96]	0.260	1.45 - 2.13 [1.31, 2.29]

[1]DW = dry weight
[2]Probability values <0.05 are statistically significant
[3]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties.

5.3.3 Fatty acids

The levels of 26 fatty acids (C8-C24) in soybean MON87705 and the parental control seed were analysed. The results of these analyses are presented in Table 8; no data are presented for 17 fatty acids that were below the limit of quantitation. Of those that were measurable, seven fatty acids are statistically significantly different in MON87705 seed, compared with the non-transgenic control, as shown in Table 8.

As a direct result of the down-regulation of the FAD2 and FATB proteins, oleic acid levels have increased over three-fold from a mean of 23% in conventional soybean to

approximately 76% (% weight of fatty acids) in MON87705 soybean seeds. In addition, the mean levels of linoleic acid (C18:2) have decreased from approximately 50% in control soybean, to 10% in MON87705 soybean. The mean levels of two saturated fatty acids (palmitic and stearic acids) are also significantly reduced in MON87705 compared to the non-transgenic control. In particular, palmitic acid (C16:0) levels decreased from a mean of 11% to approximately 2% in MON87705. As expected, the changes in the mean levels of oleic, linoleic and palmitic acids are outside the tolerance interval for conventional soybean.

Differences in stearic acid (C18:0), linolenic acid (C18:3) and arachidic acid (C20:0) were all statistically significant, however the mean levels of these fatty acids in MON87705 were within the 99% tolerance interval for commercial soybean varieties. Although there was also a significantly higher mean level of eicosenoic acid (C20:1) in MON87705 (0.34%) compared with the control (0.19% in A3525), the overall amounts are low (<1% of fatty acids), and this was not a consistent finding across all sites. Moreover, the level of eicosenoic acid in MON87705 (combined site analysis) is within historical ranges in the published literature.

Significance of compositional differences

The fatty acid composition of conventional soybean oil is known to vary widely, depending on agricultural and environmental factors and the geographical location in which the soybean crop is grown (Gunstone *et al*.). Therefore, consumption of traditional varieties of soybean with very different compositions from one another has occurred over time without safety or nutritional concerns.

Existing high oleic acid soybean varieties, such as DP-305423-1, have previously undergone safety assessments, including consideration of compositional changes brought about by genetic modification. The levels of certain fatty acids, although different from conventional soybean, are typical of other commonly consumed vegetable oils, such as olive and canola oils. Conservative dietary modelling scenarios for soybean oils have previously shown that variable fatty acid profiles would have minimal nutritional impact on the whole diet (FSANZ Application A1018 – Supporting Document 1). Overall, the compositional changes introduced into MON87705 soybean are similar to those occurring in other approved GM soybean lines, and in conventionally produced vegetable oils from other sources, and do not raise food safety concerns.

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 MON87705, control A3525 and commercial soybean varieties (OECD, 2001). The results of the combined site analysis are presented in Table 9, as aglycon equivalents.

The mean dry weight concentrations of genistein, daidzein and glycitein in seed of MON87705 soybean were not significantly different to the levels in the conventional control (A3525). None of the isoflavones showed a significant difference in any single site or in more than one site. In addition, the mean levels of daidzein, genistein and glycitein in MON87705 soybean are within the 99% tolerance intervals established for conventional soybean varieties grown at the same test sites as MON87705 and the near isogenic control.

Table 4: Summary of Fatty Acids in MON87705 and Control (A3525) Soybean Seed - Combined-site analysis

Fatty Acid (% DW)[1]*	MON87705 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value[3]	
16:0 Palmitic Acid	2.36 (0.056) [2.25 - 2.44]	10.83 (0.056) [10.51 - 11.08]	-8.47 (0.055) [-8.68 - -8.13]	-8.62, -8.31	**<0.001**	(8.78 - 11.51) [7.62, 12.55]
18:0 Stearic Acid	3.31 (0.067) [3.07 - 3.82]	4.50 (0.067) [4.24 - 4.85]	-1.19 (0.065) [-1.47 - -0.79]	-1.37, -1.01	**<0.001**	(3.82 - 7.21) [2.87, 7.15]
18:1 Oleic Acid	76.47 (0.59) [73.13 - 79.17]	22.81 (0.59) [21.41 - 25.08]	53.65 (0.22) [51.71 - 55.05]	53.17, 54.13	**<0.001**	(20.77 - 27.19) [18.40, 30.22]
18:2 Linoleic Acid	10.10 (0.39) [7.85 - 12.42]	52.86 (0.39) [51.68 - 53.89]	-42.77 (0.18) [-43.74 - -41.38]	-43.17, -42.37	**<0.001**	(48.62 - 54.74) [47.75, 56.46]
18:3 Linolenic Acid	6.69 (0.28) [5.55 - 7.81]	8.02 (0.28) [6.86 - 8.60]	-1.33 (0.072) [-1.80 - -0.59]	-1.53, -1.13	**<0.001**	(5.89 - 9.11) [4.97, 9.93]
20:0 Arachidic Acid	0.30 (0.0076) [0.28 - 0.36]	0.34 (0.0077) [0.31 - 0.36]	-0.039 (0.0071) [-0.082 - -0.022]	-0.059, -0.019	**0.005**	(0.28 - 0.54) [0.22, 0.53]
20:1 Eicosenoic Acid	0.34 (0.013) [0.27 - 0.40]	0.19 (0.013) [0.15 - 0.21]	0.15 (0.0086) [0.12 - 0.20]	0.13, 0.18	**<0.001**	(0.15 - 0.22) [0.13, 0.25]
22:0 Behenic Acid	0.29 (0.0037) [0.28 - 0.33]	0.30 (0.0038) [0.28 - 0.31]	-0.0052 (0.0051) [-0.029 - 0.020]	-0.017, 0.0070	0.346	(0.29 - 0.46) [0.22, 0.47]
24:0 Lignoceric Acid	0.14 (0.017) [0.066 - 0.17]	0.13 (0.017) [0.067 - 0.16]	0.0046 (0.0046) [-0.019 - 0.027]	-0.0084, 0.018	0.372	(0.056 - 0.21) [0.030, 0.26]

[1]DW = dry weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.
[3]Probability values <0.05 are statistically significant.
*Changes in fatty acids listed above the double line were intended as a result of the genetic modification.

Table 5: Isoflavone content of Soybean Seed – Combined Site Analysis

Analyte (µg/g DW)[1]	MON87705 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[3]	Commercial (Range) [99% Tolerance Interval][2]
Daidzein	1806.33 (229.35) [1145.72 - 2565.56]	1794.07 (229.50) [1092.43 - 2565.86]	0.748	(320.54 - 3061.22) [0, 3328.03]
Genistein	1160.30 (115.82) [809.79 - 1527.94]	1117.27 (115.95) [751.67 - 1466.21]	0.107	(433.41 - 2301.59) [0, 2727.33]
Glycitein	132.85 (12.38) [49.11 - 196.59]	126.86 (12.53) [72.10 - 167.04]	0.648	(21.67 - 354.30 [0, 376.03]

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

5.3.5 Anti-nutrients

Five key anti-nutrients were measured in seed from soybean MON87705 and the conventional control (A3525), and the results are presented in Table 10. There were no significant differences in the levels of anti-nutrients in MON87705 soybean when compared with the parental control in the combined-site analysis. At one site only, the levels of stachyose were approximately 4% higher in MON87705 than in the control, however there were no statistically significant differences found at any other sites for this analyte, nor in the combined-site analysis. Even for this one measurement, all values were within the tolerance interval for commercial soybean.

Table 6: Anti-nutrient content of Soybean Seed – Combined Site Analysis

Analyte (%DW, unless noted)[1]	MON87705 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value[3]	Commercial (Range) [99% Tolerance Interval²]
Lectin (H.U./mg DW)	2.21 (0.40) [0.72 - 3.77]	2.45 (0.41) [0.61 - 5.53]	0.686	(0.65 - 8.10) [0, 6.44]
Phytic Acid	1.82 (0.052) [1.52 - 2.13]	1.85 (0.053) [1.63 - 2.19]	0.457	(1.42 - 2.27) [1.35, 2.35]
Raffinose	0.58 (0.029) [0.48 - 0.71]	0.58 (0.029) [0.50 - 0.70]	0.981	(0.40 - 0.80) [0.27, 0.87]
Stachyose	3.87 (0.13) [3.39 - 4.48]	3.70 (0.13) [3.04 - 4.43]	0.290	(2.30 - 4.53) [1.96, 4.41]
Trypsin Inhibitor (TIU/mg DW)	38.14 (2.60) [26.73 - 52.01]	37.25 (2.61) [27.23 - 49.78]	0.408	(23.11 - 60.42) [8.75, 63.43]

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

5.3.6 Vitamins

Vitamin analysis was done only for Vitamin E (α-tocopherol). The results of the combined-site analysis are presented below, expressed as mg/100g dry weight (DW) of seed. The results indicate no differences between MON87705 and the control line in the level of vitamin E, and all values were within the commercial tolerance interval.

	MON87705 Mean (S.E.) [Range]	Control A3525 Mean (S.E.) [Range]	p-Value	Commercial (Range) [99% Tolerance Interval]
Vitamin E (mg/100g DW)	2.83 (0.43) [1.23 - 4.36]	3.27 (0.43) [1.69 - 4.19]	0.062	(1.09 - 5.10) [0, 7.36]

5.4 Composition of processed soybean fractions

Study submitted:
D.R. Lundry, S.G. Riordan, K.D. Miller and R. Sorbet. (2010).Amended Report for MSL0022167: Composition Analyses of Soybean Seed, Meal, Oil, Protein Isolate, and Lecithin Derived from MON87705 Produced in the United States during the 2007 Field Season. Monsanto report MSL0022708.

As the genetic modification results in intended changes to the fatty acid constituents of soybean oil derived from MON87705, the composition of processed soybean fractions derived from MON87705, the control line A3525, and 12 commercial non-GM soybean lines was analysed in a separate study. Seed from MON87705 and A3525 was harvested from plants grown at two field trial sites in the United States. The conventional reference soybean varieties were grown at three US field sites, to establish a range of natural variability for each analyte across different agricultural locations. The seed samples were processed according to typical industry standards into: toasted defatted (TD) soybean meal; refined, bleached and deodorised soybean oil (RBD oil); protein isolate; and crude lecithin fractions.

The TD soybean meal was analysed for proximates (moisture, protein, fat ash and carbohydrates by calculation), ADF, NDF, amino acids, trypsin inhibitors and phytic acid. The RBD oil was analysed for fatty acids and vitamin E (α-tocopherol). The protein isolate was analysed for amino acids and moisture. The crude lecithin fraction was analysed for phosphatides (α-phosphatidic acid, α-phosphatidylcholine, α-phosphatidylethanolamine and α-phosphatidylinositol).

The results of the compositional analysis of RBD oil from MON87705 largely mirror those obtained for the whole soybean seed. Oleic acid constitutes over 70% of total fatty acids (% total FA), linoleic acid is reduced to approximately 14% of total fatty acids, and palmitic acid is reduced from around 12% in the conventional control to approximately 2% in MON87705 soybean. Significant differences were found in other fatty acids measured in RBD oil, however in each case the value was within the tolerance interval for commercial soybean, or absolute levels were below 0.5% of total fatty acids, representative of less abundant, minor constituents. By way of example, similar small differences in arachidic, eicosenoic and behenic acids were detected in both seed and RBD oil from MON87705 compared with control, however the absolute levels of these fatty acids range between only 0.2% and 0.3% of total fatty acids.

Comparison of the composition of TD soybean meal prepared from MON87705 and the control soybean showed statistically significant differences in several amino acids and NDF. The magnitude of the differences in amino acids was very small (<0.1% dw) and all levels found in MON87705 were within the 99% tolerance interval for conventional soybean varieties, and also within the range of published values for conventional soybean. The mean

difference in NDF between MON87705 and the control was only 1.7% (dw) which is not of any biological or nutritional significance. TD soybean meal from MON87705 contains a small amount of fat or oil (0.78% dw), which was detected in the control at similar levels (0.86% dw).

There were no statistically significant differences between MON87705 and the conventional control for components measured in the protein isolate fraction and in the phosphatide composition of crude lecithin.

5.5 Allergenicity assessment

As outlined in previous assessments, 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 MON87705 soybean was conducted to determine whether the introduction of the *cp4 epsps* gene and suppression cassettes had any unintended effect on allergenicity, relative to conventional non-GM soybean.

Study submitted:
McClain, S., Finnessy, J., Meng, C. and Bannon, G. (2009). Quantitative ELISA Assessment of Human IgE Binding to MON87705, Control, and Reference Soybean Using Sera from Soybean-Allergic Subjects. Monsanto Study Report MSL0022044.

This *in vitro* study determined the level of IgE binding of serum obtained from clinically documented soybean allergic patients, to protein extracts prepared from MON87705 soybean seed and the conventional control A3525, using a validated ELISA method. The identity of the seed used to prepare the extracts was confirmed by event-specific PCR. Protein extracts from 17 commercial soybean varieties were also tested to establish a reference range of IgE binding. The reference varieties are commercially available lines and include high protein, high oil, and food-grade (tofu) soybean varieties that are already on the market as food for human consumption.
Sera were obtained from 13 clinically documented, soybean-allergic, and five non-allergic subjects. The soybean-allergic subjects had a documented case history of soybean allergy with anaphylaxis and a positive Double-Blind, Placebo-Controlled Food Challenge (DBPCFC) response. Each soybean extract was tested in triplicate. The level of IgE binding provided an estimate of the amount of endogenous soybean allergens present in the seeds. The results were compared with a soybean-specific IgE standard curve. The IgE binding values obtained for the 17 reference soybean extracts were used to calculate a 99% tolerance interval for each serum sample.

All of the IgE binding values to MON87705 and A3525 control extracts were within the reference tolerance limits for individual serum samples. As anticipated, no binding was seen with any of the non-allergic control sera. These results indicate that MON87705 soybean and its parental control are similar to conventional soybean varieties already on the market in terms of soybean-specific IgE binding properties.

5.6 Conclusion from compositional studies

Detailed compositional analyses were conducted to establish the nutritional adequacy of seed from soybean MON87705 and to characterise the intended as well as any unintended compositional changes. Analyses were done of proximates (ash, carbohydrates, moisture, protein and fat), fibre, amino acids, fatty acids, vitamin E, anti-nutrients and isoflavones. The levels were compared to levels in the non-GM parent A3525, and to those in 19 non-GM

commercial soybean cultivars. The genetic modification has resulted in a significant increase in oleic acid levels from around 25% in conventional soybean to approximately 76% in MON87705 soybean oil. At the same time, the levels of linoleic acid are decreased from around 50% to approximately 10% in MON87705 soybean. The suppression of the endogenous genes also results in a modest decrease in the levels of saturated fatty acids, palmitic and stearic acids. The altered fatty acid profile in MON87705 soybean oil is nevertheless within a broad natural range of variability that is typical of edible vegetable oils. There are no other compositional changes in MON87705 soybean of any biological or nutritional significance.

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 to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (FSANZ, 2007; OECD, 2003). If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment can 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. This assessment should include consideration of the bioavailability of the modified nutrient.

Soybean MON87705 exhibits a significantly altered fatty acid profile, resulting in a greater nutritional impact on the oil rather than on other processed fractions which are largely unchanged from those obtained from conventional soybean. Although the composition of the oil has changed, all components of MON87705 soybean oil are typical constituents of the human diet.

6.1 Dietary intake estimates

MON87705 soybean oil has a polyunsaturated fatty acid content that is not optimal for commercial frying, that is, repeated frying applications at high temperature. Non-frying liquid vegetable oils are largely used to manufacture margarine, and used as bottled oils for domestic use. The Applicant commissioned two dietary intake studies to determine the potential dietary effects of substituting MON87705 soybean oil for conventional soybean oil in a Western diet.

Studies submitted:
Lemke, S. and Tran, N. (2009).Estimated Daily Intake of Fatty Acids and the Effect of Substituting MON87705 Soybean Oil for Liquid Soybean Oil in the UK Adult Population. A report prepared by Exponent (Center for Chemical Regulation and Food Safety) for The Monsanto Company, St Louis, MO. Monsanto study report MSL0022302.
Wang, C. and Petersen, B. (2009). Estimated Daily Intake of Fatty Acids and the Impact of Substituting MON87705 for Liquid Soybean Oil in Four Food Categories. A report prepared by Exponent (Center for Chemical Regulation and Food Safety) for The Monsanto Company, St Louis, MO. Monsanto study report MSL0022140

United Kingdom

Dietary intake was estimated for the adult population (in the United Kingdom), for a scenario in which MON87705 soybean oil was a 100% substitute for the liquid soybean oil component of four major food categories in the UK: margarine, salad dressing, mayonnaise and spread, and soybean oil for home use. The modelling was based on food consumption data from the UK National Diet and Nutrition Survey (NDNS).
The results of this survey represent theoretical maximum changes in the intake estimates of the five fatty acids (listed in Table 11), and, because of the conservative nature of the assumptions, is a large overestimate of likely actual consumption. This analysis indicates only a modest potential impact on the diet. The small changes that were predicted were generally in line with current health recommendations (i.e. reduction in saturated fat intake and increases (as proportion of total fat) in mono- and poly-unsaturated fats).

Table 11: Mean fatty acid intakes (g/person/day) estimated for soybean oil in the UK adult population

Fatty acid	Conventional soybean oil (g/day)	MON87705 soybean oil (g/day)	Net Effect (g)
Palmitic acid	0.2	0.1	-0.1
Stearic acid	0.1	0.1	no change
Oleic acid	0.5	1.7	+1.2
Linoleic acid	1.2	0.2	-1
Linolenic acid	0.4	0.3	-0.1

Vegetable oils, including traditional non-hydrogenated soybean oil and MON87705 oil are not significant sources of *trans* fats. Therefore, replacement of liquid soybean oil with MON87705 soybean oil is not expected to have any effect on the current dietary intake of *trans* fats.

United States

A similar exposure estimate was conducted for the US population using food intake and nutrient composition data from NHANES 2003-2004 and 2005-2006 [National Health and Nutrition Examination Survey Data, Centres for Disease Control and Prevention (CDC) 2007].

The results were very similar to those found in the UK study. That is, the effects on dietary intake of different fatty acids were minimal, especially when taken as a percentage of daily energy intakes. Any estimated changes would lead to increases in monounsaturated fatty acid intake, which is in line with current dietary advice.

6.2 Feeding studies

The Applicant conducted two feeding studies in animals to support the safety and nutritional adequacy of food derived from MON87705 soybean. The studies – a 90 day in rats and a 42-day broiler study – used processed soybean meal produced from MON87705 and the conventional control. No feeding studies using soybean oil were provided.

Study submitted:
Kirkpatrick, J.B. (2010). A 90-day Feeding Study in Rats with Processed Meal from MON87705 Soybeans. **Performing Laboratories**: WIL Research Laboratories, LLC, OH; TestDiet, IN; Covance Laboratories Inc., WI; Midwest ToxPath Sciences, Inc, MO; Monsanto Company, MO. Sponsor study number WI-08-291

A 90-day toxicology assessment was conducted in rats (Crl:CD®[SD]) to evaluate the potential health effects of processed soybean meal from MON87705 as compared to

processed meal from A3525, the parental conventional soybean variety, and three reference soybean varieties.

All animals in the study survived to the scheduled necropsy. There were no clinical observations related to the MON87705 diet. There were no statistically significant differences in body weights, cumulative body weight gains, or food consumption between groups. There were no test diet-related alterations in haematology, coagulation, serum chemistry or urinalysis parameters and no effects on organ weights. There were no apparent macroscopic or histological findings that correlated with any of the test diets. The conclusion of the study was that administration of processed meal derived from MON87705 soybean for at least 90 consecutive days at a concentration of 30% (w/w) in the diet had no adverse effect on the growth or health of rats, when compared to a diet containing conventional soybean meal.

Study submitted:
Davis, S.W. (2009). Comparison of Broiler Performance and Carcass Parameters When Fed Diets Containing Soybean Meal Produced from MON87705, Control or Reference Soybean. Monsanto Study Number: CQR-08-271

A 42-day feeding assessment using 500 broiler chickens (Cobb x Cobb) was conducted to compare the nutritional value of diets containing soybean meal produced from MON87705, the conventional control (A3525), and six conventional soybean varieties representative of the population of commercial soybean.

Chick mortality during the first seven days of the study ranged from 0 to 2.5%, with an average of 1.1% across all diet groups. During the period Day 7 to Day 42, bird mortality was an average of 1.3%. Mortality in the MON87705 diet group was 1.0% during this period. Birds in all diet groups surviving to the end of the study were in good health based on twice daily pen observations.

A range of performance measurements were taken to compare dietary groups. There were no differences between groups in live bird weight (at Day 42), total feed intake, and unadjusted and adjusted feed to gain ratios. There were no unexpected effects on broiler performance when birds were fed diets containing soybean meal produced from MON87705, compared to diets containing soybean meal produced from the conventional control or reference soybean varieties. Carcass yield measurements for broilers were not different across any of the diet groups. Meat composition (moisture, fat and protein) was not significantly different for birds fed diets containing soybean meal from MON87705 compared to that from birds in the other dietary groups. The study concluded that a diet containing soybean meal from MON87705 was as wholesome as an equivalently formulated diet containing conventional soybean meal in terms of the ability of the diet to support typical rapid growth in broiler chickens.

6.3 Conclusion

Dietary exposure assessments using data from the UK and the US show that substitution of conventional soybean oil in the diet with MON87705 soybean oil would have no adverse nutritional effects caused by the changes in the fatty acid profile. The most significant change would be an increase in the intake of oleic acid, a monounsaturated fatty acid, at the expense of linoleic acid, which is already abundant in the diet. A diet containing soybean meal produced from MON87705 was not associated with any adverse health impact in rats and was equivalent to diets containing conventional soybean meal in terms of the ability of the diet to provide nutritional support to rapidly growing animals.

References and Bibliography

Axelos, M., Bardet, C., Liboz, T., Le Van, T.A., Curie, C. and Lescure, B. (1989) The gene family encoding the Arabidopsis thaliana translation elongation factor EF-1 alpha: molecular cloning, characterization and expression. *Molecular and General Genetics* 219(1-2):106-112.

Barker, R.F., Idler, K.B., Thompson D.V. and Kemp, J.D. (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. *Plant Molecular Biology* 2:335-350.

Barry, G.F., Kishore, G.M., Padgette, S.R. and Stallings, W.C. (1997) Glyphosphate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases. (5,633,435): USA.

Brookes, G. and Barfoot, P. (2009) *GM crops: global socio-economic and environmental impacts 1996-2007*. PG Economics Ltd. http://www.pgeconomics.co.uk/pdf/2009globalimpactstudy.pdf. Accessed on 5 June 2009.

Coruzzi, G., Broglie, R., Edwards, C. and Chua, N.H. (1984) Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. *EMBO Journal* 3(8):1671-1679.

Delaney, B., Astwood, J.D., Cunny, H., Eichen Conn, R., Herouet-Guicheney, C., MacIntosh, S., Meyer, L.S., Privalle, L.S., Gao, Y., Mattsson, J., Levine, M. and ILSI. (2008) Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* 46:S71-S97.

Doyle, J.J., Schuler, M.A., Godette, W.D., Zenger, V., Beachy, R.N. and Slightom, J.L. (1986) The glycosylated seed storage proteins of Glycine max and Phaseolus vulgaris. Structural homologies of genes and proteins. *Journal of Biological Chemistry* 261(20):9228-9238.

EuropaBio (2003) *Safety Assessment of GM Crops. Document 1.4: Substantial Equivalence - Soybean*. The European Association for Bioindustries. http://www.europabio.org/relatedinfo/CP8.pdf. Accessed on 23 April 2009.

FAO/WHO (1991) *Report of a joint FAO/WHO consultation: strategies for assessing the safety of food processed by biotechnology*. World Health Organisation, Geneva.

Fillatti, J.J., Bringe, N.A. and Dehesh, K. (2003) Nucleic acid constructs and methods for producing altered seed oil compositions. WO 2003/080802 A3.

FSANZ (2007) *The role of animal feeding studies in the safety assessment of gentically modified foods*. Report of a workshop hosted by Food Standards Australia New Zealand, Friday 15th June 2007, Canberra. http://www.foodstandards.gov.au/_srcfiles/Workshop%20Report%20FINAL.pdf.

Grey, D. (2006) Growing Soybeans in Northern Victoria. State of Victoria, Department of Primary Industries, http://www.dpi.vic.gov.au/DPI/nreninf.nsf/v/BB11EFFEFD5E4D17CA257427001D7C73/$file/Growing_Soybeans_in_Northern_Victoria.pdf. Accessed on 7 May 2009.

James, A.T. and Rose, I.A. (2004) Integrating crop physiology into the Australian soybean improvement program. *In: 4th International Crop Science Congress, Brisbane, Australia, September 2004*. http://www.cropscience.org.au/icsc2004/poster/3/4/6/327_jamesat.htm#TopOfPage.

John, M.E. and Keller, G. (1995) Characterization of mRNA for a Proline-Rich Protein of Cotton Fiber. *Plant Physiology* 108(2):669-676.

Klee, H.J., Muskopf, Y.M. and Gasser, C.S. (1987) Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. *Molecular and General Genetics* 210(3):437-442.

Martinell, B.J., Julson, L.S., Elmer, C.A., Huang, Y., McCabe, D.E. and Williams, E.J. (2002) Soybean Agrobacterium transformation method. (6384301): United States.

OECD (2001) *Consensus document on compositional considerations for new varieties of soybean: key food and feed nutrients and anti-nutrients*. Series on the Safety of Novel Foods and Feeds No. 2. Report No. ENV/JM/MONO(2001)15, Organisation for Economic Co-operation and Development, Paris.

OECD (2003) *Series on the safety of novel foods and feeds, no 9*. Report No. ENV/JM/MONO(2003)10, Organisation for Economic Co-operation and Development, Paris.

Padgette, S.R., Re, D.B., Barry, D.B., Eichholtz, D.A., Delannay, X., Fuchs, R.L., Kishore, G.M. and Fraley, R.T. (1996) New Weed Control Opportunities: Development of Soybeans with a Roundup Ready(TM) Gene. In: Duke, S.O. eds. *Herbicide-Resistant Crops*. CRC Press Inc, pp. 53-84.

Polevoda, B. and Sherman, F. (2000) Nα-terminal Acetylation of Eukaryotic Proteins. *Journal of Biological Chemistry* 275(47):36479-36482.

Richins, R.D., Scholthof, H.B. and Shepherd, R.J. (1987) Sequence of figwort mosaic virus DNA (caulimovirus group). *Nucleic Acids Research* 15(20):8451-8466.

Siomi, H. and Siomi, M.C. (2009) On the road to reading the RNA-interference code. *Nature* 457(7228):396-404.

The American Soybean Association. (2008) Soy Stats 2008. http://www.soystats.com/2008/Default-frames.htm. Accessed on 30 March 2009.

Zambryski, P., Depicker, A., Kruger, K. and Goodman, H.M. (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *Journal of Molecular and Applied Genetics* 1(4):361-370.