Parte 3

Estudios de casos

(en inglés)

Para mayor utilidad de los estudios de casos a los efectos de la capacitación, se ha resumido parte de la información y los datos que se ofrecen son sólo algunos de los efectivamente presentados. Los estudios de casos no recogen una aplicación íntegra ni una evaluación completa de la inocuidad.
Dichos estudios de casos se incluyen en este material de capacitación sin ninguna modificación o mejora por parte de la FAO. Las opiniones expresadas en ellos no reflejan necesariamente el parecer de la FAO.

Estudio de caso 1

Evaluación de la inocuidad del maíz genéticamente modificado resistente a los insectos, evento MON 810

Food safety assessment of genetically modified insect resistant corn event MON 810

Preface

The United States Food and Drug Administration (FDA) completed a consultation for insect resistant (protected) corn line MON 810 in 1996. Health Canada notified Monsanto that the Department had no objection to the food use of corn line MON 810 in 1997. These decisions were made by both regulatory authorities following a comprehensive assessment of MON 810 based upon internationally accepted principles for establishing the safety of foods derived from genetically modified plants. The record of review and decision-making is described for the FDA consultation in Appendix 1 and for Health Canada's assessment in Appendix 2.

The data and information in this case study have been summarized for training purposes. The case study is derived from parts of the food safety submission assessed by Health Canada. Monsanto Canada Inc. provided data on the description of the new variety, the donor organism(s), the genetic modification methods and characterization. The novel protein was identified, characterized and compared to the original bacterial protein, including an evaluation of its potential toxicity. Scientific publications and data from field testing in Canada and the United States under confined trials in 1995 and 1996 were supplied.

Note that statements in quotes are taken directly from the submission to Health Canada.

Disclaimer

Monsanto Canada Inc. has consented to the use of the information provided in their regulatory submission for event MON 810 as a training tool. It must be noted, however, that in order to enhance the utility of the case study as a training tool, liberties were taken with the information provided in the original applications. 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 any of the original submissions.

Description of the recombinant-DNA plant

Line MON 810 contains an inserted genetic fragment of the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 that produces an active delta endotoxin protein expressed in the corn tissue. The target pest, European corn borer (ECB) (*Ostrinia nubilalis*), is an important corn insect pest. Physical damage is caused by ECB feeding on various tissues of the corn plant. The tissues damaged depend on the number of generations of ECB. The damage from ECB feeding includes: a) leaf feeding, b) stalk tunneling, c) leaf sheath and collar feeding, and d) ear damage. Estimated losses range from 5-10% corn yield annually from ECB from disruption of nutrient and water translocation, secondary disease infections, stalk lodging, ear droppage and kernel damage.

The company further describes the variety and its history, "Line MON 810 was supplied to various seed companies as F1 seed of transformed genotype Hi-II crossed to several various elite inbreds. The resulting lines were subjected to multiple cycles of backcrossing to the recurrent inbred parent to recover the converted elite genotype, followed by several cycles of selfing to derive converted inbred parents for hybrid testing. Further cycles of seed increase (selfing) are required to produce parent seed for commercial hybrid seed production. Insect-protected hybrid seed will be heterozygous for the *cryIA(b)* gene since one inbred parent containing the gene is sufficient to confer the insect-protected phenotype on progeny hybrids."

MON 810 is a field corn, not a sweet corn and is intended primarily as an animal feed, but some human food uses occur for field corn. For example, MON 810 may be used either dry or wet milled in processed corn products for humans. No differences in the intended uses of MON 810 are expected as compared to existing field corn hybrids.

Description of the host plant and its use as food

The host plant used is a hybrid line of *Zea mays* with a Mo17X (Hill X B73) background. These corn lines have a long history of use in particular as animal feed, being field corn and not sweet corn.

Zea mays L. (corn, maize) has been cultivated for over 8000 years in Mexico and Central America. A versatile and responsive species, corn has increased both in productivity and geographical range over the past century

with the development of hybrids, breeding programs and fertilizer use and is now grown on every habitable continent. Corn yields prior to hybridization in the early 1930s were around 1.3 metric ton per hectare (ha). The current record high is 123.5 t/ha (with an average of around 137 bushels per acre in the US). World production of corn in 2000 is estimated at 23,800 million bushels.

Corn is used for many different products and uses, as a staple food in many parts of the world and in derived forms, such as starch, alcohol, oil, and for animal feed. Also, corn is used for production of ethanol as a renewable fuel.

Description of the donor organism(s)

The donor of the *cryIA(b)* gene that codes for the CryIA(b) protein, a delta endotoxin active against lepidopteran insect pests, is *Bacillus thuringiensis* subsp. *kurstaki* (B.t.k.) strain HD-1.

The cryIA(b) gene inserted into MON 810 originates from a *Bacillus thuringiensis* subsp. *kurstaki*. *Bacillus thuringiensis* (or Bt) species are spore-forming, gram-positive bacteria that produce a crystal with insecticidal properties. Bt species have been used commercially as pest control agents for decades.

Different strains of Bt are insecticidally active against selected insect pests:

- Bt *israelensis* strains for dipterans (mosquitoes and black flies)
- Bt var. *sandiego* and *tenebrionis* strains for coleopterans (Colorado potato beetle, elm leaf beetle, yellow mealworm)
- Bt *kurstaki*, *thuringiensis*, *sotto* and *aizawai* strains for lepidopterans (corn borer, tomato hornworms, gypsy moth, cabbage looper, tobacco budworm, cotton bollworm).

The delta endotoxin crystals are produced when the bacterium sporulates. To be active, the protein must be ingested by the insect. While the protein is insoluble at neutral or acidic pH, it is soluble at the alkaline pH that occurs in the guts of larval insects where it is activated by proteases in the gut. The activated protein (stripped of its carboxy terminal and about 28 amino acids from the amino terminal end, at approximately 600 amino acids in size) diffuses through the peritrophic membrane of the insect to the midgut epithelium. There it binds to the specific high affinity receptors on the surface of the insect midgut, inserts itself into the membrane and forms ion-specific pores (non-target insects, birds, mammals and fish do not have these receptors). The resulting pores in the membrane cause leakage of the intracellular contents into the gut lumen and water into the epithelial gut cells which swell and lyse. The gut becomes paralyzed disrupting the digestive process, which causes the insect to stop eating and die.

The protein produced in MON 810 insect protected (IP) corn is identical to that produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, which controls insect pests by the production of delta-endotoxin crystals. Data to support this claim are supplied in the submission.

B.t.k. has been used as a microbial pest control agent for decades and "the naturally occurring Bt proteins have been demonstrated to be virtually non-toxic to fish, avian species, mammals and other nontargets … no adverse effects are expected to wildlife from the commercialization of these plants."

The company's submission states: "The CryIA(b) protein is insecticidal only to lepidopteran insects. Only seven of the eighteen insects screened were sensitive … and they were all lepidopteran. This specificity is directly attributable to the presence of receptors in the target insects. Selective activity of B.t.k. endotoxin will not disrupt populations of either beneficial insects or nontarget animals (*e.g.*, birds, fish)."

Tests (cited from the literature), registration documentation and safety assessments from pesticidal registrations on commercially available microbial pesticide products, such as DIPEL®, indicate that they are "widely recognized as nontoxic for mammals, birds and fish as well as beneficial nontarget insects including predators and parasitoids of lepidopteran insect pests and honeybee."

Description of the genetic modification

Plasmid DNA was introduced into the plant tissue by particle acceleration (also known as biolistic transformation). The DNA is precipitated onto the surface of microscopic tungsten or gold particles using calcium chloride and spermidine. A drop of coated particles, placed onto a plastic macrocarrier, is accelerated at high velocity through a barrel by a gunpowder explosion. The macrocarrier flight is stopped by a plastic stopping plate allowing the DNA-coated particles to continue their journey, penetrating plant cells in the path of the explosion. The DNA is deposited and incorporates into the cell chromosome. The cells are incubated on a tissue culture medium containing 2,4-D, which supports callus growth. The cells with introduced DNA contain genes for glyphosate tolerance and are

grown in the presence of glyphosate to select the transformed cells.

Two plasmids were used during this biolistic process, PV-ZMBK07 (Figure 1) containing the cryIA(b) gene and PV-ZMGT10 (Figure 2) containing two marker genes used for selection on glyphosate, CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) and glyphosate oxidoreductase (gox). Tables 1 and 2 describe the DNA elements in the plasmids.

Only a portion of the PV-ZMBK07 plasmid vector is present in MON 810 and the final MON 810 construct does not contain the marker genes. Details on how this was determined follow in Chapter 3. "It is presumed that the genes which allow for selection on glyphosate were originally incorporated into the plant genomic DNA but were lost by segregation during backcrossing." The reason given is that these genes "integrated at a separate loci from the cryIA(b) gene and segregated out during the crossing."

While both plasmids contain the nptII gene encoding for neomycin phosphotransferase II (*nptII*) under the control of its own bacterial promoter, data shows that the *nptII* gene is not present in MON 810. This bacterial gene was used as a selectable marker during plasmid construction.

Experiments in corn transformation have demonstrated that the frequency of obtaining transformants containing glyphosate tolerance selection

Table 1. Summary of DNA elements in plasmid PV-ZMBK07 (See Fig. 1)

Genetic element	*Size Kb*	*Function*
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
hsp 70 intron	0.80	Intron from the maize *hsp70* gene (heat shock protein) present to increase the level of gene transcription
cryIA(b)	3.46	The gene encodes the CryIA(b) protein product
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
lacZ	0.24	A partial *E. coli lacI* coding sequence, the promoter P*lac* and a partial coding sequence for β-D-galactosidase or lacZ protein from pUC119
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in *E. coli*
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid

Table 2. Summary of DNA elements in plasmid PV-ZMGT10 (See Fig. 2)

Genetic element	*Size Kb*	*Function*
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region
hsp 70 intron	0.80	Intron from the maize *hsp70* gene (heat shock protein) present to increase the level of gene transcription
CTP2	0.31	Chloroplast transit peptide (CTP) isolated from *Arabidopsis thaliana* EPSPS present to direct the CP4 EPSPS protein to the chloroplast, the site of the aromatic amino acid synthesis
CP4 EPSPS	1.4	The gene for CP4 EPSPS, isolated from *Agrobacterium* sp strain CP4 which allows for the selection of transformed cells on glyphosate
CTP1	0.26	Chloroplast transit peptide (CTP) isolated from the small subunit gene of ribulose-1,5-biphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana* present to direct the GOX protein to the chloroplast, the site of the aromatic amino acid synthesis
gox	1.3	The gene encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase (GOX) isolated from *Achromobacter* sp. (new genus *Ochrobactrum anthropi*) strain LBAA
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation
lacZ	0.24	A partial *E. coli lacI* coding sequence, the promoter P*lac* and a partial coding sequence for β-D-galactosidase or lacZ protein from pUC119
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in *E. coli*
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid

was increased when both plant selectable markers were used.

The plasmid size of PV-ZMBK07 is 7794 bp and of PV-ZMGT10 is 9427 bp.

Characterization of the genetic modification

Introduction

Several methods, including Southern and Western blot analyses, were used in the molecular characterization of MON 810. Possible novel genes and potential gene products that may have been present in MON 810, based on the information in the plasmid maps, are listed in Table 3.

Molecular characterization

Molecular characterization of the integrated DNA (I-DNA) included determination of:

- The insert number (number of integration sites within the corn genome)
- Copy number (number of each gene within the integrated DNA)
- Insert integrity.

Southern blot analysis was used to determine the above parameters.

MON 810 is compared against a non-transgenic control (counterpart) MON 818, which also has a Mo17 X (Hi-II X B73) background. MON 818 does not contain the genes encoding for B.t.k. HD-1 Cry1A(b), CP4 EPSPS or GOX proteins.

Table 3. Possible novel genes and potential gene products in MON 810.

Novel gene	*Novel gene product*	*Regulatory sequence*	*Other DNA sequences*
PV-ZMBK07			
cryIA(b)	*Bt* gene	Sequence is controlled by E35S promoter (0.6Kb) and a 0.8 Kb intron from the hsp70 gene (heat shock protein) is present to increase the levels of gene transcription. A 0.24 Kb nopaline synthase 3' nontranslated terminator sequence (NOS 3') attached to the *cry* gene provides the mRNA polyadenylation signals.	
lacZ-alpha	Betagalactosidase. A polylinker (region with multiple cloning sites) which allowed the cloning of the desired genes in the plasmid vector	Bacteria controlled promoter. Joined at the 3'end of NOS.	Followed by a 0.7 Kb region of replication for the pUC plasmids (*oripUC*) which allows replication of plasmids in *E. coli*.
nptII (marker for selection during construction of the plasmid derived from procaryotic transposon Tn5)	Neomycin phosphotransferase Resistance to aminoglycoside antibiotics (*i.e.*, kanamycin and neomycin)	Has its own bacterial promoter	
PV-ZMGT10			
gox gene cloned from *Achromobacter* sp. strain LBAA	Glyphosate metabolizing enzyme, glyphosate oxidoreductase (GOX). Degrades glyphosate by conversion to aminomethylphosphonic acid and glyoxylate	Joined to CTP1 peptide which targets the gene to the plastids, a chloroplast transit peptide. Derived from a subunit of ribulose - 1,5 bisphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana*. Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	
CP4 EPSPS Isolated from *Agrobacterium* species strain CP4 which is resistant to glyphosate	5-enolpyruvylshimkimate-3-phosphate synthase	Joined to CTP2 peptide. Isolated from *Arabidopsis thaliana* EPSPS. The gene and CTP2 are about 1.7Kb in size. Under control of sequences as described above of E35S promoter, hsp70 intron and NOS 3' terminator	
Also contains the same *lacZ-alpha*, ori-pUC and *nptII* genes described above			

Insert Number

After digestion of extracted DNA with restriction enzyme NdeI, which does not cleave within either of the plasmids used to produce MON 810, analysis shows that a single band at approximately 5.5 Kilobase (Kb) was observed (Figure 3). This indicates that the DNA from the plasmid was present at one site. The rationale for this is that since there are no restriction sites inside the plasmids, the enzyme cleaves outside the inserted DNA releasing a fragment containing the inserted DNA and some adjacent genomic DNA. Since the plasmid DNA inserts randomly in the DNA of the plant, the distance between the inserted DNA and the restriction enzyme sites in the plant DNA will vary. If there are multiple insertion sites it is likely that cutting with a restriction enzyme that cleaves only outside the insert, the released fragment containing the inserted DNA would vary in size depending on the distance from the NdeI retriction site. You would expect to see multiple bands detected in the Southern if there were multiple insertion sites.

Insert Composition

Using a number of probes, tests show that the CP4 EPSPS, gox and ori-pUC sequences were not detected in MON 810, whereas nptII, E35S, hsp70 and the cryIA(b) were present within the 5.5 Kb NdeI fragment.

cryIA(b)

Digestion of DNA with NcoI/EcoRI to release the cryIA(b) gene followed by Southern blot analysis found an approximately 3.1 Kb fragment (Figure 4), which is "sufficient to encode an insecticidally active CryIA(b) protein." While "the positive hybridization control (lane 1 of figure 4) produced one 3.46 Kb fragment which corresponds to the expected size of cryIA(b) gene, the MON 818 DNA (lane 2) does not contain any bands, as expected for the control line. The MON 810 DNA contains one band of approximately 3.1 Kb."

Western blots indicate that the trypsin resistant protein of 63 Kilo-Dalton (kD) is produced by the integrated partial cryIA(b) gene in MON 810 (Figures 5 and 6). "Based on the Western blot data and efficacy of maize line MON 810, the cryIA(b) gene present produces an insecticidal CryIA(b) protein which provides effective, season long control of ECB."

CP4 EPSPS

Digestion with NcoI/BamHI would release any CP4 EPSPS genes present. Southern blots (Figure 7) indicate that MON 810 does not contain the 3.1 Kb fragment (the expected size of CP4 EPSPS) found in the gel spiked with the two plasmids. The CP4 EPSPS protein was not detected by ELISA in leaf, whole plant or grain tissues. Western blot analysis confirms the absence of the protein from leaf extracts (Figure 8, lane 9).

gox

Digestion with NcoI/BamHI would excise the gox gene, if present (NcoI to NcoI) and would be about 3.1 Kb in size. Southern blot analysis (Figure 7) indicates that MON 810 does not contain the gox gene. Neither was it detected by ELISA of plant tissues nor by Western blot analysis (Figure 9, lane 8).

Plasmid backbone

In order to detect backbone (nptII/ori-pUC) DNA, the nptII gene was used to probe a NcoI/EcoRI digestion of the Mon 810 DNA and PV-ZMBK07 plasmid DNA. When probed with the nptII gene, Southern analysis detected bands only for the plasmid at 2.5 Kb and 1.8 Kb. No signal was detected in the MON 810 DNA. Using the ori-pUC DNA a 1.8 Kb band for detected in the plasmid lane, but the ori-pUC) Southern blots (Figure 10) indicate that MON 810 contains no ori-pUC backbone sequences.

From the above information the interpretation is that one I-DNA containing approximately 4 Kb of DNA from the PV-ZMBK07 plasmid consisting of a portion of the enhanced E35S promoter (estimated to include one of two enhancer elements plus the promoter), the full length intron from the hsp70 gene (heat shock protein) and 2448 bp of the full length of 3468 bp cryIA(b) gene was inserted in the genome of MON 810, as shown in the schematic in Figure 11. No DNA from the bacterial vector backbone (*e.g.*, the pUC-origin of replication), the nptII, gox or CP4 EPSPS genes was detected. The submission states that, "MON 810 contains one integrated DNA contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, maize hsp70 intron and the cryIA(b) gene." Western analysis established that the trypsin resistant 63 kD B.t.k. HD-1 protein was produced in MON 810.

CryIA(b) gene integrity and activity

During particle acceleration plasmid DNA can be broken, resulting in integration of partial genes into the genomic DNA. Southern blots and genomic clone sequence established that the first 2448 bp of the 3468 bp cryIA(b) gene integrated into MON 810.

Modified plant expression

Molecular analysis of MON 810 "established that the line only contains cryIA(b) gene from plasmid PV-ZMBK07 and not the CP4 EPSPS, gox or nptII/ori-pUC genes. There is no evidence that any of the DNA contained in plasmid PV-ZMGT10 was inserted. MON 810 contains one integrated DNA fragment, contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, the maize hsp70 intron and the cryIA(b) gene."

The 'cry1a(b)' gene and its novel trait

The full length gene encoding for CryIA(b) protein has been described. While the genes inserted into MON 810 have been modified to enhance expression in corn, the amino acid sequence of expressed protein is identical to natural protein derived from B.t.k. The *cryIA(b)* gene fragment (Table 4) inserted into the MON 810 has been shown to be equivalent to the original bacterium source, as far as activity against insect pests. Table 4 is a summary of the gene product and its characteristics as submitted by the company.

Western analysis was used:

- To assess the protein products of the partial gene using antibodies specific to B.t.k. proteins
- To compare them to the *E. coli* produced protein standard and tissue extracts from other insect protected corn lines
- To look for any anomalous or unexpected protein products (ex. CP4 EPSPS and GOX (Figures 8, 9, and 12)), and
- To determine if the expressed B.t.k. protein was converted to the expected size of 63 kD trypsin-resistant protein product (Figures 5 and 6).

The company stated, "as is commonly observed in Western blot analysis of Bt proteins, multiple protein products were observed for line MON 810 and the other six insect protected corn lines (Figure 5, lanes 5-11). The full-length gene was not observed in line MON 810, as expected since the full-length gene was not incorporated into the corn genome. … MON 810 showed no apparent differences in the size ranges of the less than full length protein products … when compared to the other six insect protected lines produced with the same full length *cryIA(b)* gene. The predicted molecular weight of the B.t.k. HD-1 protein from the partial *cryIA(b)* gene is 92 kD but is not detected, probably due to low expression or rapid degradation to the trypsin-resistant product during the extraction process."

When the protein extracts are subjected to trypsin digestion, all seven lines show the core protein at approximately 63 kD (Figure 6).

The protein products in MON 810 and expected immuno-reactive products are similar to those in other IP corn lines, except for the lack of the full length B.t.k. HD-1 protein. No unexpected products were observed. The trypsin results demonstrate that the partial *cryIA(b)* gene inserted into MON810 produces the efficacious trypsin-resistant B.t.k. HD-1 protein.

Equivalence of bacterial and plant produced protein

Escherichia coli containing the B.t.k. gene was used to produce the quantities of the CryIA(b) protein needed to do tests, such as feeding trials. Therefore, the equivalence of the B.t.k. HD-1 protein produced in the IP corn was assessed against that from the *E. coli*. As the company states, the rationale is that: "the expression level of B.t.k. HD-1 in IP corn plants is extremely low. Therefore it is not feasible to isolate this protein from plants in sufficient quantity to conduct the various safety studies performed for the registration of this product. The best alternative was to isolate the functionally active B.t.k. HD-1 protein produced in a microbial host … and verify its physical and functional equivalence to the plant-expressed protein. Because the full length B.t.k. HD-1 protein (~ 131 kD) … would be expected to be rapidly converted to the trypsin-resistant core protein (~ 63 kD) upon ingestion … the trypsin-resistant core of the B.t.k. HD-1 protein was considered an appropriate test material to assess the full length B.t.k. HD-1 protein."

Two studies were presented. One study compares the B.t.k. HD-1 CryIA(b) from the commercial microbial

Table 4. Summary of gene products in the modified plant

Gene product	*Breakdown products, byproducts and metabolic pathways*	*Expression*	*Activity of the gene product in the plant*	*Activity of the geneproduct in the environment*
CryIA(b) delta endotoxin protein	Tryptic peptide is active ingredient	Constitutive	Does not affect other metabolic pathways	Rapidly degraded by digestion (non lepidopteran) and in soil

product DIPEL with leaf tissue samples from the plant expressed in line 754-10-1. Line 754-10-1 was produced with the same transformation plasmids as MON 810, but has higher expression of the protein and therefore it was possible to purify a greater quantity of the protein for equivalence studies. The study demonstrated that the B.t.k. HD-1 trypsin resistant core from corn and *E. coli* are equivalent in molecular weight and immunological reactivity. Both DIPEL and line 754-10-1 contain a full length B.t.k. protein band at approximately 134 kD and the same trypsin resistant core of approximately 63 kD. Western blots demonstrated that the B.t.k. HD-1 core from line 754-10-1 and MON 810 were equivalent, therefore it is concluded that the protein produced by the *E. coli* is an appropriate substitute for the protein in MON 810.

Multiple protein products occur in the plant extract, in the commercial microbial product DIPEL and in the full-length protein preparation used in the acute toxicity study. A question about other fragments in the Western blots that are reactive to the CryIA(b) antibody probes and the meaning were addressed with the following. There should be no concerns since the acute oral toxicity study would have included these fragments. Any fragments outside the trypsin resistant core 28-610 amino acids (1-28 and 611-1150) possibly present in corn tissues show no amino acid homology with known toxins or allergens. Comparison of the CryIA(b) full length protein sequence against the same sequence data base indicates there is no homology with known toxins or allergens. Digestive fate shows that the protein is rapidly digested and the commercial microbial product DIPEL contains many fragments as well.

Western blots of proteins after treatment with trypsin show equivalent bands and that the 63 kD core is in both samples. MON 810 produces a protein product whose trypsin resistant core is equivalent to the trypsin resistant core of the B.t.k. 754-10-1 protein in terms of size and activity.

In a newer test than the one for 754-10-1, the equivalency was established directly between the bacterially and plant produced proteins in MON 810 using Western blot analysis, which was, “highly sensitive, specific for B.t.k. proteins and allows for comparison of the apparent molecular weights of proteins possessing immunological cross-reactivity in complex mixtures.”

Leaf extracts of several IP lines and control lines were digested in trypsin to produce their B.t.k. HD-1 trypsin-resistant core protein and compared against the 63 kD *E. coli* produced trypsin- resistant core protein and the reference corn line MON 801 protein. The corn lines included MON 810 and its counterpart MON 818.

The Western blot analysis (Figure 6) shows a prominent band at the same molecular weight for MON 810 as the bacterial reference material. Smaller bands are also present and are assumed to be other B.t.k. HD-1 fragments. A band at 20 kD was seen in all extracts (both IP and control lines) and presumably represents a background non-specific cross-reactivity unrelated to the B.t.k. HD-1 protein.

“The results obtained in this study clearly establish that the B.t.k. HD-1 protein (as the trypsin-resistant core) produced by both *E. coli* and the IP corn lines analyzed in this study are equivalent. … the equivalence established … serves as the justification for using the safety data generated with the *E. coli*- produced (lot #I92017) protein to support the safety of the B.t.k. HD-1 protein expressed in these new insect protected corn lines.”

Expression

Samples of field-grown IP corn (MON 810) and a control (MON 818) collected from US field sites were used to assess the expression level of CryIA(b), CP4 EPSPS, GOX and NPTII proteins. The control lines (MON818 and 819) are not genetically modified, but have “background genetics representative of the test substances.” MON 818 is the counterpart for MON 810.

Leaf and grain samples were collected from six field sites distributed across the US corn growing regions, representative of the conditions where IP corn could be grown as a commercial product (2 in Illinois, 2 in Iowa, 1 each in Indiana and Nebraska). Whole plant and pollen samples were collected once from a single site (in Illinois). Over season leaf samples (taken every two weeks) were also collected from the Illinois site. Except for the pollen samples, B.t.k. HD-1, CP4 EPSPS and GOX protein levels were assessed using validated ELISAs specific for each protein. For the pollen samples, ELISA was used for the B.t.k. levels and Western blot analysis for CP4 EPSPS and GOX proteins.

Expression levels of the *cryIA(b)* gene were low in corn leaf, seed, pollen and whole plant tissues (Table 5). CP4 EPSPS, GOX and NPTII proteins were not detected. Average protein expression evaluated at six locations was 9.35 μg/g (f.w.) in leaves and 0.31 μg/g (f.w.) in seeds. Protein expression evaluated at one site was 4.15 μg/g (f.w.) in the whole plant and 0.09 μg/g (f.w.) in pollen, as determined from a single sample. Protein expression ranged from 7.93 to 10.34 μg/g (f.w.) in leaves, from 0.19 to 0.39 μg/g (f.w.) in grain and from

Table 5. Summary of levels of protein expression in MON 810 tissues[1]

Tissue	*Mean*	*Standard deviation*	*Range*
B.t.k. HD-1			
Leaf	9.35	1.03	7.93-10.34
Over season leaf[2]	9.78, 8.43, 4.91		
Pollen	0.09		
Whole plant[3]	4.15	0.71	3.65-4.65
Grain	0.31	0.09	0.19-0.39
CP4 EPSPS			
Leaf, over season leaf[2], whole plant, grain	nd	–	–
GOX			
Leaf, over season leaf[2], whole plant, grain	nd	–	–

[1] Unless indicated, values are in μg/g fwt (fresh weight). Unless indicated, the mean, standard deviation and range were over the six sites sampled. For those samples collected at one site see other notes.

[2] The numbers are means for the three separate sampling times collected at two week intervals.

[3] The mean and standard deviation were calculated from one site.

3.65 to 4.65 μg/g (f.w.) in the whole plant. Protein expression declined over the growing season as indicated by the Cry1A(b) levels present in leaves assayed over the growing season.

Tissue specificity, as stated by the company, was not expected since the *cryIA(b)* gene is "under the control of a CaMV promoter. Since this is a constitutive promoter that is not developmentally or tissue restricted, no specificity of expression to particular tissues is anticipated, although the CaMV promoter may be more or less active in certain cell types, as seen from the distribution of the CryIA(b) proteins in tissues." Neither were developmental stage specificity nor inducibility expected or found, because the CaMV promoter is a non-inducible constitutive promoter.

Western blot analysis of pollen (Figure 12) shows that the GOX gene is not expressed in MON 810 (lane 11).

For GM food assessments, expression in the consumed portion of the plant, in this case the grain, is the most important. The levels of expression in the grain of the novel protein range from 0.19 to 0.39 μg/g fresh weight.

The expression of the NPTII protein from the nptII gene, under the control of a bacterial specific promoter was tested for one of the lines used in this test (MON 801). The promoter was not active and, therefore, the gene does not express the protein in plant cells.

Breakdown products and metabolism

"The CryIA(b) protein does not have any specific breakdown products in plants. In the insect gut, the alkaline environment solubilizes the protein, which is then cleaved by proteases to yield the activated endotoxin. … As is commonly observed in Western blot analysis of Bt proteins, multiple polypeptides are apparent in extracts of plants expressing the *cryIA(b)* gene. These are recognized as breakdown products liberated as a result of protease action either in planta or during extraction."

Stability of the insert

MON 810 has been crossed into diverse corn genotypes for several generations and the efficacy of the line has been maintained. The molecular characterization of MON 810 was from the third generation of backcrossing and therefore the single insert appears to be stably integrated. Segregation data (Table 6) support a single active insert of the *cryIA(b)* gene segregating according to Mendelian genetics.

The *cryIA(b)* gene is stable through seven generations of crosses to one recurrent parent (B73) and six generations of crosses to a second, unrelated inbred (Mo17) (Table 7). The Chi square tests for the backcross to B73 and Mo17 did not deviate from expectations.

Assessment of possible toxicity

Introduction

Most of the studies were done using the insecticidally active trypsin-resistant core *E. coli* produced protein and not with plant-produced protein. The test proteins produced in *E. coli* are chemically and functionally the same as the plant-produced proteins (section 4.1.1).

Table 6. Segregation data of MON 810 progeny

Generation	*Description*	*Actual*	*Expected*	*ChiSq*
BC0F1[1]	Derived from cross of R0 with an inbred line	44:47	45.5:45.5	0.044*
BC1F1[2]	Derived from cross of BC0F1 plants to the same inbred line used to cross the R0 plant	10:4	7:7	1.786*
BC1F2 progeny[3]	Derived from cross of individual BC0F2 plants by a non-transgenic tested	69:181:77	81.75:163.5:81.75	4.138#

[1] Expressed as number of expressing plants: number of non-expressing plants based on ECB feeding assay.
[2] Expressed as number of expressing plants: number of non-expressing plants based on CryIA(b) ELISA.
[3] Expressed as number of ear rows with homozygous number of expressing plants: number of ear rows with segregating plants: number of ear rows with homozygous susceptible plants based on ECB feeding assay.
** Not significant at p=0.05 (chi square = 3.94, 1df); # not significant at p=0.05 (chi square = 5.99, 2 df).*

Table 7. Stability of gene transfer based on segregation data for backcross derivatives of MON 810 with two unrelated inbred lines (B73 and Mo17)

Generation[1]	*Actual*	*Expected*	*Chi square*
BC6F1 (B73)	8:13	10.5:10.5	0.762*
BC5F1 (Mo17)	11:11	11:11	0.045*

[1] Data expressed as number of expressing plants: number of non-expressing plants based on CryIA(b) ELISA.
** Not significant at p=0.05 (chi square = 3.84, 1 df).*

Some of the food safety considerations are based on CryIA(b) characterization and digestive fate studies in simulated gastric and intestinal fluids.

Protein specificity

The CryIA(b) protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH, however, is solubilized by the alkaline gut of larval insects. The solubilized protein is then activated by the proteases in the insect gut, which diffuses through the peritrophic membrane to the midgut epithelium, binding to specific high affinity receptors on the surface. This paralyzes the gut due to changes in electrolytes and pH causing the insect to stop feeding and die.

There are no similar receptors for the protein delta-endotoxins of Bt species on the surface of mammalian intestinal cells, therefore mammals are not susceptible to these proteins. Also, absence of adverse effects in humans is supported by numerous reviews on the safety of Bt proteins.

Comparison to toxin databases

The Cry1A(b) amino acid sequence was compared to known protein toxins. Similarity to a known toxin could trigger toxicological testing to address potential impact of the homology. B.t.k. HD-1 protein was compared to the amino acid sequences of 2632 toxins collected from public domain genetic databases (GenBank, EMBL, PIR and Swiss Prot) for homology. The results confirm that the B.t.k. HD-1 protein is homologous to Bt insecticidal crystal proteins, but no amino acid homology was detected for other protein toxins. The closest match is shown in Figure 14.

Mouse acute oral gavage

An acute oral toxicity study (7 days) was done with albino mice using *E. coli* produced protein (converted to the trypsin resistant core) and tested for purity, potency and stability. The protein was administered by gavage to mice at targeted doses of 0, 400, 1000 and 4000 mg/kg. The highest dose represents the maximum hazard dose concept outlined in US Subdivision M Guidelines for biochemical pesticides. One group was dosed with 4000 mg/kg of bovine serum albumin (BSA) as a protein control.

No treatment related adverse effects were observed (Table 8) and no statistical differences in body weight measures or food consumption were seen. No differences were seen in gross pathology between the groups. The LC50 of the B.t.k HD-1 (truncated) protein in mice is greater than 4000 mg/kg with the NOEL set at that value.

Potential toxic contaminants

In response to queries about possible changes in contaminant levels due to the introduction of the

Table 8. Results of acute mouse gavage test with CryIA(b) protein

Test group	*Weight pretest (g)*	*Weight at end (g)*	*Food consumption (mean g/day)*
Vehicle control (buffer)	31.1 [25.5]	30.8 [25.1]	5.3 [6.4]
Control (BSA 4000*)	31.1 [25.4]	31.0 [24.7]	6.2 [7.3]
400 Bt protein	31.1 [25.4]	30.5 [25.2]	5.3 [8.0]
1000 Bt protein	31.0 [25.3]	31.1 [25.0]	5.3 [8.0]
4000 Bt protein	31.0 [25.5]	30.5 [25.5]	5.5 [8.0/7.4]

*[females] / *mg/kg body weight*

Table 9. Dissipation of B.t.k. HD-1 protein insecticide activity in simulated gastric fluids

B.t.k. *HD-1*	*Tobacco budworm mortality*		*% change*
(µG/nL)	*0*	*2 minutes*	
0.75	29	3	-90
7.5	69	8	-88
75	94	24	-74

Table 10. Dissipation of B.t.k. HD-1 protein insecticide activity in simulated intestinal fluids

B.t.k. *HD-1*	*Tobacco budworm mortality*		*% change*
(µG/nL)	*0*	*19,5 hours*	
0.75	26	25	-4
7.5	76	61	-20
75	100	90	-10

cryIA(b) gene, the company notes that for alflatoxins, tests with MON 810 from the 1993 field trial did not detect alflatoxins and therefore the test was not repeated.

DIMBOA (2,4-dihodroxy-7-methoxy-1,4-bezoxanin-3-one) is not present in seeds of cereals and therefore does not pose a hazard to consumers of grain products.

Metabolic degradation in simulated gastric and intestinal fluids

Purified CryIA(b) protein (B.t.k HD-1 as expressed in *E. coli*) degrades rapidly in vitro using simulated digestive fluids. In the simulated gastric fluid, more than 90% of the protein degraded within two minutes, as detected by Western blot analysis (Figure 15). Lanes 6-11 are incubations at 0, 10, 20, 30, 60 and 120 seconds. Protein bioactivity detected using an insect bioassay also dissipated quickly with 74-90% of the added protein dissipated within two minutes (Table 9), the earliest time point measured. In a human stomach, approximately 50% of solid food empties to the intestines in two hours and liquids in about 25 minutes.

In the simulated intestinal fluid, the purified Cry1A (b) protein did not degrade substantially after 19.5 hours as assessed by Western blot (Figure 16, lanes 8-11 are incubations at 0, 60 minutes, 4 hours and 19.5 hours) and insect assay (Table 10). This was anticipated since the tryptic core of Bt insecticidal proteins is known to be relatively resistant to serine proteases like trypsin, a key protease in intestinal fluid. The insect used for the insect assay studies was the tobacco budworm.

Assessment of possible allergenicity

Humans consume large quantities of proteins daily and allergenic reactions are rare. One factor to consider is whether the source of the gene being introduced into the plants is known to be allergenic. Bt does not have a history of causing allergy. “In over 30 years of commercial use, there have been no reports of allergenicity to Bt, including occupational allergies associated with manufacture of products containing Bt.” Further, protein allergens need to be stable in peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response. Tests above show that the CryIA(b) protein does not survive under simulated gastric digestion. Another common factor of allergenic proteins is that they occur in high levels in the foods (*e.g.*, allergens in milk, soybean, peanuts). This is not the case with the CryIA(b) protein which is present at approximately 0.19-0.39 µg/g fresh weight of corn seed.

The company stated that Comparing sequences of amino acids to known allergens and gliadins is a useful first approximation of potential allergenicity or association with coeliac disease. A database of 219 protein sequences associated with allergy and coeliac disease assembled from genetic databases (GenBank, EMBL, PIR and Swiss Prot) was searched for sequences similar to B.t.k. HD-1 protein. “Most major … food allergens have been reported and the important IgE

binding epitopes of many allergenic proteins have been mapped. The optimal peptide length for binding is between 8 and 12 amino acids. T-cell epitopes of allergenic proteins and peptide fragments appear to be at least 8 amino acids in length. Exact conservation of epitope sequences is observed in homologous allergens of disparate species. … an immunologically relevant sequence comparison test for similarity … is defined as a match of at least eight contiguous identical amino acids." No biologically significant homology nor immunological significant sequence similarities were found. The best match is shown in Figure 17. The results establish that B.t.k. HD-1 protein shares no significant similarity with known allergen or gliadin proteins.

In summary, the low levels of the protein in the corn, combined with the digestive lability and the lack of homology with known allergenic sequences indicate that this protein does not possess allergenic properties. Coupled with the history of use as a microbial control agent with no allergenic concerns, this indicates that there is no reason to believe that CryIA(b) should pose any significant allergenic risks for the consumption of products produced from insect-protected corn.

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

Introduction

Nutritional data are important relative to dietary exposure to corn products. While little whole kernel or processed corn is directly consumed by humans, corn based food ingredients such as starch and corn oil are used.

Compositional data

Samples for composition analysis were collected at the same time and from the same six sites used for analysis of expression levels in corn grain for a one-time experiment.

Corn seed (grain) samples of MON 810 and the control MON 818 were analyzed for the following components and compared with available literature values:

- Proximates (moisture, protein, ash, fat, crude fibre)*
- Calories
- Carbohydrate
- Starch
- Fatty acid profile*
- Sugar profile
- Amino acid composition*
- Tocopherols*
- Phytic acid*
- Minerals (calcium, phosphorus)* as summarized in Table 11.

Parameters with an asterisk (*) are considered for feed assessments, while the other parameters (often derived from calculations) are not commonly considered.

Carbohydrates were not measured but deduced using the following calculation: % carbohydrates = 100% - (% protein + % fat + % ash + % moisture). Also, calories was a derived parameter using the following USDA approved calculation: calories (kcal/100g) = (4 * % protein) + (9 * % fat) = (4 * % carbohydrates).

There were no significant differences for the variables protein, fat, ash, carbohydrates, calories and moisture between the IP corn and its control and both were within the reported values from the literature.

MON 810 contained eight amino acids (cystine, tryptophan, histidine, phenylalanine, alanine, proline, serine and tyrosine), which were statistically different from the control. The mean values for six of these (all except cystine and histidine) are within literature ranges. Cystine and histidine for both lines were statistically higher than the literature range but within the range (1.9-2.3%) observed for two (MON 800/801) similar lines. The level of histidine for MON 810 (3.1%) is within the range of another previous study for two lines of similar genetic backgrounds.

For fatty acids and carbohydrates measured (starch, fructose, glucose, sucrose and phytic acid), no significant differences were found between the control and the IP lines. Crude fiber values in MON 810 grain (2.6%) were statistically different from MON 818, but both values were within the literature range (2.0-5.5%).

Tocopherols are naturally present in corn oil and have vitamin E potency. The gamma tocopherol is one-tenth as active as the alpha and is therefore not considered an important component of the corn grain. MON 810 values for the alpha and gamma tocopherols were statistically similar to the control but the beta tocopherol differs statistically from the control (Table 11).

For the minerals calcium and phosphorus, calcium levels in MON 810 were statistically higher than for MON 818, but within ranges reported for tests with MON 800/801. No statistical differences were found for phosphorus.

Table 11. Comparison of compositional analysis for MON 810 corn grain with control (MON 818) and literature values

Component	*MON 810[1] mean (range)[2]*	*MON 818 mean (range)[2]*	*Literature value[4] mean (range) [MON 800/801 range]*
Proximate analysis			
Protein[3]	13.1 (12.7-13.6)	12.8 (11.7-13.6)	9.5 (6.0-12.0) 12.3 (9.7-16.1) [11.2-13.6]
Fat	3.0 (2.6-3.3)	2.9 (2.6-3.2)	4.3 (3.1-5.7), 4.6 (2.9-6.1) [3.8-4.2]
Ash[3]	1.6 (1.5-1.7)	1.5 (1.5-1.6)	1.4 (1.1-3.9) [1.5-1.8]
Carbohydrate[3]	82.4 (81.8-82.9)	82.7 (81.7-83.8)	not reported [80.8-83.0]
Calories/100g	408.4 (407.0-410.1)	408.5 (406.0-410.1)	not reported [412.6-415.7]
Moisture %	12.4 (11.0-14.4)	12.0 (10.6-14.2)	16.0 (7-23) [13.0-15.8]
Amino acid composition - nutritionally essential[5]			
Methionine	1.7 (1.6-1.9)	1.7 (1.6-1.7)	1.0-2.1 [2.0-2.6]
Cystine	2.0* (1.9-2.1)	1.9 (1.8-2.0)	1.2-1.6 [1.9-2.3]
Lysine	2.8 (2.5-2.9)	2.8 (2.7-2.9)	2.0-3.8 [2.6-3.4]
Tryptophan	0.6* (0.5-0.7)	0.6 (0.4-0.6)	0.5-1.2 [0.5-0.6]
Threonine	3.9 (3.7-4.4)	3.8 (3.7-3.9)	2.9-3.9 [3.9-4.2]
Isoleucine	3.7 (3.3-4.1)	3.8 (3.6-4.0)	2.6-4.0 [3.5-3.8]
Histidine	3.1* (2.9-3.3)	2.9 (2.8-3.0)	2.0-2.8 [2.8-3.3]
Valine	4.5 (4.1-4.9)	4.6 (4.3-4.8)	2.1-5.2 [4.2-4.8]
Leucine	15.0 (14.1-16.7)	14.5 (13.8-15.0)	7.8-15.2 [13.6-14.5]
Arginine	4.5 (4.2-4.7)	4.5 (4.2-4.7)	2.9-5.9 [4.1-5.0]
Phenyalanine	5.6* (5.2-5.6)	5.4 (5.2-5.6)	2.9-5.7 [5.2-5.6]
Glycine	3.7 (3.4-4.0)	3.7 (3.5-3.8)	2.6-4.7 [3.4-4.2]
Amino acids - nonessential[5]			
Alanine	8.2* (7.8-8.9)	7.8 (7.5-8.0)	6.4-8.0 [7.8-8.2]
Aspartic acid	7.1 (6.4-8.2)	6.6 (6.3-6.8)	5.8-7.2 [6.7-7.3]
Glutamic acid	21.9 (20.4-24.4)	21.1 (201.-21.6)	12.4-19.6 [19.9-21.4]
Proline	9.9* (9.7-10.5)	9.6 (9.4-9.8)	6.6-10.3 [9.0-9.4]
Serine	5.5* (5.3-5.9)	5.2 (5.1-5.4)	4.2-5.5 [5.5-6.1]
Tyrosine	4.4* (4.1-4.8)	4.0 (3.9-4.1)	2.9-4.7 [3.8-4.3]
Fatty acids[6]			
Palmitic (16:0)	10.5 (10.2-11.1)	10.5 (10.2-10.7)	7-19 [10.2-10.9]
Stearic (18:0)	1.9 (1.7-2.1)	1.8 (1.8-1.9)	1-3 [1.6-3.1]
Oleic (18:1)	23.2 (21.5-25.4)	22.8 (21.6-23.9)	20-46 [21.2-25.9]
Linoleic (18:2)	62.6 (59.5-64.7)	63.0 (61.8-64.6)	35-70 [58.9-65.0]
Linolenic (18:3)	0.8 (0.7-0.9)	0.9 (0.8-0.9)	0.8-2 [0.9-1.1]
Carbohydrates and fiber7			
Starch %	67.6 (65.3-69.7)	66.9 (64.6-69.0)	64-78.0 [63.7-71.5]
Crude fiber %	2.6* (2.5-2.8)	2.4 (2.3-2.5)	2.0-5.5 [1.98-2.61]
Sugars[8]			
Fructose	0.32 (0.23-0.35)	0.27 (0.22-0.40)	[0.47-0.96]
Glucose	0.44 (0.34-0.47)*	0.93 (0.79-1.12)	[0.47-1.03]
Sucrose	0.93 (0.79-1.12)	0.93 (0.68-1.11)	[0.40-0.94]
Phytic acid %	0.86 (0.81-0.91)	0.84 (0.79-0.91)	0.7-1.0 [0.45-0.57]
Tocopherols (mg/kg)			
Alpha	10.4 (9.7-11.3)	10.9 (9.9-12.1)	3.0-12.1 [7.3-12.3]
Beta	8.5* (8.1-9.2)	7.5 (7.0-7.9)	[7.9-10.7]
Gamma	20.2 (15.3-24.8)	21.6 (18.8-27.8)	[21.7-42.5]

(Continued)

Table 11. (cont.)

Component	*MON 810[1] mean (range)[2]*	*MON 818 mean (range)[2]*	*Literature value[4] mean (range) [MON 800/801 range]*
Inorganic components[7]			
Calcium %	0.0036* (0.0033-0.0039)	0.0033 (0.0029-0.0037)	0.01-0.1 [0.003-0.004]
Phosphorus %	0.358 (0.334-0.377)	0.348 (0.327-0.363)	0.26-0.75 [0.311-0.368]

*1 Values with * are statistically different from MON 818.*
2 Values reported are means of six samples from six sites. Ranges are the highest and lowest values across those sites.
3 Percent dry weight of samples.
4 Where there are more than one value, this indicates more than one published source.
5 Values for amino acids reported as percent of total protein.
6 Values for fatty acids are % total lipid. Other fatty acids were below the limit of detection of the assay.
7 Values on a dry weight basis.
8 Sugars measured as g/100g. Galactose, lactose and maltose were also measured, but values were below the limit of detection.

The company concluded, "Based on these data, it was concluded that there are no meaningful compositional differences between the IP corn lines ... and the control line, MON 818."

Additionally, the company summarized its Nutritional analysis conclusions, "nutritional composition … falls within the ranges of each nutrient measures for non-modified corn lines. It can be concluded that there appears to be no meaningful effect on corn plant nutrient levels. Phenotype was not affected in any of the numerous ways that were measured. Of the vitamins and minerals measured there were no practical differences reported. In terms of nutritional composition, MON 810 may be considered to be substantially equivalent to regular corn." ●

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

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.

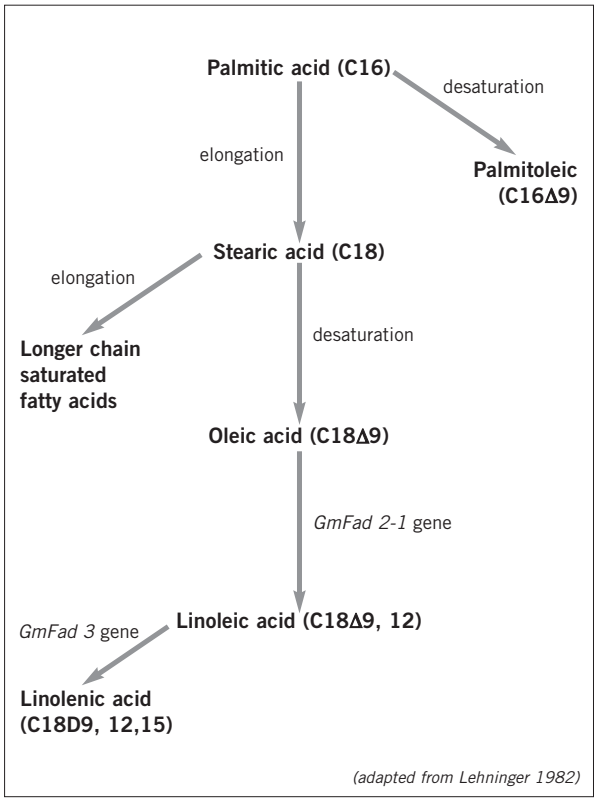
Disclaimer

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 not 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

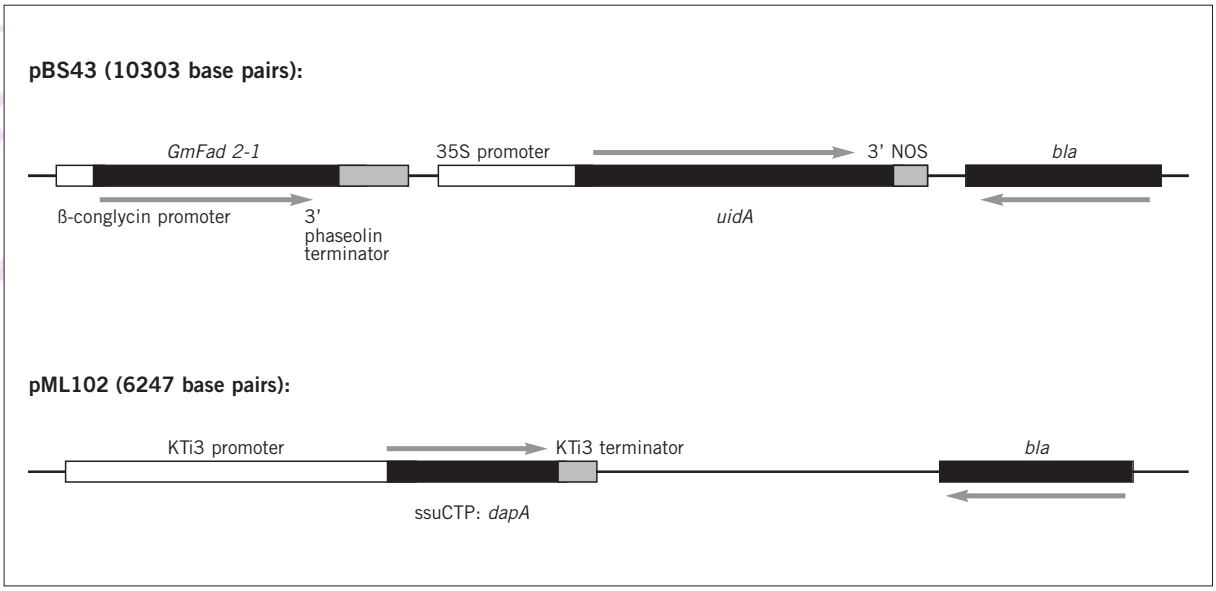


Table 1: Description of the gene expression cassettes in pBS43 and pML102

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

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

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

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.

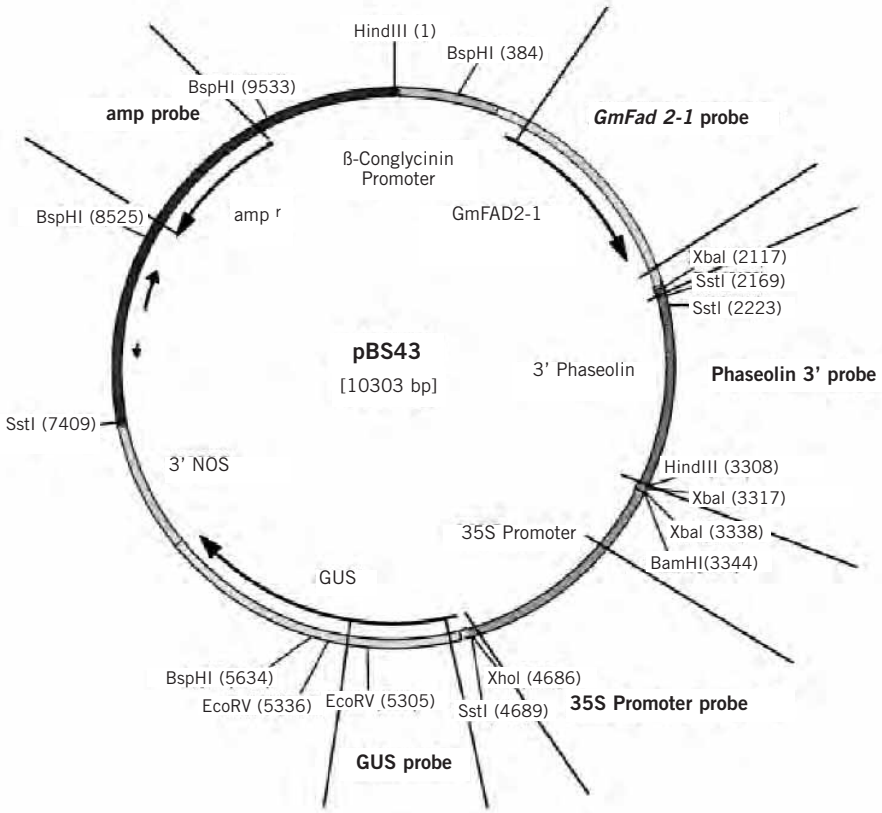


Figure 1: Plasmid map of pBS43. Figure indicates the location of hybridisation probes and restrictions enzyme sites used for Southern blot analysis of high oleic soybeans.

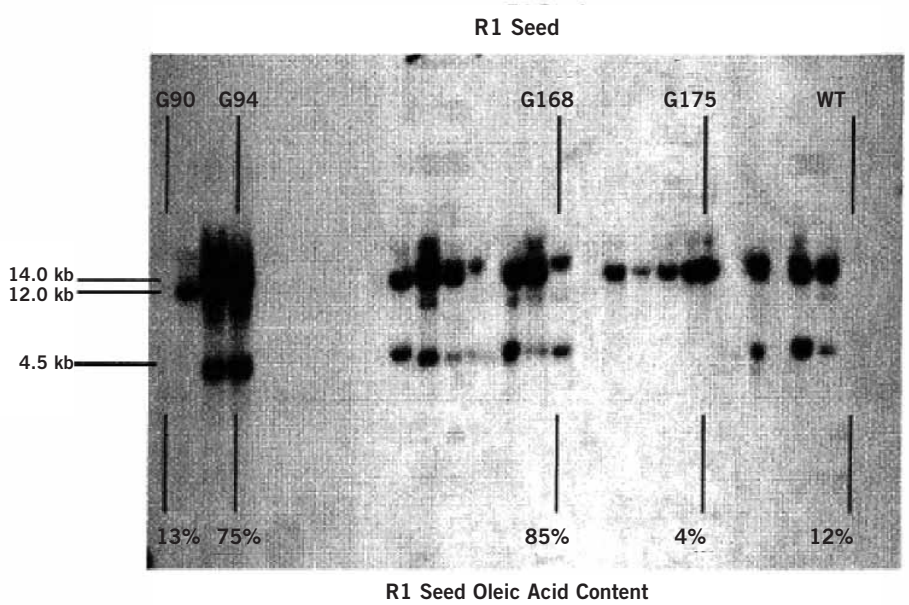


Figure2. Southern blot of DNA isolated from leaf tissue of event 260-05 R1 plants. Plants were grown from chipped seeds analysed for fatty acid composition. The genomic DNA was digested with *BamHI* and probed with the phaseolin 3' probe to detect the integration of the *GmFad 2-1* construct.

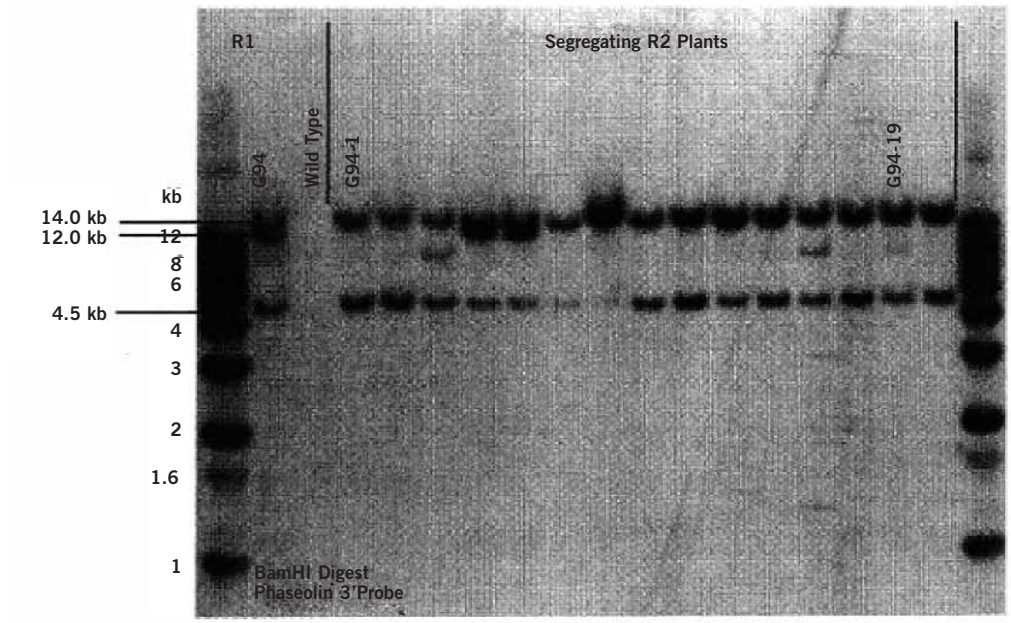


Figure 3. Southern blot on R1 and R2 leaf tissue from G94 R1 seed. The genomic DNA was digested with BamHI and probed with the phaseolin 3' probe to detect the integration of the GmFad 2-1 construct. The G94 seed has three different sized fragments of DNA that hybridise with the probe. G94-1 and G94-19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

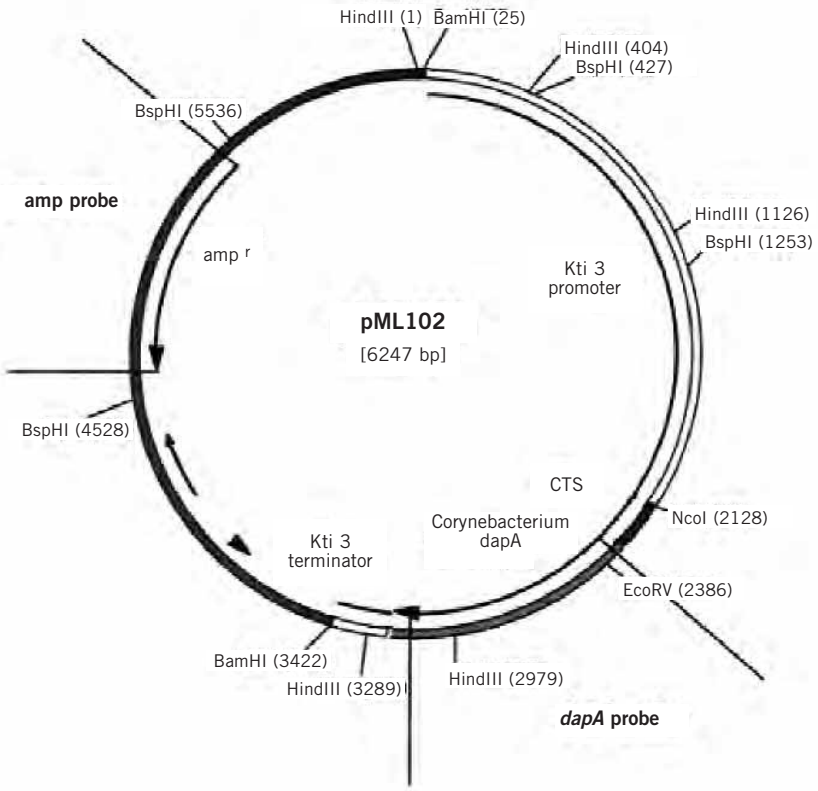


Figure 4: Plasmid map of pML102. Figure indicates the location of hybridisation probes and restriction enzyme sites used for Southern blot analysis of high oleic soybeans.

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

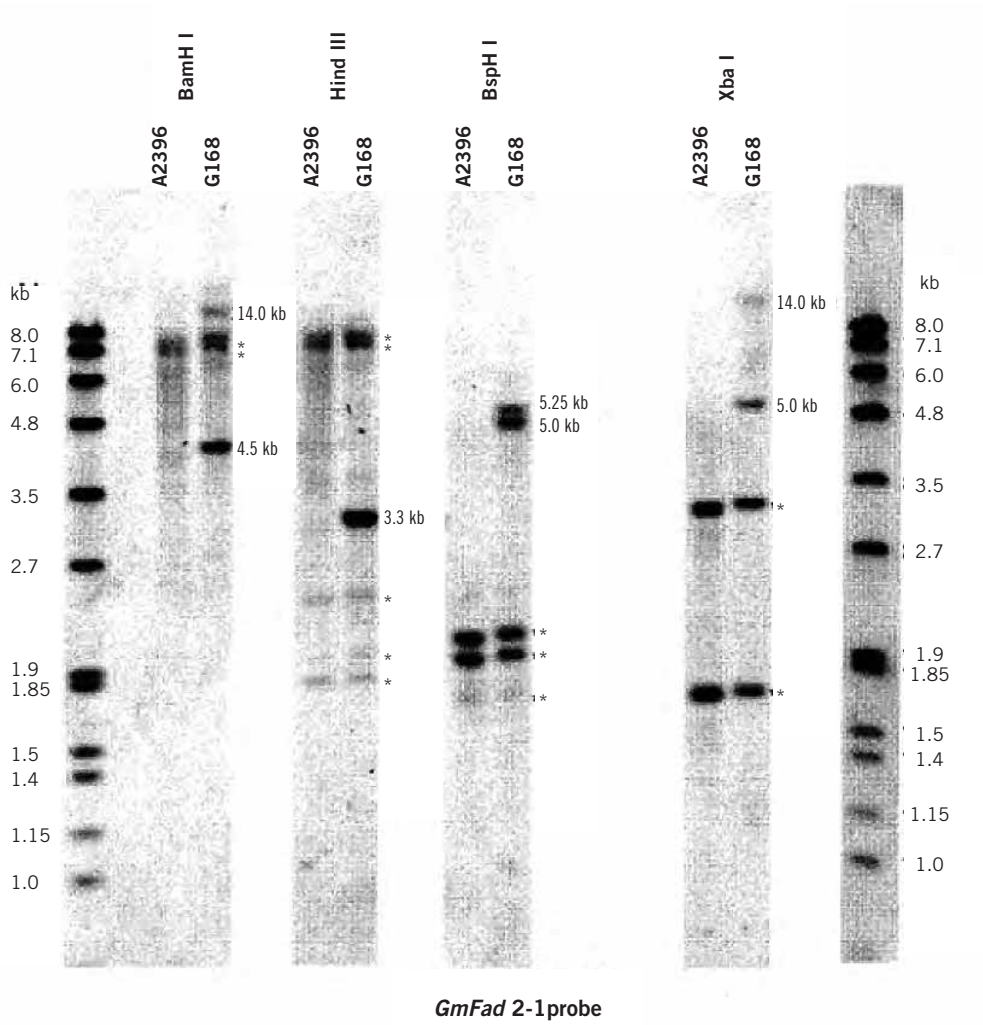


Figure 5a. Southern blot analysis of DNA isolated from R6 leaf tissue of high oleic soybean sub-line G168 and from control line A2396. Genomic DNA was digested with the indicated enzymes and hybridised with the *GmFad 2-1* probe. The underlined molecular weight sizes indicate the sizes of the hybridising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

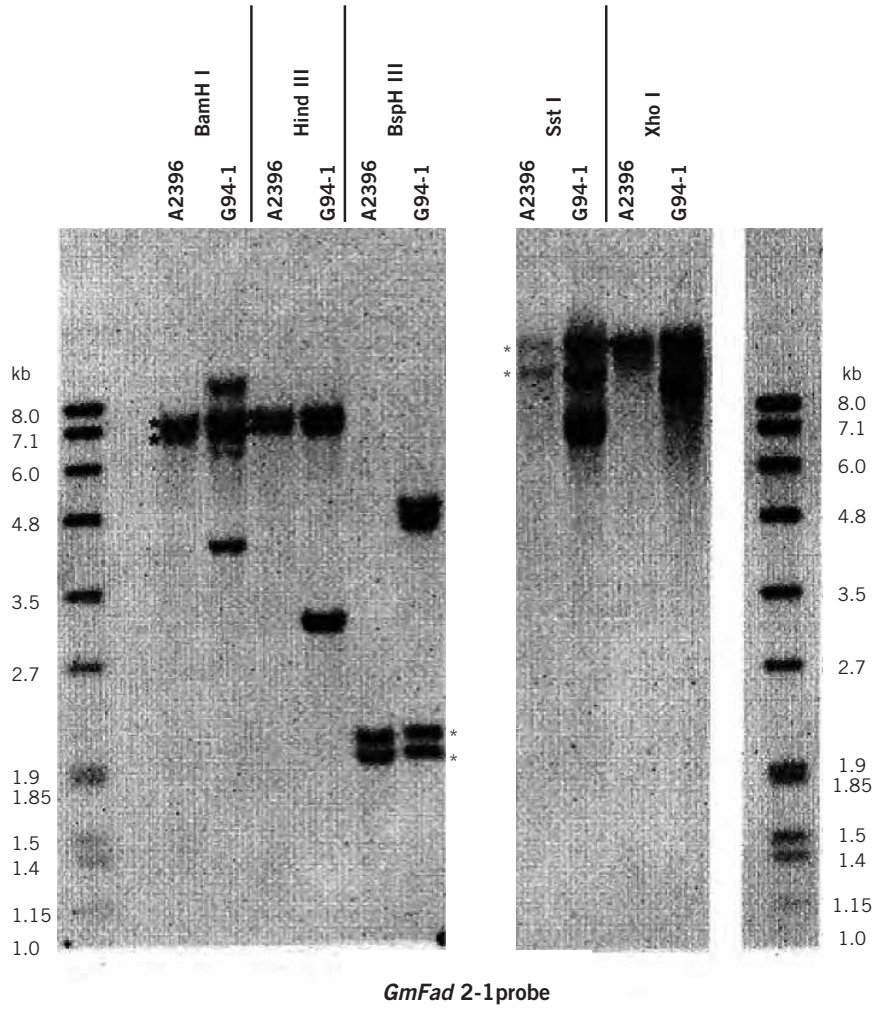


Figure 5b. Southern blot analysis of DNA isolated from R6 leaf tissue of high oleic acid soybean sub-line G94-1 and from control line A2396. Genomic DNA was hybridised with the *GmFad 2-1* probe.

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***				
	GmFad 2-1	Phaseolin 3'	GUS	35S Promoter	*amp*
HindIII	3.3	3.3	7.0	7.0	7.0
BamHI	Border fragment	Border fragment	Border fragment	Border fragment	Border fragment
BspHI	5.25	5.25	5.25	5.25	1.0
SstI	5.1	2.5	2.7	2.5	5.1
XbaI	9.1	1.2	9.1	9.1	9.1
XhoI	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*				
	GmFad 2-1	Phaseolin 3'	GUS	35S Promoter	*amp*
HindIII	3.3[2]	3.3	6.5	6.5	6.5 4.2 3.3
BamHI	14.0 4.5	14.0 4.5	6.5	6.5	14 6.5 2.8
BspHI	5.25 5.0	5.25 5.0	5.25 5.0	5.25 5.0	1.4 1.0
SstI		2.5	2.7 1.7	2.5	
XbaI	14.0 5.0	1.5	6.7	6.7	
XhoI			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

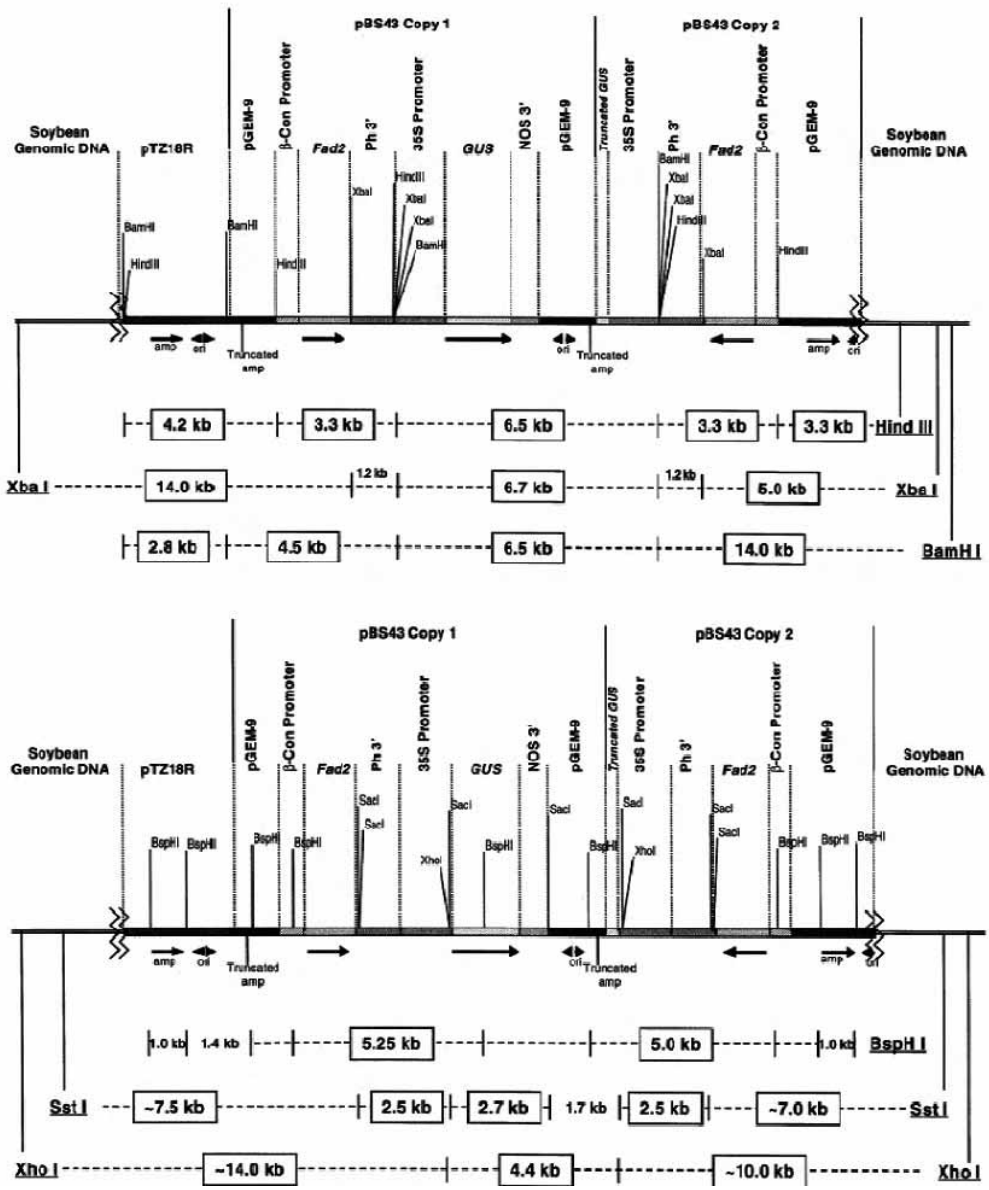


Figure 6a (top) and 6b (bottom). Schematic diagram of insert at *locus A* in high oleic acid soybeans. The top section of each diagram details the inserted genetic elements from the plasmids and their orientation. The bottom section diagrams the hybridising fragments for each restriction enzyme shown 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

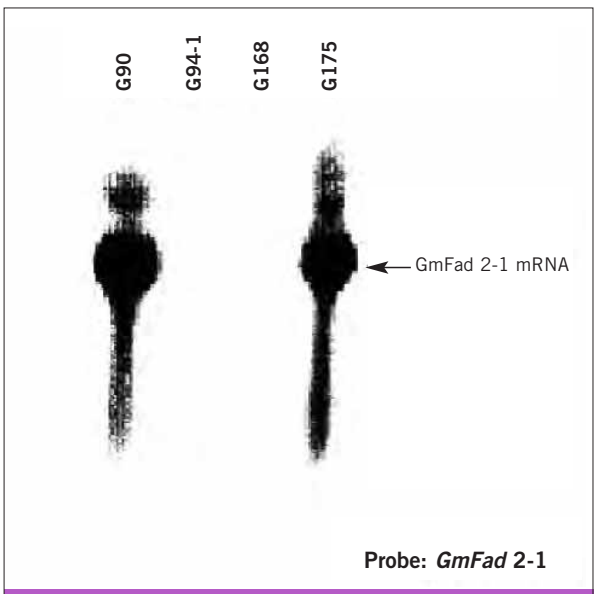


Figure 7. GmFad 2-1 Northern blot analysis on RNA isolated from developing R4 seeds at 20 days after flowering. G90 contains only the endogenous GmFad 2-1 gene and was used as a wild-type control. G94-1 and G168 contain the *GmFad 2-1 locus A* and G175 contains the *GmFad 2-1 locus B*.

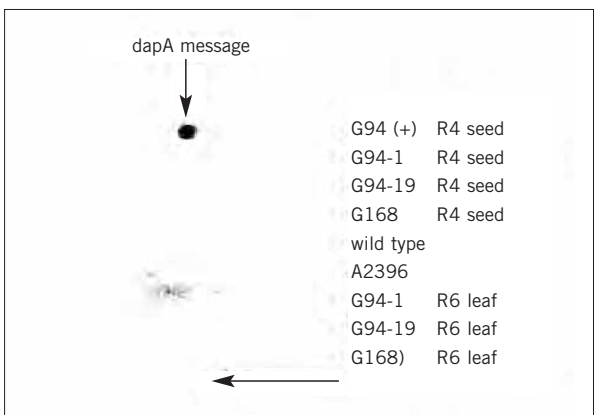


Figure 8. Northern blot analysis of high oleic soybeans. The blot was probed with the dapA coding region. Seed G94 contained the dapA gene and was used as a positive control. Two negative controls were used and labelled as wild type and A2396. The top of the gel is to the right and the bottom is to the 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 likely[37]. 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.

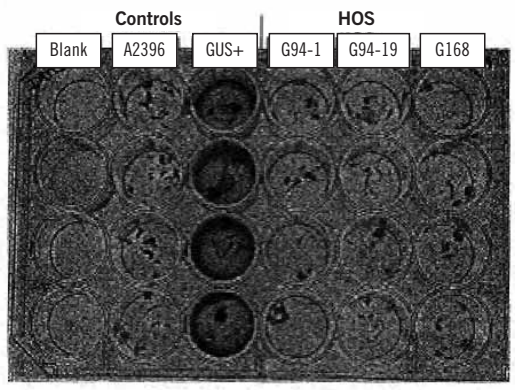
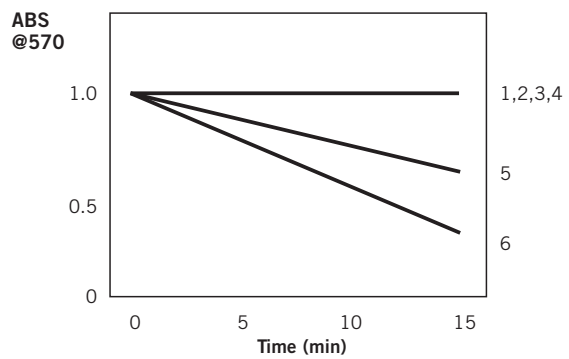


Figure 9. Colorimeteric GUS enzyme assay analysis on R6 seeds of high oleic acid soybean sub-lines G94-1, G94-19 and G168 and positive and negative (A2396) control lines. The positive control is a well-characterised GUS positive soybean line from a different transformation event. The dark colour of the solution in the wells indicates GUS enzyme activity.



1 = 50 µg BSA; 2 = 500µg A2396; 3 = 500µg G94-1;
4 = 2500 µg G94-1; 5 = 50 µg *E. coli*; 6 = 100 µg *E. coli*

Figure 10. b-lactamase activity in high oleic soybeans, elite control A2396 soybeans and in *E. coli* transformed with pBS43.

- 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 (Sjoblad *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 (Metcalfe *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, stacchyose and phytic acid content as well as isoflavone content. In the second study conducted in the summer of 1996, lines G94-1, G94-19 and G168 were grown in parallel with the parental line A2396 at four locations in the United States: Redwood Falls, Minnesota, Kalamazoo, Michigan, Prairie City, Iowa and Cedar Rapids, Iowa. Seeds, representing the R6 generation, were analysed from each of the four locations. Values were obtained from duplicate assays on three replicates from each of the four locations. Analyses were done of proximate, trypsin inhibitor, amino acid, fatty acid, vitamin and mineral, and tocopherol content.

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 content[1] of control and high oleic acid soybeans

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

[1] *Mean values, the range in brackets.*

Table 6. Amino acid content[1] of parental and high oleic acid soybeans

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

[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-octadecadeinoic acid), normally found only in hydrogenated soybean oils and butterfat, was also detected. This isomer is not present in the oil of the parental soybean line A2396.

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

The applicant provided data on the occurrence of the 9,15 isomer of linoleic acid in commonly used oils and fats for frying and baking in Europe. This data is presented in Table 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 content[1] of the control and high oleic acid soybeans

Vitamin or mineral[2]	*Parental control*	*High oleic acid lines*	*Literature range*
	(mg/100 g dry weight unless noted)		
Minerals			
Calcium	264 (245-302)	232 (212-251)	132.7-326.3
Copper	0.64 (0.30-1.00)	0.67 (0.24-1.02)	0.9-5.1
Iron	5.6 (4.2-7.4)	5.8 (3.8-7.9)	3.2-7.9
Magnesium	247 (232-260)	236 (215-261)	
Manganese	2.9 (1.9-4.0)	2.7 (2.2-3.6)	0.4-6.8
Phosphorous	621 (516-742)	636 (501-771)	378-1836
Potassium	1755 (1468-1950)	1689 (1492-1896)	859-1784
Sodium	3.1 (1.1-6.5)	4.3 (2.2-8.7)	
Zinc	4.0 (3.2-4.7)	4.3 (3.0-5.8)	
Vitamins			
Vitamin B6	0.115 (0.098-0.131)	0.125 (0.110-0.141)	
ß-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 (°g/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 content[1] of parental and high oleic acid acid soybean lines

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

[1] *Mean values, range in brackets.*

Table 12. Lectin content[1] of parental and high oleic acid soybean lines

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

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

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 content[1] for parental and high oleic acid soybeans

Anti-nutrient	*Parental control*	*High oleic acid lines*	*Literature range*
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. Stacchyose and raffinose content[1] of parental and high oleic acid soybeans

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

[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 stacchyose, are present in soybeans and can be responsible for the production of unpleasant side effects, such as flatulence, when soybeans and soybean products are ingested. The processing of soybean flours into concentrates and isolates removes these oligosaccharides. Seeds representing the R4 and R5 generations of lines G94-1 and G94-19 were analysed for raffinose and stacchyose content. The results of the analyses are summarised in Table 14.

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

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

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

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

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 ± standard deviations)

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

[1] *No high oleic acid soybean oil usage.*

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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Estudio de caso 3

Evaluación de la inocuidad de la soja genéticamente modificada tolerante a herbicidas

Food safety assessment of a genetically modified herbicide tolerant soybean