FOOD DERIVED FROM HIGH OLEIC ACID SOYBEAN LINES G94-1, G94-19 and G168

A Toxicological Review and Risk Assessment

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SUMMARY

Food derived from high oleic acid soybean lines G94-1, G94-19 and G168 has been evaluated to determine its suitability for human consumption. The evaluation criteria included characterisation of the transferred genes, analysis of changes at the DNA, protein and whole food levels, and assessment of the potential allergenicity and toxicity of any newly expressed proteins. Examination of these criteria has enabled both intended and unintended changes to be identified, characterised and evaluated for safety.

Nature of the genetic modification

Three lines of a new variety of soybean (G94-1, G94-19 and G168), high in the monounsaturated fatty acid oleic acid, were generated by the transfer of a second copy of a soybean fatty acid desaturase gene (*GmFad 2-1*) to a high yielding commercial variety of soybean (line A2396). The fatty acid desaturase is responsible for the synthesis of linoleic acid, which is the major polyunsaturated fatty acid present in soybean oil. The presence of a second copy of the fatty acid desaturase gene causes a phenomenon known as "gene silencing" which results in both copies of the fatty acid desaturase gene being "switched off", thus preventing linoleic acid from being synthesised and leading to the accumulation of oleic acid in the developing soybean seed.

Other genes transferred along with the *GmFad 2-1* gene were the *uidA* gene and the *bla* gene. The *uidA* gene is a colourimetric marker used for selection of transformed plant lines during the soybean transformation procedure. It codes for the enzyme β-glucuronidase and is derived from the bacterium *Escherichia coli*. The *bla* gene is a marker used to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells. It codes for the enzyme β-lactamase and confers resistance to some β-lactam antibiotics, such as penicillin and ampicillin.

The transferred genes were all found to be stably integrated into the genome of the high oleic acid soybean lines and are all phenotypically and genetically stable over multiple generations and in various environments.

History of use

Soybeans are grown as a commercial crop in over 35 countries worldwide and have a long history of safe use as human food. The major food product to be derived from the high oleic acid soybeans will be the oil. High oleic acid soybean oil will be predominantly used in spraying and frying applications and might replace heat stable fats and oils such as hydrogenated soybean and rapeseed oil or palm olein/vegetable oil blends.

Antibiotic resistance genes

One of the important issues to consider in relation to genetically modified foods is the impact on human health from the presence of antibiotic resistance genes. In the case of the high oleic acid soybeans, it was concluded that the *bla* gene would be extremely unlikely to transfer to bacteria in the human digestive tract because of the number and complexity of the steps that would need to take place consecutively. More importantly, however, in the highly unlikely event that transfer did occur, the human health impacts would be negligible because

ampicillin resistant bacteria are already commonly found in the human gut and in the environment.

Characterisation of novel protein

Extensive analyses of the high oleic acid soybeans demonstrated that none of the transferred genes give rise to a protein product, meaning no new proteins are expressed in any of the high oleic acid soybean lines.

Comparative analyses

The composition of the high oleic acid soybeans was compared to that of the elite soybean line from which they were derived. These comparisons looked at the key nutrients, toxicants and anti-nutrients of soybeans, as well as the protein profile.

Soybeans contain the toxicant lectin as well as the anti-nutrients trypsin inhibitor and phytate. The high oleic acid soybean lines exhibit slightly elevated lectin levels when compared to the control but these levels are well within the literature reported range for soybeans. As lectins are readily degraded upon heating and there are no human food uses for raw soybeans, the slightly elevated levels observed are not a cause for concern. No differences were seen in the levels of the anti-nutrients.

Comparisons were also made with the levels of various macro- and micro-nutrients. Proximate (crude fat/protein, fibre, ash), amino acid, fatty acid, vitamin and mineral, and isoflavone levels were measured. These analyses confirmed that the high oleic acid soybeans are significantly changed with respect to their fatty acid profile. The mean oleic acid content has been increased from 23.1% in the parental soybean to 83.8% in the high oleic acid soybean lines and the linoleic acid content has been concomitantly decreased from a mean level of 55.4% to a mean level of 2.2%. Small reductions in the levels of palmitic and linolenic acid were also observed. High oleic acid levels are found in other commonly consumed premium edible oils (e.g., olive oil and high oleic acid sunflower and canola oil). The consumption of high levels of oleic acid is not considered to pose any safety concerns.

The compositional analyses revealed the unexpected occurrence of trace amounts (less than 1%) of an isomer of linoleic acid in the high oleic acid soybeans. This isomer is not present in the parental soybean line but is normally found in commonly consumed foods such as hydrogenated soybean oils and butterfat. It is present at levels in the high oleic acid soybeans that are comparable to the levels found in hydrogenated soybean oils and butterfat. Its presence is not considered to pose any toxicological or nutritional concerns.

The seed storage proteins of soybeans, which comprise a number of naturally occurring allergens were also compared. Although no new proteins are expressed in any of the high oleic acid soybean lines, they were found to exhibit a slightly altered seed storage protein profile. Allergenicity testing confirmed, however, that the altered protein profile does not give rise to any significant differences between the allergen content of the high oleic acid soybeans and the parental soybean line A2396. Nor did the altered protein profile lead to significant changes to the total protein content of the high oleic acid soybeans.

In all other respects, the high oleic acid soybeans were found to be compositionally equivalent to the parental soybean line and other commercial varieties of soybean.

Nutritional impact

Two animal feeding studies, with pigs and chickens, were done with the high oleic acid soybeans. These studies confirmed that the high oleic acid soybeans are equivalent to other commercial varieties of soybean with respect to its ability to support typical growth and well-being.

A study was also undertaken to assess the human nutritional impact of the use of high oleic acid soybean oil as a replacement for frying fats. The study concluded that the use of high oleic acid soybean oil might lower dietary linoleic acid intake somewhat (by an absolute maximum of 29%), but it would not do so to any level that would be a public health concern in terms of cardiovascular disease. Overall, the conclusion of the study was that the nutritional impact of the use of high oleic acid soybean oil was likely to be beneficial because diets incorporating high oleic acid soybean oil show decreased saturated fatty acid intakes and this is likely to reduce risk factors for cardiovascular disease.

Conclusion

Based on currently available data, the high oleic acid soybeans are significantly changed with respect to their fatty acid profile but are comparable to non-GM soybeans in terms of their safety and nutritional adequacy.

FOOD DERIVED FROM HIGH OLEIC ACID SOYBEAN LINES G94-1, G94-19 AND G168

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on soybeans that have been genetically modified to contain increased levels of oleic acid, a monounsaturated fatty acid. The soybeans are referred to as *high oleic acid soybean* lines G94-1, G94-19 and G168.

The high oleic acid soybeans were generated by the transfer of a second copy of a soybean fatty acid desaturase gene (*GmFad 2-1*) to a high yielding commercial soybean variety. The fatty acid desaturase is an enzyme responsible for the synthesis of linoleic acid, which is the major polyunsaturated fatty acid present in soybean oil. The presence of a second copy of the fatty acid desaturase gene causes a phenomenon known as "gene silencing" which results in both the endogenous and introduced fatty acid desaturase genes being "switched off". This blocks the step in the metabolic pathway that leads to the synthesis of linoleic acid and results in the accumulation of oleic acid in the developing soybean seed.

Traditional soybean oil has poor oxidative stability due to naturally high levels of polyunsaturated fatty acids, such as linoleic acid. High oleic acid soybean oil is considered to have superior properties to that of traditional soybean oil because of its reduced levels of the oxidatively unstable polyunsaturated fatty acids. High oleic acid soybean oil can thus be used for a number of food applications, including deep fat frying, without the need for additional processing, such as chemical hydrogenation. High oleic acid soybean oil is also considered to offer improved nutritional properties compared to traditional soybean oil or partially hydrogenated soybean oil because of the increased levels of monounsaturated fatty acids.

HISTORY OF USE

Soybean (*Glycine max*.) is grown as a commercial crop in over 35 countries worldwide and has a long history of safe use for both human food and stockfeed. The elite soybean cultivar A2396, which has been used as the host for the high oleic acid trait described in this paper, is an Asgrow Seed Company early Group II maturity soybean variety that has high yield potential. The protein and oil characteristics of line A2396 are similar to that of other soybeans, that is, 40% protein and 22% oil on a dry weight basis.

There are three major soybean commodity products: seeds, oil and meal. Unprocessed soybeans, which contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors, have only limited feed use, and no food use. Appropriate heat processing inactivates these compounds. Whole soybeans are used to produce soy sprouts, baked soybeans, and roasted soybeans. The soybean hulls can be processed to create full fat soy flour and the traditional soy foods such as miso, tofu, soymilk and soy sauce.

Before processing, soybeans are graded, cleaned, dried and de-hulled. The soybean hulls are further processed to create fibre additives for breads, cereals and snacks and are also used for stockfeed. After de-hulling, soybeans are rolled into full fat flakes that may be either used in stockfeed or processed further into full fat flour. Crude soybean oil is then extracted from the

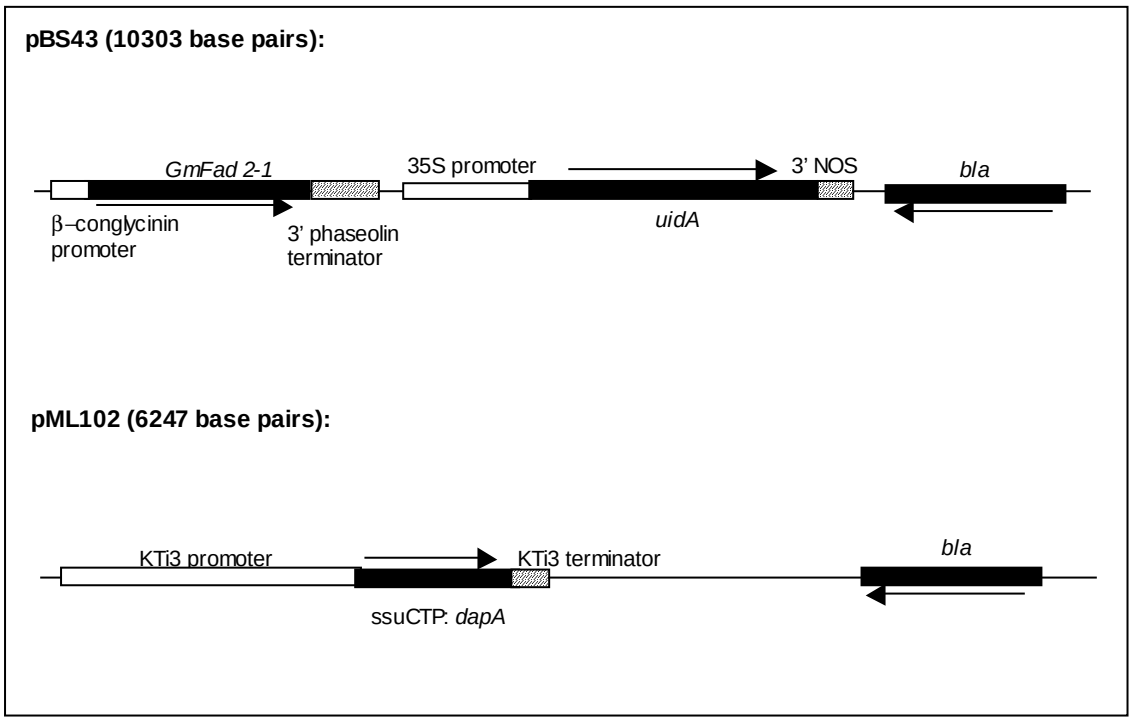
flakes by immersing them in a solvent bath. Crude lecithin is then separated from the oil, which is further refined to produce cooking oil, margarine and shortening. After the oil is extracted from the flakes, the solvent is removed and the flakes are dried for use in the production of soy flour, soy concentrates and soy isolates. De-fatted soy flakes are also used in stockfeed.

Oil from high oleic acid soybeans is intended to be used predominantly for spraying and frying applications in the food industry and food services and might replace heat stable fats and oils such as hydrogenated soybean and rapeseed oil or palm olein/vegetable oil blends.

NATURE OF THE GENETIC MODIFICATION

Methods used in the genetic modification

The method of microprojectile bombardment was used to transfer purified plasmid DNA, carrying the genes of interest, into meristem tissue of elite soybean line A2396. The two plasmids used were pBS43 and pML102 (see diagram below).



Function and regulation of the novel genes

Co-transformation of soybean with plasmids pBS43 and pML102 resulted in the transfer of three gene expression cassettes – *GmFad 2-1*, *uidA* (otherwise known as GUS), and *dapA*. The expression cassettes are described in Table 1 below.

Table 1: Description of the gene expression cassettes in pBS43 and pML102

Cassette	Genetic element	Source	Function
GmFad 2-1 **expression cassette (pBS43)**	β-conglycinin promoter	α^1-subunit of β-conglycinin seed storage protein of soybean (Barker *et al* 1988).	Seed specific promoter that allows high level gene expression during seed development.
	GmFad 2-1 coding region	Protein coding sequence of the δ-12 fatty acid desaturase from soybean (Okuley *et al* 1994, Heppard *et al* 1996).	The endogenous enzyme adds a second double bond to oleic acid thus converting it to linoleic acid.
	phaseolin 3' terminator	The 3' terminator region from the phaseolin seed storage protein of green bean *Phaseolis vulgaris* (Doyle *et al* 1986).	Contains signals for termination of transcription and directs polyadenylation.
GUS expression cassette (pBS43)	35S promoter	A promoter derived from the cauliflower mosaic virus (CaMV) (Odell *et al* 1985).	Promoter of high level constitutive gene expression in plant tissues.
	Cab 22L non-translated leader	The 5' untranslated leader from the photosynthetic *22L* chlorophyll a/b binding protein (*Cab22L*) promoter of *Petunia hybrida* var. Mitchell (Harpster *et al* 1988).	The untranslated leader sequence helps to stabilise mRNA and improve translation.
	uidA coding region	Protein coding sequence of the enzyme β-glucuronidase (*uidA* gene) from *Escherichia coli* (Jefferson *et al* 1985).	Colourimetric marker used for selection of transformed plant lines.
	NOS 3'	The 3' terminator region of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Depicker *et al* 1982, Bevan *et al* 1983).	Contains signals for termination of transcription and directs polyadenylation.
dapA **expression cassette (pML102)**	Kti3 promoter	Promoter from Kunitz trypsin inhibitor gene 3 of soybean (Jofuki and Goldberg 1989).	Seed specific promoter that allows high level gene expression during seed development.
	ssu CTP	The N-terminal chloroplast transit peptide sequence from the soybean small subunit of Rubisco (Berry-Lowe *et al* 1982).	Directs the protein into the chloroplast which is the site of lysine biosynthesis.
	dapA coding region	Coding sequence of the *Corynebacterium dapA* gene encoding the lysine insensitive version of the enzyme dihydrodipicolinic acid synthase (DHDPS) (Bonnassie *et al* 1990, Yeh *et al* 1988).	Expression of *Corynebacterium* DHDPS deregulates the lysine biosynthetic pathway resulting in accumulation of free lysine (Falco *et al* 1995).
	Kti3 3' terminator	The 3' terminator region from Kunitz trypsin inhibitor gene 3 from soybean (Jofuki and Goldberg 1989)..	Contains signals for termination of transcription and directs polyadenylation.

The GmFad 2-1 gene

In soybean, there are two Fad 2 genes, but only the *GmFad 2-1* gene is expressed in the developing seed (Heppard *et al* 1996). The expression of *GmFad 2-1* increases during the period of oil deposition, starting around 19 days after flowering, and its gene product is responsible for the synthesis of the polyunsaturated fatty acids found in the oil fraction. The second Fad 2 gene (*GmFad 2-2*) is expressed in the seed, leaf, root and stem at a constant level and its gene product is responsible for the synthesis of the polyunsaturated fatty acids present in cell membranes.

The presence of a second copy of the *GmFad 2-1* gene in the soybean causes a phenomenon known as "gene silencing" which results in both copies of the *GmFad 2-1* gene (the transferred copy as well as the original soybean copy) being "switched off", thus preventing linoleic acid from being synthesised and leading to the accumulation of oleic acid in the developing soybean seed.

Gene silencing, also known as co-suppression, is a phenomenon that is sometimes observed when plants are genetically modified to contain new or additional copies of genes and is a means by which plant genes can be deliberately "switched off" so that they no longer give rise to a protein product in the cell (US patent 5034323). Gene silencing is thought to occur by one of two mechanisms (Reviewed in Matzke and Matzke 1995, Finnegan and McElroy 1996, Stam *et al* 1997). In one, inactivation occurs by repression of RNA transcription and is associated with methylation of the promoter. The second results in the failure to accumulate messenger RNA in the cytoplasm, probably due to targeted degradation of mRNA.

The dapA gene

The *dapA* gene codes for the enzyme dihydrodipicolinic acid synthase (DHDPS), which is responsible for catalysing the first step in the metabolic pathway for the synthesis of the essential amino acid lysine (Brock *et al* 1984). The DHDPS found in plants is inhibited by lysine, whereas the *dapA* gene transferred to the soybeans, which was derived from *Corynebacterium,* codes for a form of DHDPS that is insensitive to inhibition by lysine.

In previous experiments it has been shown that expression of the lysine-insensitive DHDPS, encoded by the *Corynebacterium dapA* gene, will result in more than a 100-fold increase in the accumulation of free lysine in the seeds, essentially doubling total seed lysine content (Falco *et al* 1995).

The objective of transforming soybean with both the soybean *GmFad 2-1* gene and the *Corynebacterium dapA* gene was to produce transgenic soybeans with increased lysine in their meal fraction, due to expression of the lysine insensitive form of DHDPS, and a reduced level of polyunsaturated fatty acids in their oil fraction, due to silencing of the *GmFad 2-1* gene (described above).

Other genetic elements

In addition to the gene expression cassettes described above, a number of other genetic elements, including an antibiotic resistance gene, were also present in the plasmid DNA and were therefore also subsequently transferred to the soybeans. These genetic elements are described in Table 2 below.

Table 2: Description of other genetic elements transferred to high oleic acid soybeans

Genetic element	Source	Function
lac	An incomplete copy of the *lac* operon which contains a partial *lac*I coding sequence, the promoter P_{lac}, and a partial coding sequence for β-D-galactosidase (*lac*Z′).	These genes are not intact and no longer function in *E.coli.*
ori	Origin of replication from the high copy number *E. coli* plasmid pUC19.	Allows plasmids to replicate in *E. coli.*
bla	Gene coding for the enzyme β-lactamase from *E. coli.*	Confers ampicillin resistance to *E. coli.*
f1 ori	Bacteriophage f1 origin of replication.	Origin of replication recognised by bacteriophage f1 to produce single stranded DNA. The f1 origin is not recognised unless a phage f1 is present.

These genetic elements are present in most *E. coli* cloning vectors and are well described (Sambrook *et al* 1981). They are used to assist in the manipulation of DNA sequences as well as direct gene expression in *E.coli.*

Characterisation of the genes in the plant

Selection of plant lines

From the initial population of transformed plants, one plant (Event 260-05) was selected which exhibited GUS activity and which was also shown, using the polymerase chain reaction (PCR), to contain the *GmFad 2-1* gene. Small samples were taken from the R1 seeds of plant 260-05 and screened for fatty acid composition and lysine content. Four different fatty acid profiles in combination with lysine changes were identified among the R1 seeds:

(i) seeds with ≥ 80% oleic acid content and normal lysine levels (G168);

(ii) seeds with about 72% oleic acid content and increased lysine levels (G94);

(iii) seeds with about 4% oleic acid content and increased lysine levels (G175); and

(iv) seeds with oleic acid and lysine levels similar to that of line A2396 (G90).

R2 seeds from (i) and (ii) above were further characterised by fatty acid composition and lysine assays. Southern blot analysis was also done on R1 and R2 leaves.

Southern blotting is a sensitive technique that enables the detection of specific sequences among DNA fragments that have been separated using gel electrophoresis (Southern 1975). . The overall pattern of the specific fragments detected can be used to characterise the nature of the T-DNA insertion into the genome (e.g. how many loci in the genome has the T-DNA have inserted into, whether the inserted copies are intact, etc).

Southern blotting of genomic DNA extracted from R1 and R2 leaves revealed that the *GmFad 2-1* construct had become integrated at two different loci in the genome of the original transformant (line 260-05). At one locus (*locus A*), the *GmFad 2-1* construct was causing silencing of the endogenous *GmFad 2-1* gene, resulting in seeds like G168 with a high oleic acid content only. *Locus A* was characterised using Southern blotting and shown to contain two copies of the *GmFad 2-1* expression cassette as indicated by two hybridising bands on the Southern blot. The second locus (*locus B*) contained a copy of *GmFad 2-1* that was over-expressing, thus decreasing the oleic acid levels to around 4% (G175). This locus also contained a functioning *dapA* gene as evidenced by an increase in the seed lysine levels. *Locus B* contained only a single copy of the *GmFad 2-1* expression cassette as indicated by a single hybridising band on the Southern blot. In seeds with both *locus A* and *locus B* (G94), oleic acid levels were increased but not as high as *locus A* alone and lysine levels were increased.

Lines G94 and G168 were selected for further characterisation as they contained the silencing *locus A* with the high oleic acid phenotype. As G94 plants contained both *locus A* and *locus B*, an additional round of selection was used on the segregating R2 plants to isolate plants containing *locus A* and not *locus B*. Southern blot analysis on R2 leaf tissue grown from G94 R2 seed identified two sub lines, G94-1 and G94-19, that contained *locus A* without *locus B* which had been removed through segregation. *Locus B* was not further characterised for the purposes of this application.

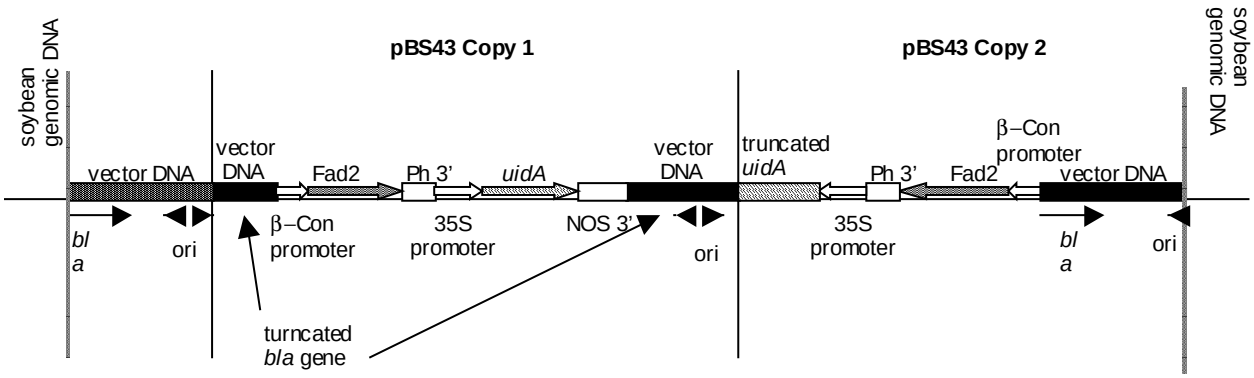
The three sub lines, G94-1, G94-19 and G168, identified as containing the *GmFad 2-1* silencing *locus A*, were selected as the high oleic acid soybeans for subsequent analyses. The application for food use relates to these sub lines only.

Molecular characterisation of the gene insertion in sub lines G94-1, G94-19 and G168

Studies evaluated:
Luckring, A. *et al* (1999). Southern blot analysis of high oleic acid soybean sublines G94-1, G94-19 and G168 derived from Event 260-05. Optimum Quality Grains Study No. 99 Southern-HOS-02.

A set of six different DNA hybridisation probes, specific for different parts of the *GmFad 2-1* expression cassette, were used to fully characterise and map the DNA insertion at *locus A* of R6 leaf tissue.

The mapping of *locus A* shows that one copy of pBS43, opened in the *bla* gene, inserted intact into the genome. A second copy of pBS43, opened in the *uidA* gene, inserted as an inverted repeat relative to the first copy. At the 5' end of *locus A*, proceeding from the soybean genomic DNA junction to the first copy of pBS43, a fragment of pML102, containing only the vector region with the *bla* gene, was inserted. Therefore, the insertion at *locus A* consists of two intact copies of the *GmFad 2-1* expression cassette, one intact copy of the *uidA* expression cassette and a truncated copy of the *uidA* gene, and at least two intact copies of the *bla* gene plus one truncated copy. A diagram of the gene organisation at *locus A* is presented in below.



A series of Northern blots (for RNA expression), Western blots (for protein expression) and amino acid profiles were done on sub lines G94-1, G94-19 and G168 to confirm that the functional *dapA* gene at *locus B* was absent. However, additional Southern blots, using a *dapA* probe, indicated that a truncated *dapA* gene expression cassette had become integrated into another locus in the genome (*locus C*). This locus segregates independently of *locus A*. The truncated *dapA* gene is non-functional as indicated by Northern, Western and amino acid analyses.

Stability of the genetic changes

Sub lines G94-1, G94-19 and G168 differ from the parent line A2396 in that the fatty acid profile has been altered to produce oil containing about 82-85% oleic acid with consequent low levels of linoleic (< 1%) and linolenic acids (< 2.5%). This compares to a range of 19 – 30% oleic acid reported for standard edible soybean oil (Codex Alimentarius 1989).

To evaluate the genetic and phenotypic stability of the sub lines, genomic DNA from a number of generations of high oleic acid soybeans, homozygous for the *GmFad 2-1* silencing *locus A,* were subject to detailed Southern blot analyses. The applicant reports that sub lines G94-1, G94-19 and G168 had been kept separate for six generations and all were shown to maintain identical Southern banding patterns over that period. Analysis of the oleic acid content of seeds from eight different generations also showed that the fatty acid phenotype was stable over this period, with average oleic acid content greater than 80%. In addition, the high oleic acid trait is also reported by the applicant to be stable over a number of different growing environments when compared to the elite parent line and a high oleic acid soybean line derived through conventional breeding methods.

Conclusion

The inserted genes in the three sub lines of high oleic acid soybeans are stably integrated and all three lines are phenotypically and genetically stable over multiple generations and in various environments.

ANTIBIOTIC RESISTANCE GENES

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA *per se* (WHO 1993). There have been concerns expressed, however, that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of antibiotics.

This section of the report will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from high oleic acid soybeans to microorganisms present in the human digestive tract.

The two plasmids used to transform soybean line A2396 – pBS43 and pML102 – both contained a copy of the *bla* gene under the control of a bacterial promoter. The *bla* gene encodes the enzyme β-lactamase and confers resistance to a number of β-lactam antibiotics such as penicillin and ampicillin. Analysis of the high oleic acid soybean lines has confirmed the presence of two intact copies of the *bla* gene along with its bacterial promoter. The *bla* gene is not itself expressed in the high oleic acid soybean lines.

The first issue that must be considered in relation to the presence of an intact *bla* gene in the high oleic acid soybeans is the probability that this gene would be successfully transferred to, and expressed in, microorganisms present in the human digestive tract. The following steps would be necessary for this to occur:

- excision of DNA fragments containing the *bla* gene and its bacterial promoter;
- survival of DNA fragments containing the *bla* gene in the digestive tract;
- natural transformation of bacteria inhabiting the digestive tract;
- survival of the bacterial restriction system by the DNA fragment containing the *bla* gene;
- stable integration of the DNA fragment containing the *bla* gene into the bacterial chromosome or plasmid;
- maintenance and expression of *bla* gene by the bacteria.

The transfer of a functional *bla* gene to microorganisms in the human digestive tract is therefore considered to be highly unlikely because of the number and complexity of the steps that would need to take place consecutively.

The second and most important issue that must be considered is the potential impact on human health in the unlikely event successful transfer of a functional *bla* gene to microorganisms in the human digestive tract did occur.

In the case of the *bla* gene, the human health impacts are considered to be negligible because ampicillin-resistant bacteria are commonly found in the digestive tract of healthy individuals (Calva *et al* 1996) as well as diseased patients (Neu 1992). Therefore, the additive effect of a *bla* gene from the high oleic acid soybeans being taken up and expressed by microorganisms

of the human digestive tract would be insignificant compared to the population of ampicillin resistant bacteria already naturally present.

Conclusion

It is extremely unlikely that the ampicillin resistance gene will transfer from high oleic acid soybeans to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. In the highly unlikely event that the ampicillin resistance gene was transferred to bacteria in the human digestive tract the human health impacts would be negligible because ampicillin resistant bacteria are already commonly found in the human gut and in the environment.

CHARACTERISATION OF NOVEL PROTEIN

Studies evaluated:

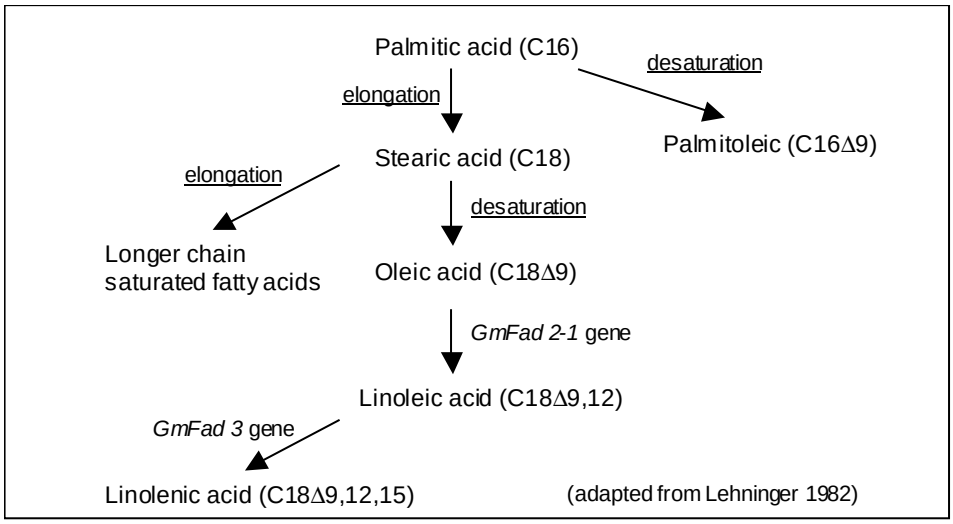
Stecca, K. (1996). Northern blot analysis of high oleic acid soybean sublines G94-1, G94-19 and G168 derived from Event 260-05. Optimum Quality Grains Study No. 96 Northern-HOS-33.

Sanders, C. (1997). Analysis of protein expression in high oleic acid soybean sublines G94-1, G94-19 and G168 derived from Event 260-05 by enzyme assays, western blots and protein gel electrophoresis. Optimum Quality Grains Study No. 97 Protein-HOS-02.

Biochemical function and phenotypic effects

δ-12 desaturase

The synthesis of polyunsaturated fatty acids in developing oilseeds is catalysed by two membrane-associated desaturases that sequentially add a second and third double bond to oleic acid (Kinney 1994). The pathway for the synthesis of long chain fatty acids in plants is depicted below.



The second double bond, converting oleic acid to linoleic acid, is added at the δ-12 (n-6) position by a δ-12 desaturase, encoded by the *GmFad 2-1* gene (Okuley *et al* 1994, Heppard *et al* 1996). The third double bond, converting linoleic acid to linolenic acid, is added at the

n-3 (δ-15) position by an n-3 desaturase, encoded by the *GmFad 3* gene (Yadav *et al* 1993). The *GmFad 2-1* gene used to genetically modify the soybeans is itself derived from soybean.

Dihydrodipicolinic acid synthase

Dihydrodipicolinic acid synthase (DHDPS) is responsible for catalysing the first step in the metabolic pathway for the synthesis of the essential amino acid lysine (Brock *et al* 1984). DHDPS catalyses the condensation of aspartate semi-aldehyde with pyruvate to form 2,3-dihydrodipicolinate. The reaction takes place in the chloroplast of higher plants as well as in many bacteria. In plants, DHDPS is inhibited by lysine and is the major regulatory enzyme of lysine biosynthesis. Animals are incapable of synthesising lysine; therefore they must obtain their lysine through dietary sources.

β-glucuronidase

The *uidA* gene from *E. coli* encodes the enzyme β-glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31), which is an acid hydrolase that catalyses the cleavage of a wide variety of β-glucuronides. Many glucuronide substrates can be used for spectrophotometric, fluorometric and histochemical analyses. Very little, if any, β-glucuronidase activity has been detected in higher plants (Jefferson *et al* 1986), therefore fusions of the *uidA* gene to plant genes or promoters can be used as a visual marker of plant transformation. In the case of plants that have been transformed with the *uidA* gene, the colourimetric substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide is used as an indicator of β-glucuronidase activity.

β-lactamase

The bacterial *bla* gene codes for the enzyme β-lactamase and confers resistance to some β-lactam antibiotics, such as penicillin and ampicillin. The gene is used as a marker to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells. Only those bacterial cells that express the β-lactamase will grow in the presence of antibiotic. As the *bla* gene is under the control of a bacterial promoter it would not be expected to be expressed in transformed plant cells.

Protein expression analyses

δ-12 desaturase

Northern blot analysis, using the *GmFad 2-1* gene as a probe, was done on RNA isolated from developing R4 seeds of the high oleic acid soybeans at the time when the endogenous *GmFad 2-1* would normally be expressed (during seed development, 20 days after flowering). The β-conglycinin promoter, linked to the transferred copy of the *GmFad 2-1* gene, is also active during this period. The data shows that seeds containing *GmFad 2-1* silencing *locus A* (G94-1, G168) do not have any detectable *GmFad 2-1* mRNA, whereas, seeds that contain the *GmFad 2-1* over expressing *locus B* (G175) or seeds that only contain the endogenous *GmFad 2-1* gene (G90) have significant levels of mRNA. This demonstrates that neither of the *GmFad 2-1* genes is transcribed in the high oleic acid soybeans.

Dihydrodipicolinic acid synthase

Northern blot analysis, using the *dapA* probe, was done on RNA isolated from R6 leaves and R4 immature seeds of the high oleic acid soybeans. The data show that there is no detectable expression of *dapA* mRNA in sub lines G94-1, G94-19 and G168. Western blot analysis, using a polyclonal anti-*Corynebacterium* DHDPS antibody, was done on total protein isolated from leaves and seeds of the three sub lines. The data show that DHDPS protein can only be detected in seeds of the high lysine positive control line and not in any of the high oleic acid sub lines under consideration.

Amino acid analyses were done on three replicates of each of the high oleic acid soybean sub lines. These show that there are no differences in the lysine levels of the high oleic acid soybeans when compared to the parental soybean line (A2396).

β-glucuronidase

An intact *uidA* expression cassette is present in sub lines G94-1, G94-19 and G168, however, colourimetric analyses of R6 seeds and leaves from these lines show that the *uidA* gene is not expressed. The original transformant, line 260-05, was selected on the basis of its GUS expression therefore the *uidA* gene has become 'switched off' in subsequent generations. The applicant has not speculated as to the reason for the inactivation of the *uidA* gene, however, the inactivation of transgenes is relatively common in plants (Kilby *et al* 1992, Ingelbrecht *et al* 1994, Brusslan and Tobin 1995).

β-lactamase

All of the lines derived from event 260-05, which contain only the *GmFad 2-1* silencing *locus A,* also contain two intact copies of the *bla* gene. These two copies are under the control of a bacterial promoter and, therefore, should not be expressed in the plant cell. To confirm this, the activity of β-lactamase was measured in cell free extracts of leaf tissue from sub line G94-1. The results of this study, which show that there is no detectable β-lactamase activity in sub line G94-1, confirm that the *bla* gene is not expressed in plant cells.

Conclusion

Detailed Northern and protein analyses have demonstrated that no new proteins are expressed in sub lines G94-1, G94-19 and G168. Therefore, there are no toxicological or allergenicity issues to consider in relation to any new proteins.

COMPARATIVE ANALYSES

Studies evaluated:

Anon (1996). Compositional analysis of high oleic acid soybean sub lines G94-1, G94-19 and G168 derived from Event 260-05. Optimum Quality Grains Study No. 96 Field-Comp-HOS-11.

There are concerns that transformation will affect the overall nutritional composition of a food, or cause unintended changes that could adversely affect the safety of the product. Therefore a safety assessment of food produced from transgenic plants must include analysis of the composition of the food, based on a comparison with other commercial varieties of the

crop. Generally, comparisons are made not only with the parental line but also with other non-transformed lines. If the parameter for the transformed line is within the normal range for non-transformed lines, this is considered acceptable (Hammond and Fuchs 1998).

Key nutrients

The applicant undertook two separate field studies of the high oleic acid soybeans. In the first study, lines G94-1 and G94-19 were grown at two locations in the United States: Slater, Iowa, and Isabella, Puerto Rico during the summer of 1995 and the Winter of 1995/1996. Seeds, representing the R4 and R5 generation, were analysed from each location. Values were obtained from duplicate assays on single samples from each of the four locations. Analyses were done of raffinose, stacchyose and phytic acid content as well as isoflavone content. In the second study conducted in the summer of 1996, lines G94-1, G94-19 and G168 were grown in parallel with the parental line A2396 at four locations in the United States: Redwood Falls, Minnesota, Kalamazoo, Michigan, Prairie City, Iowa and Cedar Rapids, Iowa. Seeds, representing the R6 generation, were analysed from each of the four locations. Values were obtained from duplicate assays on three replicates from each of the four locations. Analyses were done of proximate, trypsin inhibitor, amino acid, fatty acid, vitamin and mineral, and tocopherol content.

Proximate analyses

Proximate analysis includes the measurement of crude fat/oil, protein, fibre, and ash content and is done to determine if there have been any changes to the primary constituents of the soybean seed. The results of the proximate analysis are presented in Table 3 below.

Table 3: Proximate content[#] of control and high oleic acid soybeans

	Parental control	High oleic acid lines	Literature range
	(g/100 g dry weight unless noted)		
Moisture (g/100 g fresh wt)	7.69 (7.00-8.20)	7.85 (7.20-8.40)	7-11
Crude fat/oil	25.37 (21.62-28.29)	23.90 (19.74-29.28)	13.2-22.5
Protein	40.11 (38.41-41.68)	40.76 (38.85-42.97)	36.9-46.4
Fibre	6.11 (5.44-7.14)	6.76 (5.00-7.26)	4.7-6.8
Ash	5.13 (4.53-5.85)	4.81 (4.13-5.54)	4.61-5.37

[#] mean values, the range in brackets

The results show that there are no significant differences in proximate composition between the parental soybean line and the high oleic acid soybeans. The values obtained are also comparable to those reported in the literature for soybeans.

Amino acid composition

Amino acid content was determined for 17 out of the 20 amino acids. The three amino acids not analysed were proline, asparagine and glutamine. A summary of the results of the amino acid analysis appears in Table 4 below.

Table 4: Amino acid content[#] of parental and high oleic acid soybeans

Amino acid	Parental control	High oleic acid lines	Literature range
		(g/100 g dry weight)	
Tryptophan	0.44 (0.41-0.46)	0.47 (0.42-0.51)	0.53-0.54
Lysine	2.45 (2.27-2.63)	2.38 (2.17-2.67)	2.35-2.86
Histidine	0.96 (0.90-1.05)	0.93 (0.83-1.09)	0.89-1.08
Arginine	2.64 (2.42-2.91)	2.64 (2.37-2.88)	2.45-3.49
Aspartic acid	4.3 (3.98-4.58)	4.45 (4.14-4.93)	3.87-4.98
Threonine	1.37 (1.24-1.50)	1.52 (1.38-1.70)	1.33-1.79
Serine	1.79 (1.61-1.95)	1.84 (1.65-2.02)	1.81-2.32
Glutamic acid	7.13 (6.58-7.81)	7.03 (6.50-7.79)	6.10-8.72
Cysteine	0.55 (0.51-0.60)	0.58 (0.52-0.71)	0.56-0.66
Glycine	1.57 (1.44-1.68)	1.71 (1.56-1.85)	1.88-2.02
Alanine	1.54 (1.43-1.68)	1.67 (1.50-1.84)	1.49-1.87
Valine	1.73 (1.61-1.86)	1.84 (1.58-2.05)	1.52-2.24
Methionine	0.47 (0.44-0.50)	0.54 (0.47-0.60)	0.49-0.66
Isoleucine	1.72 (1.48-1.87)	1.76 (1.54-2.00)	1.46-2.12
Leucine	2.86 (2.64-3.05)	2.91 (2.70-3.18)	2.71-3.20
Tyrosine	1.45 (1.35-1.54)	1.51 (1.38-1.62)	1.12-1.62
Phenylalanine	1.82 (1.71-1.97)	1.86 (1.72-2.03)	1.70-2.08

[#] mean values, the range in brackets

No significant differences were observed in amino acid content between the parental line and the high oleic acid soybeans for any of the 17 amino acids analysed. The values determined were comparable to the literature reported ranges.

Fatty acid composition

A complete fatty acid analysis of oil from the high oleic acid soybean lines G94-1 and G94-19 and control soybean lines grown in field trials in 1995/1996 was done and compared to the ranges specified by Codex Alimentarius for soybean oil. The results of the analysis are presented in Table 5 below.

Table 5: Complete fatty acid analysis of control and high oleic acid soybean lines from 1995/96 field trials

Fatty acid	Parental control	G94-1	G94-19	Codex range
	(g/100 g fatty acid, mean values presented, ranges not provided)			
C14:0 myristic	<0.1	<0.1	<0.1	<0.5
C16:0 palmitic	10.1	6.3[#]	6.6	7.0-14.0
C16:1 palmitoleic	0.1	0.12	0.12	<0.5
C16:2 hexadienoic	<0.1	<0.1	<0.1	
C16:3 hexatrienoic	<0.1	<0.1	<0.1	
C18:0 stearic	3.2	3.7	3.6	1.4-5.5
C18:1 oleic	14.7	84.6	84.9	19.0-30.0
C18:2 (9,12) linoleic	61.6	0.9	0.6	44.0-62.0
C18:2 (9, 15) linoleic	<0.1	0.8	0.7	
C18:3 linolenic	9.5	2.4	1.9	4.0-11.0
C20:0 arachidic	0.2	0.4	0.5	<0.1
C20:1 eicosenoic	0.2	0.4	0.4	<0.1
C20:2 eicosadienoic	not done	not done	not done	
C22:0 behenic	0.3	0.4	0.5	<0.5
C22:1 erucic	<0.1	<0.1	<0.1	
C24:0 lignoceric	0.1	0.1	0.2	

[#] Underlined values are significantly different from the parental control

A further, but more limited analysis of fatty acid content was done on all three high oleic acid soybean lines and the parental control soybean line grown in field trials in 1996. The results of the analysis are presented in Table 6 below.

Table 6: Fatty acid composition[#] of oil from high oleic acid and control soybean lines from 1996 field trials

Fatty acid	Parental control	High oleic acid lines	Literature Range
		(g/100 g fatty acid)	
C16:0 palmitic	10.25 (9.94-10.59)	6.55 (6.22-6.96)	7-12
C18:0 stearic	3.95 (3.57-4.27)	3.43 (3.04-3.81)	2-5.5
C18:1 oleic	23.09 (22.07-23.91)	83.84 (80.02-85.38)	20-50
C18:2 linoleic	55.36 (53.61-56.48)	2.23 (1.19-4.83)	35-60
C18:2 9,15 linoleic isomer	0.00	0.48 (0.37-0.56)	-
C18:3 linolenic	7.35 (6.81-8.35)	3.47 (2.87-4.51)	2-13

[#] mean values, the range in brackets

The results from the two separate analyses demonstrate that the high oleic acid soybeans differ significantly from the parental soybean line in the levels of oleic, linoleic, linolenic and palmitic acid present in the oil. Oleic acid levels have been significantly increased and this has resulted in concomitant decreases in the levels of palmitic, linoleic and linolenic acids. The levels of other fatty acids present in the oil were similar between the parental and high oleic acid soybean lines and were comparable to the Codex Alimentarius ranges for soybean oil. High levels of oleic acid are commonly consumed in other premium edible oils (e.g., olive oil, high oleic acid sunflower and canola oils). The increased oleic acid levels do not pose a safety concern.

In addition to the expected changes to the fatty acid composition of oil from the high oleic acid soybean lines, a trace amount (less than 1% of the total fatty acid content) of the 9,15 isomer of linoleic acid (cis-9, cis-15-octadecadeinoic acid), normally found only in hydrogenated soybean oils and butterfat, was also detected. This isomer is not present in the oil of the parental soybean line A2396.

The applicant speculates that the presence of the isomer is the result of activity of a δ-15 (n-3) desaturase (*GmFad3*), which normally inserts a δ-15 double bond into 9,12-linoleic acid. In the transgenic plants, the linoleic acid content is reduced from >50% of the total fatty acids to <2% and therefore they speculate that the *GmFad3* enzyme probably creates a small amount of the isomer by putting a δ-15 double bond into 9-oleic acid. The applicant provided data to support this hypothesis where the high oleic acid soybeans were crossed with a soybean containing a suppressed *GmFad3* gene. In the resulting progeny, the isomer is either reduced or virtually eliminated.

The applicant provided data on the occurrence of the 9,15 isomer of linoleic acid in commonly used oils and fats for frying and baking in Europe. This data is presented in Table 7 below.

Table 7: Occurrence of the 9,15 linoleic acid isomer in commonly used oils and fats for frying and baking

	Fatty acid composition (g/ 100 g fatty acid)					
Oil/fat	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:2 (9,15)**	**C18:3**
Palm olein, partially hydrogenated	20.8	4.0	48.3	22.4	1.3	0.8
Soybean oil, partially hydrogenated	10.8	5.8	44.8	21.4	3.4	0.7
Rapeseed oil, partially	5.6	3.8	72.0	8.9	2.7	1.3

hydrogenated						
Butter fat	34.8	11.7	26.6	2.6	0.4	0.8

This data shows that the 9,15 isomer of linoleic acid is commonly found in other edible sources of fat such as butterfat and partially hydrogenated vegetable oils at a range of 0.4-3.4% of the total fatty acids. Therefore, its occurrence in high oleic acid soybean oil at a level of 0.5% of the total fatty acids (representing about 25% of the linoleic acid fraction) is not considered to pose any safety concerns.

Vitamins and minerals

The high oleic acid soybean lines G94-1, G94-19 and G168 and the parental soybean line A2396 were analysed for their mineral and vitamin content including tocopherols. The tocopherols, also known as vitamin E, exist as four isomers (α-, β-, γ-, and δ-tocopherol). The four isomers are not equivalent, with α-tocopherol being the most important in terms of bioactivity. The Recommended Daily Intake (RDI) for vitamin E is normally presented as α-tocopherol equivalents. The results of the vitamin and mineral analyses are summarised in Table 8 below.

Table 8: Vitamin and mineral content[*] of the control and high oleic acid soybeans

Vitamin or mineral[#]	Parental control	High oleic acid lines	Literature range
	(mg/100 g dry weight unless noted)		
Minerals:			
Calcium	264 (245-302)	232 (212-251)	132.7-326.3
Copper	0.64 (0.30-1.00)	0.67 (0.24-1.02)	0.9-5.1
Iron	5.6 (4.2-7.4)	5.8 (3.8-7.9)	3.2-7.9
Magnesium	247 (232-260)	236 (215-261)	
Manganese	2.9 (1.9-4.0)	2.7 (2.2-3.6)	0.4-6.8
Phosphorous	621 (516-742)	636 (501-771)	378-1836
Potassium	1755 (1468-1950)	1689 (1492-1896)	859-1784
Sodium	3.1 (1.1-6.5)	4.3 (2.2-8.7)	
Zinc	4.0 (3.2-4.7)	4.3 (3.0-5.8)	
Vitamins:			
Vitamin B6	0.115 (0.098-0.131)	0.125 (0.110-0.141)	
β-carotene (IU/100 g dry wt)	8 (5-12)	10 (5-16)	
Vitamin B1	0.96 (0.74-1.17)	0.89 (0.63-1.24)	
Vitamin B2	0.29 (0.26-0.30)	0.30 (0.27-0.35)	
Vitamin E (IU/100 g dry wt)	1.2 (1.1-1.6)	1.1 (0.9-1.7)	
Niacin	2.6 (2.28-2.88)	2.74 (2.38-3.15)	
Pantothenic acid	1.051 (0.936-1.132)	0.961 (0.794-1.063)	
Folic acid (μg/100 g dry wt)	274 (184-379)	284 (186-384)	
Tocopherols:			
Total	20.11 (18.01-22.50)	18.57 (16.36-21.16)	
alpha	1.37 (1.11-1.62)	1.32 (1.06-1.62)	1.09-2.84
beta	0.17 (0.07-0.20)	0.22 (0.15-0.30)	<0.5
gamma	16.17 (14.03-18.81)	15.42 (13.12-17.58)	15.0-19.1
delta	1.72 (1.52-2.11)	1.88 (1.61-2.28)	2.46-7.25

[#] all samples contained less than 0.1 μg/100 g vitamin B12, less than 1.0 mg/100 g vitamin C and less than 5 IU/100 g retinol

[*] mean values, the range in brackets.

No significant differences in mineral or vitamin content, including tocopherols, were observed between the high oleic acid soybeans and the parental soybean line. The mineral content of the high oleic acid soybeans was within the literature reported ranges. With the exception of the tocopherols, literature ranges for vitamin content was not provided. The

delta tocopherol content was lower than the literature reported range for both the parental control and high oleic acid soybean lines. The content of the other tocopherols in the high oleic acid soybeans were within the literature reported ranges for soybeans.

Isoflavones

Soybeans naturally contain a number of isoflavone compounds reported to possess biochemical activity, including estrogenic and hypocholesterolemic effects, in mammalian species. Isoflavones (known to include phytoestrogens) have, in the past, also been regarded as anti-nutrients, however, this is no longer universally accepted as isoflavones have also been reported to have beneficial anti-carcinogenic effects. The major isoflavones in soybeans and soybean products include daidzin, genistin, and their corresponding aglycons, daidzein and genistein. Glycitin and glycitein also occur in trace amounts.

High oleic acid soybean lines G94-1 and G94-19 and parental soybean line A2396 were analysed for isoflavone content. The results are summarised in Table 9 below.

Table 9: Isoflavone content[#] of parental and high oleic acid acid soybean lines

Isoflavone	Parental control	High oleic lines	Literature range
		(µg/g dry weight)	
Total daidzein	693 (623-762)	612 (525-694)	295-1527
Total genistein	714 (574-854)	724 (548-910)	416-2676
Total glycitein	192 (188-196)	273 (261-287)	149-341

[#] mean values, range in brackets

There are no significant differences between the parental soybean and the high oleic acid soybean lines G94-1 and G94-19 in either total daidzein or genistein content which is also within the literature reported ranges for soybeans. In relation to total glycitein content, however, the high oleic acid soybean lines exhibit slightly elevated levels compared to the control. The level reported for total glycitein however is within the literature reported range therefore this slightly elevated level compared to the control is not considered to pose any safety concerns.

Key toxicants

The only naturally occurring toxicants in soybeans are lectins. Lectins are proteins that bind to carbohydrate-containing molecules and which inhibit growth and sometimes cause death in animals. It is reasonable to assume that similar effects would occur in humans. Lectins, however, are rapidly degraded upon heating, and therefore only become an issue when raw soybeans are consumed. There are no human food uses for raw soybeans.

Notwithstanding that there are no human food uses for raw soybeans, the applicant undertook compositional analyses for lectin content of seeds from the high oleic acid soybean lines. The seeds represent the R6 generation of the high oleic acid soybean lines. Lines G94-1, G94-19 and G168 were grown in parallel with the parental line A2396 at four locations in the United States in the summer of 1996. To obtain the data, three replicates were analysed in duplicate from each of the four locations. The results of these analyses are summarised in Table 10 below.

Table 10: Lectin content[#] of parental and high oleic acid soybean lines

Lectin	Parental control	High oleic acid soybeans	Literature range
HU[1]/mg extracted protein	6.36 (4.09-7.90)	7.83 (5.37-9.70)	2.7-12.5
HU/mg total protein	2.98 (2.30-3.90)	3.67 (2.77-4.73)	1.2-6.0
HU/mg sample (FW basis)	1.03 (0.70-1.30)	1.32 (0.97-1.67)	0.5-2.4

[1] HU = haemagglutinating unit, # mean values, the range in brackets

The high oleic acid soybean lines exhibit slightly elevated lectin levels when compared to the control. The values reported however are well within the literature reported range for soybeans. As lectins are readily degraded upon heating, and the levels reported are still within the literature reported range, the slightly elevated levels do not represent a safety concern.

Key anti-nutrients

Soybeans contain two well-described anti-nutritional factors. These are trypsin inhibitors and phytic acid. Trypsins inhibitors are heat labile anti-nutrients which interfere with the digestion of proteins and result in decreased animal growth. Because they are heat labile, however, they are destroyed during the processing of soy products by heat treatment. Phytic acid, on the other hand, remains stable through most soybean processing steps and has been implicated in interfering with the bioavailability of minerals such as calcium, magnesium and zinc.

Seed representing the R6 generation of lines G94-1, G94-19 and G168 were analysed for trypsin inhibitor and phytic acid content. The results are summarised in Table 11 below.

Table 11: Anti-nutrient content[#] for parental and high oleic acid soybeans

Anti-nutrient	Parental control	High oleic acid lines	Literature ranges
Trypsin inhibitor (TIU/mg dry wt)	31.67 (22.84-40.47)	30.20 (14.21-42.43)	26.4-93.2
Phytic acid (g/100 g dry wt)	1.42 (1.32-1.53)	1.42 (1.25-1.69)	1.3-4.1

[#] mean values, the range in brackets

No significant differences were observed between the parental soybean line and the high oleic acid soybean lines for either of the anti-nutrients. The values reported are comparable to the literature reported ranges.

Other constituents

The fermentable galacto-oligosaccharides, raffinose and stacchyose, are present in soybeans and can be responsible for the production of unpleasant side-effects, such as flatulence, when soybeans and soybean products are ingested. The processing of soybean flours into concentrates and isolates removes these oligosaccharides. Seeds representing the R4 and R5 generations of lines G94-1 and G94-19 were analysed for raffinose and stacchyose content. The results of the analyses are summarised in Table 12 below.

Table 12: Stacchyose and raffinose content[#] of parental and high oleic acid soybeans

Constituent	Parental control	High oleic acid soybean	Literature range
		(μmoles/g dry weight)	
Stacchyose	63 (60-67)	68 (65-75)	44.8-68.8
Raffinose	14 (14-14)	15 (14-16)	8.6-18.5

[#] mean values, the range in brackets

No significant differences were observed between the parental soybean line and the high oleic acid soybean lines for stacchyose and raffinose content. The values reported are comparable to the literature reported ranges.

Naturally occurring allergenic proteins

Study evaluated:
Lehrer, S. (1996). Allergenicity of high oleic acid soybeans. Tulane University School of Medicine, Section of Allergy and Clinical Immunology, New Orleans, LA, USA.

The protein profile of the high oleic acid soybeans was found to be different in a number of respects to that of the parental soybean line A2396.

Soybean 7S and 11S globulins are two major storage proteins accounting for about 70% of total meal protein. The 7S fraction is made up of the α, α^1, and β subunits of β-conglycinin. The 11S fraction is made up of the acidic (A) and basic (B) subunits of glycinin. The high oleic acid soybeans were found to have reduced concentrations of the α and α^1 subunits of β-conglycinin, when compared with the parental A2396 soybean lines. This was coincident with an increase in the concentration of the A and B subunits of glycinin in addition to an increase in the concentration of the A2B1A glycinin precursor. The profile of other storage proteins appears to be identical to that of A2396.

The applicant speculates that the reduction in concentration of the β-conglycinin α and α^1 subunits is due to co-suppression by the α^1 promoter sequence used in the *GmFad 2-1* vector (pBS43). The phenomenon of co-suppression has been observed for other genes and plants and is well documented in the literature (Brusslan and Tobin, 1995).

A study was done to determine whether alterations to the protein profile of the high oleic acid soybeans had changed their allergenicity relative to the parental soybean line.

Radioallergosorbent (RAST) reactivity

Extracts were made of the parental soybean line A2396 and high oleic acid soybean line G94-1. Sera were used from 31 subjects with a history of documented soybean or food allergy, a positive skin test to soybean extract, and/or a positive IgE antibody response to soybean extract. Control sera were obtained from soybean tolerant individuals with a negative skin test and/or RAST to soy extract with total IgE levels similar to those sera of soybean-sensitive subjects.

In RAST reactivity assays many of the sera demonstrated significant IgE antibody reactivity to soybean extracts. Twenty-one of the 31 sera tested had IgE antibody % binding greater than or equal to 4 %. Eleven of the 21 positive sera had IgE antibody binding in excess of 20%. The sera with the most significant RAST reactivity were pooled for RAST inhibition studies.

RAST inhibition

Both the parental and high oleic acid soybean extracts yielded virtually identical RAST inhibition curves to the parental soybean RAST.

Immunoblot analysis

The 21 most potent RAST positive sera were selected for immunoblot analyses of soybean allergens. The immunoblot analysis showed, as expected, that there are a number of proteins in the soybean extract that bind IgE antibodies from soybean allergic sera. Some sera were more reactive than others, so six of the most reactive sera were selected and pooled for further study of the allergens present in the parental and high oleic acid soybeans. Both colourimetric and chemiluminescence techniques were used for the detection of reactive protein bands.

The results show that there are no significant differences in the number of protein bands to which the sera react or to the intensity of the IgE reactivity.

Conclusion

There are no significant differences in the allergen content of the high oleic acid soybeans compared to the parental soybean line A2396.

NUTRITIONAL IMPACT

Animal feeding studies

In assessing the safety of food produced using gene technology, 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, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further re-assurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

In the case of the high oleic acid soybeans, the extent of the compositional and other data provided in the application is considered adequate to establish the safety of the food. Nonetheless, the applicant also provided two animal feeding studies to compare the wholesomeness of the high oleic acid soybeans with controls. Although not considered essential for establishing safety in this instance, these animal feeding studies have been reviewed as additional supporting data.

Studies evaluated:

Loughmiller, J.A. *et al* (1997). Influence of soybean meal variety and processing temperature on the growth performance of pigs from 25 to 45 lb. Animal Science Department, Kansas State University, Kansas, United States.

Araba, M. and Lohrmann, T.T. (1997). The effect of heat processing on two soy varieties as measured in growing broiler chicks. Poultry Science Department, University of Georgia, Athens, Georgia, United States.

Pig feeding study

This study was done to determine if soybean meal produced from high oleic acid soybeans would provide similar levels of growth performance in pigs as soybean meal from traditional varieties.

Three hundred and ninety (39/group) high-lean growth pigs (Newsham Hybrids) were fed diets consisting of processed soybean meal from either the high oleic acid soybean lines or a standard check-line soybean. The soybeans used to make the meal were processed at four different temperature ranges (80-85, 85-90, 90-95, 100-105 °C) under conditions that simulated commercial processing. Positive and negative control diets were made using commercially available soybean meal (46.5% crude protein). The positive control diet was formulated to contain dietary 1.3% lysine whereas the negative control diet was formulated to contain 0.95% dietary lysine. All test diets also contained 0.95% lysine so that any differences in growth performance could be readily attributable to the processing temperature or the amino acid availability. All pigs were fed a common 3 stage diet series until being placed on the test diets at 21 days post weaning. All test diets were corn-soybean meal based and were fed until 38 days post weaning.

Growth performance of the pigs is indicated by the average daily gain (ADG) as well as the F/G ratio, which is a measure of the amount of the feed consumed (the average daily feed intake - ADFI) / ADG or, in other words, is an indication of how much food (in pounds) it takes to put on 1 lb of body weight in the animal. The F/G ratios obtained over the course of the study are provided in Table 13 below.

Table 13: Effect of soybean meal varieties and processing temperature on pig F/G ratios

	Day 0 to7	Day 7 to 14	Day 14 to 17	Day 0 to 17
Commercial meal:				
1.3% lysine	1.44	1.49	1.69	1.50
0.95% lysine	1.71	1.74	1.92	1.75
High oleic acid meal (0.95% lys):				
80-85°C	2.38	2.42	3.56	2.49
85-90°C	1.72	1.84	1.96	1.80
90-95°C	1.84	1.74	1.83	1.78
100-105°C	1.79	1.86	1.86	1.83
Check-line meal (0.95% lys):				
80-85°C	1.75	1.86	2.03	1.84
85-90°C	1.92	1.79	1.86	1.83
90-95°C	1.82	1.82	1.87	1.81
100-105°C	1.95	1.80	2.28	1.91

Pigs fed the positive control diet (commercially available soybean meal formulated to contain 1.3% dietary lysine) had increased performance (as measured by the ADG and the F/G ratio) than pigs fed any other treatment. This indicates that a dietary lysine content of 0.95% was insufficient to maximise growth performance of the pigs.

Pigs fed diets containing high oleic acid soybean meal were shown to have a similar growth performance compared to pigs fed diets containing either commercial soybean meal or meal

derived from the check-line soybean formulated to similar lysine levels, when the high oleic acid soybean meal is processed at temperatures above 80-85 °C. The reason for the decreased performance, compared to the control, of pigs fed the high oleic acid soybeans processed at 80-85 °C is not readily apparent. The applicant speculates that the difference may be due to difficulties experienced with the processing of the soybeans in the pilot processing plant.

Chicken feeding study

This study was done to determine the effects of five different processing temperatures on the feeding value of the parental soybean line compared to the high oleic acid soybean lines.

Six hundred and sixteen (56/group) 1-day-old broiler chicks (Peterson x Arbor Acre) were randomly allotted to one of 11 dietary treatments. The chicks were fed diets consisting of soybean meal obtained from either a standard check-line soybean or the high oleic acid soybean lines and which had been processed at five different processing temperatures (raw, 80-85, 85-90, 90-95, and 100-105 °C). A positive control diet was included using commercially obtained high protein soybean meal. Test diets using the check-line soybean meal or the high oleic acid soybean meal were formulated to meet all nutrient requirements except for the amino acid concentration. The positive control diet contained 23% crude protein and 1.2% lysine, while diets containing check-line or high oleic acid soybean meal contained 20% crude protein and 1.03% lysine. Growth performance was measured by daily weight gain, the feed conversion ratio (feed:gain), and final body weight. The results are summarised in Table 14 below.

Table 14: Effects of processing temperature and soybean meal source on chick performance

	Daily gain 0-18 d (g)	Feed intake 0-18 d (g)	Feed:gain 0-18 d (g)	Body weight 0-7 d (g)	Body weight 0-18 d (g)
Raw:					
Commercial	26.95	37.86	1.417	148.2	525.1
High oleic	15.35	30.25	1.953	101.8	316.3
Check-line	17.57	33.28	1.897	111.4	356.2
80-85 °C:					
High oleic	23.60	36.66	1.570	129.6	464.8
Check-line	23.85	38.19	1.598	134.7	469.3
85-90 °C:					
High oleic	24.96	38.83	1.558	136.5	489.3
Check-line	22.51	34.96	1.561	129.5	445.1
90-95 °C:					
High oleic	25.71	39.53	1.540	1.45.4	502.7
Check-line	23.66	36.95	1.564	126.8	465.9
100-105 °C:					
High oleic	24.03	39.07	1.628	135.0	472.5
Check-line	22.40	35.89	1.604	122.4	443.3

The results show that birds fed the 1.2% lysine diets (commercial soybean meal) performed significantly better in terms of their daily weight gain, feed conversion (feed:gain) and final body weight when compared to the test diets. This result is most likely attributable to the lower amino acid content of the test diets, although may also be due to differences in processing.

No significant differences in performance, in either the daily weight gain or the feed conversion, between the parental soybean line and the high oleic acid soybean line were observed.

Conclusion

Interpretation of both feeding studies is complicated by the fact that they were designed to look at the effect of a number of different parameters, other than soybean variety, on feeding performance (e.g., lysine content, processing temperature). Nevertheless, both demonstrate that the high oleic acid soybeans are equivalent to the commercial varieties of soybean in their ability to support typical growth and well-being in pigs and chickens.

Human nutritional impact

Studies evaluated:
The effect of using high oleic acid soybean oil to replace frying fats in targeted foods on the fatty acid composition of the diets of British adults. 1998. Study undertaken by Nutriscan Ltd, a non-profit making campus company of Trinity College, Dublin, Ireland.

To assess the nutritional impact of high oleic acid soybean oil the applicant commissioned a study on the effect of high oleic acid soybean oil on the balance of dietary fats in the human diet using dietary and nutritional survey data for British adults.

The fatty acid composition of high oleic acid soybean oil was compared with those of commercial shortenings and frying oils sourced from Europe and the United States. The key findings of these comparisons are:

- the level of saturated fatty acids in high oleic acid soybean oil is similar to that in non-hydrogenated or lightly hydrogenated oils and is considerably lower than most European shortenings;
- compared with frying oils with comparable levels of monounsaturated fatty acids, high oleic acid soybean oil has higher levels of n-6 polyunsaturated fatty acids (primarily linoleic acid);
- high oleic acid soybean oil is comparable with other frying oils for n-3 polyunsaturated fatty acids (primarily linolenic acid);
- high oleic acid soybean oil does not contain any of the trans isomers of unsaturated fatty acids found in many commercial shortenings.

For the dietary analysis two scenarios were modelled on the assumption that high oleic acid soybean oil replaced all oils present in savoury snacks, fried potatoes including chips and vegetables. It also assumed that frying oil accounted for 17% of the fat in all fried meat, eggs and fish. Because the composition of endogenous fat in the fried animal foods was not known, it had to be estimated for each food by difference between total fatty acids and a frying oil of known composition. In scenario I, a worst-case scenario, all the oil used for frying meat, eggs and fish was assumed to be a high n-6 polyunsaturated fatty acid (52.8%) corn oil. In scenario II, a more realistic scenario, the oil was assumed to be a

palmolein/rapeseed (80:20) blend (12.3 % n-6 polyunsaturated fatty acids). Assumptions also had to be made about the level of n-6 polyunsaturated fatty acids in high oleic acid soybean oil as this level can be influenced by crop growth conditions. Commercially available high oleic acid soybean oil is anticipated to contain 2.2% n-6 polyunsaturated fatty acids but batches as low as 0.9% have been observed under certain field conditions. A n-6 polyunsaturated fatty acid content of 0.9% for high oleic acid soybean oil was assumed for scenario I and 2.2% was assumed for scenario II.

A summary of the main findings of the analysis is presented in Table 15 below.

Table 15: The effect of replacing all oils and fats used in the domestic and commercial frying with high oleic acid soybean oil (values are means ± standard deviations)

		High oleic acid soybean oil usage	
% energy from:	**Current diet[1]**	**Scenario I**	**Scenario II**
Saturated fatty acids	17.24 ± 3.44	16.61 ± 3.44	16.43 ± 3.43
Monounsaturated fatty acids	12.63 ± 2.15	14.97 ± 2.98	14.68 ± 2.86
n-3 polyunsaturated fatty acids	0.78 ± 0.27	0.73 ± 0.23	0.78 ± 0.23
n-6 polyunsaturated fatty acids	5.51 ± 2.15	3.89 ± 1.98	4.33 ± 1.92
Trans unsaturated fatty acids	2.24 ± 0.83	2.15 ± 0.83	2.12 ± 0.83

[1] no high oleic acid soybean oil usage

The analysis shows that the impact of the high oleic acid soybean oil use on the intakes of saturated fatty acids is quite small, equivalent to a 5% reduction at best, with little difference between the two scenarios. The intake of monounsaturated fatty acids would increase at best by 19%, with again little difference between the two scenarios. The intake of n-6 polyunsaturated fatty acids would fall by 29% for scenario I and by 21% for scenario II. The analysis also shows that there would be little or no change to the intakes of n-3 polyunsaturated fatty acids or trans unsaturated fatty acids with either scenario.

To put the use of high oleic acid soybean oil into context, the analysis was repeated using a low n-6 olive oil (79.3% monounsaturated fatty acids, 0.7% n-3 polyunsaturated fatty acids and 6% n-6 polyunsaturated fatty acids) to replace all of the fats and oils considered in the analysis. The results of this analysis are presented in Table 16 below.

Table 16: A comparison of the effect of replacing all oils and fats used in frying and in the manufacture of savoury snacks with either high oleic acid soybean oil or olive oil (values are means)

		% energy from			
Oil	**Scenario**	**Mono**	**n-6 poly**	**n-3 poly**	**Saturated**
High oleic	I	15.7	3.2	0.8	16.6
Olive	I	15.6	3.3	0.7	16.7
High oleic	II	15.1	4.2	0.8	16.1
Olive	II	15.0	4.3	0.8	16.2
CURRENT UK DIET		12.6	5.5	0.8	17.2

This analysis shows that, were low n-6 olive oil to replace all the fats considered in the analysis, the impact would be very similar to that of high oleic acid soybean oil under similar conditions.

The study concluded that while the use of high oleic acid soybean oil might lower dietary linoleic acid intake somewhat (by an absolute maximum of 29%), it would not do so to any level that would be a public health concern in terms of cardiovascular disease. Moreover, it

was concluded that such a reduction could apply equally to many existing commercially available low n-6 polyunsaturated frying oils, such as olive oil.

Therefore, the overall finding of the study was that the nutritional impact of the use of high oleic acid soybean oil as a replacement for frying fats was likely to be beneficial because diets incorporating high oleic acid soybean oil show decreased saturated fatty acid intakes and this is likely to reduce risk factors for cardiovascular disease.

The general conclusion of this report can be applied to the Australian context although the magnitude of the changes is likely to be reduced. Table 17 shows a comparison of the fatty acid profiles of the United Kingdom and Australia from recent national dietary surveys.

Table 17: A comparison of mean percentage energy from fatty acids in British and Australian diets

Country	Mean % Energy from fatty acid type		
	Mono	**Poly**	**Saturated**
United Kingdom	12.6	6.3	17.2
Australia	11.8	5.0	12.7

The fall in mean polyunsaturated intakes quoted for the British case above assumes 100% replacement. In reality, this is unlikely to happen, and data given in the report show that, with successive reductions in the % replacement, intakes progressively increase towards original levels. For example at 25% percent replacement, percentage energy from PUFA decreases to 6.0%.

There are some high monounsaturated oils available or soon to be available on the Australian market that have been created through conventional plant breeding and selection techniques from sunflower and rapeseed stock. These types of oils have been successful in replacing a proportion of palm oil mixes in food manufacture and retail frying. Olive oil has also become a popular oil for domestic use.

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