FOOD DERIVED FROM INSECT-PROTECTED COTTON LINE COT102

A SAFETY ASSESSMENT

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SUMMARY

Food derived from insect-protected cotton line COT102 has been assessed for its safety for human consumption. This line has been developed primarily for agricultural purposes, to provide growers with a variety of cotton that is resistant to attack by cotton bollworm (*Helicoverpa armigera*) and native budworm (*H. punctigera*), two significant pests of cotton crops in Australia. The evaluation criteria included characterisation of the transferred genes, analysis of changes at the DNA, protein and whole food levels, stability of the introduced genes and assessment of the potential allergenicity or toxicity of any newly expressed proteins. Examination of these criteria enables both the intended and unintended changes to be identified, characterised and evaluated for safety.

History of Use

Cotton is grown primarily for the value of its fibre with cottonseed and its processed products being a by-product of the crop. Cottonseed oil, the major product of cottonseed, has been consumed by humans for decades. Cottonseed oil is considered to be a premium quality oil, valued for its high unsaturated fatty acid content. The other food use of cottonseed is the linters, which are composed of greater than 99% cellulose. Cottonseed itself and the meal fraction are not presently used in Australia and New Zealand as a food for human consumption because they contain naturally occurring toxic substances. These toxins are removed in the production of oil and linters, making them fit for human consumption.

The types of food products likely to contain cottonseed oil are frying oils, mayonnaise, salad dressing, shortening, and margarine. After processing, linters may be used as high fibre dietary products and thickeners in ice cream and salad dressings.

Nature of the Genetic Modification

Cotton line COT102 was generated through the transfer of the *vip3A* gene to the non-transgenic cotton line Coker 312. The *vip3A* gene encodes the vegetative insecticidal protein 3A, denoted VIP3A, which is selectively toxic to certain insect pests of cotton. The *vip3A* gene is derived from the soil and plant bacterium *Bacillus thuringiensis* from which the Cry family of insecticidal proteins is also derived. An antibiotic resistance gene *hph* was also transferred to COT102. The *hph* gene, which encodes the enzyme hygromycin B phosphotransferase (APH4), confers resistance to the antibiotic hygromycin and was used in selecting transformed cotton cells.

Detailed molecular and genetic analyses of cotton line COT102 indicate that the transferred *vip3A* and *hph* genes are stably integrated into the plant genome at a single insertion site and are stably inherited from one generation to the next.

Characterisation of Novel Protein

Cotton line COT102 expresses two novel proteins – VIP3A and APH4. Protein expression analyses indicate that VIP3A is expressed in COT102 cottonseed at low

levels, the highest level recorded being 3.23 µg VIP3A protein/g dry weight. APH4 levels in COT102 cottonseed ranged from undetectable to 150 ng/g dry weight. Neither protein was detected in refined cottonseed oil or cotton fibres. Therefore exposure to the protein through consumption of oils and linters derived from cotton line COT102 would be unlikely and if it did occur the levels of protein would be extremely low.

A number of studies have been done with VIP3A and APH4 to determine their potential toxicity and allergenicity. These studies demonstrate that both proteins are non-toxic to mammals, and have limited potential as food allergens.

Comparative Analyses

Compositional analyses were done to establish the nutritional adequacy of cotton line COT102, and to compare it to the non-transformed control line Coker 312 and commercial varieties of cotton. The constituents measured were protein, fat, carbohydrate, ash, moisture, fibre, fatty acids, amino acids, minerals and the anti-nutrients gossypol and cyclopropenoid fatty acids.

No differences of biological significance were observed between the transgenic cotton line and its non-transgenic counterpart. Several differences in key nutrients and other constituents were noted, however these differences were minor and do not to raise any food safety concerns. On the whole, it was concluded that food from cotton line COT102 is equivalent in composition to that from other commercial cotton varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of the food and indicate that food derived from cotton line COT102 is equivalent in composition to food from non-GM cotton varieties. Small differences in composition were all within normal variation for cotton and would not be expected to have any impact on nutrition.

Conclusion

No potential public health and safety concerns have been identified in the assessment of food produced from cotton line COT102. On the basis of all available information, food produced from this cotton line can be considered as safe and as wholesome as food produced from other cotton varieties.

FOOD DERIVED FROM INSECT-PROTECTED COTTON LINE COT102

A SAFETY ASSESSMENT

BACKGROUND

A safety assessment has been conducted on food derived from cotton that has been genetically modified to be protected against attack from insects. The genetically modified (GM) cotton variety is known as cotton line COT102.

Protection against cotton bollworm (*Helicoverpa armigera*) and native budworm (*H. punctigera*), two significant pests of cotton crops in Australia, is conferred by the expression in the plant of a bacterially derived protein toxin (a *Bt*-toxin) that is specific for these two insects. This protein is known as the vegetative[1] insecticidal protein 3A (VIP3A) and is encoded by the *vip3A* gene. The *vip3A* gene in COT102 is a synthetic version of the *vip3A* gene derived from *Bacillus thuringiensis* subspecies *kurstaki*. The VIP3A protein is an exotoxin and is structurally, functionally and biochemically distinct from the *Bt* delta endotoxins (or Cry proteins), which have been widely used in other insect protected crops.

Cotton line COT102 also contains the hygromycin resistance gene, *hph*, from *Escherichia coli*, expressing the enzyme hygromycin B phosphotransferase (APH4), which confers resistance to the antibiotic hygromycin.

Cottonseed is processed into four major by-products: oil, meal, hulls and linters. Only the oil and the linters are used in food products. Cottonseed oil is used in a variety of food including cooking, salad and frying oils: mayonnaise, salad dressing, shortening, margarine and packaging oils. Cotton linters are used as a cellulose base in high fibre dietary products as well as viscosity enhancers in toothpaste, ice cream and salad dressing. Cottonseed meal is primarily used for stock food, is not currently sold for human consumption in Australia or New Zealand.

HISTORY OF USE

Host Organism

Cotton (*Gossypium hirsutum* L.) is grown as a commercial crop worldwide and has a long history of safe use for both human food and stock feed.

Cotton is grown typically in arid regions of the tropics and sub-tropics. It is primarily grown as a fibre crop with the resulting cottonseed being processed as a by-product. Cottonseed is processed into four major by-products: oil, meal, hulls and linters, but only the oil and the linters are used in food products. Food products from cottonseed are limited to highly processed products due to the presence of the natural toxicants, gossypol and cyclopropenoid fatty acids in the seed. These

[1] VIP3A Insecticidal proteins are expressed vegetatively, that is, in the vegetative stage of growth starting at mid-log phase, as well as during sporulation, establishing a clear distinction from the Cry proteins which are expressed only during sporulation

substances are removed or reduced by the processing of the cottonseed into oil and linters.

Cottonseed oil is regarded as a premium quality oil and has a long history of safe food use. It is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine and packing oil. It is considered to be a healthy oil as it contains predominantly unsaturated fatty acids. Cottonseed oil has been in common use since the middle of the nineteenth century (Jones and King 1990, 1993) and achieved GRAS (Generally Recognised As Safe) status under the United States Federal Food Drug and Cosmetic Act because of its common use prior to 1958. In the USA, it ranks third in volume behind soybean and corn oil, representing about 5-6% of the total domestic fat and oil supply.

Cotton linters are short fibres removed from the cottonseed during processing and are a major source of cellulose for both chemical and food uses. They are used as a cellulose base in products such as high fibre dietary products as well as a viscosity enhancer (thickener) in ice cream, salad dressings and toothpaste.

The other major products derived from cottonseed are the meal and hulls, which are used as stock feed. Cottonseed meal is not used for human consumption in Australia or New Zealand. Although it has permission to be used for human food (after processing) in the USA and other countries, it is primarily sold for stock feed. Human consumption of cottonseed flour has been reported, particularly in Central American countries and India where it is used as a low cost, high quality protein ingredient in special products to help ease malnutrition. In these instances, cottonseed meal is inexpensive and readily available (Ensminger 1994, Franck 1989). Cottonseed flour is also permitted for human consumption in the United States, provided it meets certain specifications for gossypol content, although no products are currently being produced.

Donor Organisms

The source of the *vip3A* gene used in this GM cotton is the ubiquitous soil and plant bacterium *Bacillus thuringiensis* (*Bt),* subspecies *kurstaki*. There are no documented cases of *Bt* causing any adverse effects in humans when present in drinking water or food (IPCS, 2000).

More than 60 serotypes and hundreds of different subspecies of *B. thuringiensis* have been described. Several of these subspecies have been extensively studied and commercially exploited as the active ingredients in a number of different insecticide products for use on agricultural crops, harvested crops in storage, ornamentals, bodies of water and in home gardens. The majority of described *B. thuringiensis* strains have insecticidal activity, mediated via the Cry proteins, predominantly against Lepidopteran insects (moths and butterflies) although a few have activity against Dipteran (mosquitoes and flies), Coleopteran (beetles), and Hemipteran (bugs, leafhoppers etc) insects. Other Cry proteins with toxicity against nematodes, protozoans, flatworms and mites have also been reported (Feitelson et al 1992, Feitelson 1993). The subspecies that served as the source of the *vip3A* gene expressed in cotton COT102 is selectively active against the cotton bollworm

(*Helicoverpa armigera*) and native budworm (*H. punctigera*), two significant pests of cotton in Australia (OGTR, 2002).

Bt proteins are used widely as an insecticide in both conventional and organic agriculture. In Australia, various *Btk* insecticidal products containing VIP3A protein are registered with the Australian Pesticides and Veterinary Medicines Authority (APVMA) for use on cotton, vegetables, fruits, vines, oilseeds, cereal grains, herbs, tobacco, ornamentals, forestry and turf. The very wide use of *Bt* insecticidal proteins indicates that people eating and handling fresh foods may regularly come into contact with this protein.

Insecticidal products using *Bt* were first commercialised in France in the late 1930s (Nester *et al.*, 2002) and were first registered for use in the United States by the Environment Protection Agency (EPA) in 1961 (EPA, 1998). The EPA thus has a vast historical toxicological database for *B. thuringiensis*, which indicates that no adverse health effects have been demonstrated in mammals in any infectivity/ pathogenicity/ toxicity study (Betz *et al.*, 2000, McClintock *et al*., 1995; EPA, 1998). This confirms the long history of safe use of *Bt* formulations in general, and the safety of *B. thuringiensis* as a donor organism.

Escherichia coli

The source of the *hph* gene is the bacterium *Escherichia coli*. *E. coli* belongs to the Enterobacteriaceae, a relatively homogeneous group of rod-shaped, Gram-negative, facultative aerobic bacteria.

Members of the genus *Escherichia* are ubiquitous in the environment and found in the digestive tracts of vertebrates, including humans. The vast majority of *E. coli* strains are harmless to humans, although some strains can cause diarrhoea in travellers and *E. coli* is also the most common cause of urinary tract infections. More recently, a particularly virulent strain of *E. coli*, belonging to the enterohaemorrhagic *E. coli* group, known as 0157:H7, has come to prominence as a food-borne pathogen responsible for causing serious illness.

This particular group of pathogenic *E. coli* are however distinct from the strains of *E. coli* (the K-12 strains) that are used routinely in laboratory manipulations. The K-12 strains of *E. coli* have a long history of safe use and are commonly used as protein production systems in many commercial, including pharmaceutical and food ingredient, applications (Bogosian and Kane, 1991).

Agrobacterium tumefaciens

The species *Agrobacterium tumefaciens* is a Gram-negative, non-spore forming, rod-shaped bacterium commonly found in the soil. It is closely related to other soil bacteria involved in nitrogen fixation by certain plants.

Agrobacterium naturally contains a plasmid (the *Ti* plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally therefore, *Agrobacterium* is a plant pathogen causing root deformation mainly with sugar beets, pome fruit and viniculture crops. However, adaptation of this natural

process has now resulted in the ability to transform a broad range of plant species without causing adverse effects in the host plant.

DESCRIPTION OF THE GENETIC MODIFICATION

Method used in the genetic modification

COT102 was produced via *Agrobacterium*-mediated transformation of *Gossypium hirsutum* L. cultivar Coker 312, using the transformation vector pCOT1, containing the *vip3a* and *hph* genes.

Transformation was carried out by incubating *Agrobacterium* cells, containing the transformation vector pCOT1, with cotton hypocotyl tissue and subsequent plating of the tissue onto synthetic culture medium containing hygromycin B. Plants were regenerated and individually analysed for the presence of the *vip3A* gene by polymerase chain reaction (PCR) techniques and for insecticidal bioactivity. The selected T_0 transformed plants were self-pollinated to produce T_1 seed, and a single homozygous plant designated line COT102 was selected from the T_1 generation for further breeding.

Function and regulation of novel genes

The section of plasmid (the expression cassette) transferred into cotton line COT102 is illustrated in Figure 1. This portion of the pCOT1 plasmid contains the genes that encode the VIP3A and APH4 proteins and the regulatory elements that control the expression of these genes in the transgenic cotton. All the genetic elements present in the expression cassette are described in Table 1.

Table 1: Genetic elements present in the expression cassette in COT102

Genetic Element	Size (kb)	Source	Function
Left border	0.025	*Agrobacterium tumefaciens* nopaline Ti plasmid	Required for transfer of T-DNA in to the plant cell. No function in the plant cell.
nos terminator (2 copies)	0.254	*Agrobacterium tumefaciens* nopaline Ti plasmid	Transcription terminator for *vip3A* and *hph* genes (Bevan *et al*., 1983)
hph gene	1.025	*E. coli*	Antibiotic resistance marker (hygromycin) used to select for transformed plant cells. (Waldron, 1997 and Kaster *et al.*, 1983)
ubiquitin-3 promoter + first intron of the ubiquitin gene	1.720	*Arabidopsis thaliana*	Confers constitutive expression of the *hph* gene in the cotton plant. (Norris *et al*., 1993)
actin-2 promotor	1.407	*Arabidopsis thaliana*	Confers constitutive expression of the *vip3A* gene in the cotton plant. (An *et al*., 1996)
Synthetic *vip3A* gene	2.369	Synthetic version of gene from *B. thuringiensis* (Murray *et al*., 1989)	Gene for production of the VIP3A protein, which is toxic to certain insect pests of cotton. (Estruch *et al*., 1996)
Right border	0.025		Required for transfer of T-DNA in to the plant cell. No function in the plant cell.

The vip3A gene

The *vip3A* gene was derived from *B. thuringiensis* strain AB88 (Estruch *et al*., 1996). It encodes the vegetative insecticidal protein 3A (VIP3A), which is an exotoxin specific to certain lepidopteran pests. Two homologues of the *vip3A* gene have been isolated from *B. thuringiensis*, *vip3A(a)* and *vip3A(b*), and are 98% identical. Cotton line COT102 contains a synthetic version of the bacteria *vip3A(a)* gene, which has been modified to accommodate the preferred codon usage for plants. The synthetic gene encodes a protein that differs by a single amino acid from the protein encoded by the native *vip3A(a).* The native *vip3A(a)* gene encodes a lysine at amino acid position 284 whereas the synthetic version of the gene encodes a glutamine. The substitution is conservative in that lysine and glutamine are polar amino acids having a molecular weight of 146 kDa.

The COT102 encoded VIP3A protein differs from the native VIP3A protein because the first published sequence of this gene (Estruch *et al*., 1996) incorrectly ascribed a glutamine residue at position 284, resulting from an incorrect nucleotide in the corresponding codon at this position in the *vip3A(a)* gene. This error was due to a sequencing error and was only noted after the paper had been published.

The *vip3A* gene is under the regulatory control of the actin-2 promoter (An *et al.*, 1996) derived from *Arabidopsis thaliana*, which confers constitutive expression of the VIP3A protein throughout the plant. The nos terminator from *Agrobacterium*

tumefaciens has been used to terminate transcription and to provide a polyadenylation site.

The hph gene

The transformation construct also contained an antibiotic resistance marker (the *hph* gene) that confers resistance to the antibiotic hygromycin. This was used as a selectable marker during the plant transformation process to select for transformed cells. The *hph* gene is under the control of the ubiquitin promoter and the first intron of the ubiquitin-3 gene of *Arabidopsis thaliana* and the nos terminator from *Agrobacterium tumefaciens.*

Characterisation of the genes in the plant

Traditional molecular techniques were used to analyse the inserted DNA in line COT102. Southern blot analysis was used to determine the insert copy number, intactness of both the VIP3A and APH4 coding regions, intactness of both the VIP3A and APH4 expression cassettes, and to assess whether vector backbone sequences were introduced during the transformation process.

Insert and copy number

Southern hybridisation was used to determine the number and nature of DNA insertions in line COT102. COT102 genomic DNA, non-transgenic Coker 312 genomic DNA and pCOT1 plasmid DNA were digested with restriction enzymes, processed by gel electrophoresis, transferred by blotting to nylon membranes, and probed with eight different probes covering the entire plasmid DNA sequence. Four of the probes were specific to the *vip3A* gene, the *hph* gene, the ubiquitin-3 promoter, and the actin-2 promoter and the other four probes were specific to regions outside of the T-DNA region of pCOT1 (the spectinomycin gene, the repA gene, the VS1 Ori, and the ColE1 Ori). Only the T-DNA region is expected to be transferred. None of the four probes specific to regions outside of the T-DNA hybridised with the COT102 genomic DNA, confirming that no sequences from this region were transferred. The four probes specific to sequences within the T-DNA region (*vip3A*, *hph*, ubiquitin-3 promoter, and actin-2 promoter probes) all hybridised with the COT102 genomic DNA, indicating that all these elements had been transferred as expected.

The copy number was determined by digesting COT102 genomic DNA with HindIII, which cuts once only within the insert, and probing the Southern blot with all four probes. Only one band was visible for each probe, indicating that a single insert is present.

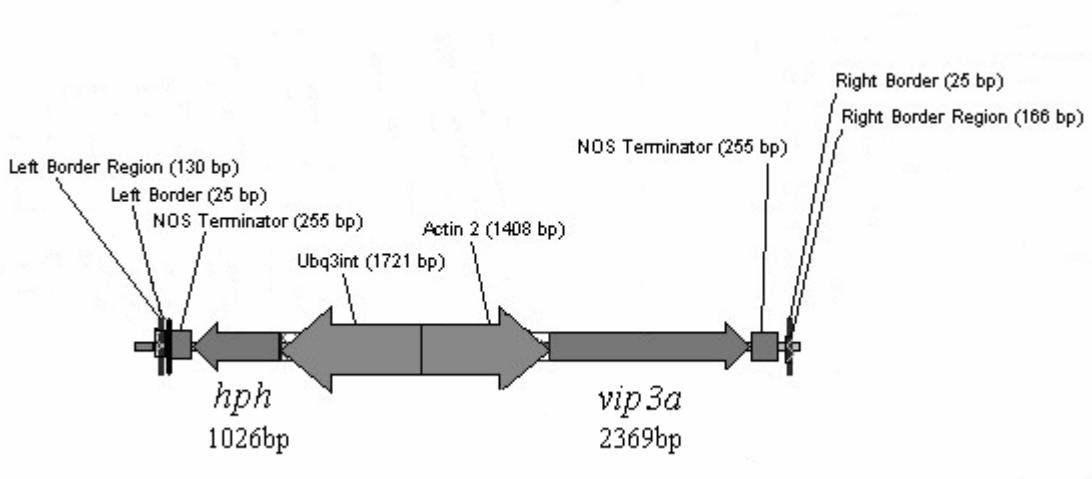

Figure 1: Linear map of insert in cotton line COT102 (7476 base pairs)

PCR and sequence analysis

The COT102 insert DNA and flanking regions were sequenced using a combination of the Universal Genome Walker kit and polymerase chain reaction (PCR) amplification respectively. The sequence of the insert DNA was then compared to the sequence of pCOT1.

The alignment of the transgenic COT102 sequence and the pCOT1 plasmid sequence resulted in an exact alignment of all functional elements contained within the T-DNA. Some truncation occurred to the border sequences during the transformation of Coker 312 producing line COT102. The left border sequence has a truncation of 19-25 bases and the right border sequence has a truncation of 24-25 bases. These deletions have no impact on the expression of the APH4 or VIP3A proteins, and this phenomenon has also been previously observed in *Agrobacterium* transformation and been cited in the literature (Tinland and Hohm, 1995).

Flanking regions and putative Open Reading Frame analysis

Sequence analysis was done on 1385bp of cotton genomic DNA flanking the left border and 461bp of cotton genomic DNA flanking the right border of line COT102. The entire 9356bp sequence including the flanking regions and the insert DNA was analysed in all six reading frames for open reading frames (ORFs) beginning with ATG and exceeding 50 codons. In addition to the *vip3A* and *hph* genes, ten additional ORFs were identified, however further bioinformatic analysis indicated these ORFs lack the appropriate upstream transcriptional regulatory sequences, so are very unlikely to be transcribed.

Conclusion

Detailed molecular analyses have been performed on cotton line COT102 to characterise the novel genes present in the genome. Results indicate that one copy of the T-DNA has been introduced at a single locus in the cotton genomic DNA.

The *vip3A* and *hph* genes are intact and no changes occurred to the DNA sequence of the insert during the transformation process. No other functional ORFs were identified.

Stability of the genetic changes

Breeding process

The transformed cotton cells were selected on hygromycin and regenerated into T_0 cotton plants. These plants were analysed phenotypically, and for expression of the *vip3A* protein. The T_0 plants were self pollinated to produce T_1 seed and a single homozygous plant designated COT102 was selected from the T_1 generation for seed increase and backcrossing into elite varieties. Coker 312 is not grown commercially, so the plants that have been assessed are the progeny from crosses between the genetically modified Coker 312 (COT102) and commercial cultivars. Table 2 indicates the genetic lineage of COT102 and analysis carried out at each generation.

Table 2: Genetic Lineage and analysis of COT102

Generation	Method Produced	Analysis
T_0	Primary transformant in Coker 312 background	
T_1	Result of self pollinated T_0	Homozygous chosen and designated line COT102
T_2	Result of self pollinated T_1	Field evaluations - USA
T_3	Result of self pollinated T_2	Field evaluations - USA
T_4	Result of self pollinated T_3	Field evaluations; fibre quality analysis, cottonseed compositional analysis; molecular characterisation - USA
T_5	Result of self pollinated T_4	Field evaluations -USA
F_1	Result of T_1 generation backcross to Coker 312	Mendelian inheritance analysis
BC_1F_1	Result of F_1 generation backcross to non transgenic commercial germplasm	
BC_1F_2	Result of self-pollinated BC_1F_1	Mendelian inheritance analysis
BC_2F_1	Result of BC_1F_1 generation back cross to non-transgenic commercial germplasm	Mendelian inheritance analysis
BC_2F_2	Result of self pollinated BC_2F_1	Mendelian inheritance analysis
BC_3F_1	Result of BC_2F_1 generation back cross to non-transgenic commercial germplasm	Mendelian inheritance analysis

Segregation analysis

The stability of the insert in COT102 was analysed over five generations (F_1, BC_1F_2, BC_2F_1, BC_2F_2, and BC_3F_1) by examining the expression of the VIP3A protein using an enzyme linked immunosorbent assay (ELISA).

Segregation data comparing the frequency of observed-to-expected numbers of progeny expressing the VIP3A protein were analysed statistically using Chi square analysis. As can be seen in Table 3, all generations segregated as expected for a single insertion site. The F_1 progeny were produced from a homozygous population and, as expected, all expressed the VIP3A protein. All four of the other generations tested produced the expected results with regard to expression of the VIP3A protein, consistent with a Mendelian pattern of inheritance.

Table 3: Segregation data and analysis of progeny of line COT102

Generation	Expected		Observed[1]		Chi square
	Positive	Negative	Positive	Negative	
F_1 (all +)	122	0	122	0	
BC_1F_2 (3:1)	82.5	27.5	85	25	0.1939^{ns}
BC_2F_1 (1:1)	54.5	54.4	47	62	1.7982^{ns}
BC_2F2 (3:1)	36	12	33	15	0.5277^{ns}
BC_3F_1 (1:1)	24	24	26	22	0.1875^{ns}

[1] Number of plants that are positive or negative for VIP3A based on ELISA.
[ns] not significant at p = 0.05 (chi square = 3.84, 1 degree of freedom)

Conclusion

The results of the segregation analysis are consistent with a single site of insertion for the *vip3A* gene and confirm the results of the molecular characterisation. ELISA analysis of a total of five generations indicates that the inserted DNA is stably inherited from one generation to the next.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the presence in the food of the 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 some antibiotics. This section of the report will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from cotton line COT102 to microorganisms present in the human digestive tract.

The COT102 line expresses the hygromycin resistance gene, (*hph*) which was used as a selectable marker in the plant transformation. When used as a selectable marker, the hygromycin resistance gene produces a protein that protects plants from hygromycin B, an aminoglycoside antibiotic produced by *Hygromyces hygroscopicus*.

The first issue that must be considered in relation to the presence of the *hph* gene in cotton line COT102 is the probability that this gene would be successfully transferred

to and expressed in microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

1. a fragment of DNA, containing the coding region of the *hph* gene, would have to be released, probably as a linear fragment, from the DNA in the GM food;

2. the DNA fragment would then have to survive exposure to various nucleases excreted by the salivary glands, the pancreas and the intestine;

3. the DNA fragment would have to compete for uptake with dietary DNA and would have to be available at a time and place in which competent bacteria develop or reside;

4. the recipient bacteria would have to be competent for transformation;

5. the DNA fragment would have to be stably integrated into the bacterium, either as a self-replicating plasmid or through a rare recombination event with the bacterial chromosome;

6. the *hph* gene would have to be expressed, that is, would have to be integrated into the bacterial chromosome in close association with a promoter or would need to already be associated with a promoter that will function in the recipient bacterium;

7. the *hph* gene would have to be stably maintained by the bacterial population.

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

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

In the case of transfer of the *hph* gene, the human health impacts are considered to be negligible. Hygromycin is not used in clinical medicine and has a limited and declining veterinary use in food producing animals. There are no current registered uses of hygromycin B listed with Australian Pesticides and Veterinary Medicines Authority (APVMA).

Conclusion

It is extremely unlikely that the *hph* gene would transfer from cotton line COT102 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 *hph* gene were transferred, the human health impacts would be negligible because the antibiotic to which it confers resistance has no clinical use in Australia and New Zealand.

CHARACTERISATION OF NOVEL PROTEINS

Biochemical function and phenotypic effects

The only novel proteins in cotton line COT102 are VIP3A and APH4.

Studies evaluated:
Artim, L. 2002. Characteristics of Bacillus thuringiensis Vip3A protein and Vip3A cotton plants derived from Event. Dated September 18, 2002. MRID No. 45766501. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

VIP3A

The VIP3A protein (789 amino acids, 89-kDa) is a vegetative insecticidal protein that is toxic to certain insect pests through a novel mode of action. VIP3A is a secreted protein (exotoxin), which can be detected in vegetative growth stages. VIP3A shares no sequence homology with the better-known *B.t.* insecticidal δ-endotoxins (Estruch *et al.*, 1996), also known as the Cry proteins.

The Cry1 toxins, the most studied of the *Bt* δ-endotoxins, are solubilised in the alkaline pH of the lepidopteran midgut and activated by midgut proteases. In sensitive larvae, the activated toxin then binds to specific receptors located on the epithelial cell brush border membranes. After binding, the toxin is integrated into the midgut membrane to form pores, which result in ion imbalances and cause insect death.

The 89-kDa VIP3A full-length protein is also proteolytically activated to an approximately 62-kDa core toxin either by trypsin or gut secretions in the lepidopteran larval midgut and forms pores (ion channels) in the gut membrane of sensitive species, a mechanism that appears to be correlated with its toxicity (Lee *et. al.*, 2003).

In voltage clamping assays with dissected midgut from a susceptible insect, tobacco hornworm (*Manduca sexta)*, activated VIP3A clearly formed pores, whereas the full-length VIP3A protein was incapable of pore formation (Lee *et. al.*, 2003).

VIP3A has been shown to have significantly different receptor binding properties than the Cry proteins; in ligand blotting experiments with brush border membrane vesicles from *M. sexta*, activated Cry1Ab bound to 120-kDa amino peptidase N-like and 250-kDa cadherin-like molecules, whereas activated VIP3A bound to 80-kDa and 120-kDa molecules which are distinct from the known Cry1Ab receptors (Lee *et. al.*, 2003). In addition, separate blotting experiments with activated VIP3A did not show binding to isolated Cry1A receptors (Lee *et. al.*, 2003).

The general symptomatology displayed by sensitive lepidopteran larvae following ingestion of VIP3A protein resembles that caused by *Bt* δ-endotoxins, i.e., cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death (Yu et al, 1997). Histopathological examination of intoxicated larvae reveals that VIP3A specifically impacts the midgut epithelium, which is also the target of the δ-endotoxins. Following exposure of *Agrotis ipsilon* (black cutworm) larvae to 100-200

ng VIP3A/cm^2 diet cube for 24 hours, Yu *et al*. (1997) observed morphological changes in columnar and goblet cells. By 48 hours, the midgut lumen was filled with cellular debris, and by 72 hours, desquamation of the epithelial layer was complete and the larvae were dead. Similar histopathology was observed for *Spodoptera frugiperda* (fall armyworm), but not for *Ostrinia nubilalis* (European corn borer), which is relatively insensitive to VIP3A.

The expression of the VIP3A protein in cotton line COT102 confers protection against lepidopteran insect larvae including black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*), cotton bollworm (*H. armigera*) and native budworm (*H. punctigera*) (Estruch, *et al.*, 1996).

Other than its demonstrated insecticidal activity, VIP3A is not known to have any other biological or catalytic function. Although VIP3A shares no homology with known Cry proteins, extensive testing has established that VIP3A is similarly very specific in its activity, and has demonstrated toxicity only to the larvae of certain lepidopteran species, including key pests of cotton. Further, because VIP3A appears to target a different receptor than Cry proteins in sensitive species, it represents a potentially useful tool in the management of pest resistance to Cry proteins.

APH4

The hygromycin B phosphotransferase protein, APH4, is expressed by the *hph* gene. APH4 is an aminocyclitol phosphotransferase that catalyses the phosphorylation of hygromycin and some closely related aminoglycoside antibiotics. Expression of the APH4 gene in plant cells allows for growth and selection of transformed cells in the presence of hygromycin B.

APH4 has a molecular weight of approximately 42kDa and catalyses the phosphorylation of hygromycin B, thereby inactivating it. The enzyme has a narrow range of substrates, in that it phosphorylates hygromycin B, hygromycin B_2, and the closely-related antibiotics destomycin A and destomycin B, but does not phosphorylate other aminocyclitol or aminoglycoside antibiotics.

The APH4 protein has no role in the final cotton crop.

Protein expression analysis

In cotton line COT102 the only novel proteins expected to be expressed are the VIP3A protein and the APH4 protein. Expression levels of these proteins were determined using an enzyme linked immunosorbent assay (ELISA) and are reported below.

Studies evaluated:
Artim, L. (2002). Analysis of processed COT102 cottonseed products for yield and presence of gossypol and VIP3A protein. Report SSB-017-02. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

Privalle, L. (2002d) Quantification of VIP3A and APH4 proteins in cotton tissues and whole plants derived from transformation Line COT102. Report SSB-001-2. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report

VIP3A and APH4 protein expression levels

With regard to the safety of cotton line COT102 and foods derived from this line, it is important to determine the level of expression of VIP3A and APH4 in order to establish potential dietary exposure to this protein.

Two studies were done to determine the expression levels of VIP3A and APH4 in different plant tissues. The first study (Artim, 2002) was undertaken to evaluate and compare the processing of COT102 with its non-transgenic counterpart, Coker 312 and determine levels of the VIP3A protein in the defatted meal and processed oil.

Cottonseed from line COT102 and Coker 312 were obtained from field grown plants produced in Leland, Mississippi during the 2001 planting season. Approximately 1.4 kilograms of fuzzy cottonseed from each line were sent to the Food Protein Research and Development Center, Texas A&M University for processing into defatted meal and cottonseed oil. Samples of non-toasted meal, toasted meal and refined oil were quantitatively analysed for the presence of the VIP3A protein by ELISA using immunoaffinity-purified polyclonal goat and protein A-purified rabbit antibodies specific for VIP3A.

As shown in Table 4, VIP3A protein was present in the defatted non-toasted meal from COT102 at a level of 2.75 µg/g and was not detected in the control. After toasting the meal, the VIP3A protein concentration dropped significantly to 0.23 µg/g toasted meal.

VIP3A protein was not detected in the COT102 refined oil. In fact, no protein of any type was detected in the oil using a standard Coomassie blue protein assay.

Table 4: VIP3A Levels in Processed Cottonseed Products

VIP3A µg/g	Non-Toasted meal[1]	Non-Toasted meal[2]	Toasted meal[2]	Refined Oil
COT102	2.75 ± 0.12	2.57 ± 0.03	0.23 ± 0.02	Nd
Coker 312	Nd	Nd	Nd	Nd

[1] ELISA analysis completed on May 9, 2002.
[2] ELISA analysis completed on November 15, 2002.
Nd = not detected (the mean absorbance generated during ELISA did not exceed that of the controls). The limit of detection was from 40 ng/g – 270 ng/g fresh weight, depending on the tissue and developmental stage.

The second study (Privalle, 2002d) aimed to characterise the level and site of expression of transgenic proteins in the cotton plants derived from line COT102. The concentrations of VIP3A protein (the active insecticidal protein) and APH4 protein (the selectable marker protein) were determined by ELISA for whole plants and designated plant tissues obtained from three separate field locations (Georgia, Texas and Arizona in the United States of America) at six developmental stages:

Four leaf stage, *ca*. 2 weeks post emergence

Squaring, *ca*. 4 weeks post emergence
First White Bloom, *ca*. 9 weeks post emergence
Peak Bloom, *ca*. 13 weeks post emergence
First Open Boll, *ca*. 15 weeks post emergence
Pre-harvest, *ca*. 22 weeks post emergence
(Sampling time varied depending on environmental conditions)

In most cases, ten whole plants, including roots, from the transgenic COT102 line, plus two plants from the control Coker 312 line, were harvested. Plant tissues that were analysed were leaves, roots, bolls, squares, and whole plants. In addition, pollen and nectar were collected from 15-25 greenhouse-grown plants to produce a single pooled sample of pollen and a single pooled sample of nectar for each genotype.

For most tissues and sampling stages, VIP3A concentrations were generally comparable across all locations. Across all developmental stages and locations, mean VIP3A concentrations measured in whole-plant samples ranged from *ca*. 1-13 µg/g fresh weight (1-73 µg VIP3A/g dry wt). Leaves had the highest mean VIP3A levels. Mean concentrations measured in all plant tissues measured are shown in Table 5. The limit of quantitation (LOQ) of the ELISA was estimated based on the lowest concentration of pure reference protein lying on the linear portion of the standard curve, the maximum volume of a control extract that could be analysed without background interference, and the corresponding weight of the sample that the aliquot represented. The LOQ ranged from 40 ng/g -270 ng/g fresh weight, depending on the tissue and developmental stage.

Table 5: Mean VIP3A concentrations across all developmental stages and locations

Mean VIP3A concentration	Fresh weight (µg/g)	Dry weight (µg/g)
Whole plant	1-13	1-73
Leaves	3-22	5-118
Squares	<4	<17
Roots	<2	<7
Bolls	<1	<9

For all test locations, mean VIP3A concentration measured in seeds (Table 6) were *ca*. 3 µg/g on a fresh weight and dry weight basis. The VIP3A concentration measured in pollen was *ca*. 1 µg/g air dried pollen. The values reported were not corrected for extraction efficiency however, the estimated extraction efficiencies for the VIP3A quantitation method ranged from 80-90% across the various plant tissues analysed.

APH4 was either not detectable in most COT102 plant tissues or the levels were too low to quantify. Pollen was the only tissue in which quantifiable levels, *ca*. 2.3 µg APH4/g air dried pollen, were measured. Refined oil was not tested specifically for APH4 protein.

As APH4 was not detected or detected at very low levels in COT102 seeds, and given the absence of any detectable protein of any sort in the refined oil, human exposure to the APH4 protein through consumption of oil and linters derived from

cotton line COT102 would be unlikely and if it did occur the levels of protein would be extremely low.

Table 6: VIP3A and APH4 protein levels in seeds and cotton fibre from the pre-harvest stage during the development of COT102 plants. (n=5)

Tissue	VIP3A Levels [1]		APH4 Levels [1]	
	Mean µg VIP3A/g fresh weight ± SD (Range)	Mean µg VIP3A/g dry weight ± SD (Range)	Mean ng APH4/g fresh weight ± SD (Range)	Mean ng APH4/g dry weight ± SD (Range)
Seeds				
Georgia	2.88 ± 0.28 (2.52 – 3.28)	3.23 ± 0.31 (2.86 – 3.65)	<55[2] (nd[3] -<137)	<60 (nd - <150)
Texas	2.70 ± 0.27 (2.41 –3.05)	2.99 ± 0.29 (2.65 – 3.65)	nd	nd
Arizona	2.51 ± 0.25 (2.14 – 2.82)	2.72 ± 0.28 (2.33 – 3.08)	nd	nd
Cotton Fibre				
Georgia		nd		nd
Texas		nd		nd
Arizona		nd		nd

[1] Values were determined by ELISA and were not corrected for extraction efficiency. Values for all control plants corresponded to 0 ng VIP3A or APH4/g fresh or dry weight.
[2] Where traces of APH4 were found, but could not be quantitated, the value is indicated as less than the lower limit of quantification.
[3] 'nd' VIP3A or APH4 was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.
[4] Only dry weight values for cotton fibre were calculated.

Potential toxicity of novel protein

Studies evaluated:

Glaza, S.M. (2000) Single Dose Oral Toxicity Study with VIP3A-0199 in Mice; Covance Laboratories Inc. Study No 7012-100. Novartis Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

Glaza, S.M. (2002a) Single Dose Oral Toxicity Study with VIP3A-0100 in Mice; Covance Laboratories Inc. Study No 7012-103. Novartis Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

Glaza, S.M. (2002b) Single Dose Oral Toxicity Study with LPPACHA-0199 in Mice; Covance Laboratories Inc. Study No 7012-102. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

Kuhn, J.O. (1997) Acute Oral Toxicity Study of VIP3A Protein in Mice; Sample No. VIP3A-0196; Stillmeadow, Inc. Study No. 2989-96; Novartis Seeds Inc., Research Triangle Park, North Carolina, USA. Unpublished Syngenta Report.

Privalle, L. (2002a) Characterization of VIP3A Protein Produced in COT102-Derived Cotton and Comparison with VIP3A Protein Expressed in Both Maize (Corn) Derived From Line PACHA and Recombinant *Escherichia coli*. Syngenta Seeds Biotechnology Report No. SSB-015-02. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA. Unpublished Syngenta Report.

Privalle, L (2002e) Characterisation of the VIP3A protein produced in Pacha derived maize (corn) and comparison with VIP3A protein expressed in recombinant E. coli. Report SSB-004-00. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA. Unpublished Syngenta Report.

Equivalence of VIP3A protein used in acute toxicity studies to the VIP3A produced in cotton line COT102.

It was not possible to extract sufficient quantities of VIP3A from transgenic cotton plants to provide the high doses required for acute oral toxicity testing, therefore VIP3A was produced in a recombinant *E. coli* over-expression system for use in these studies. Additionally, as part of a separate testing program to evaluate the safety of VIP3A expressed in maize plants (which are not the subject of this assessment), a VIP3A test substance was prepared from transgenic corn plants expressing VIP3A protein (line Pacha), by extracting corn leaf protein and enriching for VIP3A content. This provided a source of plant-derived VIP3A at a concentration that was several fold higher than the VIP3A concentrations in plant tissue, however the final protein sample was only 0.36% VIP3A by weight compared to up to 73.5% VIP3A by weight in the bacterially produced VIP3A protein sample.

Two studies were submitted demonstrating that the bacterially derived VIP3A and maize (line Pacha) derived VIP3A are equivalent to cotton line COT102 derived VIP3A (Privalle, 2002a, 2002e). VIP3A protein from these three sources, recombinant *E. coli* (test substance VIP3A-0199, described below), VIP3A corn (line Pacha test substances LLPACHA-0199) and COT102 cotton (test substance LPCOT102-0102), were analysed by SDS PAGE and Western blotting to determine if the plant-expressed VIP3A has been subject to any post-translational modification that would be detected as altered molecular weight, e.g. glycosylation. Visual analysis of the Western blot demonstrated that the VIP3A from all three sources have the same apparent molecular weight, as determined by visual analysis of the Western blot. VIP3A from all three sources have a predicted molecular weight of approximately 89kDa and cross-reacted immunologically with the same anti-VIP3A antibody.

Mass spectral analysis and N-terminal sequencing were also performed on VIP3A from *E. coli*, Pacha corn and COT102 cotton. Through these analyses 85% of the complete cotton VIP3A protein (test substance LPCOT102-0102) was sequenced. This sequence corresponded exactly with the predicted amino acid sequence of VIP3A and no evidence of any post-translational modifications was observed. The cotton expressed VIP3A protein had the predicted N-terminal amino acids, beginning with asparagine 18. The 17 N-terminal amino acids not detected in the cotton-expressed VIP3A could represent *in plant* proteolysis or *in vitro* degradation. However, as the size of the cotton VIP3A protein was equivalent to the bacterially produced protein (based on Western blot analysis), it was thought that the N-terminal peptide may yet be identified with continued effort.

Ninety-five percent of the complete *E. coli*-derived VIP3A sequence (test substance VIP3A-0199) and 93% of the complete Pacha-derived VIP3A sequence (test substance IAPACHA-0100) was identified using mass spectrometry. These peptide sequences were identical to the predicted VIP3A amino acid sequence and revealed no evidence of post-translational modifications. N-terminal sequencing identified the expected amino acid sequence, starting at methionine-1 for the *E. coli* produced VIP3A and at lysine-3 for the Pacha produced VIP3A protein.

Comparisons of the biological activity of *E. coli*-expressed and cotton-expressed VIP3A protein in larval diet bioassays with several lepidopteran species

demonstrated very similar activities and rank order of VIP3A sensitivity among the four species surveyed.

Based on the various functional and biochemical parameters evaluated, it can be concluded the VIP3A proteins from recombinant *E. coli*, Pacha maize, and COT102 cotton are equivalent and therefore results of the acute oral toxicity studies on the VIP3A protein derived from *E. coli* and Pacha corn are applicable to COT102 derived VIP3A protein.

Potential toxicity of VIP3A

When proteins are toxic, they are known to act via acute mechanisms and at very low doses (Sjoblad *et al*., 1992). Therefore, if a protein demonstrates no acute oral toxicity in high-dose testing using a standard laboratory mammalian test species, it is unlikely to be toxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposures.

Despite the expectation that there will be very little or no human exposure to the VIP3A and APH4 proteins as they are not present at detectable levels in either the oil or linters of cotton line COT102, four acute oral toxicity studies in mice were submitted to demonstrate that the VIP3A protein is non-toxic. The four different test substances are described below.

Three different *E. coli* produced VIP3A test substances were used as well as a corn produced VIP3A. The three bacterially produced VIP3A proteins are from three slightly different constructs as described below.

VIP3A-0196; VIP3A protein produced in an *E. coli* expression system. The VIP3A protein encoded by the expression vector is identical in amino acid sequence to that encoded by the synthetic *vip3A* gene in line COT102 cotton and line Pacha corn, except that as a result of the addition of a restriction site in cloning, the second amino acid is aspartate instead of asparagine and at amino acid 284, the native lysine residue is present instead of the glutamine residue encoded by the *vip3A* gene in COT102 cotton and Pacha corn. Test substance VIP3A-0196 was estimated to contain 32% VIP3A by weight and 53% of the total protein as measured by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation and ELISA.

VIP3A-0199; VIP3A protein produced in an *E. coli* expression system. This preparation was used for the equivalence studies described above. The VIP3A protein encoded by the expression vector is the native *vip3A* gene from *B. thuringiensis* and as such differs from test substance VIP3A-0196 above in that it contains a lysine residue at amino acid position 284. Test substance VIP3A-0199 was estimated to contain 54% VIP3A by weight and 84% of the total protein as measured by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation and ELISA.

VIP3A-0100; VIP3A protein produced in an *E. coli* expression system. The expression vector contains the same synthetic *vip3A* gene that was used to produce cotton line COT102 and Pacha corn. Test substance VIP3A-0100 was estimated to

contain 73.5% VIP3A by weight and 81% of the total protein as measured by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation and ELISA.

LPPACHA-0199; VIP3A-enriched protein extracted from corn leaves. Test substance IAPPACHA-0100, which was used in the N-terminal sequencing and mass spectrometry described above, is also a VIP3A-enriched protein sample extracted from corn line Pacha leaves. Test substance LPPACHA-0199 was estimated to contain 0.36% VIP3A by weight and 0.39% of the total protein as measured by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation and ELISA.

Four acute oral toxicity studies in mice were evaluated, one with each of the test substances above. These are summarised below.

Test Material	Vehicle Control[1]	Test Species	Dose (by gavage)	Results
VIP3A-0196 (32% VIP3A by weight)	2% w/v	6 female and 6 male HSD:ICR mice	5050 mg/kg body weight (1616 mg VIP3A/kg bw)	No toxicity
VIP3A-0199 (54% VIP3A by weight)	0.5% w/v	12 male and 12 female CD-1®(ICR)BR mice; 6 – 8-weeks old	5000 mg/kg body weight (2700 mg VIP3A/kg bw)	No toxicity
VIP3A-0100 (73.5% VIP3A by weight)	0.5% w/v	8 male and 8 female Crl-1®(ICR)BR mice; 4 – 5-weeks old	5000 mg/kg body weight (3675 mg VIP3A/kg bw)	No toxicity
LPPACHA-0199 (0.36% VIP3A by weight)	0.5% w/v	5 male and 5 female CD-1®(ICR)BR mice; 4 – 6-weeks old	5000 mg/kg body weight (18 mg VIP3A protein/kg bw)	No toxicity

[1]carboxymethylcellulose

Overall LD_{50} Estimate for VIP3A Protein

Among the four acute oral toxicity studies, the highest dose tested was *ca*. 3675 mg VIP3A/kg body weight. Because toxicity was not observed at this does, it can be concluded that the LD_{50} for pure VIP3A is >3675 mg/kg body weight.

Potential toxicity of APH4

Study evaluated:
Johnson, I. R. (2002) APH4-0102: Acute Oral Toxicity of APH4 Protein in the Mouse; Central Toxicology Laboratory Study No. AM7143; Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA. Unpublished Syngenta Report.

In most tissues of COT102 plants, APH4 has not been detected or the levels have been too low to quantify by ELISA (<150 ng/g dry weight), so it was not possible to extract sufficient APH4 protein from COT102 transformed plants for toxicology

studies. Instead, APH4 protein was expressed from the inducible over-expression pET3a vector in *E. coli* BL21DE3pLysS cells. The APH4 protein encoded by this vector was identical in amino acid sequence to that encoded by the plant transformation vector pCOT1, except for an additional 11 amino acids at the C-terminus from the T7 tag and three amino acids at the N-terminus from the vector polylinker. Following purification from *E. coli* the resulting sample, designated Test Substance APH4-0102, was estimated by ELISA to contain *ca.* 42.6% APH4 protein by weight. The material was confirmed to be enzymatically active.

Although it was not possible to confirm the equivalence of the APH4 protein in the Test substance APH4-0102 with that produced in line COT102 plants as it was not possible to extract sufficient APH4 from the plants for these analyses, the sequence identity, combined with the presence of enzymatic activity, indicates that the two proteins can be considered to be equivalent.

Test Material	Vehicle Control	Test Species	Dose (by gavage)	Results
APH4-0102 (42.6% APH4 by weight)	1% methylcellulose	5 male and 5 female AP_fCD-1 mice	1828 mg/kg body weight (779 mg APH4 /kg bw)	No toxicity

There is no evidence of toxicity of the test substance at 1828 mg/kg body weight, representing 799 mg APH4 protein/kg body weight. The estimated LD_{50} value for pure APH4 protein in male and female mice is >779 mg/kg body weight, the single dose tested.

Similarities with known protein toxins

Bioinformatic analyses were done to determine if the VIP3A and APH4 proteins have any similarity with known protein toxins.

The VIP3A protein sequence (GenBank Accession Number AAC37036.1) was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI) GenBank Database (NCBI, 2002) containing all publicly available protein sequences. The procedure used allowed a determination of whether any proteins in the database showed significant amino acid homology to the VIP3A protein, indicating they may be closely related to VIP3A, and whether any sequences with significant homology to VIP3A were known to be toxins.

The amino acid sequence homology search was performed using the BLASTP search program, carried out by comparing the complete amino acid sequence of the VIP3A protein with all protein sequences present in the reference databases.

The VIP3A protein showed no significant homology with any non-VIP3A proteins in the public GenBank database, including other proteins from *B. thuringiensis* and proteins identified as toxins.

Similarly, extensive bioinformatics searches revealed no significant amino acid homology between the APH4 protein and proteins known to be toxic in the GenBank

database.

Potential allergenicity of novel proteins

There are concerns that new proteins introduced into food will cause allergic reactions in some individuals. The potential allergenicity of a novel protein is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on the source of the novel protein, any significant amino acid similarity between the novel protein and that of known allergens, and the structural properties of the novel protein, including susceptibility to degradation in simulated digestion models. Applying such criteria systematically provides reasonable evidence about the potential of the newly introduced proteins to act as an allergen (Lehrer and Reese 1998; Jones and Maryanski 1991).

The two novel proteins expressed in cotton line COT102 are VIP3A and APH4. These proteins were assessed using these criteria for their potential allergenicity.

Studies evaluated:
Vlachos, D. (2002a) Summary of Mammalian Toxicity Data for the VIP3A and APH4 proteins Produced by Transgenic VIP3A Cotton Line COT102. An unpublished Syngenta summary report, dated September 16, 2002, submitted by Syngenta Seeds, Inc. to US EPA on September 24 2002.

Vlachos, D. (2002b) Supplement to Summary of Mammalian Toxicity Data for VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Line COT102. An unpublished Syngenta summary report, dated December 21. 2002.

Similarity to known allergens

VIP3A

An extensive bioinformatics search was performed to determine whether the amino acid sequence of the VIP3A protein shows homology with proteins known or suspected to be allergens. Three different similarity searches were performed comparing the VIP3A protein to the entries in the Syngenta Biotechnology Incorporated (SBI) Allergen Database. This database was compiled from entries identified as allergens or putative allergens in public protein databases, and was supplemented with additional amino acid sequences identified from the scientific literature. First, the entire VIP3A protein sequence was compared to the allergen sequences using the FASTA search algorithm. Second, contiguous VIP3A peptides of 80 amino acids, overlapping by 10 amino acids, were compared to the allergen sequences using the FASTA search algorithm. Third, the VIP3A protein sequence was screened for matches of eight contiguous amino acids between VIP3A and the allergen sequences. The results of these analyses revealed no significant similarity of the VIP3A protein to known or putative allergens for which amino acid sequences were available. This indicates that VIP3A does not have linear amino acid homology to any known allergens.

APH4

Similarly, these same three bioinformatic searches were performed comparing the APH4 protein sequence to the SBI Allergen Database. The results of these analyses revealed no significant similarity of the APH4 protein to known or putative allergens for which amino acid sequences are available.

In vitro digestibility

Studies evaluated:
Privalle, L. (2002b) *In vitro* Digestibility of VIP3A Protein Under Simulated Mammalian Gastric Conditions. Syngenta Seeds Biotechnology Report No. SSB-008-001. Syngenta Seeds. Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

Privalle, L. (2002c) *In vitro* Digestibility of APH4 Protein Under Simulated Mammalian Gastric and Intestinal Conditions. Syngenta Seeds Biotechnology Report No. SSB-001-2. Syngenta Seeds Inc., Research Triangle Park, North Carolina, USA, Unpublished Syngenta Report.

Vlachos, D. Summary of Mammalian Safety Data for the VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Event COT102. An unpublished Syngenta summary report, dated 16 September 2002.

Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergic response (Kimber et al 1999; Astwood et al 1996; Metcalfe et al 1996). The VIP3A and APH4 proteins were therefore investigated for their digestibility in simulated digestion models.

VIP3A

The susceptibility of VIP3A protein to proteolytic degradation was tested in simulated mammalian gastric fluid (SGF) containing pepsin. VIP3A from two sources, recombinant *E. coli* (VIP3A-0100) and leaves of transgenic corn plants (Line Pacha), was evaluated. VIP3A from these sources has been shown to be equivalent to that produced in COT102 VIP3A cotton. VIP3A from both sources was susceptible to pepsin degradation. No intact VIP3A (89kDa) was detected upon immediate sampling of the digestion reaction mixtures, as assessed by SDS-PAGE followed by Western blot analysis or staining with Coomassie blue. Using *E. coli* produced VIP3A (at a significantly higher concentration than was possible for corn-produced VIP3A, two lower molecular weight bands (9kDa and 6kDa) were still detectable as minor bands after 2 minutes in SGF. A progressive decline in intensity of these bands during the 60 minute incubation in SGF indicated that they represented transient VIP3A degradation products that were susceptible to pepsin digestion. These data support a conclusion that VIP3A expression in transgenic plants will be as readily digested as conventional dietary protein under typical mammalian gastric conditions.

In addition, many food allergens are stable to heat and food processing and may be glycosylated. However, mass spectral analysis of VIP3A peptides from cotton line COT102 showed no evidence of glycosylation or other post-translational modifications. Further, the stability of the VIP3A (VIP3A-0199) protein under a range of heat and pH conditions was evaluated. Instability of the protein was measured as the loss of bioactivity against VIP3A-sensitive fall armyworm larvae. Although incubation of VIP3A at ambient temperature or at 37ºC for 30 minutes had no apparent effect on its bioactivity, VIP3A protein was inactivated by heating at 55ºC

for 30 minutes.

Also, when cottonseed meal prepared from COT102 cotton was subjected to a standard toasting procedure that included a steam heat treatment of 110ºC for 40 minutes, the VIP3A concentration measured in the toasted cottonseed meal by ELISA was reduced to less than one-tenth of the concentration prior to testing. Therefore it can be seen that VIP3A is unstable to heat and food processing. This information suggests that VIP3A has limited potential to become a food allergen.

APH4

The susceptibility of APH4 to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin and simulated mammalian intestinal fluid (SIF) containing pancreatin. APH4, produced in recombinant *E. coli* (identical in amino acid sequence to that encoded by the plant transformation vector pCOT1, except for an additional 11 amino acids from the T7 tag and 3 amino acids from the vector polylinker), was rapidly degraded in both SGF and SIF. No intact APH4 (42kDa) was detected upon immediate sampling of the reaction mixtures, as assessed by SDS PAGE followed by Coomassie staining and Western blot analysis. All lower molecular weight bands disappeared after two minutes in SGF and five minutes in SIF. These data support a conclusion that APH4 expressed in transgenic plants will be as readily digested as conventional dietary protein under typical mammalian gastric conditions. Furthermore, in the unlikely event that APH4 protein survives the gastric environment, it will be degraded rapidly in the intestines.

Summary and conclusion

Cotton line COT102 expresses two novel proteins – VIP3A and APH4. VIP3A is expressed in COT102 cottonseed at low levels with the highest expression level recorded being 3.23 µg VIP3A protein/g dry weight. APH4 levels in COT102 cottonseed ranged from undetectable to 150 ng/g dry weight. Neither protein was detected in cottonseed oil or cotton fibres.

A number of studies have been done with the VIP3A and APH4 proteins to determine their potential toxicity and allergenicity. These studies demonstrate that both proteins are non-toxic to mammals, and have limited potential to be allergenic.

COMPARATIVE ANALYSES

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 critical components to be measured are determined by identifying key nutrients, key toxicants and anti-nutrients for the food source in question (FAO 1996). 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 cottonseed that have been considered in this comparison include proximates, amino acids, fatty acids, minerals, and the toxicants gossypol and cyclopropenoid fatty acids.

Nutrient analysis

To determine whether unexpected changes had occurred in the nutrient composition of cotton line COT102 as a result of the genetic modification, and to assess the nutritional adequacy of this line, compositional analysis was done on whole cottonseed from this line and from its non-transgenic counterpart, Coker 312. A total of 47 components were analysed - proximate content (moisture, fat, protein, fibre, ash and carbohydrate), amino acids, fatty acids, minerals, gossypol, and cyclopropenoid fatty acids.

Transgenic cotton line COT102 and its non-transgenic control were grown in 2001 and 2002. In 2001, cottonseed was produced at three locations (Lubbock Texas, Leland Mississippi and Maricopa Arizona, in the United States of America). Plot size was limited due to seed availability and therefore, a single sample was collected at each location from both COT102 and the non-transgenic parental cultivar Coker 312. In 2002, cottonseed was produced at two locations (Leland, Mississippi, and Visalia, California, in the United States of America). At both locations four samples of cottonseed were collected and analysed for each genotype.

For the analysis of the data collected from the 2001 sites, each location was treated as a replicate for the purposes of statistical analysis. The data were subjected to analysis of variance across sites and the effect of genotype was evaluated using a standard F-test. The corresponding “F-test Probability” or “p-value” for each analysis is shown in the attached tables. A p-value <5% would indicate that the effect of genotype was statistically significant at the customary 5% level.

Data from the 2002 sites was also subjected to analysis of variance both within and between locations in order to compare the two genotypes in regard to nutrient content.

The analysis is done across locations to (i) assess whether the relationship between genotypes changes from one location to another, and if not (ii) provide a more accurate and concise summary of the relationship and to provide a more powerful test for the effect of transformation. The issue of whether the relationship between genotypes changes from one location to another is addressed by the location x genotype interaction, which indicates whether it is appropriate to compare genotypes averaged across locations. The corresponding “F-test Probability” for each analysis is shown in the attached tables. The single standard deviation presented for each analysis summarises the random plot-to-plot variation present in the data after effects due to genotype or location have been accounted for by the analysis of variance, and applies to both genotypes.

The issue of whether the relationship between genotypes changes from one location to another is addressed by the location x genotype interaction. Where the F-test

probability is greater than 5%, this indicates that there is nothing in the data to suggest that the relationship between genotypes changes from one location to another, and consequently that it is justifiable to compare genotypes averaged over locations. Where the corresponding F-test probability for genotype is also greater than 5% this indicates there is no convincing evidence that the transformation has affected that parameter and that there is no significant difference between the conventional and GM cotton.

Had the interaction F-test probability been low (i.e. <5%) it would have suggested that the relationship between genotypes does indeed change from one location to another, in which case the value of comparing genotypes averaged over locations is dubious.

Looking at the 2002 dataset as a whole, there were very few instances in which the analysis detected a significant interaction between location and genotype, and so, in general, presenting results averaged across locations is justified.

Proximate analysis

A summary of the proximate analyses is shown in Tables 7, 8 and 9. No statistically significant differences between cotton line COT102 and the control line grown in 2001 were observed in any of the parameters measured (F-test probability < 5%).

In 2002, there were no significant differences observed between the transgenic cotton and the control cotton for any of the proximates at Visalia. At Leland, the only statistically significant difference was in ash content, however both the transgenic and control plants were within the reference range for this parameter. When the data were analysed across locations, there was no significant difference between the two genotypes for any of the proximates.

However, although there was no significant difference between the two genotypes for any of the proximates when analysed across locations, in the 2002 analysis of fibre content, the F-test interaction result was significant at 3.2%. As explained above, there were very few instances in which the comparative analysis detected a significant interaction between location and genotype, and so presenting results averaged across locations is justified. In this case, there was no significant difference between the transgenic cotton and the control cotton (F-test = 30.8%) when analysed across locations.

It was also noted that the fibre content in both the COT102 and the Coker 312 cottonseed was outside the literature range. However, as both COT102 and Coker 312 showed this result and as the fibre was within the literature range in the 2001 trials this difference is not due to the genetic modification.

Mineral Analysis

The minerals iron, phosphorus, potassium, calcium, zinc, copper, sodium, magnesium, manganese, and chromium were analysed and compared between the transgenic cottonseed and the control cottonseed. A summary of the mineral analyses is shown in Tables 10, 11 and 12. No statistically significant differences

were observed in any of the minerals measured in 2001.

In 2002, no significant differences were observed between the transgenic cotton and the control cotton for the mineral potassium. For the other eight minerals analysed, some significant differences were observed as can be seen in Tables 11 and 12. However, the differences were small and were not consistent within and between locations. When the minerals were observed across locations, the means for all minerals were within the reference range and are not expected to have a nutritional effect.

Fatty Acid Analysis

The fatty acids myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidic acid (20:0), and behenic acid (22:0) were analysed in cotton line COT102 and compared with Coker 312. A summary of the fatty acid analyses is shown in Tables 13, 14 and 15.

No statistically significant differences were observed in any of the fatty acids measured in 2001.

In 2002, there were no significant differences between the transgenic and control cottonseed at either location or when the data were analysed across locations. The only statistically significant difference between the transgenic and control cottonseeds was for myristic acid when analysed for a genotype x location interaction. However, as the within and across location analysis for this fatty acid showed no significant difference this interaction is not considered to be biologically significant.

Amino Acid Analysis

Eighteen amino acids were analysed in cotton line COT102 and compared with the non-transgenic control, Coker 312. A summary of the amino acid analysis is presented in Tables 16, 17 and 18. No statistically significant differences were measured in amino acids from the 2001 field trials.

In 2002, no statistically significant difference between COT102 and the control were observed for 15 of the 18 amino acids analysed either within or across locations. Of the three amino acids for which a significant difference was observed, threonine and serine showed no difference when analysed within locations. The statistically significant difference between the lysine content of the transgenic and control cotton is due to a very small percentage decrease in the amount of lysine in the transgenic cotton compared to the control (less than 4% decrease).

Table 7. 2001 Proximate analysis of COT102 and Coker 312 cottonseed from three locations (n = 3)

Component[1]	COT102	Coker 312	SD[2]	C of V[3]	F-test prob	Reference Range 1[4]	Reference Range 2[5]
Moisture	8.84 (8.06-9.27)	9.27 (8.01-11.47)	1.25	13.8%	71.5%	3.97-7.49	3.97-8.47
Fat	21.90 (20.89-23.47)	22.12 (21.78-22.35)	0.82	3.7%	77.4%	15.44-23.64	15.44-23.83
Protein	29.87 (28.92-31.72)	29.34 (27.73-31.02)	0.54	1.8%	35%	21.76-27.79	21.76-28.15
Fibre	15.25 (14.79-15.98)	15.81 (14.13-17.05)	1.49	9.6%	69.1%	15.38-19.31	15.38-20.89
Ash	4.06 (3.37-4.69)	4.21 (3.85-4.63)	0.21	5.0%	47.5%	3.76-4.85	3.76-4.85

[1] All values (mean and range) expressed as % dry weight except moisture, which is % fresh weight.
[2] SD = Standard deviation
[3] C of V = Coefficient of variation
[4] Range includes data from four commercially available non-transgenic cotton varieties.
[5] Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table 8. Analysis of variance (F-Test) for 2002 proximate analysis of COT102 and Coker 312 cottonseed within locations (n = 4)

Location	Visalia, CA					Leland, MS				
Component[1]	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability
Moisture	6.43 (6.1-6.6)	6.68 (6.3-7.3)	0.30	4.5%	32%	6.88 (6.6-7.3)	7.28 (6.9-7.5)	0.40	5.7%	25.6%
Fat	24.63 (23.2-26.8)	23.58 (20.9-24.7)	2.30	9.5%	56.4%	21.20 (19.9-22.5)	21.83 (21.1-22.3)	1.02	4.7%	45%
Protein	28.80 (25.7-30.7)	28.68 (27.7-29.6)	1.27	4.4%	89.8%	29.23 (27.9-30.1)	29.43 (28.4-30.5)	0.94	3.2%	78.3%
Fibre	31.55 (31.1-32.7)	32.93 (31.3-36.2)	1.85	5.7%	37%	36.15 (34.2-38.0)	32.93 (31.9-33.5)	1.45	4.2%	5.1%
Ash	3.85 3.6-4.3)	3.85 (3.6-4.1)	0.25	6.5%	100%	4.03 (3.9-4.1)	4.35 (4.2-4.5)	0.09	2.1%	**1.4%**
Carbo-hydrate	36.30 (34.8-37.2)	37.23 35.3-37.1)	1.87	5.1%	53.5%	38.68 (37.8-40.3)	37.13 (35.5-38.4)	1.17	3.1%	15.8%

[1] All values (mean and range) expressed as % dry weight except moisture, which is % fresh weight.

Table 9. Analysis of variance (F-test) for 2002 proximate analysis of COT102 and Coker 312 cottonseed across locations (n = 2)

Component[1]	COT102	Coker 312	SD[2]	C. of V.[3]	F-test Genotype[4]	F-test Interaction[5]	Reference Range 1[6]	Reference Range 2[7]
Moisture	6.65 (6.1-7.3)	6.98 (6.3-7.5)	0.35	5.2%	11.7%	68.7%	3.97-7.49	3.97-8.47
Fat	22.91 (19.9-26.8)	22.70 (20.9-24.7)	1.78	7.8%	81.9%	38.2%	15.44-23.64	15.44-23.83
Protein	29.01 (25.7-30.7)	29.05 (27.7-30.5)	1.12	3.9%	94.9%	78.1%	21.76-27.79	21.76-28.15
Fibre	33.85 (31.1-38.0)	32.93 (31.3-36.2)	1.66	5.0%	30.8%	**3.2%**	15.38-19.31	15.38-20.89
Ash	3.94 (3.6-4.3)	4.10 (3.6-4.5)	0.19	4.7%	13.6%	13.6%	3.76-4.85	3.76-4.85
Carbo-hydrate	37.49 (34.8-40.3)	37.18 (35.3-41.0)	1.56	4.2%	70.3%	16.4%	45.64-53.62	45.64-53.62

[1] All values (mean and range) expressed as % dry weight except moisture, which is % fresh weight.
[2] SD = Standard Deviation
[3] C. of V. = Coefficient of Variation
[4] F-test probability for genotype
[5] F-test probability for interaction
[6] Range includes data from four commercially available non-transgenic cotton varieties.
[7] Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table 10. Mineral analysis of COT102 and Coker 312 cottonseed from 2001(n = 3)

Component[1]	COT102	Coker 312	SD[2]	C of V[3]	F-test prob	Reference Range 1[4]
Phosphorus (%)	0.64 (0.54-0.72)	0.68 (0.64-0.75)	0.04	6.0%	33%	0.61-0.88
Calcium (%)	0.11 (0.10-0.12)	0.12 (0.09-0.15)	0.02	16.3%	58%	0.12-0.33
Sodium (ppm)	969 (562-1300)	929 (529-1300)	212	22.3%	83.9%	54-3000
Iron (ppm)	82.1 (79.3-84.3)	81.7 (67.4-93.7)	7.6	9.3%	95.8%	41.84-72.15
Magnesium (%)	0.33 (0.33-0.34)	0.34 (0.33-0.37)	0.02	5.5%	58%	0.37-0.49
Manganese (ppm)	13.6 (13.2-14.1)	13.6 (13.3-14.1)	0.6	4.4%	95.2%	11.17-18.31
Potassium (%)	0.81 (0.72-0.88)	0.82 (0.76-0.89)	0.01	1.8%	30%	1.08-1.25
Zinc (ppm)	30.3 (29.3-32.0)	31.6 (31.5-31.6)	1.0	3.3%	27.5%	27.39-51.20
Copper (ppm)	9.14 (8.7-9.43)	9.4 (9.1-9.8)	0.5	5.7%	61.3%	4.39-10.35
Chromium (ppm)	<1	<1	-	-	-	-

[1] mean and range.
[2] SD = Standard deviation
[3] C of V = Coefficient of variation
[4]Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table 11. Analysis of variance (F-Test) for 2002 mineral analysis of COT102 and Coker 312 cottonseed within locations (n = 4)

Location	Visalia, CA					Leland, MS				
Component[1]	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability
Phosphorus	656 (562-785)	680 (626-766)	87	13.0%	72.3%	638 (602-680)	745 (694-795)	13	1.9%	**0.2%**
Calcium	133 (118-144)	147 (140-154)	7	5.2%	8.4%	126 (118-132)	119 (110-127)	5	3.9%	11.5%
Sodium	18.1 (17.0-19.3)	41.5 (27.9-60.9)	9.6	32.1%	**4%**	23.8 (16.6-32.2)	14.6 (<10-26.8)	10.3	53.7%	29.3%
Iron	4.17 (4.10-4.25)	4.38 (3.91-4.74)	0.32	7.5%	42.6%	5.16 (4.85-5.23)	5.92 (5.50-6.12)	0.24	4.4%	**2.1%**
Magnesium	379 (352-427)	378	30	8%	96.6%	391 (385-399)	412 (403-418)	2	0.6%	**0.1%**
Manganese	1.16 (1.09-1.20)	1.23 (1.03-1.35)	0.09	7.8%	38.3%	1.23 (1.15-1.26)	1.31 (1.25-1.39)	0.03	2.7%	**4.1%**
Potassium	1063 (1030-1130)	1036 (992-1060)	55	5.2%	53.4%	1103 (1090-1140)	1153 (1090-1210)	40	3.5%	17.2%
Zinc	3.33 (3.15-3.69)	3.79 (3.26-4.14)	0.43	12.2%	23.2%	3.14 (2.75-3.42)	3.57 (2.94-3.79)	0.17	5.0%	**3.6%**
Copper	0.487 (0.464-0.535)	0.614 (0.539-0.693)	0.065	11.7%	6.9%	0.643 (0.600-0.719)	0.704 (0.654-0.749)	0.03	4.5%	6.4%
Chromium	<0.2	<0.2	-	-	-	<0.2	<0.2	-	-	-

[1] All values (mean and range) expressed as mg/100g.

Table 12. Analysis of variance(F-test) for 2002 mineral analysis of COT102 and Coker 312 cottonseed across locations (n = 2)

Component[1] mg/100 gm	COT102	Coker 312	SD[2]	C. of V.[3]	F-test Genotype[4]	F-test Interaction[5]	Reference Range[6]
Phosphorus	647	712	62	9.2	8.1%	23.4%	610-880
Calcium	130	133	6	4.7	39%	**1.6%**	120-330
Sodium	20.9	28.0	9.9	40.6	20.2%	**1.7%**	5.4-300
Iron	4.66	5.15	0.28	5.8	**1.4%**	9.6%	4.184-7.215
Magnesium	385	395	21	5.5	38.2%	34%	370-490
Manganese	1.19	1.27	0.07	5.7	7.7%	83.8%	1.117-1.831
Potassium	1083	1094	48	4.4	64.6%	15.7%	1080-1250
Zinc	3.23	3.68	0.33	9.5	**3.6%**	91.9%	2.739-5.120
Copper	0.565	0.659	0.05	8.2	**1.0%**	23.7%	0.439-1.035

[1] Mean across locations.
[2] SD = Standard deviation
[3] C of V = Coefficient of variation
[4] F-test probability for genotype
[5] F-test probability for genotype x location interaction
[6]Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table 13. Fatty acid analysis of COT102 and Coker 312 cottonseed from 2001(n = 3)

Component[1] (g/100g)	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test prob
14:0 myristic	0.837 (0.59-0.99)	0.813 (0.54-0.96)	0.022	2.6%	31.7%
16:0 palmitic	24.84 (22.81-25.87)	24.27 (22.59-25.64)	0.20	0.8%	15.2%
16:1 palmitoleic	0.587 (0.57-0.62)	0.570 (0.55-0.59)	0.022	3.7%	44.4%
18:0 stearic	2.51 (2.39-2.58)	2.51 (2.41-2.58)	0.01	0.3%	42.3%
18:1 oleic	15.25 (13.53-16.14)	15.51 (13.94-16.73)	0.34	2.2	44%
18:2 linoleic	55.04 (52.97-59.14)	55.94 (52.59-58.14)	0.27	0.5%	70.5%
18:3 linolenic	0.393 (0.27-0.53)	0.513 (0.48-0.58)	0.058	12.8%	12.6%
20:0 arachidic	0.240 (0.21-0.26)	0.237 (0.20-0.27)	0.008	3.4%	66.7%
22:0	0.120	0.123	0.008	6.7%	66.7%

behenic	(0.1-0.13)	(0.11-0.14)			

[1] mean and range.

Table 14. Analysis of variance (F-Test) for 2002 fatty acid analysis of COT102 and Coker 312 cottonseed within locations (n = 4)

Location	Visalia, CA					Leland, MS				
Component[1]	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability
14:0 myristic	0.185 (0.18-0.19)	0.165 (0.14-0.18)	0.010	5.7%	6.6%	0.150 (0.14-0.16)	0.155 (0.15-0.16)	0.0009	6.0	49.5%
16:0 palmitic	5.75 (5.53-6.12)	5.42 (4.73-5.70)	0.50	9.0	42.3%	4.69 (4.43-5.07)	4.96 (4.76-5.09)	0.28	5.8	26.9%
16:1 palmitoleic	0.130 (0.13-0.13)	0.128 (0.11-0.14)	0.009	6.9	71.8%	0.118 (0.11-0.12)	0.120 (0.12-0.12)	0.004	3.0	39.1%
18:0 stearic	0.553 (0.52-0.59)	0.523 (0.48-0.55)	0.039	7.2	35.3%	0.443 (0.41-0.48)	0.463 (0.45-0.49)	0.024	5.3	32%
18.1 oleic	3.75 (3.65-3.93)	3.68 (3.32-3.86)	0.26	7.0	71.8%	3.00 (2.79-3.09)	2.91 (2.82-2.96)	0.12	4.2	41.3%
18:2 linoleic	12.48 (11.80-13.80)	11.68 (10.50-12.30)	1.18	9.8	40.8%	10.68 (10.10-11.50)	11.08 (10.80-11.30)	0.54	4.9	36.8%
18:3 linolenic	0.058 (0.05-0.06)	0.063 (0.06-0.07)	0.004	6.8	18.2%	0.068 (0.06-0.07)	0.068 (0.06-0.07)	0.006	8.6	100%
20:0 arachidic	0.065 (0.06-0.07)	0.058 (0.05-0.06)	0.007	11.1	21.5%	0.053 (0.05-0.06)	0.058 (0.05-0.06)	0.007	12.9	39.1%
22:0 behenic	0.043 (0.04-0.05)	0.040 (0.04-0.04)	0.004	8.6	39.1%	0.038 (0.03-0.04)	0.040 (0.04-0.04)	0.004	9.1	39.1%

[1] All values (mean and range) expressed as g/100g.

Table 15. Analysis of variance (F-test) for 2002 fatty acid analysis of COT102 and Coker 312 cottonseed across locations (n = 2)

Component[1]	COT102	Coker 312	SD[2]	C. of V.[3]	F-test Genotype[4]	F-test Interaction[5]
14:0 myristic	0.168	0.160	0.01	5.8%	16.8%	**4%**
16:0 palmitic	5.22	5.19	0.41	7.9%	88.8%	19.3%
16:1 palmitoleic	0.124	0.124	0.007	5.5%	100%	48.8%
18:0 stearic	0.498	0.493	0.032	6.5%	76.6%	17.1%
18.1 oleic	3.37	3.30	0.20	6.1%	47.3%	96.2%
18:2 linoleic	11.58	11.38	0.92	8.0%	67.8%	23.8%
18:3 linolenic	0.063	0.065	0.005	7.8%	35.6%	35.6%
20:0 arachidic	0.059	0.058	0.007	11.9%	73.0%	12.1%
22:0 behenic	0.040	0.040	0.004	8.8%	100%	20.7%

[1] All values (mean and range) expressed as g/100 gm cottonseed.
[2] SD = Standard Deviation
[3] C. of V. = Coefficient of Variation
[4] p-value Genotype
[5] p-value interaction

Table 16. Amino acid analysis of COT102 and Coker 312 cottonseed from 2001(n = 3)

Component [1] (mg/100g)	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test probability
Asp	423 (360-460)	400 (360-460)	27	6.5%	52.5%
Thr	400 (370-420)	403 (380-430)	36	9.0%	92.1%
Ser	2340 (2160-2490)	2270 (2180-2420)	85	3.7%	41.9%
Glu	787 (720-840)	770 (740-820)	25	3.2%	49.8%
Pro	1057 (950-1130)	1023 (950-1110)	29	2.8%	30.0%
Gly	4597 (4060-5000)	4450 (4090-4930)	159	3.5%	37.6%
Ala	880 (800-960)	850 (800-950)	31	3.6%	35.6%
Cys	963 (880-1030)	940 (890-1010)	25	2.6%	36.9%
Val	953 (900-1010)	950 (900-1020)	29	3.1%	90.2%
Met	1013 (920-1090)	987 (930-1090)	39	3.9%	49.0%
Ile	733 (670-790)	710 (670-780)	23	3.2%	33.6%
Leu	1330 (1200-1430)	1297 (1220-1410)	43	3.3%	44.4%
Tyr	540 (480-590)	520 (490-560)	19	3.5%	32.1%
Phe	1197 (1060-1300)	1153 (1060-1280)	41	3.5%	32.9%
His	657 (590-710)	643 (600-700)	18	2.7%	45.6%
Lys	1003 (930-1070)	990 (960-1040)	27	2.7%	60.4%
Arg	2630 (2280-2890)	2523 (2290-2800)	89	3.5%	28.0%
Trp	310 (280-330)	313 (310-320)	11	3.5%	74.2%

[1] mean and range.
[2] SD = Standard deviation
[3] C of V = Coefficient of variation

Table 17. Analysis of variance (F-Test) for 2002 amino acid analysis of COT102 and Coker 312 cottonseed within locations (n = 4)

Location	Visalia, CA					Leland, MS				
Component[1]	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test probability	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test probability
Asp	2525 (2250-2710)	2605 (2460-2730)	83	3.2	26.5%	2480 (2280-2610)	2488 (2280-2600)	51	2.1	85%
Thr	785 (730-840)	805 (760-840)	17	2.2	20.1%	773 (700-820)	745 (700-770)	20	2.6	14%
Ser	1113 (1050-1180)	1170 (1080-1250)	29	2.6	6.8%	1098 (980-1210)	1190 (1050-1250)	51	4.5	8.4%
Glu	5740 (5160-6180)	5925 (5410-6230)	201	3.5	28.4%	5780 (5300-6140)	5883 (5650-6050)	157	2.7	42.5%
Pro	1085 (940-1160)	1078 (1000-1140)	32	3.0	76.5%	1025 (950-1070)	1083 (1120-1180)	36	3.4	11.1%
Gly	1155 (1060-1220)	1168 (1090-1220)	30	2.6	60%	1128 (1050-1180)	1155 (1120-1180)	38	3.3	37.7%
Ala	1068 (980-1130)	1085 (1020-1130)	29	2.7	44.9%	1063 (980-1110)	1095 (1060-1120)	39	3.6	32.7%
Cys	443 (420-460)	470 (450-520)	35	7.7	35.1%	468 (450-510)	463 (450-480)	14	2.9	63.8%
Val	1313 (1170-1400)	1325 (1220-1380)	35	2.7	65.1%	1288 (1200-1380)	1323 (1280-1370)	42	3.2	32.6%
Met	388	390	24	6.2	89.3%	413	405	12	3.0	44.4%

	(380-400)	(360-430)				(390-440)	(400-410)			
Ile	920 (830-980)	925 (850-970)	22	2.4	76.9%	905 (840-970)	925 (890-950)	33	3.6	45.6%

[1] All values (mean and range) expressed as mg/100g.

Table 17: continued

Location	Visalia, CA					Leland, MS				
Component[1]	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test probability	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test probability
Leu	1608 (1460-1710)	1633 (1500-1720)	33	2.0	36.2%	1600 (1470-1690)	1640 (1600-1670)	64	4.0	44.2%
Tyr	718 (650-770)	735 (690-780)	20	2.8	31%	703 (650-750)	735 (720-750)	30	4.2	22.6%
Phe	1465 (1310-1580)	1503 (1380-1590)	41	2.	29%	1450 (1330-1540)	1498 (1470-1530)	51	3.5	28.3%
His	780 (690-840)	795 (740-840)	22	2.8	40.6%	760 (700-800)	780 (770-790)	30	3.9	41.5%
Lys	1223 (1140-1300)	1270 (1170-1350)	9	0.7	**0.5%**	1205 (1110-1260)	1250 (1190-1320)	39	3.1	19.7%
Arg	3148 (2740-3430)	3175 (3030-3450)	141	4.4	29.1%	3098 (2860-3310)	3228 (3180-3290)	114	3.6	20.6%
Trp	263 (240-280)	270 (270-270)	12	4.5	44.4%	268 (260-280)	275 (270-280)	4	1.3	5.8%

[1] All values (mean and range) expressed as mg/100g.

Table 18. Analysis of variance (F-test) for 2002 amino acid analysis of COT102 and Coker 312 cottonseed across locations (n = 2)

Component[1]	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test Genotype[2]	F-test Interaction[3]
Asp	2503	2546	69	2.7	25.1%	33.3%
Thr	779	775	18	2.4	69.8%	**4.2%**
Ser	1105	1180	42	3.7	**1.2%**	43.4%
Glu	5760	5904	181	3.1	16.2%	66.4%
Pro	1055	1080	34	3.2	19.6%	10.7%
Gly	1141	1161	34	3.0	28.5%	67.5%
Ala	1065	1090	34	3.2	19.6%	67.8%
Cys	455	466	27	5.8	43.2%	27.0%
Val	1300	1324	39	3.0	26.8%	58.4%
Met	400	398	19	4.8	80.2%	61.8%
Ile	913	925	28	3.1	40.9%	61.3%
Leu	1604	1636	51	3.1	24.9%	77.8%
Tyr	710	735	26	3.6	10.0%	58.1%
Phe	1458	1500	47	3.2	11.9%	83.8%
His	770	788	26	3.4	23.2%	85.6%
Lys	1214	1260	28	2.3	**1.6%**	93.2%
Arg	3123	3251	128	4.0	9.2%	98.5%
Trp	265	273	9	3.3	14.3%	100.0%

[1] Mean expressed as mg/100g cottonseed.
[2] F-test probability for Genotype
[3] F-test probability for Genotype x Location interaction

Key toxicants

Cotton contains two naturally occurring toxic compounds – gossypol and cyclopropenoid fatty acids. These compounds have been analysed in cottonseed from line COT102 and compared with the non-transgenic parental line Coker 312 (Tables 19, 20 and 21)

Gossypol

Gossypol is a biologically active terpenoid aldehyde that is present in discrete glands in all plant tissues, including seed (Abou-Donia, 1976; Jones, 1991). Gossypol can cause a number of toxic effects on mammals including reduced appetite, body weight loss, and dyspnoea (difficult and laboured breathing) (Berardi and Goldblatt, 1980), adverse effects on the protein nutritive value of food by rendering lysine metabolically unavailable (Yannai and Bensai, 1983) and damage to normal mitochondrial functioning (Cuellar and Ramirez, 1993; Randel *et al.,* 1992; Risco *et al.,* 1993).

The levels of gossypol and related terpenoids in cottonseed varies with variety and environmental conditions, which can include factors as diverse as soil and air temperature, disease infections, moisture stress and the presence of chemicals (Bell, 1991).

Any presence of gossypol limits the use of cottonseed as a protein source for humans or in animal feed, except for ruminants where bacteria in the rumen are able to detoxify gossypol (Randel *et al.,* 1992; Poore and Rogers, 1998; Nikokyris *et al.,* 1991). Processing of cottonseed is therefore essential for it to have feed or food value.

Gossypol exists in two forms, free and bound. The free form is toxic, while the bound form is considered non-toxic since it is not released in the animal rumen. In whole unprocessed cottonseed almost all of the gossypol is in the free form. During processing, gossypol partitions into the meal and oil components. Although some of the gossypol in meal remains as the free form, much of it becomes bound to proteins and therefore detoxified. Gossypol in oil is eliminated during the refining process.

There were no significant differences in the levels of total and free gossypol in the cottonseed between COT102 and Coker 312.

Cyclopropenoid fatty acids

Cyclopropenoid fatty acids are unique fatty acids that are naturally present in cotton, crude cottonseed oil and in the meal (because of the residual oil in the meal fractions). Refinement of cottonseed oil includes deodorisation and bleaching, which greatly reduces the cyclopropenoid fatty acid content of the oil due to extreme pH and temperature conditions.

The major types are sterculic acid (C-17), malvalic acid (C-18) and dihydrosterculic acid (C-19). Cyclopropenoid fatty acids are considered to be undesirable, anti-nutritional compounds of concern for food safety. They have unfavourable biological effects including the inhibition of biodesaturation of stearic to oleic acid affecting phospholipid biosynthesis (Rolph *et al.,* 1990; Cao *et al.,* 1993, Gunstone *et al.,* 1994), and have been reported to induce termination of embryo development in sheep through inhibition of progesterone production in the *corpus luteum* (Tumbelaka *et al.,* 1994).

The cyclopropenoid fatty acids are destroyed either by hydrogenation or by heating the oil in the presence of free fatty acids for deodorisation purposes (Gunstone *et al.,* 1994).

There were no significant differences in cyclopropenoid fatty acids between the COT102 cottonseed and its control Coker 312 as can be seen in Tables 20 and 21.

Table 19. Gossypol analysis of COT102 and Coker 312 cottonseed from 2001(n = 3)

Component[1] (g/100g)	COT102	Coker 312	Standard Deviation	Coefficient of Variation	F-test
Gossypol	0.877 (0.824-0.907)	0.939 (0.893-1.010)	0.077	8.4%	42.6%

[1] mean and range.

Table 20. Analysis of variance (F-Test) for 2002 gossypol and cyclopropenoid analysis of COT102 and Coker 312 cottonseed within locations (n = 4)

Location	Visalia, CA					Leland, MS				
Component[1]	COT102	Coker 312	SD	C of V	F-test	COT102	Coker 312	SD	C of V	F-test
Total Gossypol	0.864 (0.771-1.060)	0.856 (0.760-0.939)	0.128	14.9%	92.9%	0.949 (0.841-1.030)	1.025 (0.963-1.140)	0.071	7.2%	22.3%
Free Gossypol	0.674 (0.600-0.826)	0.684 (0.618-0.724)	0.061	9.0%	84.1%	0.727 (0.692-0.811)	0.773 (0.728-0.820	0.049	6.6	27.6%
Sterculic	0.248 (0.141-0.475)	0.255 (0.188-0.400)	0.049	19.6%	84.3%	0.278	0.260	0.089	33.2%	80%
Malvalic	0.320 (0.273-0.422)	0.320 (0.269-0.392)	0.038	11.8%	100%	0.378	0.408	0.037	9.4%	33.4%
Dihydro-sterculic	<0.1 (<0.1-<0.1)	0.105 <0.1-0.11	0.004	4%	18.2%	0.108	0.113	0.019	17%	73.1%

[1] All values (mean and range) expressed as g/100g of cottonseed.

Table 21. 2002 Analysis of variance (F-test) for gossypol and cyclopropenoid fatty acid analysis of COT102 and Coker 312 cottonseed across locations (n = 2)

Component[1]	COT102	Coker 312	SD[2]	C. of V.[3]	F-test Genotype[4]	F-test Interaction[5]
Total Gossypol g/100g	0.906	0.940	0.104	11.2%	53.8%	44.2%
Free Gossypol g/100g	0.700	0.728	0.056	7.8%	35.5%	53.3%
Sterculic	0.263	0.258	0.072	27.7%	89.4%	74.1%
Malvalic	0.349	0.364	0.037	10.5%	45.3%	45.3%
Dihydro-sterculic	0.104	0.109	0.014	12.7%	48.8%	100%

[1] All values (mean and range) expressed as g/100g cottonseed.
[2] SD = Standard Deviation
[3] C. of V. = Coefficient of Variation
[4] F-test probability for Genotype
[5] F-test probability for interaction between genotype and location

Conclusion

Most crops, including oilseed crops, exhibit considerable variability in their nutrient composition. Environmental factors and the genotype of the plant have an enormous impact on composition. Thus, variation in these nutrient parameters is a natural phenomenon and is considered to be normal. Furthermore, some differences in composition may be expected given the different susceptibility of the GM versus the non-GM cotton to insect pests. It is thought that insect predation leads to changes in the composition of plants and this may well be a component of the minor differences seen in these studies.

The comparative analyses do not indicate that there are any compositional differences in cottonseed from transgenic cotton line COT102, compared to the non-GM control (Coker 312) that would lead to food safety or nutritional problems. Several minor differences in key nutrients and other constituents were noted, however the levels observed were within the range of natural variation for commercial cotton lines and do not indicate an overall pattern of change that would warrant further investigation. On the whole, it can be concluded that COT102 cottonseeds are equivalent in composition to non-GM cottonseeds.

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 wellbeing. 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. Feeding studies with feeds derived from the approved GM plants have shown equivalent animal performance to that observed with the non-GM feed.

Thus the evidence to date is that for GM varieties shown to be compositionally equivalent to conventional varieties, feeding studies with target livestock species will add little to a safety assessment and generally are not warranted.

For plants engineered with the intention of significantly changing their composition/ nutrient bioavailability and thus their nutritional characteristics, however, 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 for the animal.

In the case of cotton line COT102, the extent of the compositional and other available data is considered adequate to establish the nutritional adequacy of the food.

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