

Estudio de caso 2

Evaluación
de la inocuidad
de la soja
genéticamente
modificada
de alto contenido
en ácido oléico

Safety
assessment of
genetically modified
high oleic acid
soybeans

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Preface

The sale of food derived from high oleic acid soybean lines G94-1, G94-19 and G168 (Application A387) was approved in Australia and New Zealand in November 2000, following completion of a comprehensive safety assessment. Food Standards Australia New Zealand (FSANZ) conducts the safety assessments of genetically modified foods based upon internationally accepted principles for establishing the safety of foods derived from GM plants.

The findings of the FSANZ safety assessment were published as the “Final Risk Analysis Report: Application A387 - Food derived from high oleic soybean lines G94-1, G94-19, and G168”.

Parts of the data and information on high oleic acid soybeans provided to FSANZ for assessment have been summarised into this case study for training purposes.

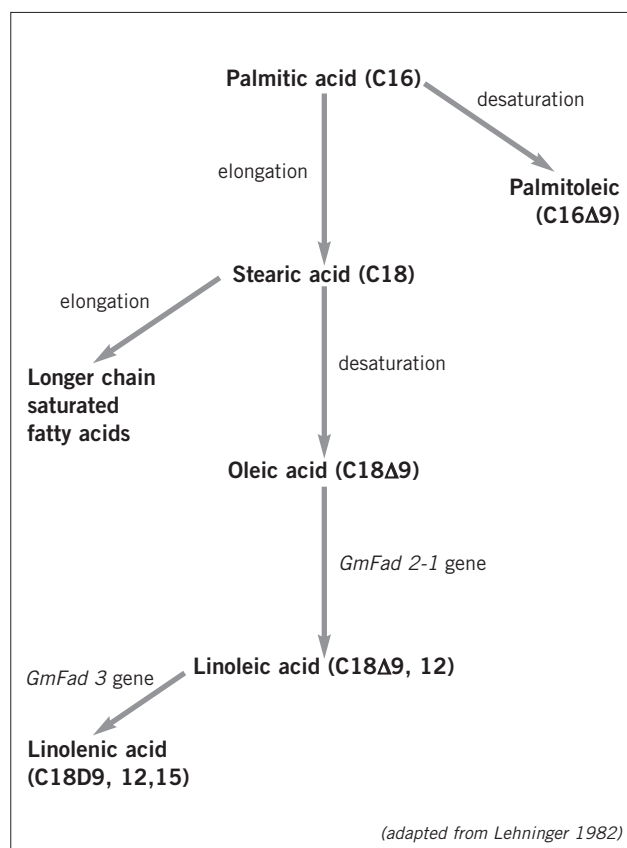
Disclaimer

In order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original application. Certain information has been reduced to summaries and the present data as presented in the case study are only a subset of that actually submitted. The case study in no way constitutes a complete application nor is it to be considered a complete safety assessment. To that end, the use of this information in the form of a training tool does not constitute an endorsement of the information or product nor should it be considered a reflection of the original submission.

Description of the recombinant-DNA plant

Optimum Quality Grains LLC (a joint venture between DuPont and Pioneer Hi-Bred International, Inc) originally intended to develop soybeans with two introduced traits: (a) increased lysine in the meal fraction and (b) increased oleic acid, a monounsaturated fatty acid, in the oil fraction. However, during development, it was decided not to pursue the high-lysine trait. The new variety therefore has been genetically modified only to contain increased levels of oleic acid. The soybeans are referred to as high oleic acid soybeans.

The high oleic acid trait was 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. 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. The pathway for the synthesis of long chain fatty acids in plants is depicted below.

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 standard soybean oil because of its reduced levels of the oxidatively unstable polyunsaturated fatty acids. This means that high oleic acid soybean oil may 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 conventional soybean oil or partially hydrogenated soybean oil because of the increased levels of monounsaturated fatty acids.

Oil from high oleic 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 oil/vegetable oil blends.

Description of the host plant and its use as food

Soybeans (*Glycine max*) are grown as a commercial crop in over 35 countries worldwide and have a long history of safe use as both human food and stockfeed. The major producers of soybeans are the United States, Argentina, Brazil and China, accounting for 90% of world production.

There are three major soybean commodity products: seeds, oil and meal. There is only limited feed use, and no food use, for unprocessed soybeans, as they contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors, making them unsuitable for human consumption. 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.

Finished food products containing soybean ingredients therefore include beer, noodles, breads, flours, sausage casings, pastries, crackers, meat substitutes, milk substitutes and confectionery among other things.

The elite soybean cultivar A2396, which has been used as the host for the high oleic acid trait described in this application, is an Asgrow Seed Company early Group II maturity soybean variety that has high yield potential. Protein and oil characteristics are said to be similar to other soybeans at 40% protein and 22% oil on a dry weight basis.

Description of the genetic modification

Methods used in the genetic modification

Plasmid DNA carrying the genes of interest, was introduced into meristem tissue of elite soybean line A2396 by microprojectile bombardment, or biolistic transformation. The bombarded cells are incubated on a tissue culture medium, which supports callus growth. The cells that have taken up the DNA were selected by picking those that express an introduced marker gene, GUS (a fluorescent marker protein).

Novel genes

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 in plants can occur at both transcriptional (TGS) and post-transcriptional (PTGS) levels. The primary mechanism of TGS is thought to be methylation of the promoter sequences. Methylation of promoters is thought to block their interaction with transcription factors or alter the chromatin structure of the DNA thus suppressing transcription, however these mechanisms remain unclear (Wang and Waterhouse, 2001). PTGS was initially referred to as ‘co-suppression’ because in experiments involving the transformation of petunia with a sense chalcone synthase transgene the expression of both the transgene and the corresponding endogenous gene was suppressed. PTGS involves the

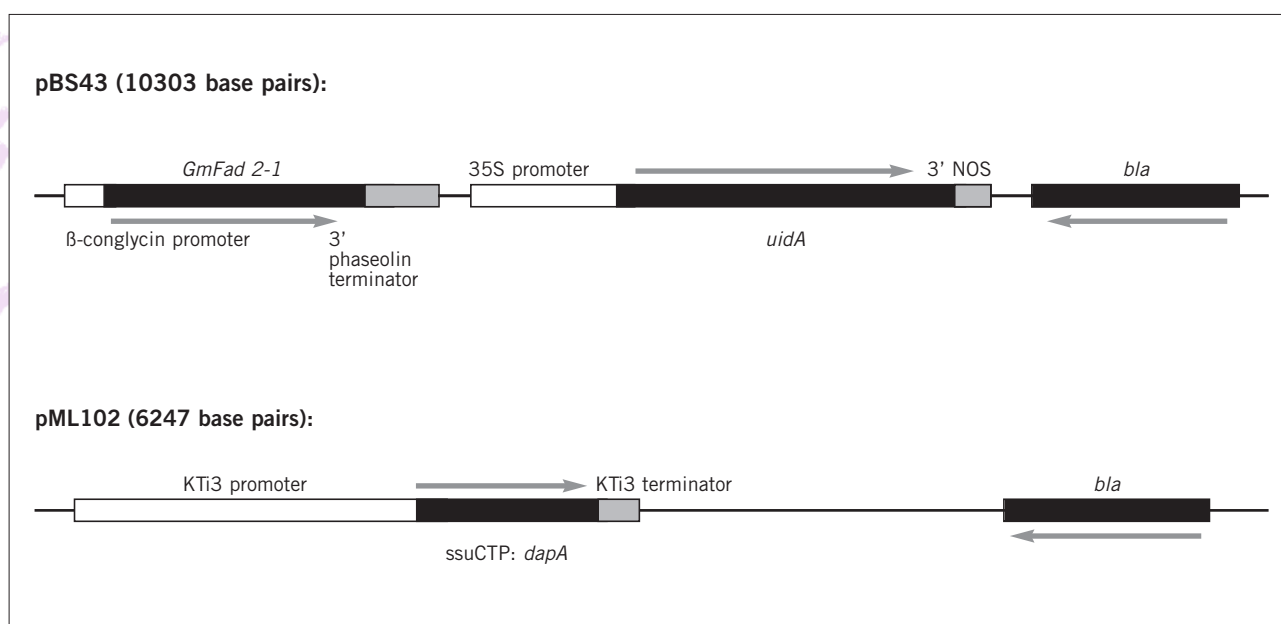


Table 1: Description of the gene expression cassettes in pBS43 and pML102

Cassette	Genetic element	Source	Function
<i>GmFad 2-1</i> expression cassette (pBS43)	β -conglycinin promoter	α^1 -subunit of β -conglycinin seed storage protein of soybean (Barker <i>et al.</i> 1988)	Seed specific promoter that allows high level gene expression during seed development
	<i>GmFad 2-1</i> coding region	Protein coding sequence of the δ -12 fatty acid desaturase from soybean (Okuley <i>et al.</i> 1994, Heppard <i>et al.</i> 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 <i>Phaseolis vulgaris</i> (Doyle <i>et al.</i> 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 <i>et al.</i> 1985)	Promoter of high level constitutive gene expression in plant tissues
	<i>Cab 22L</i> non-translated leader	The 5' untranslated leader from the photosynthetic 22L chlorophyll a/b binding protein (<i>Cab22L</i>) promoter of <i>Petunia hybrida</i> var. Mitchell (Harpster <i>et al.</i> 1988)	The untranslated leader sequence helps to stabilise mRNA and improve translation
	<i>uidA</i> coding region	Protein coding sequence of the enzyme β -glucuronidase (<i>uidA</i> gene) from <i>Escherichia coli</i> (Jefferson <i>et al.</i> 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 <i>Agrobacterium tumefaciens</i> (Depicker <i>et al.</i> 1982, Bevan <i>et al.</i> 1983)	Contains signals for termination of transcription and directs polyadenylation
<i>dapA</i> 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 <i>et al.</i> 1982)	Directs the protein into the chloroplast which is the site of lysine biosynthesis
	<i>dapA</i> coding region	Coding sequence of the <i>Corynebacterium</i> <i>dapA</i> gene encoding the lysine insensitive version of the enzyme dihydrodipicolinic acid synthase (DHDPS) (Bonnassie <i>et al.</i> 1990, Yeh <i>et al.</i> 1988)	Expression of <i>Corynebacterium</i> DHDPS deregulates the lysine biosynthetic pathway resulting in accumulation of free lysine (Falco <i>et al.</i> 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

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

Cassette	Genetic element	Source	Function
<i>lac</i>	An incomplete copy of the <i>lac</i> operon which contains a partial <i>lacI</i> coding sequence, the promoter P_{lac} , and a partial coding sequence for β -D-galactosidase (<i>lacZa'</i>)		These genes are not intact and no longer function in <i>E. coli</i>
<i>ori</i>	Origin of replication from the high copy number <i>E. coli</i> plasmid pUC19		Allows plasmids to replicate in <i>E. coli</i>
<i>bla</i>	Gene coding for the enzyme β -lactamase from <i>E. coli</i>		Confers ampicillin resistance to <i>E. coli</i>
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

failure to accumulate messenger RNA in the cytoplasm and thus no expression products are produced. It is now widely accepted that double stranded RNA can cause PTGS in plants through a process that involves sequence-specific RNA degradation (Voinnet, 2002).

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).

uidA gene

In addition to the primary genes, the soybeans also contain a visual marker gene, the *uidA* gene from *Escherichia coli* (Jefferson *et al.*, 1985). The protein product of this gene, β -glucuronidase (GUS), is an enzyme that can be used to catalyse a colourimetric

reaction resulting in the production of a blue colour in transformed plant tissues.

Gene constructs

Two circular plasmids were used in the transformation, pBS43 and pML102, containing the three gene expression cassettes, one for each gene of interest, *GmFad 2-1* and *dapA*, and one for the reporter gene, *uidA*. Both plasmids pBS43 and pML102 also contained the antibiotic resistance marker gene, *bla*. The plasmids are shown in the diagram (Fig. 1) in linear form, with the novel genes in black. Table 1 contains a description of each gene and its regulatory elements.

Other genetic elements

In addition to the gene expression cassettes described in Table 1 above, a number of other genetic elements, including the antibiotic resistance marker gene, were also present in the plasmid DNA. These genetic elements are described in Table 2.

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 genetic modification

Selection of plant lines

The method used in the transformation did not necessarily result in the successful transfer of both plasmids to the soybeans, therefore a large number of transformed plants needed to be screened to identify those with the two traits of interest.

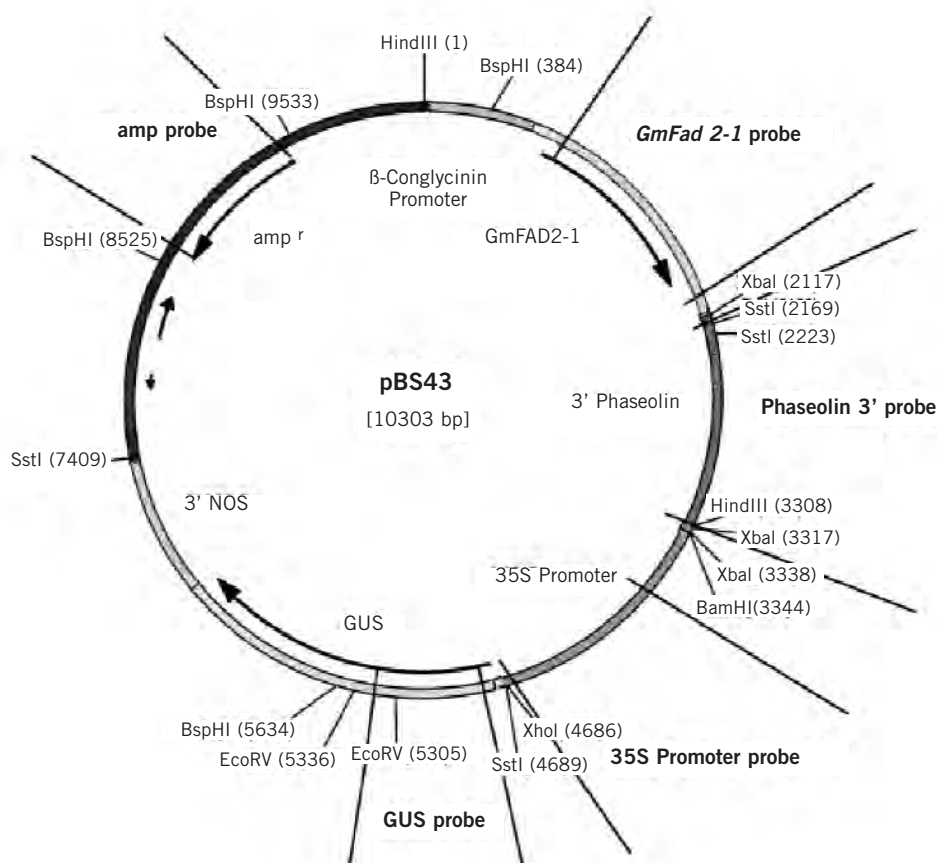


Figure 1: Plasmid map of pBS43. Figure indicates the location of hybridisation probes and restrictions enzyme sites used for Southern blot analysis of high oleic soybeans.

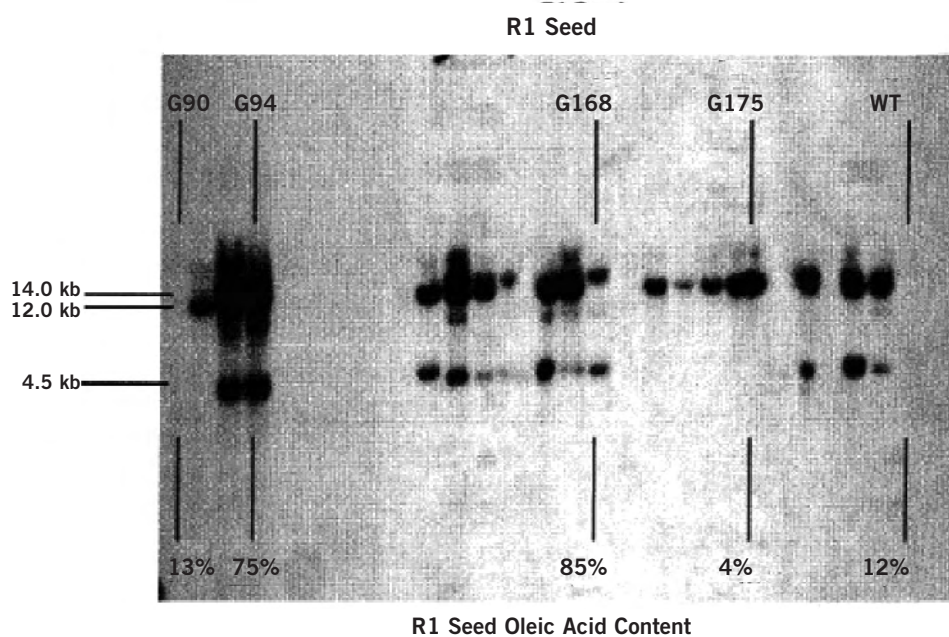


Figure 2. Southern blot of DNA isolated from leaf tissue of event 260-05 R1 plants. Plants were grown from chipped seeds analysed for fatty acid composition. The genomic DNA was digested with *Bam*HI and probed with the phaseolin 3' probe to detect the integration of the *GmFad 2-1* construct.

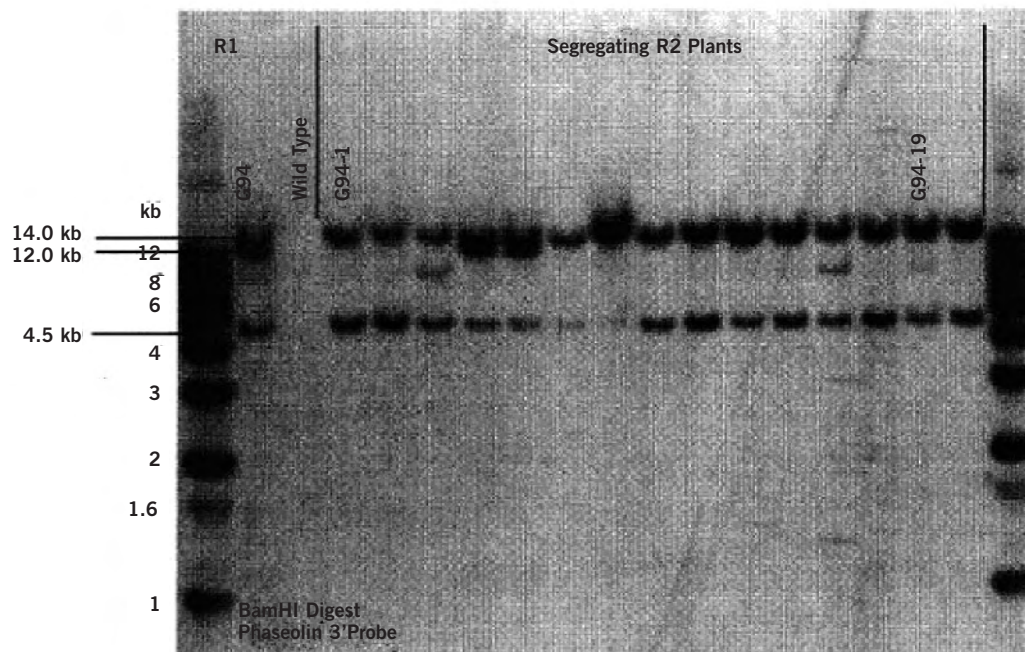


Figure 3. Southern blot on R1 and R2 leaf tissue from G94 R1 seed. The genomic DNA was digested with BamHI and probed with the phaseolin 3' probe to detect the integration of the GmFad 2-1 construct. The G94 seed has three different sized fragments of DNA that hybridise with the probe. G94-1 and G94-19 have only two – at 14.0 Kb and 4.5 Kb.

As the GUS reporter gene is linked to the *GmFad 2-1* gene, the population of transformed plants was first screened for GUS activity. The GUS-positive plants were then tested using the polymerase chain reaction (PCR), for the presence of the *GmFad 2-1* gene. From this initial screening one plant (event 260-05) was identified. Small samples were taken from the seeds of plant 260-05 (the R1 generation) 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:

1. Seeds with $\geq 80\%$ oleic acid content and normal lysine levels (G168);
2. Seeds with about 72% oleic acid content and increased lysine levels (G94);
3. Seeds with about 4% oleic acid content and increased lysine levels (G175); and
4. Seeds with oleic acid and lysine levels similar to that of the untransformed line A2396 (G90).

Southern blot hybridisation was used to analyse genomic DNA from seeds from the four transformed lines described above. Southern blotting is a sensitive technique used to detect specific sequences within DNA

fragments that have been separated according to size using gel electrophoresis (Southern, 1975). This provides information on the number of inserts of the T-DNA, and the number of insertion sites (i.e., the number of loci) in the genome of the soybean plants. It is also possible to some extent to determine whether the inserted T-DNA copies are whole (intact) or partial copies.

Genomic DNA was extracted from the seed samples, digested with the restriction enzyme BamHI and probed with the 3' region of the phaseolin terminator to detect the *GmFad 2-1* gene expression cassette. BamHI cuts once in the plasmid pBS43 and would be expected to result in one hybridizing band for each copy of the plasmid inserted into the genome. The map of pBS43 with restriction sites and locations of probes is shown in Figure 1. The results of the Southern blot are shown in Figure 2.

Three different banding patterns can be seen in Figure 2. The results for G168 show two hybridising bands of 14.0 Kb and 4.5 Kb, indicative of two *GmFad 2-1* genes. G175 has one band only, corresponding to 12.0 kb. All three hybridising fragments are present in G94.

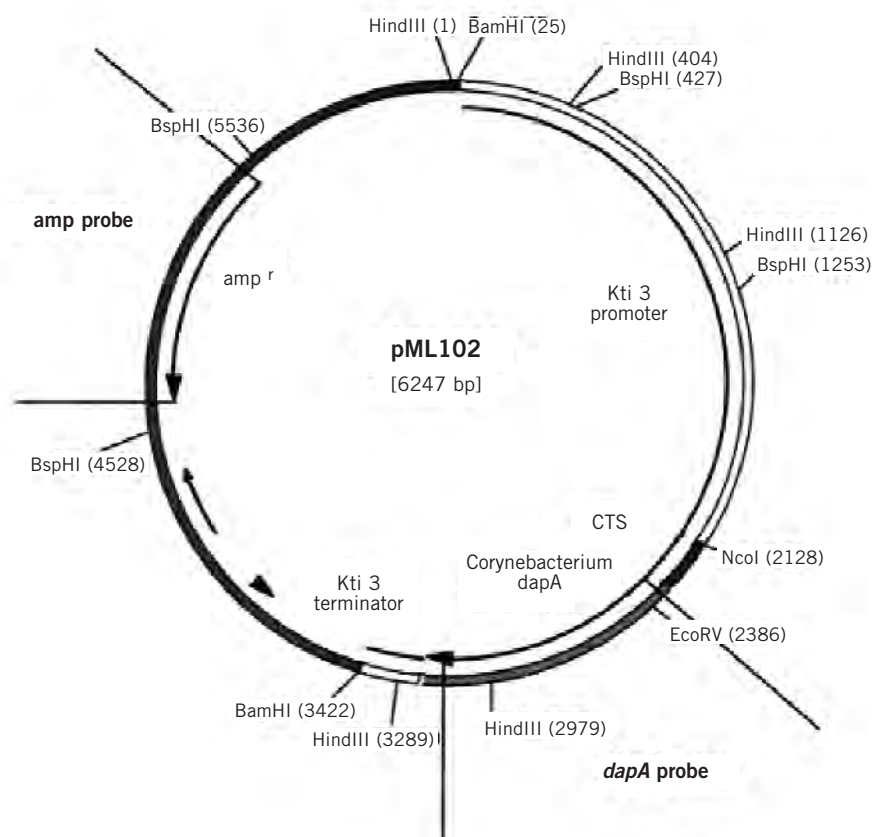


Figure 4: Plasmid map of pML102. Figure indicates the location of hybridisation probes and restriction enzyme sites used for Southern blot analysis of high oleic soybeans.

Interpretation of this DNA hybridisation pattern in Figure 2 suggests that in the original transformation event (event 260-05) the *GmFad 2-1* construct was integrated at two different loci in the soybean genome. Line G168 contains one of the loci (designated *locus A*) consisting of two linked *GmFad 2-1* genes as indicated by the two hybridising fragments of 14.0 kb and 4.5 kb. Line G175 contains the second locus (*locus B*) consisting of a single *GmFad 2-1* gene. G94 contains both loci and thus showed all three hybridising fragments. Only G168 and G94 were selected for further analysis because these showed the desired phenotype of high oleic acid content. Southern blotting of G94 also showed the presence of the *dapA* gene responsible for the increased lysine phenotype.

As G94 plants contained both *locus A* and *locus B*, an additional round of selection was necessary 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* (Figure 3) without *locus B*, which had been removed through segregation. *Locus B* was not further characterised.

The two sub-lines, G94-1, G94-19 and line G168, identified as containing the *GmFad 2-1* locus A, were selected as the high oleic acid soybeans for subsequent analyses. The application for food use relates to these sub-lines only. None of these three lines express the high lysine trait.

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

To fully characterise the insertion in G94-1, G94-19 and G168, six different DNA hybridisation probes based on the genetic fragments in pBS43 (Figure 1) and pML102 (Figure 4) were used for Southern blot analysis. The six probes used were *GmFad 2-1*, phaseolin 3', GUS, 35S

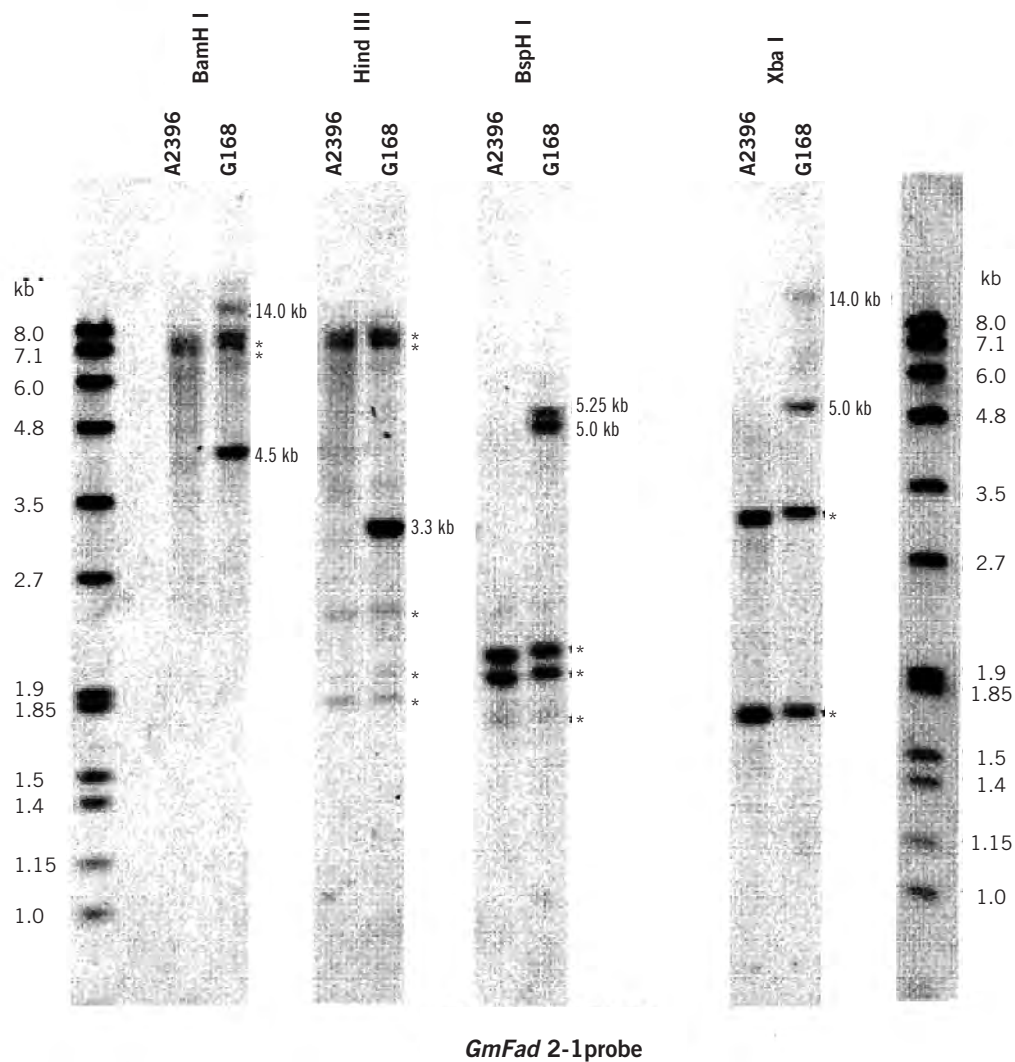


Figure 5a. Southern blot analysis of DNA isolated from R6 leaf tissue of high oleic soybean sub-line G168 and from control line A2396. Genomic DNA was digested with the indicated enzymes and hybridised with the *GmFad 2-1* probe. The underlined molecular weight sizes indicate the sizes of the hybridising transgene for each digest and the asterisks indicated the hybridising endogenous *GmFad 2-1* bands.

promoter, Amp, and *dapA*. Genomic DNA was isolated from R6 leaf tissue from two plants each of G94-1, G94-19, and G168 and the control line A2396. The DNA was digested with six different restriction enzymes to fully characterise the insertions. The results of the Southern blot analysis are presented in Figures 5a and 5b. Table 3 shows the sizes of DNA fragments expected from the different digestions, if it is assumed that one intact copy of plasmid pBS43 was inserted into the genome. For comparison, the sizes of fragments actually obtained in

the Southern blot analyses are shown in Table 4.

From the information obtained in these Southern blot analyses, it was possible to deduce a map of the inserted DNA present in the soybean lines (Figures 6a and 6b).

Characterisation of the R6 generation also revealed that a truncated *dapA* gene had been integrated into another locus in the genome of the G94 sub-lines and G168 (*locus C*). These Southern data are not presented in this case study.

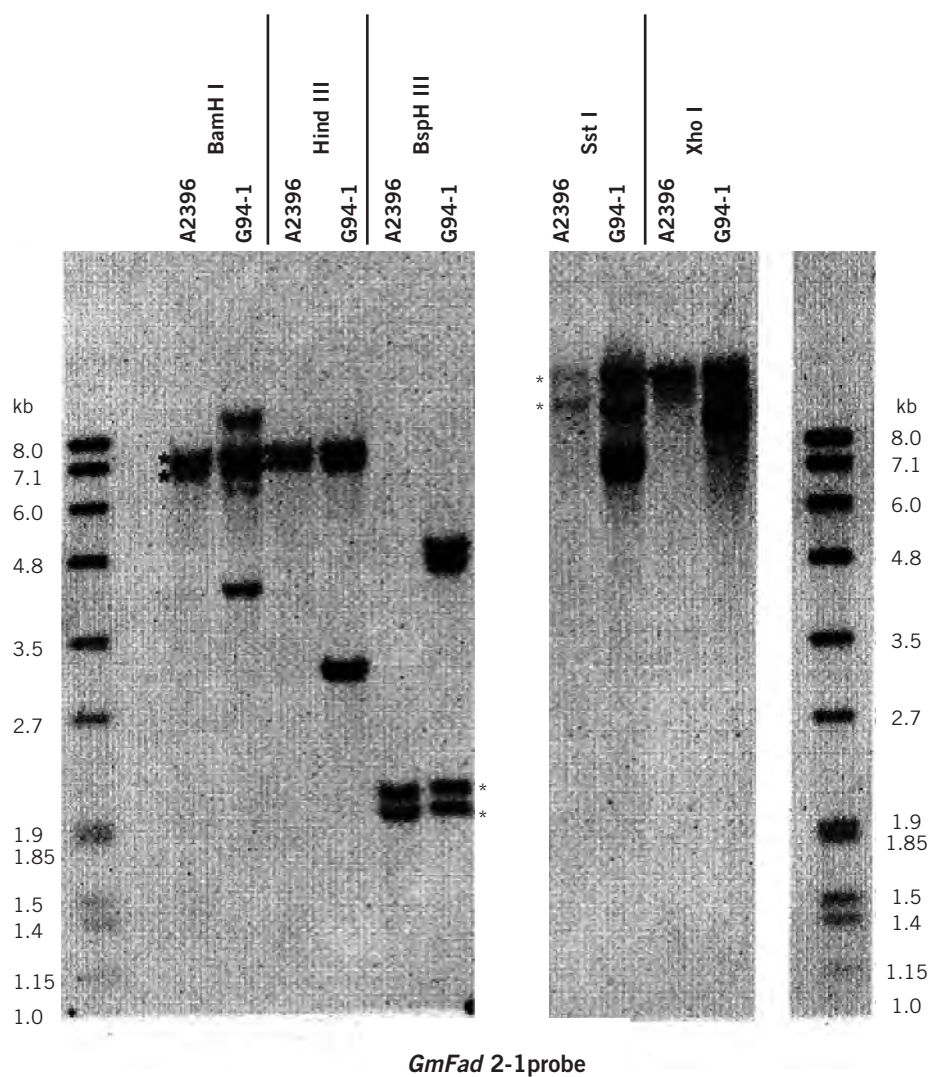


Figure 5b. Southern blot analysis of DNA isolated from R6 leaf tissue of high oleic acid soybean sub-line G94-1 and from control line A2396. Genomic DNA was hybridised with the *GmFad 2-1* probe.

Table 3. Expected fragment sizes (kb). Summary chart of expected hybridising fragment sizes based on the sequence of pBS43 if inserted into the genome as one intact copy

Restriction Enzyme	Hybridisation Probe				
	<i>GmFad 2-1</i>	Phaseolin 3'	GUS	35S Promoter	<i>amp</i>
<i>HindIII</i>	3.3	3.3	7.0	7.0	7.0
<i>BamHI</i>	Border fragment	Border fragment	Border fragment	Border fragment	Border fragment
<i>BspHI</i>	5.25	5.25	5.25	5.25	1.0
<i>SstI</i>	5.1	2.5	2.7	2.5	5.1
<i>XbaI</i>	9.1	1.2	9.1	9.1	9.1
<i>XhoI</i>	Border fragment	Border fragment	Border fragment	Border fragment	Border fragment

Table 4. Actual fragment sizes (kb)¹. Summary chart of Southern blot results describing the DNA fragment sizes that hybridised to the indicated probes when high oleic soybean genomic DNA was digested with the listed restriction enzymes

Restriction Enzyme	Hybridisation Probe				
	<i>GmFad 2-1</i>	Phaseolin 3'	GUS	35S Promoter	<i>amp</i>
<i>HindIII</i>	<u>3.3</u> ²	<u>3.3</u>	6.5	6.5	6.5 4.2 3.3
<i>BamHI</i>	14.0 4.5	14.0 4.5	6.5	6.5	14 6.5 2.8
<i>BspHI</i>	5.25 5.0	5.25 5.0	5.25 5.0	5.25 5.0	1.4 <u>1.0</u>
<i>SstI</i>		<u>2.5</u>	2.7 1.7	<u>2.5</u>	
<i>XbaI</i>	14.0 5.0	<u>1.5</u>	6.7	6.7	
<i>XhoI</i>			4.4		

¹ Hybridising fragments larger than 10 kb should be considered as approximate sizes due to the limitations of the gel system for separating large fragments.

² Fragment sizes that are bold and underlined indicate two copies of the fragment are released by digestion with the listed enzyme. These fragments may give stronger hybridisation signals.

Figure 6a and 6b: Schematic diagram of insert at locus A in high oleic acid soybeans. The top section of each diagram details the inserted genetic elements from the plasmids and their orientation. The bottom section diagrams the hybridising fragments for each restriction enzyme shown in Table 4. The inserted DNA is drawn to scale whereas the bordering soybean genomic DNA is not drawn to scale.

Summary of 'Locus A'

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 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 (data not shown), 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 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

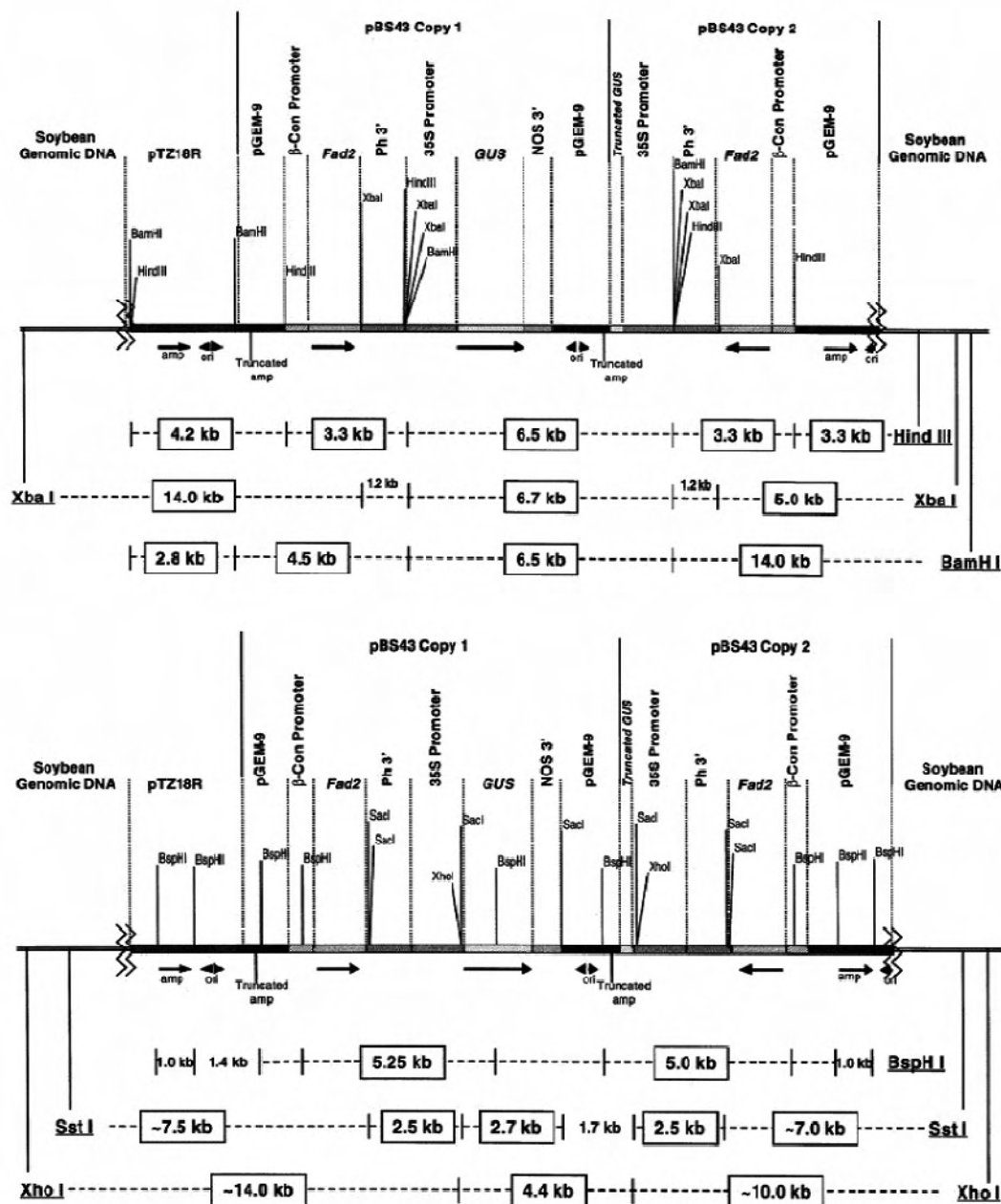


Figure 6a (top) and 6b (bottom). Schematic diagram of insert at *locus A* in high oleic acid soybeans. The top section of each diagram details the inserted genetic elements from the plasmids and their orientation. The bottom section diagrams the hybridising fragments for each restriction enzyme shown in Table 3.4. The inserted DNA is drawn to scale whereas the bordering soybean genomic DNA is not drawn to scale.

soybean line derived through conventional breeding methods.

Conclusion

The *GmFad 2-1* 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 case study therefore concentrates 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. Molecular characterisation 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 (see Section 6.7).

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 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. In addition, ampicillin has now largely been replaced by more potent forms of β -lactam antibiotics or is only used in combination with drugs that work to inactivate β -lactamase (Walsh 2000).

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 and ampicillin is rarely used clinically.

Characterization of novel protein

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 in the introductory chapter.

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 (Figure 7). The δ -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 locus A* (G94-1, G168) do not have any detectable *GmFad 2-1* mRNA, whereas, seeds that contain the *GmFad 2-1 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 (Figure 8). The data show that there is no detectable expression of *dapA*

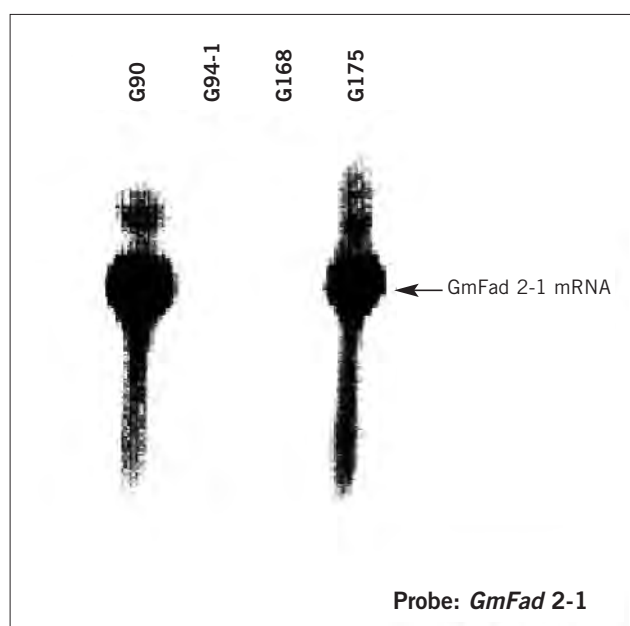


Figure 7. *GmFad 2-1* Northern blot analysis on RNA isolated from developing R4 seeds at 20 days after flowering. G90 contains only the endogenous *GmFad 2-1* gene and was used as a wild-type control. G94-1 and G168 contain the *GmFad 2-1 locus A* and G175 contains the *GmFad 2-1 locus B*.

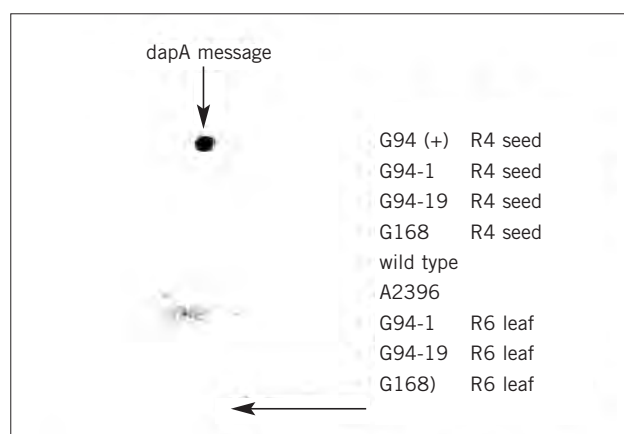


Figure 8. Northern blot analysis of high oleic soybeans. The blot was probed with the *dapA* coding region. Seed G94 contained the *dapA* gene and was used as a positive control. Two negative controls were used and labelled as wild type and A2396. The top of the gel is to the right and the bottom is to the left.

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 (Figure 9). 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 *GmFad 2-1 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 (Figure 10).

Assessment of possible toxicity

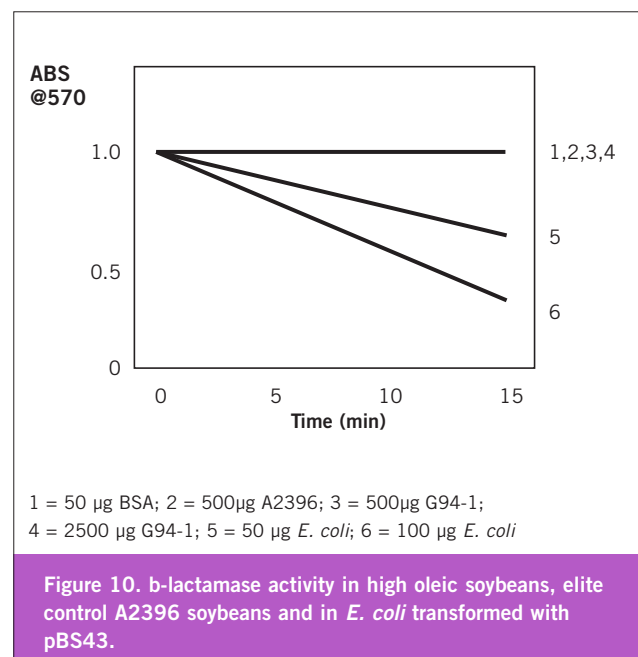
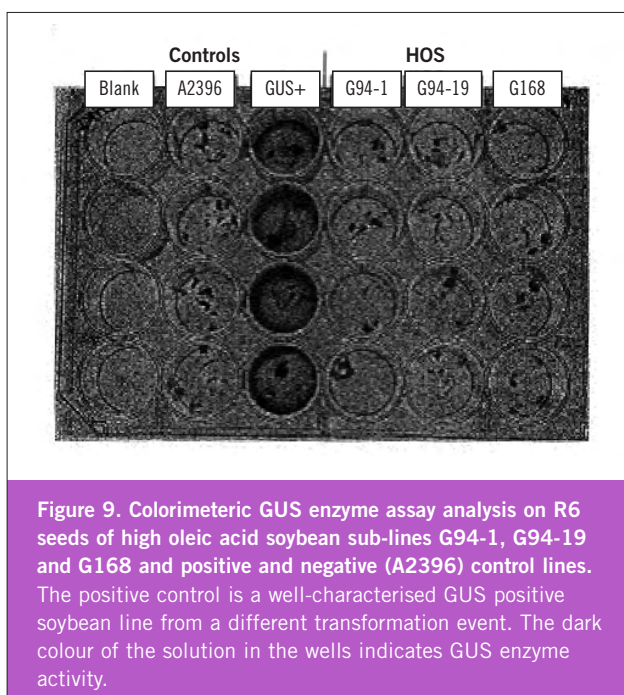
If the GM food differs from its traditional counterpart by the presence of one or a few novel proteins, it is usually possible to assess the potential toxicity of these proteins in a manner analogous to traditional toxicity testing (WHO 2000). That is, the assessment is applied to the novel protein itself, rather than the whole food.

In considering the potential toxicity of a novel protein it is first important to determine whether it is likely to be present in the food as consumed, and thus whether exposure is likely³⁷. Once likely human exposure to a novel protein is established, a number of different pieces of information can collectively be used to demonstrate there is a reasonable certainty that no harm will result from that exposure.

An assessment of potential toxicity of a novel protein should consider the following:

- Whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food;

³⁷ Even if it can be demonstrated that a protein will not be present in the edible portion, proteins known to be toxic to humans should never be deliberately introduced into another organism to be used for food because of the risk of accidental carryover into the edible portion.



- Whether there is any amino acid sequence similarity between the novel protein and known protein toxins and anti-nutrients;
- Whether the novel protein causes any adverse effects in acute oral toxicity testing;
- Whether the novel protein is resistant to heat and/or processing;
- Whether the novel protein is resistant to degradation in simulated digestion models.

It should be noted that, unlike many other substances that are added to foods, the majority of proteins have a predictable metabolic fate in the digestive system, that is, they are typically broken down into their constituent amino acids and then assimilated. For novel proteins, it is therefore important to establish that they will behave like any other dietary protein. One method that can be used to demonstrate this is an *in vitro* digestibility assay. This assay should be able to establish if a novel protein has any characteristics unusual in dietary protein, such as resistance to digestive fluids.

Acute oral toxicity testing is an important component of the safety assessment of novel proteins and is particularly useful in circumstances where there is no prior history of safe consumption of the protein. Acute tests should be sufficient since - if toxic - proteins are known to act via acute mechanisms and laboratory animals have been shown to exhibit acute toxic effects from exposure to proteins known to be toxic to humans (Sjoblod *et al.*, 1992). The acute toxicity tests are done using purified protein that is administered at very high dose levels, usually orders of magnitude above what the human exposure level would be. Ideally, the protein to be tested should be that which has been directly purified from the new organism. Where this is not possible, usually because it is difficult to obtain sufficient quantities of purified protein, it is essential to ensure that the protein tested is biochemically and functionally equivalent to that present in the GM food.

If a novel protein is found to have no significant sequence similarities to known protein toxins, is not stable to heat and/or processing and is readily digested in conditions that mimic mammalian digestion and either has a prior history of safe human consumption and/or does not cause any toxic effects in acute toxicity testing then it can be reasonably concluded that the protein is non-toxic to humans and no further toxicological investigations would be required.

If a novel protein fails one or more of the criteria discussed above then further investigation of the novel protein may be required. For example, if adverse effects

were noted in acute toxicity testing then additional toxicity testing would be required to determine a safe level of human exposure.

As part of the assessment of the potential toxicity of a novel protein it is important to also determine if the activity of the novel protein in the organism is likely to produce any secondary effects, such as the accumulation of other substances. If other substances are found to accumulate as a result of the activity of a novel protein, *e.g.*, the accumulation of a metabolite as a result of the detoxification of a herbicide in a plant, it is important to also include an assessment of the potential toxicity of such substances.

Assessment of possible allergenicity

Virtually all food allergens are proteins, but only a small fraction of the many proteins found in food are allergenic. Therefore, even though foods can contain tens of thousands of different proteins, relatively few are allergenic. As the use of recombinant-DNA techniques can result in additional protein diversity being added to the food supply, the potential allergenicity of any new protein should be a part of the safety assessment. It should be noted however that additional protein diversity could also be introduced into the food supply through conventional breeding techniques.

The prediction of the allergenic potential of a novel protein is not a simple matter and there are presently no validated animal models for the assessment of allergenicity. Because of this, the potential for a novel protein to be allergenic must be evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity.

The assessment focuses on the source of the novel protein, any significant amino acid similarity between the novel protein and that of known allergens, and the structural properties of the novel protein, including susceptibility to digestion. Applying such criteria systematically provides reasonable evidence about the potential of a novel protein to act as an allergen (Lehrer and Reese 1998; Jones and Maryanski 1991).

The source of the novel protein and its amino acid sequence similarity to known allergens are key considerations in the allergenicity assessment. If the novel protein comes from a source known to be allergenic or has sequence similarity to a known allergen, further immunological testing, using sera from



individuals with a clinically validated allergy to the source of the protein, can be used to determine if the novel protein is likely to illicit an allergic response in affected individuals. A negative result may necessitate additional testing, such as skin tests in appropriate subjects.

Resistance to digestion has been observed in several food allergens, therefore such information will also be useful in making an overall determination about the potential for a novel protein to be allergenic to humans. The ability of food allergens to reach and cross the intestinal mucosal barrier in immunologically intact form appears to be a prerequisite to allergenicity (Metcalf *et al.*, 1996). Simulated gastric and intestinal digestive models of mammalian digestion are typically used to assess the digestive stability of proteins (Astwood *et al.*, 1996).

As with potential toxicity, exposure to the novel protein is also an important consideration, which will contribute to an overall conclusion about the potential for a novel protein to be allergenic to humans. In this regard, the nature of the food product intended for consumption should be taken into consideration in determining the types of food processing which would be applied and its effects on the presence of the protein in the final food product. A classic example where this is relevant is in the case of refined oils, which typically do not contain any detectable protein.

Compositional analyses of key components, evaluation of metabolites, food processing and nutritional modification

A comparative approach, focussing on the determination of similarities and differences between the GM food and its conventional counterpart, aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO 2000). The compositional analysis, where the key nutrients, key toxicants and anti-nutrients are measured in the GM food, is an important part of the comparative assessment. The key nutrients and toxicants/anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. These may be major constituents (*e.g.*, fats, proteins, carbohydrates) or minor components (*e.g.*, minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be

significant to health (*e.g.*, solanine in potatoes if the level is increased). The key components of soybeans that should be considered in the comparison include protein, fat, carbohydrates, amino acids, fatty acids, phytic acid, trypsin inhibitors, lectins and isoflavones (OECD 2001). The composition of the high oleic acid soybeans was compared to that of the elite soybean line from which they were derived (A2396).

Field studies and data collection

Two separate field studies of the high oleic acid soybeans were conducted. 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, stachyose 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.

Key nutrients

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 major constituents of the soybean seed. The results of the proximate analysis are presented in Table 5.

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

Table 5. Proximate content¹ of control and high oleic acid soybeans

	<i>Parental control</i>	<i>High oleic acid lines</i>	<i>Literature range</i>
	(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

¹ Mean values, the range in brackets.

Table 6. Amino acid content¹ of parental and high oleic acid soybeans

<i>Amino acid</i>	<i>Parental control</i>	<i>High oleic acid lines</i>	<i>Literature range</i>
	(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

¹ Mean values, the range in brackets.

proline, asparagine and glutamine. A summary of the results of the amino acid analysis appears in Table 6.

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 7.

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 8.

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

Table 7. 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 ¹	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	

¹ Complete fatty acid analysis of control and high oleic acid soybean lines from 1995/96 field trials.

Table 8. Fatty acid composition¹ 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

¹ Mean values, the range in brackets.

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-octadecadienoic 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 9.

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

Oil/fat	Fatty acid composition (g/ 100 g fatty acid)					
	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 hydrogenated	5.6	3.8	72.0	8.9	2.7	1.3
Butter fat	34.8	11.7	26.6	2.6	0.4	0.8

Table 10. Vitamin and mineral content¹ of the control and high oleic acid soybeans

<i>Vitamin or mineral²</i>	<i>Parental control</i>	<i>High oleic acid lines</i>	<i>Literature range</i>
	(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

¹ Mean values, the range in brackets.

² 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.

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

Table 11. Isoflavone content¹ of parental and high oleic acid soybean lines

<i>Isoflavone</i>	<i>Parental control</i> (µg/g dry weight)	<i>High oleic acid lines</i>	<i>Literature range</i>
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

¹ Mean values, range in brackets.

Table 12. Lectin content¹ of parental and high oleic acid soybean lines

<i>Lectin</i>	<i>Parental control</i>	<i>High oleic acid lines</i>	<i>Literature range</i>
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.

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 10.

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 11.

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,

Table 13. Anti-nutrient content¹ for parental and high oleic acid soybeans

<i>Anti-nutrient</i>	<i>Parental control</i>	<i>High oleic acid lines</i>	<i>Literature range</i>
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

¹ Mean values, the range in brackets.

Table 14. Stachyose and raffinose content¹ of parental and high oleic acid soybeans

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

¹ Mean values, the range in brackets.

three replicates were analysed in duplicate from each of the four locations. The results of these analyses are summarised in Table 12.

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 13.

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

stachyose, 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 stachyose content. The results of the analyses are summarised in Table 14.

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

Summary of the compositional analysis

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.

Analysis of the levels of various macro- and micronutrients 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.

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 soybeans.

Endogenous allergenic proteins

A separate part of the comparative analysis also considered the seed storage proteins of soybeans, which comprise a number of naturally occurring allergens. 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 and so 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 (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).

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.

No significant differences were observed in the number of protein bands to which the sera react or to the intensity of the IgE reactivity.

Conclusion

The altered protein profile in the high oleic acid soybeans does not give rise to any significant differences in their allergen content compared to 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.

Table 15. Effect of soybean meal varieties and processing temperature on pig F/G ratios

	Day 0 to 7	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

Nutritional impact

In assessing the safety and suitability 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.

To date, all approved GM plants with modified agronomic production traits (*e.g.*, herbicide tolerance) have been shown to be compositionally equivalent to their conventional counterparts. Animal feeding studies with feeds derived from the approved GM plants have shown equivalent animal nutritional performance to that observed with the non-GM feed. Thus the evidence to date is that where GM varieties have been shown to be compositionally equivalent to conventional varieties, feeding studies using target livestock species will add little to a safety assessment and generally are not warranted (OECD 2003).

For plants engineered with the intention of significantly changing their composition or nutrient bioavailability and thus their nutritional characteristics, however, it is recognised that suitable comparators may not be available for a nutritional assessment based solely on compositional analysis. In such cases, feeding trials with one or more target species may be useful to demonstrate wholesomeness in the test animals.

In the case of the high oleic acid soybeans, significant compositional changes have been deliberately introduced into the food. The applicant therefore provided two animal feeding studies to compare the

wholesomeness of the high oleic acid soybeans to controls and also undertook a study to estimate the human nutritional impact of high oleic acid soybean oil in the diet.

Animal feeding studies

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

Table 16. Effects of processing temperature and soybean meal source on chick performance

	<i>Daily gain 0-18 d (g)</i>	<i>Feed intake 0-18 d (g)</i>	<i>Feed:gain 0-18 d (g)</i>	<i>Body weight 0-7 d (g)</i>	<i>Body weight 0-18 d (g)</i>
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	145.4	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

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 15.

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 16.

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.

Table 17. The effect of replacing all oils and fats used in the domestic and commercial frying with high oleic acid soybean oil (values are means \pm standard deviations)

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

¹ No high oleic acid soybean oil usage.

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

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 were:

- 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 17.

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

Table 18: 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)

Oil	% energy from				
	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

Table 19. 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

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 18.

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 were then applied to the Australian context and indicate that the

magnitude of the changes is likely to be reduced. Table 19 shows a comparison of the fatty acid profiles of the United Kingdom and Australia from recent national dietary surveys.

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.

Conclusions

The information summarised in this case study was used for safety assessment in Australia and New Zealand.

FSANZ stated the following as a summary of their evaluation of the high oleic acid soybeans:

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.

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.

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 use of the bla gene as a selectable marker was not considered to pose any safety concerns.

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.

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.

The composition of the high oleic acid soybeans was compared to that of the elite soybean line from which they were derived. These comparisons examined 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 micronutrients. 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.

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.

Overall it was concluded that 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.

On the basis of this safety assessment, food from high oleic soybean lines G94-1, G94-19 and G168 was approved in Australia and New Zealand in November 2000.

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