FOOD DERIVED FROM BROMOXYNIL-TOLERANT COTTON TRANSFORMATION EVENTS 10211 AND 10222

A Safety Assessment

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SUMMARY AND CONCLUSIONS

Food from bromoxynil-tolerant cotton has been evaluated to determine its safety for human consumption. The evaluation criteria used in this assessment included characterisation of the transferred genes, the modifications at the DNA, protein and whole food levels, compositional analyses, and the potential allergenicity and toxicity of the newly expressed proteins. This enables the intended as well as any significant unintended changes to be identified, characterised and evaluated for their safety.

History of use

Cotton (*Gossypium hirsutum*) is grown primarily for the value of its fibre; cottonseed (and its processed products) is very much a by-product of the crop. Cottonseed itself is not used as a food for human consumption because it contains naturally occurring toxic substances. These toxic substances can however be removed or reduced by the processing of the cottonseed into various fractions of which it is really only the oil and linters that are used for human consumption. Both the oil and linters have been routinely used in foods and have an established history of safe use. The types of food products likely to contain cottonseed oil are frying oils, mayonnaise, salad dressing, shortening, and margarine. After processing, linters, which are >99% cellulose, may be used as high fibre dietary products, sausage casings and thickeners in ice cream and salad dressings.

Nature of the genetic modification

Cotton transformation events 10211 and 10222 were made tolerant to the herbicide bromoxynil through the *Agrobacterium*-mediated transfer of a single copy of the *oxy* gene from the soil bacterium *Klebsiella pneumoniae* subspecies *ozaenae*. The bromoxynil-tolerant cotton lines derived from these transformation events are known commercially as either BXN or OXY cotton.

The *oxy* gene is responsible for the production of the enzyme nitrilase that hydrolyses bromoxynil to an inactive, non-phytotoxic compound. Low concentrations of bromoxynil kill conventional cotton varieties therefore the purpose of the genetic modification is to enable bromoxynil-containing herbicides to be used for weed control in cotton crops.

Both cotton transformation events also each contain a single copy of the *nptII* gene that was used as a marker for selection of transformed plant lines during the cotton transformation procedure. The *nptII* gene codes for the enzyme neomycin phosphotransferase II (NPTII) and confers resistance to the antibiotics neomycin, kanamycin, and geneticin (G418).

Both genes are stably integrated into the cotton genome and the bromoxynil-tolerant trait is stably maintained from one generation to the next in a variety of different genetic backgrounds.

One of the important issues to consider in relation to genetically modified foods is the impact on human health from potential transfer of antibiotic resistance genes to microorganisms in the human digestive tract. The assessment found that the *nptII* gene would be extremely unlikely to transfer to bacteria in the human digestive tract because refined oil and linters are essentially devoid of DNA. Even were DNA to be present in refined oil and linters, horizontal DNA transfer would be extremely unlikely because the number and complexity of steps that would be required to take place consecutively. Regardless of the above, the human health impacts of such a transfer would be negligible because kanamycin resistant bacteria are already commonly found in the human digestive tract and in the environment.

Characterisation of novel protein

Transformation events 10211 and 10222 express two novel proteins — nitrilase and NPTII. While both proteins can be readily detected in leaf tissue as well as in cottonseed and meal, neither could be detected in crude cottonseed oil at a detection limit of 0.1 ppm.

In relation to the potential toxicity and allergenicity of nitrilase and NPTII, it was concluded from the protein expression data that humans are highly unlikely to be exposed to either protein through the consumption of refined cottonseed oil and cellulose products from BXN cotton. Moreover, the absence of toxicity of nitrilase and NPTII has been confirmed through acute toxicity testing in mice, and neither protein demonstrates any potential to become a food allergen.

The assessment also considered the potential toxicity of 3,5-dibromo-4-hydroxybenzoic acid (DBHA), a by-product of the detoxification of bromoxynil by nitrilase. It was concluded that DBHA is likely to be no more toxic than its parent compound, bromoxynil, which is considered to pose negligible risk to human health at expected exposure levels.

Comparative analysis

Detailed compositional analyses were done to establish the safety and nutritional adequacy of the food products derived from BXN cotton and also to demonstrate that unintended changes to the composition of the cotton plants had not occurred as a result of the genetic modification. Analyses were done of the key nutrients, toxicants and anti-nutritional factors in both herbicide-sprayed and unsprayed plants. The most important analyses, in terms of nutritional adequacy, were those of the oil, which is the principal human food product. On the basis of the data provided, cotton transformation events 10211 and 10222 were found to be compositionally equivalent to other commercially available cotton varieties.

Conclusion

Based on the data submitted in the present application, refined oil and linters from bromoxynil-tolerant cotton transformation events 10211 and 10222 are equivalent to refined oil and linters from other commercially available cotton varieties in terms of their safety and nutritional adequacy.

INTRODUCTION

A safety assessment has been conducted on food derived from cotton, which has been genetically modified to be tolerant to the oxynil family of herbicides comprising bromoxynil and ioxynil. The genetically modified cotton is known commercially either as OXY cotton or BXN cotton.

The oxynil family of herbicides act by inhibiting electron transport in photosystem II in plants. Inhibition of electron transport causes superoxide production resulting in the destruction of cell membranes and an inhibition of chlorophyll formation, leading to plant death (Comai and Stalker 1986). Tolerance to either bromoxynil (3,5-dibromo-4-hydorxybenzonitrile) or ioxynil (3,5-di-iodo-4-hydroxybenzonitrile) is achieved through expression in the plant of a bacterial nitrilase enzyme that hydrolyses the herbicide to an inactive, non-phytotoxic compound. The nitrilase is derived from the bacterium *Klebsiella pneumoniae* subspecies *ozaenae* and is responsible for rapidly degrading bromoxynil in soil. The nitrilase enables the bacterium to utilise bromoxynil as a sole source of nitrogen (McBride *et al* 1986).

The oxynil herbicides are primarily used on field corn, wheat and grain crops to control a variety of grasses and broadleaf weeds. Low concentrations of bromoxynil-containing herbicides kill conventional cotton varieties. Therefore, current weed control practices in cotton involve either prophylactic pre-plant, pre-emergence herbicide application or post-directed herbicide sprays to avoid crop injury. The rationale for engineering cotton to be bromoxynil-tolerant is to enable bromoxynil-containing herbicides to be used for the post-emergence control of dicotyledonous weeds in cotton crops.

The major human food products obtained from cotton are refined oil and linters. Cottonseed oil is a premium quality oil that may be used in a variety of foods including frying oil, mayonnaise, salad dressing, shortening, margarine and packing oil. Linters are short fibres removed from the cottonseed during processing (delinting). After extensive processing at alkaline pH and high temperatures, the linters may be used as high fibre dietary products, sausage casings and thickeners in ice cream and salad dressings. The linters consist primarily of cellulose (>99%).

The BXN cotton lines currently in commercial production, or planned for future commercial release, are derived from transformation events 10222 (current lines) and 10211 (future lines). The currently available BXN cotton lines include BXN 47 and BXN 16. The first of these, BXN 47 cotton, was commercialised in 1997. Therefore, cottonseed oil derived from BXN cotton or processed products containing cottonseed oil or linters derived from BXN cotton may have been imported into Australia and New Zealand since that time.

HISTORY OF USE

Cotton is grown primarily for the value of its fibre; cottonseed (and its processed products) is very much a by-product of the crop. Cottonseed itself is not used as a food for human consumption because it contains naturally occurring toxic substances known as gossypol and the cyclopropenoid fatty acids. These harmful substances can however be removed or reduced with processing which means that a number of products derived from cottonseed are suitable for animal as well as human food uses. The four main products derived from cottonseed are oil, meal, hulls and linters. Processing of cottonseed typically yields by weight: 16% oil, 45% meal, 9% linters, and 26% hulls, with 4% lost during processing (Cherry and Leffler 1984).

The main products destined for human consumption are the oil and linters. These products are routinely used in foods and have a history of safe use. Cottonseed oil has been in common use since the middle of the nineteenth century (Jones and King 1990) 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. Cottonseed meal and hulls are typically used for livestock feed. Cottonseed oil is premium quality oil that is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil. Linters are a major source of cellulose for both chemical and food uses. Food uses include casings for sausages and frankfurters and as a thickener in products such as ice cream and salad dressings.

Some human food uses for cottonseed flour have 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 where 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.

Cottonseed processing steps

After the majority of the fibre is removed at the cotton gin, a significant amount of "fuzzy" fibre remains associated with the seed. These short fibres, known as linters, are removed from the seed during de-linting. After extensive processing at alkaline pH and high temperatures, the linters can be used as a high fibre dietary product. After this processing, the fibre does not normally contain any detectable genetic material or protein. Once the lint is removed from the seed, the hulls are cut and separated from the seed. After hulling, the cottonseed is flaked by a rolling process to facilitate oil removal. Prior to oil extraction, the flakes are heated to: (i) break down the cell walls; (ii) reduce the viscosity of the oil; (iii) coagulate the protein; (iv) inactivate proteins and kill any microbial contamination; (v) detoxify gossypol by the combination of heat and moisture; and (vi) fix certain phosphatides in the cake to minimise refining losses.

After cooking, the oil is typically removed from the meal by direct solvent extraction with hexane. The material left over after the crude oil is extracted is the cottonseed meal. After extraction the gossypol levels in the oil are reduced by about half. Crude

cottonseed oil is then further processed, depending on the end use of the product. A winterisation step is added to produce cooking oil, whereas for solid shortening, a hydrogenation step is added to transform the liquid oil into a solid fat. Further processing (refining) for all the uses of cottonseed oil includes deodorization and bleaching. Deodorization greatly reduces the cyclopropenoid fatty acid content of the oil due to the extreme pH and temperature conditions and the resulting oil generally contains no detectable protein (Jones and King 1990).

DESCRIPTION OF THE GENETIC MODIFICATION

Methods used in the genetic modification

Cotton (*Gossypium hirsutum*) line Coker 315 was transformed with plasmid pBrx75 (see Figure 1 below), using the method of *Agrobacterium tumefaciens*-mediated transformation as described by Fillatti *et al* (1990) and Radke *et al* (1990). The transformation resulted in the selection of nine independent transformant events, two of which, 10211 and 10222, are the subject of this application and have been, or will be, used to derive the BXN cotton lines for commercial production.

Function and regulation of the novel genes

The transformation of cotton with plasmid pBrx75 resulted in the transfer of two gene expression cassettes denoted *oxy* and *nptII*. These gene expression cassettes are described in Table 1 below.

Table 1: Description of the gene expression cassettes in pBrx75

Cassette	Genetic element	Source	Function
oxy	35S promoter	The cauliflower mosaic virus (CaMV) 35S promoter region (Gardner *et al* 1981).	A promoter for high level constitutive (occurring in all parts of the plant and at all stages of development) gene expression in plant tissues
	oxy	Gene isolated from *Klebsiella pneumoniae* subspecies *ozaenae* encoding the enzyme nitrilase (Stalker *et al* 1988).	Inactivates the herbicide bromoxynil and confers bromoxynil tolerance when expressed in plants.
	tml 3'	The 3' non-translated region of the *tml* gene from *Agrobacterium tumefaciens* plasmid pTiA6 (Barker *et al* 1983).	Contains signals for termination of transcription and directs polyadenylation.
nptII	35S promoter	as above	as above
	nptII	The gene coding for neomycin phosphotransferase II from Tn5 in *Escherichia coli* (Beck *et al* 1982).	Confers resistance to the antibiotics kanamycin and neomycin. Used as a selectable marker for plant transformation (Horsch *et al* 1984, DeBlock *et al* 1984).
	tml 3'	as above	as above

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The oxy gene

The *oxy* gene was isolated from the soil bacterium *Klebsiella pneumoniae* subsp. *ozaenae* and encodes an enzyme that metabolises the herbicide bromoxynil (Stalker and McBride 1987). The *oxy* gene has been fully sequenced and its encoded enzyme, nitrilase, has been fully characterised (Stalker *et al* 1988). When transferred into plants, the gene, through its encoded protein, confers tolerance to the oxynil family of herbicides including bromoxynil and ioxynil. The mechanism of tolerance involves the detoxification of the herbicide by the nitrilase enzyme. This degradation effectively inactivates the herbicide and enables the normally bromoxynil-sensitive plant to survive and grow when treated with applications of the herbicide.

The nptII gene

The *nptII* gene is widely used as a selectable marker in the transformation of plants (Kärenlampi 1996). The gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation. It codes for the enzyme neomycin phosphotransferase II (NPTII) and confers resistance to the aminoglycoside antibiotics, neomycin, kanamycin, and geneticin (G418). The *nptII* gene is transferred along with the *oxy* gene, enabling those plant cells successfully transformed with the *oxy* gene to grow in the presence of kanamycin. Those cells that lack the *nptII* gene, and hence the *oxy* gene, will not grow and divide in the presence of kanamycin.

Other genetic elements

The plasmid pBrx75 is a 16.1 kb double border binary plant transformation vector derived from the *Agrobacterium* binary vector pCGN1559 (McBride and Summerfelt 1990).   The plasmid contains well characterised DNA segments required for its selection and replication in bacteria as well as the right and left borders delineating the region of DNA (T-DNA) which is transferred into the plant genomic DNA. This is the region into which the gene of interest, and the plant cell selectable marker, is inserted. DNA residing outside the T-DNA region does not normally get transferred into plant genomic DNA (Zambryski 1992). The additional genetic elements contained within pBrx75 are described in Table 2 and a map of the T-DNA region is provided in Figure 1. The host for all DNA cloning and vector construction was *E. coli* strain MM-294, a derivative of the common laboratory *E. coli* K-12 strain.

Table 2: Description of other genetic elements contained within pBrx75

Genetic element	Source	Function
aac (resides outside the T-DNA)	Gene derived from *Escherichia coli* coding for gentamicin-3-N-acetyltransferase (Hayford *et al* 1988, Carrer *et al* 1991).	Confers resistance to the antibiotic gentamicin. Used as a marker to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken in the laboratory prior to transformation of the plant cells.
LB	A DNA fragment of the pTiA6 plasmid containing the 24 bp nopaline-type T-DNA left border (LB) region from *A. tumefaciens* (Barker *et al* 1983).	Terminates the transfer of the T-DNA from *A. tumefaciens* to the plant genome.
pRi *ori* (resides outside the T-DNA region)	Origin of replication region derived from the *Agrobacterium rhizogenes* plasmid pRiHRI (Jouanin *et al* 1985).	Allows the binary vectors to be stably maintained in *A. tumefaciens* without antibiotic selection.
***ori-322/rop* region** (resides outside the T-DNA region)	A 1.8 kb segment of the plasmid pBR322 which contains the origin of replication region and the *bom* site for the conjugational transfer.	Allows for autonomous replication of plasmids in *E. coli* as well as their conjugal transfer into *A. tumefaciens* cells (Bolivar *et al* 1977, Sutcliffe 1978).
RB	A DNA fragment from the pTiA6 plasmid containing the 24 bp nopaline-type T-DNA right border (RB) region from *A. tumefaciens*. (Barker *et al* 1983).	The RB region is used to initiate T-DNA transfer from *A. tumefaciens* to the plant genome.

Figure 1: Map of the T-DNA region of pBrx75
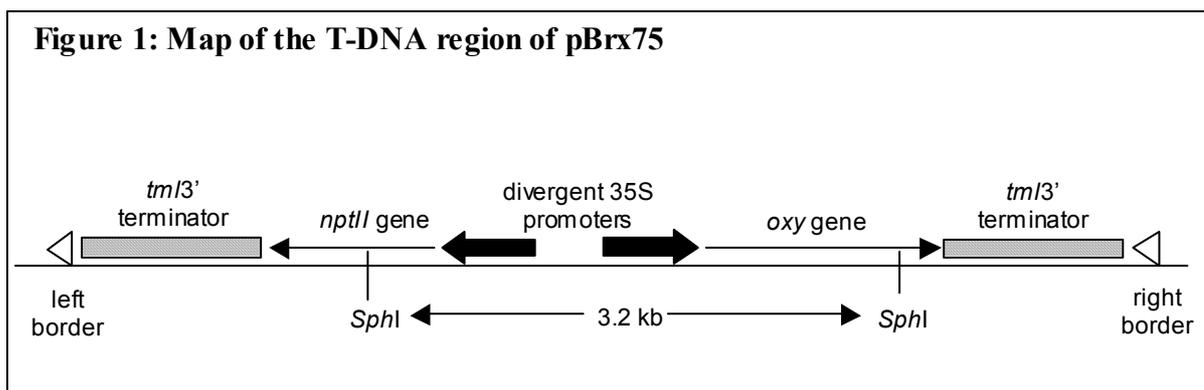
Characterisation of the genes in the plant

Selection of the plant lines

<u>Initial screen of T_1 plants</u>

The plants resulting from the *Agrobacterium*-mediated transformation (the T_1 generation) were tested for the presence of a functional T-DNA insert by a bromoxynil dab assay. Observations of plant morphology were made, including (but not limited to) leaf size, internode distance, plant stature, flower morphology, fertility of flowers, relative flower and boll abortion rates, boll size, seed per boll and total seed per plant. This information was then used to select individuals for field-testing and for comparison with field observations on subsequent generations. Infertility, due to flower structure, pollen inviability, premature flower abortion or boll abortion, are morphological criteria used to drop non-commercial lines from the product development program before contained field testing of the T_2 generation.

<u>Production and analysis of T_2 material</u>

A total of nine transformation events passed the initial T_1 screen (events 10103, 10109, 10206,10208, 10209, 10211, 10215, 10222, and 10224) and were self-fertilised to produce T_2 seed. T_2 progeny were then planted in the field and sprayed with Buctril® (a proprietary herbicide containing bromoxynil as the active ingredient) when the plants were between a two leaf stage and 12 inches tall. In early spray experiments, four rows of T_2 progeny from each event were planted and each row was sprayed at different herbicide rates up to 3 times the recommended field application rate. Only plants that exhibited tolerance to Buctril® at 3 times the recommended field application rate were selected for further development.

Counts were made of tolerant (alive, no symptoms) and susceptible (dead) individuals to Buctril® to determine the segregation ratio of the trait. Individual tolerant T_2 plants were selected from agronomically promising events that segregated in a 3:1 or 15:1 ratio of tolerant to susceptible to bromoxynil (consistent with one or two independently segregating loci, respectively). Seed from each individual plant was then harvested and maintained separately. Ideally, a single genetic locus is preferred because, while not essential for the performance of the cotton or the *oxy* gene, it simplifies the breeding of the trait into other elite commercial cultivars.

<u>Production of T_3 material</u>

Progeny rows from each T_2 selection were grown in the next field generation and were again sprayed with Buctril®. Events which segregated 3:1 in the T_2 generation are expected to produced progeny rows, one third of which are 100% tolerant to Buctril® (indicating the individual parent was homozygous for the *oxy* gene) and two thirds segregating 3:1 (indicating that the individual parent was heterozygous for the *oxy* gene).

Events that segregated 15:1 in the T_2 generation are expected to produce progeny rows that segregate 3:1, 15:1 or that are 100% tolerant. In this case, the rows segregating 3:1 were the ones of most interest because they have inherited only one of the two loci originally in the T_1 plant. Selections from these 3:1 progeny rows were harvested to identify T_4 homozygous lines, as was the case with the events segregating 3:1 in the T_2 generation.

Homozygous T_3 progeny rows from 3:1 segregating T_2 events were evaluated for potential agronomic acceptability. Individual plant selections were made and these were advanced to the next generation. Advanced progeny rows were then grown from these selections. A bulk harvest of the remaining plants from promising progeny rows were also made for initial yield and quality testing. In all, five events were selected for further testing. The best homozygous rows were selected from each of these events and individual plant selections were made from within each selected row.

Subsequent generations

In subsequent generations, more stringent selection based on yield, fibre quality and good agronomic performance (including earliness, height, and pest and disease resistance) were used to further select and reduce the candidates for commercial release.

Thus, although nine independent transformation events were originally selected, this application only relates to events 10211 and 10222. These events have been, or are being used to derive the BXN lines for commercial release.

Characterisation of the inserted T-DNA

Progeny of the nine independently derived transformation events were analysed, using a combination of genetic analyses and Southern blot analysis, to characterise the genes from the T-DNA region of pBrx75 that had been inserted into the plant genome. The data for events 10211 and 10222 are presented below.

Genetic analysis

As described above, the total number of functional (bromoxynil-tolerant) loci that have been integrated into an individual transformed plant can be determined by spraying seedlings with the herbicide Buctril® and determining the Mendelian segregation ratios of the bromoxynil tolerant trait. Progeny of single plants are grown and sprayed with the herbicide and plants whose progeny segregate with a ratio of 3 tolerant to 1 susceptible are assumed to contain one functional locus or insertion site. This method cannot however determine the number of copies of the *oxy* gene that have been inserted into the single site, nor can it be used to determine if there has been an insertion of non-functional copies of the *oxy* gene because this method detects functional expression of the trait only. The results of the spray analyses of the T_2 generation of events 10222 and 10211 are provided in Table 3.

Table 3: Segregation ratios[1] for events 10211 and 10222 sprayed with Buctril®

	Event 10211	Event 10222
1.5 lb/acre:		
No. of tolerant plants	61	53
No. of susceptible plants	18	17
Chi-Square value 3:1	0.21	0.02
Chi-Square value 15:1	36.86	38.86
3.0 lb/acre:		
No. of tolerant plants	62	66
No. of susceptible plants	13	18
Chi-Square value 3:1	2.35	0.57
Chi-Square value 15:1	15.72	33.03
4.5 lb/acre:		
No. of tolerant plants	65	69
No. of susceptible plants	22	17
Chi-Square value 3:1	0.00	1.26
Chi-Square value 15:1	53.81	26.82
All spray rates:		
No. of tolerant plants	188	188
No. of susceptible plants	53	52
Chi-Square value 3:1	1.16	1.42
Chi-Square value 15:1	101.92	97.35

[1] Chi-Square values of 3.84 or less fit the expected ratios with a 95% level of confidence

The results of these analyses show that the bromoxynil tolerant trait in events 10222 and 10211 segregates as a single functional locus. Further analysis of the transferred T-DNA was done using Southern blot analysis (Southern 1975).

Southern blot analysis

Southern blotting is a sensitive technique that enables the detection and characterisation of specific sequences among DNA fragments separated using gel electrophoresis.

For events 10222 and 10211 the Southern analyses were used to characterise the inserted T-DNA in terms of insert number (number of integration events), copy number (number of T-DNA copies at a particular genetic locus), insert integrity (gene size, composition and linkage), and sequences outside the T-DNA borders (including the gentamicin resistance gene). Genomic DNA was isolated from leaf tissue of non-transformed control *G. hirsutum* (var. Coker 315) plants and from the homozygous T_3 progeny of BXN cotton events 10222 and 10211 transformed with pBrx75.

To determine the copy number of each of the genetic elements genomic DNA was digested with the restriction enzyme *Sph*I and probed with DNA corresponding to each of the regions of interest (see Table 4). Because the *Sph*I restriction sites in the T-DNA were known (see Figure 1), the size of the hybridising fragments that would be expected to result from a single copy inserted at a single genomic location could be predicted. The expected fragment sizes are detailed in Table 4.

Table 4: Expected fragment sizes for a single copy of T-DNA inserted at a single genomic location

Probe	Expected fragment size
oxy	3.2 kb + 1 larger right border fragment
nptII	3.2 kb + 1 larger left border fragment
tml 3'	right and left border fragments
35S	3.2 kb fragment

Hybridising DNA fragments of the expected size (as indicated above), without any additional fragments, were detected using Southern analysis for both 10222 and 10211 indicating that a single copy of each genetic element is present at a single insertion site in the genome. These results confirm the findings of the genetic analysis above. This experiment also demonstrates physical linkage between the *oxy* and *nptII* genes (both genes inserted at the same site within the genome) because of the common 3.2 kb fragment identified when either the *oxy* or the *nptII* probe is used.

To further confirm the number of insertion sites as well as the T-DNA copy number, analyses were done to determine the number of border fragments that represent the junctions of the inserted genes with plant DNA. A plant would be suspected of having multiple copies of T-DNA at an insertion site if the number of right border fragments was not equal to the number of left border fragment, and/or if the intensity of the hybridisation signal was much stronger for some DNA fragments than for others. As indicated by Table 4, the *oxy* and *nptII* probes can be used to identify the right and left borders, respectively. This approach is valid because physical linkage between the *oxy* and *nptII* genes has been demonstrated. In addition, plants transformed with pBrx75 have copies of the *tml* 3' polyadenylation signal at each T-DNA border. Hybridisation with the *tml* 3' probe was used to further confirm the number of right and left border fragments in each event. The Southern analyses demonstrated that there is one left border and one right border only in both 10222 and 10211 thus confirming that one copy of each gene had been integrated at a single site in the genome.

The two events were also analysed for the transfer of DNA sequences from outside the T-DNA region. Three hybridisation probes were used. The first was the entire binary plasmid pCGN1532 (a precursor to pBrx75) that consists of the *A. rhizogenes* replicon region, the pBR322 origin of replication and the gentamicin resistance gene (*aac*; see Table 2). The second probe was the *aac* gene itself and the third probe was to the *nptII* region (the positive control). If the pCGN1532 probe hybridises to any of the genomic DNA then transfer beyond the T-DNA region has occurred. Southern analysis showed that neither 10222 nor 10211 contains any sequences that hybridise to pCGN1532 indicating that transfer of DNA beyond the T-DNA borders has not occurred. To confirm this finding specifically in relation to the gentamicin resistance gene, the same Southern blot was re-probed with the *aac* probe. Once again, no hybridising sequences were detected in either 10222 or 10211.

Conclusion

A single copy of T-DNA, containing the *oxy* and *nptII* gene cassettes, has been integrated at a single site in transformation events 10222 and 10211. All transferred genes appear to be intact and no re-arrangements of the T-DNA were detected. An

analysis of segregating plant populations using bromoxynil treatment indicated that the *oxy* gene is functional in both events and that the bromoxynil-tolerant trait is segregating according to standard Mendelian genetics. No sequences residing outside the T-DNA region had been transferred during the transformation.

Stability of the genetic changes

Analysis of integrated sequences

Southern analysis was done on later generations of events 10211 and 10222 to confirm that the DNA banding pattern observed in the homozygous T_3 plants was maintained in subsequent generations. Two plant lines, derived from transformation events 10211 and 10222, were analysed at the T_5 generation. As plants from these two events had been previously analysed in the T_3 generation it enabled a direct comparison. In addition, events 10211 and 10222 had also been used in a backcrossing program to integrate the *oxy* gene into elite commercial cotton varieties, therefore the stability of the T-DNA in different genetic backgrounds could also be determined.

The pattern of hybridising DNA fragments from plants of the T_5 generation for lines 10211-20 and 10222-1 was shown by Southern analysis to be identical to that observed in DNA from T_3 generation plants. Southern analysis of late generations of these crosses between events 10211 and 10222 with elite cotton varieties also showed no difference compared to the analysis of the T_3 generation.

Inheritance of the bromoxynil tolerance trait by BXN cotton

The genetic stability and segregation of the bromoxynil tolerance trait was monitored using data obtained from field sprayed plants.

BXN cotton lines were screened for bromoxynil tolerance by spraying plants of each generation with the herbicide and selecting lines for commercialisation. As part of the normal screening process in the breeding program of BXN cotton, events with consistent segregation patterns and desirable characteristics are advanced, and those with unusual segregation patterns (not fitting classic Mendelian inheritance patterns) are not developed further.

The *oxy* gene has been maintained for at least six seed generations (self-pollinated plants) and at least 5 generations of backcrossing with commercial varieties in the breeding program. Inheritance of the BXN tolerance trait was found to be consistent, not only with progeny produced by self-pollination but also in a backcross program involving introgression of the *oxy* gene into a variety of genetic backgrounds.

T_3 seed was collected from individual T_2 plants and processed separately. The seed from each plant was planted in an individual row and sprayed with a bromoxynil containing herbicide. The plant numbers obtained from the experiment should fit either a 3:1 tolerant to susceptible ratio or be 100% tolerant. The 3:1 ratio rows come from T_2 plants that were heterozygous for the insertion and the 100% rows come from T_2 plants that were homozygous tolerant.

Table 5 gives the fit to Mendelian inheritance in the T_2 generation for transformation events 10211 and 10222. Both events were found to fit an expected 3:1 ratio for one gene insertion site (as described above for the molecular characterisation).

Table 5: Segregation ratios for BXN cotton events

Event No.	Total:susceptible T_2 plants	Chi-Square fit for 3:1 ratio[a]	Chi-Square fit for 15:1 ratio[a]
10211	241:53	1.163	101.922
10222	240:52	1.422	97.351

[a] Chi-Square of < 3.84 has a 95% probability of a 3:1 or 15:1 segregation ratio

The vast majority of T_3 rows were found to fit reasonably closely to the expected ratios, the few rows that did not fit had too few plants to verify the fit statistically or were suspected to contain contaminant seed from processing.

The second statistic to verify expected segregation is the number of individual rows falling into each class. In the T_2 generation, a single insertion event is expected to segregate 3 tolerant: 1 susceptible. This is the observed phenotype, but genetically, the genotypes are 1 homozygous tolerant: 2 heterozygous tolerant: 1 homozygous susceptible. By examining the next generation from each surviving plant, it is possible to determine how many of the 3 tolerant T_2 plants were heterozygous and how many were homozygous. It was found that the ratio of genotypes was as expected, that is 1:2:1.

Overall, the T_2 and T_3 data presented support normal gene segregation for transgenes inserted into cotton plants. After the T_3 or T_4 generation, homozygous lines are selected, meaning these lines will no longer display segregation of the BXN trait. Screening with bromoxynil is then only done to monitor seed purity. The consistency of the tolerance trait in these lines is a good measure of the level of genetic stability (providing there is no contamination from bromoxynil-susceptible lines).

Table 6 shows the percentages of bromoxynil-sensitive plants found in the field of T_6 plants derived from events 10211 and 10222.

Table 6: The percentages of bromoxynil-sensitive plants found in the field of T_6 plants

Line	Field	Open-pollinated[a]		Self-pollinated[b]	
		Population size	% susceptible	Population size	% susceptible
10211-1	Empire 1874#3	153 536	0.5	412 508	0.02
10211-20	Harlan Bohne	1 093 251	0.97	670 057	0.07
10211-1	Somerset	482 853	0.22	875 172	0.05
10211-20	Indianola	446 533	1.09	210 133	0.04
10222-1	Empire 1074			1 822 538	0.001

[a] open-pollinated in South Africa 1991-92 nursery
[b] self-pollinated in South Africa 1991-92 nursery

The populations of T_6 plants were split into open pollinated and self-pollinated. This refers to pollination done three generations (T_3) earlier in a counter season location. Rows from self-pollinated seed of individual plants in the T_3 generation were then

grown at T_4 progeny rows in the United States nursery without self-pollination in the following season. T_5 bulk seed harvested from these rows were planted under isolation from other cotton in the next counter season. The T_6 generation was then grown in several different field locations in the United States. Self-pollinated seed should not produce any susceptible plants. The small number of bromoxynil susceptible plants found in the self-pollinated lots most likely came from crossing which occurred in the T_4 generation grown in the US nursery. The number of bromoxynil susceptible plants found in the open pollinated populations is still carryover from the nursery in the counter season when the lines were T_3s.

These data are consistent with the conclusion that the BXN tolerance trait is stably inherited and maintained in BXN cotton.

Conclusion

Stability of the transferred *oxy* gene was studied by backcrossing of plants containing transformation events 10211 and 10222 with commercially available cotton varieties and by self-crossing followed by propagation. The BXN gene was determined to be stable over at least six generations through observed tolerance to bromoxynil treatment. Additionally, Southern blot analysis demonstrated that both the *oxy* and *nptII* genes were stably transferred from generation to generation in a variety of genetic backgrounds.

Antibiotic resistance genes

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

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

In transformation events 10211 and 10222, Southern analysis demonstrated that a single antibiotic resistance gene has been transferred – the *nptII* gene. Both transformation events contain the *nptII* gene under the control of the 35S promoter. The gentamicin resistance gene, which was also present in plasmid pBrx75, was not transferred to the cotton genome in the transformation process.

The first issue to be considered is the probability that the *nptII* gene would be successfully transferred to and expressed in microorganisms present in the human digestive tract. There are two considerations in relation to this issue.

Firstly, DNA is not present in refined oil and linters, which are the only products intended for human consumption. Processed linters are essentially pure cellulose (>99%) and are subjected to heat and solvent treatment that would be expected to remove and destroy DNA. The refining process for cottonseed oil also includes heat, solvent and alkali treatments that would be expected to remove and destroy DNA, and intact fragments of the *nptII* gene are unlikely to survive the processing steps. The processing steps can also lead to the release of cellular enzymes (nucleases) that are responsible for degrading DNA into smaller fragments.

Refined oil from another genetically modified cotton – glyphosate-tolerant cotton line 1445 (see Technical Report No. 6) – was analysed to ascertain if any intact DNA could be detected using a highly sensitive technique called the Polymerase Chain Reaction (PCR). No DNA could be detected in refined oil produced from the cotton. The detection limit of the assay was 1ng of DNA.

The lack of intact DNA in the intended food products, cottonseed oil and cellulose from linters reduces any risk of horizontal transfer of genetic material to cells in the human digestive tract as a result of the ingestion of these foods.

The second consideration is the steps necessary for horizontal DNA transfer to occur. These are:

- excision of DNA fragments containing the *nptII* gene;

- survival of DNA fragments containing the *nptII* gene in the digestive tract;

- natural transformation of bacteria inhabiting the digestive tract;

- survival of the bacterial restriction system by the DNA fragment containing the *nptII* gene;

- stable integration of the DNA fragment containing the *nptII* gene into the bacterial chromosome or plasmid;

- maintenance and expression of *nptII* gene by the bacteria

The transfer of the *nptII* gene from refined BXN cotton seed oil or cellulose from linters to microorganisms in the human digestive tract is therefore considered to be highly unlikely because: (i) DNA would not be present in the food as consumed; and (ii) 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 extremely unlikely event successful transfer of a functional *nptII* gene to microorganisms in the human digestive tract did occur.

The human health impacts are considered to be negligible. The *nptII* gene occurs naturally in bacteria inhabiting the human digestive tract therefore the additive effect of

an *nptII* gene entering the human gastrointestinal flora from a genetically modified plant would be insignificant compared to the population of kanamycin resistant microorganisms naturally present.

Conclusion

It is extremely unlikely that the *nptII* gene would transfer from BXN cotton to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively and because the food products, refined oil and linters, are unlikely to contain any DNA. In the highly unlikely event that the *nptII* gene was transferred, the human health impacts would be negligible because kanamycin resistant bacteria are already commonly found in the human digestive tract and in the environment.

CHARACTERISATION OF NOVEL PROTEIN

Biochemical function and phenotypic effects

Nitrilase

The *oxy* gene was isolated from *Klebsiella pneumoniae* subspecies *ozaenae* (McBride *et al* 1986, Stalker and McBride 1987, Stalker *et al* 1988) and encodes a 37 kDa nitrilase (EC. 3.5.5.6).   This enzyme hydrolyses the oxynil herbicides into non-phytotoxic compounds: 3.5-dibromo-4-hydroxybenzoic acid or 3,5-diiodo-4-hydroxybenzoic acid and ammonia (Figure 2).

Figure 2: Oxynil degradation by the oxy-encoded nitrilase. (Hal is either Br or I)
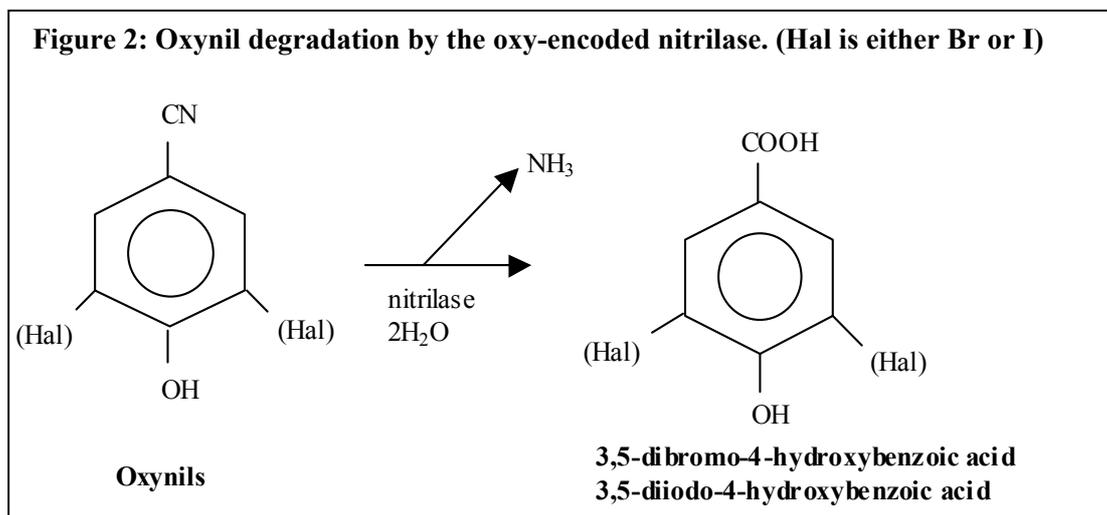
Purified nitrilase has optimal activity at pH 9.2 and at a temperature of 35°C. The pH optimum remains relatively constant at different substrate concentrations. Nitrilase activity declines to 15% at pH 7.0 and also in temperatures of 10 and 55°C. The *oxy*-encoded nitrilase is highly specific for its substrates, exhibiting a K_m of 0.31nM and a V_{max} of 15μmole of NH_3 released/min/mg protein for bromoxynil.

Neomycin phosphotransferase II

NPT II (also known as aminoglycoside 3'-phosphotransferase II) is an enzyme with a molecular mass of 29 kDa that catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics, including neomycin, kanamycin and gentamicin A and B, thereby inactivating the antibiotics (Davies *et al* 1986). The enzyme is encoded by the *nptII* gene, which is derived from transposon Tn5 from the bacterium *E. coli* (Beck *et al* 1982).

Protein expression analyses

All of the plants used for the analyses had been sprayed with an agronomic dose of Buctril® to monitor seed purity.

Nitrilase

The concentration of the nitrilase enzyme was determined in leaves, acid delinted cottonseed, decorticated cottonseed kernels, cottonseed hulls, processed cottonseed meal and crude oil using Western blot analysis. This assay detects both active and inactive nitrilase protein. Protein extractions were made of each of the tissues or fractions and these were separated electrophoretically on an SDS-polyacrylamide gel.

A positive nitrilase signal on the Western blot consists of a single band at 37 kDa. The protein level was quantitated by comparing the intensity of the signal in the tissue extracts from plants containing transformation events 10211 and 10222 with the extracts from the non-transgenic control, Coker 315 spiked with purified nitrilase of known concentrations. Each assay was repeated at least three times to obtain an estimate of the maximum nitrilase concentration in each of the transformation events. A summary of the protein expression data is provided in Table 7.

Table 7: Summary of nitrilase expression data for BXN cotton

Sample	Event 10211	Event 10222
Leaf tissue		
µg/g total protein	20	20
% total protein	0.002%	0.002%
Seed, kernels, hulls		
µg/g total protein	< 0.6	max. of 0.6
% total protein	<0.00006%	0.00006%
Meal		
µg/g total protein	0.12	0.12
% total protein	0.000012%	0.000012%
Crude oil		
µg/g total protein	Not detected[1]	Not detected[1]
% total protein		

[1] limit of detection was 0.1 ppm.

Neomycin phosphotransferase II (NPTII)

Western blot analysis was also used to determine the level of NPTII expressed in leaf tissue, cottonseed, meal and crude oil. The NPTII protein is a monomer of 29 kDa. NPTII was not detected in protein extracts from the non-transformed control, Coker 315.

The results of these studies are summarised in Table 8.

Table 8: Summary of NPTII expression data for BXN cotton

Sample	Event 10211	Event 10222
Leaf tissue		
μg/g total protein	80	80
% total protein	0.008%	0.008%
Seed, kernels, hulls		
μg/g total protein	< 30	max of 27
% total protein	< 0.003%	0.0027%
level in cottonseed	6.6 ppm	5.9 ppm
Meal		
μg/g total protein	14	7
% total protein	0.0014%	0.0007%
level in meal	5.7 ppm	2.9 ppm
Crude oil		
μg/g total protein	Not detected[1]	Not detected[1]
% total protein		

[1] limit of detection was 0.1 ppm

Conclusion

The results show that the levels of nitrilase and NPTII are highest in cotton leaf tissue, the levels being about 80μg/ g total protein for NPTII (equivalent to 0.008% of total leaf protein) and about 20μg/ g total protein for nitrilase (equivalent to 0.002% of total leaf protein). The levels of both proteins decline in the seed and meal. In the crude oil fraction, which is the fraction destined for human consumption, neither proteins can be detected at a limit of detection of 0.1 ppm. Therefore, as it is known that the refining process further removes any protein, it can be concluded that the refined oil produced from BXN cotton is extremely unlikely to contain any detectable nitrilase or NPTII.

Potential toxicity of novel proteins

The protein expression data demonstrates that transformation events 10211 and 10222 express two novel proteins – nitrilase and neomycin phosphotransferase II. This section of the report will therefore assess the potential toxicity of these two proteins.

Presence of the novel proteins in the food as consumed

It should be noted that the products intended for human consumption – refined cottonseed oil and cellulose from the linters – do not normally contain any detectable amounts of protein. Furthermore, when crude cottonseed oil from BXN cotton was analysed for the presence of both nitrilase and neomycin phosphotransferase II neither could be detected at a detection limit of 0.1 ppm. Therefore, it is highly unlikely that

humans ingesting refined oil or cellulose products derived from BXN cotton would be exposed to any appreciable amounts of the two novel proteins.

Nitrilase

Studies evaluated: Dange, M. (1996) Nitrilase: sub-acute oral toxicity study in the mouse. Rhône-Poulenc Study SA 96267.   Astwood, J.D. (1997). *Klebsiella ozaenae* nitrilase (BXN) has no significant sequence similarity to known allergens or toxins. Monsanto Study Report No. MSL-15120.

Sub-acute oral toxicity study in mice

To obtain sufficient quantities of nitrilase for toxicity testing, the enzyme was expressed in *Escherichia coli* BL21 and subsequently purified as an inclusion body pellet.

The applicant reports that an acute oral toxicity study was planned to be performed using doses up to 2000mg/kg body weight, using a suspension of nitrilase at 200mg/ml. However, the consistency of the suspension once prepared did not allow the total dose to be administered at one time. Therefore, the suspension was administered over four consecutive days at 500mg/kg body weight/day.

Four consecutive oral doses (500mg/kg body weight) of nitrilase (Batch No. JHJ0001) were administered to groups of OF1 mice (5/sex) at a dose volume of 20ml/kg. The purified nitrilase was suspended in 0.25% methylcellulose in distilled water.

All animals were checked daily for clinical signs over a period of 15 days, and their body weight recorded weekly. At termination of the study period, all animals were killed and subject to necropsy. The necropsy included the macroscopic examination of abdominal and thoracic cavities, major organs and tissues.

No clinical signs were observed during the study and there were no unscheduled deaths. The body weight gain of the animals was unaffected by the treatment and no gross findings were recorded at necropsy. The LD_{50} was designated as >500mg/kg body weight.

Similarity with known protein toxins

A database of protein toxin amino acid sequences was assembled from the public domain genetic databases, which included GenPept ver. 92 (a protein database extracted from GenBank and EMBL), PIR ver. 45, and SwissProt ver. 31. Amino acid sequences were retrieved from the databases using the STRINGSEARCH program supplied with the GCG sequence analysis package version 7 (Devereux *et al* 1984). Using the DATASET program, the sequences of toxins were combined into a single database called TOXIN3.

The keyword "toxin" identified and retrieved 2662 amino acid sequences from the public domain genetic databases – this comprised the TOXIN3 database. There were no toxins in the TOXIN3 database that showed significant similarity to nitrilase.

History of human exposure to nitrilases

Nitrilase enzymes, similar to that encoded by the *oxy* gene from *Klebsiella pneumonia,* have been found in a number of plant and microbial species. Although substrates and pathways differ, it appears as though nitrilases share common functions such as hydrolysis of nitriles to carboxylic acids. Plant nitrilases can also confer resistance to some of the nitrile containing herbicides. Nitrilases have been found in a number of important food crops such as wheat, cabbage, barley, and bananas (Buckland *et al* 1973, Thimann and Mahadevan 1964), therefore, humans have a history of exposure to similar types of proteins with no apparent ill effects ever being documented.

Potential toxicity of bromoxynil metabolites

Bromoxynil has recently been re-registered for use in the United States as a contact herbicide to control broadleaf weeds in BXN cotton (US EPA 1998). The bromoxynil-tolerant plants hydrolyse bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid (DBHA), a carboxylic acid. It is reported that significant residues of DBHA can be present on BXN cotton as a result of the enzymatic activity of the bacterial-derived nitrilase (US EPA 1998). As this metabolite is a by-product resulting from the activity of an introduced enzyme it is important that a consideration of its toxicity be included in any safety evaluation of BXN cotton.

The US Environment Protection Agency, in its evaluation of bromoxynil, stated that the human health risk from bromoxynil is negligible (US EPA 1998). As part of its evaluation of bromoxynil the US EPA also evaluated the toxicity of the DBHA metabolite of bromoxynil and concluded "there was no concern that DBHA would exhibit significant toxicity over that of the parent bromoxynil".

Bromoxynil and DBHA are extremely similar in structure, varying only in that bromoxynil has a cyano (-CN) group that has been converted to a carboxyl (-COOH) group in the DBHA metabolite. Conversion to a carboxyl group is generally considered to decrease the toxicity of a molecule (US EPA 1998). The conversion to the carboxyl group should cause the DBHA to be more polar and therefore more soluble in water and less in fats. Additionally, the presence of the carboxyl group will allow DBHA to combine with certain water molecules (such as glucuronic acid) which should further increase DBHA's water solubility and further decrease its solubility in fats. This increased water solubility, combined with the decreased fat solubility means that DBHA should be eliminated faster from the organism than its parent compound, bromoxynil. It is likely that these characteristics would also limit the amount of DBHA residue likely to be present in cottonseed oil.

To date, the US EPA has concluded that DBHA is likely to be no more toxic than bromoxynil, which the US EPA has recently determined poses negligible risk to human health at expected exposure levels.

Conclusion

The evidence from sub-acute toxicity studies in mice does not indicate that there is any potential for nitrilase from *Klebsiella pneumoniae* subsp. *ozaenae* to be toxic to humans. Furthermore, humans are extremely unlikely to be exposed to this enzyme through the consumption of refined oil and cellulose from BXN cotton as both food products are devoid of any detectable protein. The metabolite of bromoxynil, DBHA, also does not show any potential to be toxic to humans at the predicted exposure levels.

Neomycin phosphotransferase II

The potential toxicity of neomycin phosphotransferase II (NPTII) has been investigated previously where acute oral toxicity studies in mice have been evaluated. The safety of this protein has also been considered on numerous occasions in the peer reviewed scientific literature (Flavell *et al* 1992, Nap *et al* 1992, Fuchs *et al* 1993a, Fuchs *et al* 1993b). In all instances it has been concluded that NPTII is non-toxic to humans. This conclusion also applies to NPTII in BXN cotton, which is identical to the NPTII assessed for toxicity on previous occasions. Furthermore, humans are extremely unlikely to be exposed to this enzyme through the consumption of refined oil and cellulose from BXN cotton as both food products are devoid of any detectable protein.

Potential allergenicity of novel proteins

The concerns regarding potential allergenicity of novel proteins are two fold. Firstly, there are concerns that the ability to express new or different proteins in food will result in the transfer of allergens from one food to another, thereby causing some individuals to develop allergic reactions to food they have not previously been allergic to. Secondly, there are concerns that the transfer of novel proteins to food will lead to the development of new allergies in certain individuals. The former is more easily addressed than the latter because if an allergen is already known it is possible, using human sera or human skin tests, to test if it has been transferred. There are no reliable tests or animal models, however, which enable the prediction of the allergenic potential of novel proteins. Instead, potential allergenicity can only be indicated by examination of a number of characteristics of the novel protein, such as whether it is derived from a known allergenic source, its physical/chemical characteristics (resistance to acid and protease degradation, amino acid sequence similarity with known allergens) and whether it is likely to be present in large amounts in the food as consumed and therefore have potential for allergic sensitisation.

Presence of the novel proteins in the food as consumed

As humans would be extremely unlikely to be exposed to either nitrilase or NPTII through the consumption of refined oil or cellulose products derived from BXN cotton there is virtually no potential for the two novel proteins to become food allergens.

Nitrilase

Studies evaluated:

Astwood, J.D. (1997). *Klebsiella ozaenae* nitrilase (BXN) has no significant sequence similarity to known allergens and toxins. Monsanto Study Report No. MSL-15120.

Aasen, E., *et al* (1997). Assessment of the digestibility of purified BXN nitrilase protein *in vitro* using mammalian digestive fate models. Monsanto Study Report No. MSL-15148.

Similarity to known allergens and gliadins

A search for amino acid sequence similarity with known allergens and gliadins is a useful first approximation of potential allergenicity and potential association with coeliac disease (Fuchs and Astwood 1996, Metcalf *et al* 1996). Many protein allergens have been characterised and their amino acid sequences are known, and importantly, their IgE binding epitopes have been mapped (Elsayad and Apold 1983, Elsayad *et al* 1991, Zhang *et al* 1992). The binding epitopes are generally between 8 and 12 amino acids in length.

To undertake the amino acid sequence comparison between nitrilase and known protein allergens and gliadins, a database of allergen and gliadin sequences was assembled from the standard public domain databases containing protein sequences (GenPept ver. 86.0, PIR ver. 41, SwissProt ver. 30). In addition, DNA sequences were retrieved from GenBank/EMBL ver. 86 as some allergen sequence entries do not appear in the protein sequence databases. The amino acid sequences of the allergens retrieved from the GenBank/EMBL database were either obtained from the GenEMBL flat files or were obtained by translation of the open reading frames in the DNA sequences. Therefore the assembled database consisted of two parts: (1) a dataset of protein sequences and (2) a supplemental database of protein sequences initially retrieved as DNA sequences. Duplicates were deleted from the assembled database and irrelevant sequences were identified by examining complete flat files or by reference to the scientific literature. The resulting database of 219 allergens and gliadins has been published in the scientific literature (Astwood *et al* 1996).

The allergen and gliadin database was then searched for sequences similar to nitrilase. A significant sequence similarity was defined as a sequence identity of greater than seven contiguous amino acids. No significant similarity between nitrilase and any of the known allergens or gliadins was identified.

Digestibility of nitrilase

If proteins are to be allergenic they must be stable to the peptic and tryptic digestion and acid conditions of the digestive system if they are to pass through the intestinal mucosa to elicit an allergenic response.

The digestibility of nitrilase was determined experimentally using *in vitro* mammalian digestion models. *In vitro* studies with simulated digestion solutions have been used as models for animal digestion for a number of years and have had wide application.

To obtain sufficient quantities of purified nitrilase for testing, the enzyme was expressed in *Escherichia coli* from a cloned *Kelbsiella ozaenae* DNA fragment and purified to homogeneity (Stalker *et al* 1988). The coding region used to express nitrilase in *E. coli* was therefore identical to that transferred into BXN cotton. The molecular mass of nitrilase is approximately 37 kDa, however, the active form of the enzyme is as a dimer composed of two identical 37 kDa subunits.

Nitrilase was added to simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) and incubated at 37°C over a series of time points. The time points for SGF were 0 sec, 15 secs, 30 secs, 1 min, 5 mins, 10 mins, 30 mins, 1 hour and for SIF the time points were 0 sec, 1 min, 5 mins, 15 mins, 30 mins, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours.

Analysis of nitrilase after incubation in SGF showed that the protein is degraded to below the limit of detection within 15 seconds. Nitrilase was found to be stable in an inactive test system over the time period tested confirming that the degradation of nitrilase in the active test system is due to proteolytic activity, not to any molecular instability of nitrilase.

In SIF, nitrilase was degraded within 5 minutes of exposure. Once again, nitrilase was shown to be stable in an inactive SIF system.

The results of these studies demonstrate that nitrilase is rapidly degraded in conditions that mimic mammalian digestion, greatly minimising any potential for intact nitrilase to be absorbed by the intestinal mucosa.

Neomycin phosphotransferase II

The potential allergenicity of neomycin phosphotransferase II (NPTII) has been investigated previously where simulated mammalian digestion studies have been evaluated as well as studies comparing its amino acid sequence with known allergens. None of these has revealed any potential for NPTII to be a food allergen. In addition, the safety of this protein, including its potential allergenicity, has also been considered on numerous occasions in the peer reviewed scientific literature (Flavell *et al* 1992, Nap *et al* 1992, Fuchs *et al* 1993a, Fuchs *et al* 1993b). In all instances it has been concluded that NPTII has limited potential to be a food allergen. This conclusion also applies to NPTII in BXN cotton, which is identical to the NPTII assessed for potential allergenicity on previous occasions.

Conclusion

Humans are highly unlikely to be exposed to either nitrilase or NPTII through the consumption of refined cottonseed oil and cellulose products from BXN cotton. Moreover, neither of the proteins possesses any of the characteristics of known allergens. It is therefore concluded that nitrilase and NPTII have very limited potential to become food allergens.

COMPARATIVE ANALYSES

Key nutrients

There are concerns that genetic modification will affect the overall nutritional composition of a food, or cause unintended changes that could adversely affect the safety of the product. Therefore a safety assessment of food produced from transgenic plants must include analysis of the composition of the food, based on a comparison with other commercial varieties of the crop. Generally, comparisons are made not only with the parental line but also with other non-transformed lines. If the parameter for the transformed line is within the normal range for non-transformed lines, this is considered acceptable (Hammond and Fuchs 1998).

Three separate compositional analyses of the BXN cotton lines were done using cottonseed samples collected from three separate field trials. In all field trials, each replicate represents a field plot (at least 150 m^2) planted in a randomised complete block design.

For the first set of compositional analyses, T_3 cottonseed was collected from T_2 BXN cotton plants (derived from the transformation events 10211 and 10222) grown at a single location in the United States in 1991. Homozygous seed from the same transgenic event were pooled and processed as a single line. Bromoxynil treatment had been used to identify the homozygous seed lots but the seed samples used for the analyses had themselves been obtained from unsprayed plants. The seed was shipped to the Engineering Biosciences Research Centre at Texas A&M University for small scale processing under Good Laboratory Practice to obtain cottonseed meal and crude oil for the analyses. Control samples were bulk seed of the non-transgenic control Coker 315. Two seed sample lots of Coker 315 came from the same field as the BXN cotton and a third sample lot came from plants grown at a different site in the same year. Constituents analysed were: fatty acid composition of the crude oil; and protein, nitrogen, fibre, residual oil and amino acid content of the meal.

For the second set of compositional analyses, seed was harvested from a BXN cotton line (derived from transformation event 10222) and a number of commercial cotton varieties grown at four locations in the United States in 1996. The plants were grown in two replicated plots per location. The BXN cotton plants were unsprayed. Constituents analysed were: moisture, fat, protein and fibre content of the seed; amino acid content of the meal; and major fatty acid composition of the crude oil.

For the third set of compositional analyses, seed was harvested from a BXN cotton line (derived from transformation event 10222) and a commercial cotton variety grown at two locations in Spain in 1997. The BXN cotton plants had been sprayed with 563 g a.i./ha of Buctril®, which is representative of an agronomic dose. Constituents analysed were: moisture, ash, fat, protein, and fibre content of delinted seed; amino acid content of the meal; and major fatty acid composition of the crude oil.

Cottonseed

1991 field trial data - unsprayed

In samples collected from the 1991 field trial, the only constituent measured in whole cottonseed was the fibre content. Crude fibre, acid detergent fibre, and neutral detergent fibre provide measurements of relative digestibility and bioavailability for cottonseed products. The results of these analyses are presented in Table 9.

Table 9: Crude fibre, acid detergent fibre and neutral detergent fibre composition[1] of whole cottonseed from 1991 field trials in the United States

Sample	Crude fibre	Acid detergent fibre	Neutral detergent fibre
Coker 315	14.7	21.6	27.0
Event 10211	14.3	25.0	29.8
Event 10222	15.1	21.9	27.4

[1] values are percent of whole cottonseed

The levels of crude fibre, acid detergent fibre and neutral detergent fibre in the BXN cotton were comparable to the levels obtained for the Coker 315 control.

1996 field trial data - unsprayed

The BXN cotton line grown in this field trial was derived from transformation event 10222. Control samples were obtained from commercial cotton varieties (LA887, ST132 and ST474) grown at the same location. The constituents measured in cottonseed samples collected from the 1996 field trials were moisture, fat/oil, protein and crude fibre content. The results of these analyses are presented in Table 10.

Table 10: Major constituents[1] of cottonseed harvested from plants grown in the field in 1996 in the United States

Sample	Moisture content	Fat/oil content	Protein content	Crude fibre content
Event 10222	6.77 a[2]	16.18 b	20.27 ac	31.36 a
LA 887	6.49 a	16.94 a	20.09 a	31.59 a
ST132	6.88 a	16.08 b	20.79 b	31.02 a
ST 474	6.73 a	16.14 b	20.56 bc	32.06 a

[1] values are percent by weight of whole cottonseed and are the means of single analyses of two replicates from four locations
[2] values in a column marked with the same letter are not significantly different at a 95% confidence level

The levels of major constituents in the BXN cotton line are equivalent to those in standard commercial varieties of cotton.

1997 field trial data – sprayed with Buctril®

The BXN cotton variety (OXY47) grown in this field trial is a variety developed from transformation event 10222 in a ST474 genetic background. The control, ST474, is a current commercial variety of cotton. The constituents measured in delinted cottonseed samples collected from the 1997 field trials were moisture, ash, fat/oil, protein and crude fibre content. The results of the analyses are summarised in Table 11.

Table 11: Major constituents[1] of cottonseed harvested from plants grown in the field in 1997 in Spain

Sample	Moisture (% weight)	Ash	Fat/oil	Protein	Crude fibre
OXY47	10.23 a[2]	4.58 b	32.87 b	37.94 a	4.58 a
ST474	10.18 a	4.98 a	32.51 a	37.81 a	4.98 a

[1] except for moisture, values presented are percent dry weight of sample and are the average of two replicates from two sites

[2] values in a column marked with the same letter are not significantly different at a 95% confidence level

With the exception of ash and fat/oil, the levels of major constituents in OXY47 are equivalent to those measured for the parental cotton line. The differences in ash and fat/oil content are minor and have no biological significance.

Cottonseed meal

1991 field trial data - unsprayed

The constituents measured in meal obtained from cottonseed samples collected from the 1991 field trial were % total nitrogen, % total protein, % residual oil and amino acid content. Toasted cottonseed meal was analysed for % crude protein and residual oil content. Control values were obtained from meal produced from the non-transgenic control line, Coker 315. The results of these analyses are summarised in Table 12.

Some significant differences were observed between the BXN cotton and control lines with the untoasted meal from BXN cotton containing significantly increased levels of total protein (and hence total nitrogen) compared to the Coker 315 control. The total protein levels recorded for events 10222 and 10211 were however comparable to the literature reported range for total protein. Significantly increased total protein in a food generally does not represent a cause for concern. Moreover, as the refining process essentially removes all traces of protein from the food products in question (i.e. the oil and linters), this finding does not have any significance from a food safety perspective.

The levels of amino acids in meal derived from BXN cotton are equivalent to the levels measured for the control and are comparable to the literature values where these exist – the differences observed in total protein content of the meal are not reflected in the amino acid content because the levels of each amino acid were calculated as percentage of the crude protein.

Table 12: Nitrogen, protein, residual oil and amino acid content[1] of cottonseed meal obtained from plants grown in the field in 1991 in the United States

Constituent	Event 10211	Event 10222	Coker 315	Literature values[2]
Untoasted meal:				
% total nitrogen	7.21	8.55	4.37 (2.96 - 5.77)	
% total protein[3]	45.06	53.41	27.31 (18.51 - 36.03)	(22 – 50)
% residual oil	1.74	3.78	1.92 (0.574 - 4.19)	
Toasted meal:				
% crude protein	53.73	40.08	47.62	45.2[4]
% residual oil	3.16	1.26	2.68	
Amino acids[5]:				
Cysteine	1.7	1.8	1.7	2.2 (1.7 – 2.6)
Proline	3.7	3.7	3.7	4.2
Aspartic acid	10.5	10.3	10.1	10.8
Serine	5.0	5.0	5.1	4.7 (4.2 – 5.0)
Threonine	3.5	3.5	3.6	3.5 (2.9 – 4.1)
Glutamic acid	21.5	21.8	21.5	24.8
Glycine	3.9	3.9	4.0	4.8 (4.0 – 5.6)
Alanine	3.8	3.9	4.0	4.6
Valine	5.2	5.2	5.6	5.1 (4.3 – 7.4)
Methionine	1.3	1.5	1.5	1.5 (1.4 – 1.9)
Isoleucine	3.6	3.4	3.6	3.7 (3.5 – 4.3)
Leucine	6.2	6.2	6.4	6.1 (4.5 – 6.8)
Tyrosine	3.3	3.6	3.1	3.0 (1.6 – 3.6)
Phenylalanine	5.8	5.7	5.8	5.5 (3.5 – 6.6)
Histidine	3.0	3.0	3.0	2.8 (2.4 – 3.3)
Lysine	4.8	4.9	5.1	4.3 (3.2 – 5.1)
Arginine	13.3	12.8	12.3	11.4 (9.1 – 13.5)

[1] values are presented as means with the range in parentheses (where provided)
[2] values presented as means with the range in parentheses, values taken from Ensminger *et al* (1990), McCarthy and Matthews (1984) and National Research Council (1982)
[3] calculated from % nitrogen
[4] solvent extracted
[5] values are percent by weight of amino acid in cottonseed meal protein

1997 field trial data – sprayed with Buctril®

The BXN cotton line (OXY47) grown in this field trial was derived from transformation event 10222 and is in a ST474 genetic background. Control samples were obtained from the commercial cotton variety ST474, which was grown at the same location. Meal obtained from cottonseed samples harvested from the 1997 field trial were analysed for amino acid content. The results of these analyses are presented in Table 13.

The amino acid levels for OXY47 cotton sprayed with Buctril® were equivalent to those obtained for the ST474 parental control and are comparable to the literature values for amino acid levels.

Table 13: Mean amino acid content[1] of cottonseed meal from control and BXN cotton (sprayed with Buctril®) grown in the field in 1997 in Spain

Amino acid	Literature values[2]	ST474	OXY47
Cysteine	2.2 (1.7 – 2.6)	1.9	2.0
Proline	4.2	3.9	3.9
Aspartic acid	10.8	10.2	10.2
Serine	4.7 (4.2 – 5.0)	4.7	4.6
Threonine	3.5 (2.9 – 4.1)	3.5	3.5
Glutamic acid	24.8	21.2	21.1
Glycine	4.8 (4.0 – 5.6)	4.3	4.2
Alanine	4.6	4.1	4.1
Valine	5.1 (4.3 – 7.4)	4.6	4.8
Methionine	1.5 (1.4 – 1.9)	1.7	1.6
Isoleucine	3.7 (3.5 – 4.3)	3.3	3.3
Leucine	6.1 (4.5 – 6.8)	6.2	6.2
Tyrosine	3.0 (1.6 – 3.6)	3.2	3.2
Phenylalanine	5.5 (3.5 – 6.6)	5.8	5.8
Histidine	2.8 (2.4 – 3.3)	2.9	3.0
Lysine	4.3 (3.2 – 5.1)	4.6	4.7
Arginine	11.4 (9.1 – 13.5)	12.5	12.5
Tryptophan	1.4 (1.2 – 1.7)	1.4	1.3

[1] values are percent by weight amino acid in cottonseed meal protein and are the average of four samples, two from each field site
[2] values presented as means with the range in parentheses, values taken from Ensminger *et al* (1990), McCarthy and Matthews (1984) and National Research Council (1982)

Crude cottonseed oil

Crude cottonseed oil was analysed, rather than refined cottonseed oil, because of the small amount of BXN cottonseeds available for processing.

1991 field trial data – unsprayed

Fatty acid composition was determined for crude cottonseed oil obtained from seed harvested from BXN cotton plants grown in the field in the United States in 1991. The fatty acid levels obtained were compared to those measured in oil obtained from the control line, Coker 315 and in a commercial cottonseed oil product – House of Tsang wok oil. The results are summarised in Table 14.

The fatty acid levels determined for oil derived from BXN cotton are equivalent to those levels obtained for oil derived from the non-transformed control line and are comparable to the levels measured in a commercial cottonseed oil product. With the exception of palmitoleic acid (C 16:1), the fatty acid levels determined for BXN cotton are also all within the Codex specified ranges for cottonseed oil. The levels of palmitoleic acid in transformation events 10211 and 10222 are only marginally outside the Codex specified range and this finding is not considered to have any biological or food safety significance.

Table 14: Fatty acid composition[1] of crude cottonseed oil obtained from BXN cotton plants and non-transformed control plants grown in the field in the United States in 1991

Fatty acid	Codex standard[2]	Wok oil	Coker 315	Coker 315	Coker 315	Event 10211	Event 10222
C <14	< 0.1	0.07	0.02	0.03	0.03	0.05	0.03
C 14:0	0.4-2.0	0.90	0.70	0.90	0.90	0.69	0.72
C 16:0	17.0-31.0	22.53	25.68	26.26	26.36	24.50	24.65
C 16:1	0.5-2.0	0.63	0.52	0.57	0.58	0.46	0.47
C 18:0	1.0-4.0	2.62	2.82	2.64	2.69	2.78	2.83
C 18:1	13.0-44.0	19.65	15.51	15.58	15.79	14.28	13.72
C 18:2	33.0-59.0	52.37	53.87	53.05	52.65	56.30	56.72
C 18:3	0.1-2.1	0.43	0.17	0.17	0.17	0.19	0.18
C 20:0	< 0.7	0.33	0.31	0.34	0.35	0.33	0.30
C 20:1	< 0.5	0.11	0.07	0.08	0.08	0.09	0.08
C 22:0	< 0.5	0.21	0.16	0.20	0.20	0.21	0.17
C 22:1	< 0.5	0.03	0.04	0.03	0.03	0.03	0.04
C 24:0	< 0.5	0.07	0.11	0.13	0.14	0.00	0.00

[1] values are percent of total lipids and are the average of six replicates
[2] ranges adopted by the FAO/WHO Codex Alimentarius Committee on fats and oils (Jones and King 1993)

1997 field trial data – sprayed with Buctril®

Fatty acid and tocopherol content was determined for crude cottonseed oil obtained from seed harvested from BXN cotton line OXY47 grown in the field in Spain in 1997 and sprayed with Buctril®.

OXY47 is a BXN cotton variety developed from transformation event 10222 in a ST474 genetic background. The fatty acid and tocopherol levels obtained were compared to those measured in oil obtained from a current commercial cotton variety (ST474). The results are summarised in Tables 15 and 16.

Table 15: Fatty acid composition[1] of crude cottonseed oil from BXN cotton sprayed with Buctril®, and a commercial variety of cotton, grown in the field in Spain in 1997.

Fatty acid	Codex ranges[2]	Literature values[3,4]	OXY47	ST474
Myristic (14:0)	0.4-2.0	0.68-1.16	0.85	0.85
Palmitic (16:0)	17.0-31.0	21.63-26.18	22.68	22.70
Palmitoleic (16:1)	0.5-2.0	0.56-0.82	0.55	0.58
Stearic (18:0)	1.0-4.0	2.27-2.88	2.15	2.25
Oleic (18:1)	13.0-44.0	15.17-19.94	16.00	16.35
Linoleic (18:2)	33.0-59.0	49.07-57.64	55.58	55.10
Linolenic (18:3)	0.1-2.1	0.23	0.20	0.20
Arachidic (20:0)	< 0.5	0.41	0.28	0.30
Eicosenoic (20:1)	< 0.5		0.10	0.10
Behenic (22:0)	< 0.5		0.13	0.18
Lignoceric (24:0)	< 0.5		0.10	0.10

[1] values are percent of total lipids and are an average of 4 replicates
[2] ranges adopted by the FAO/WHO Codex Alimentarius committee on fats and oils (Jones and King 1993)
[3] Cherry and Leffler (1984), [4] Cherry (1983)

Table 16: Tocopherol levels[1] in crude cottonseed oil from BXN cotton sprayed with Buctril® and a commercial variety of cotton grown in the field in Spain in 1997

Line	Location[2]	α-tocopherol	δ-tocopherol	Total
OXY47 (U)	a	724	408	1131
OXY47 (T)	a	711	439	1150
ST474	a	770	400	1170
OXY47 (U)	b	810	377	1187
OXY47 (T)	b	816	375	1190
ST474	b	788	374	1162
Literature values[3]		402	572	1050.5

[1] values are expressed in mg tocopherols/kg oil extracted from whole cottonseed and are the average of duplicate analyses. OXY47 was either treated (T) with Buctril® at the agronomic dose of 563 g a.i./ha , or not treated (U).
[2] two replicates per location
[3] Jones and King (1990)

The fatty acid and tocopherol levels determined for OXY47 (both sprayed with Buctril® and unsprayed) are equivalent to those obtained for the parental cotton line. The fatty acid levels reported are also comparable to the Codex specified ranges for cottonseed oil. The levels reported for the α- and γ-tocopherols in both the OXY and control cottons however are significantly different compared to those reported in the literature for crude oil, although the total tocopherol levels are comparable. This difference is probably a reflection of agronomic conditions and has no relevance for food safety.

Conclusion

On the basis of the data provided in the present application, food from BXN cotton is compositionally equivalent to food from other commercial cotton varieties. The spraying of BXN cotton with a bromoxynil-containing herbicide does not result in any significant changes to the levels of the key nutrients.

Key toxicants

Cotton contains two naturally occurring toxic compounds – gossypol and cyclopropenoid fatty acids.

Gossypol is a biologically active terpenoid aldehyde that exists within the puncta or 'glands' found in all parts of the cotton plant, including seeds (Abou-Donia 1976). Gossypol can cause toxic effects such as reduced appetite, body weight loss, and dyspnoea (difficult and laboured breathing) (Berardi and Goldblatt 1980) and also has adverse effects on the protein nutritive value of food by rendering lysine metabolically unavailable (Yannai and Bensai, 1983). The 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).

Several derivatives and isomers of gossypol have been described (Berardi and Goldblatt 1980, Altman *et al* 1989). The concentration of gossypol and related terpenoids varies in cotton depending on both genetic and environmental factors (Altman *et al* 1990,

Dilday and Shaver 1980 and 1981, Hanny 1980). Unprocessed seed contains gossypol in the 'free' or unbound form, in the pigment glands (Jones 1991). Processing whole cottonseed into meal converts varying amounts of free gossypol to the bound form, thus eliminating much of its biological activity (Jones 1991). The removal or inactivation of gossypol during processing enables the use of some cottonseed meal in feed for fish, poultry and pigs. Some human food uses for cottonseed flour, derived from finely ground cottonseed meal, have also been reported, where the meal has been specially processed to minimise the toxicological properties of gossypol. The use of such products appears to be largely confined to Central American countries where it is used as protein enricher in special products to help ease malnutrition (Ensminger 1994, Franck 1989). Refined cottonseed oil is free of gossypol (Gunstone *et al* 1994). The gossypol that partitions into the oil is essentially completely eliminated during subsequent refining of the oil, through inactivation by heat and alkali treatment. The reduction of free gossypol in oil is a measure of the food quality and processing efficiency.

Cyclopropenoid fatty acids are naturally present in cottonseed, crude cottonseed oil and in the meal (because of the residual oil in the meal fractions). The principal forms of these fatty acids are sterculic and malvalic acid (Cherry and Leffler 1984). These fatty acids produce undesirable 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). In two studies of cyclopropenoid fatty acids from several domestic varieties, ranges were found of 0.56 to 1.17% in crude oil (Bailey *et al* 1966), and 0.07 to 0.32% in refined oil (Lawhon *et al* 1977). In another study cyclopropenoid fatty acids were found at levels up to 2% of crude oil, and 0.64% of refined oil (Jones and King 1990).

Gossypol

Free and, in some cases, total gossypol levels were measured in de-linted whole cottonseed samples taken from homozygous BXN cotton, and from the Coker 315 control line which were grown in the field in the United States in 1991 and 1993, and in Spain in 1997. The values obtained were compared to values obtained for common commercial varieties of cotton grown at the same site. Data was obtained for both bromoxynil-sprayed and unsprayed cotton. The data are presented below in Tables 17 – 19.

1991 field trial data

The samples taken from the BXN cotton for this study were from plants that had been sprayed with Buctril® once at 1.5 lb.ai/acre at the two and six-leaf stages. Free and total gossypol measurements were done on whole seed samples by Woodson-Tenant Laboratories, Inc using standard procedures. Four separate replicated field plots, planted in a randomised complete block design, were harvested from each genotype at each of three locations.

Table 17: Free and total gossypol levels[1] in whole cottonseed in BXN cotton sprayed with Buctril® in 1991 field trials

	Total gossypol	Free gossypol
Coker 315 control	0.999	0.851
Event 10211	1.03	1.14
Event 10222	1.05	1.04
Natural range[2]	0.002-6.64	0.002-6.64

[1] Values presented are the percentage of free and total gossypol in whole seed and are the average of replicate samples, analysed in duplicate
[2] Price *et al* 1993

1993 field trial data

Additional studies were done on free gossypol levels in three lines derived from events 10211 and 10222 and the values compared to free gossypol levels in both the non-transformed control as well as current commercial varieties of cotton (DPL5415, LA 887 and Stoneville 453). In this study, the BXN cotton had not been sprayed with bromoxynil. The plants were grown in four separate replicated field plots planted in a randomised complete block design at three locations. The measurements were done by Dr Millard Calhoun from the Texas A+M University. The data from three field locations are presented in Table 18.

Table 18: Free gossypol levels[1] in BXN cotton and commercial varieties of cotton grown in the United States in 1993

Line	Mississippi	South Carolina	Arizona	Overall mean
10211-1	0.900 bc[2]	0.864 bc	1.019 bcd	0.93 bcd
10211-20	0.922 bc	0.869 bc	1.077 b	0.96 abc
10222-1	0.812 cd	0.756 cd	1.003 bcd	0.89 cd
C315 control	0.889 bc	0.788 cd	1.035 bc	0.90 cd
DPL5415	0.730 d	0.819 bcd	0.954 d	0.83 d
LA 887	0.968 b	0.963 a	1.169 a	1.03 ab
Stoneville 453	1.099 a	0.897 ab	1.198 a	1.06 a

[1] The values are the percentage of free gossypol in whole seed and are the average of four replicate samples analysed in duplicate
[2] lines containing the same letter are not significantly different at a 95% confidence level

The overall mean demonstrates that none of the BXN lines are significantly different in free gossypol from the non-transformed control, Coker 315. When a comparison of individual locations is done, no BXN line has a significantly greater level of gossypol than Coker 315. These results show that in general, growing regions have an impact on the free gossypol level of the seed produced but varietal rankings stay relatively consistent from location to location.

1997 field trial data

Samples were taken from OXY 47, which is a BXN cotton variety developed from transformation event 10222 in a Stoneville 474 genetic background. Free gossypol values for OXY 47 were compared to those obtained for Stoneville 474, which had been grown at the same sites. The BXN cotton had been sprayed with Buctril® at the rate of 563 g ai/hectare, which is representative of an agronomic dose. The plants were grown

in two replicates planted in a randomised complete block design at two different field locations. The data are presented in Table 19.

Table 19: Free gossypol levels[1] in BXN cotton sprayed with Buctril® and a commercial variety of cotton grown in Spain in 1997

Line	Rep. #	Site	Gossypol content[1]	Mean
OXY47	1	a	0.590	
OXY47	1	b	0.700	
OXY47	2	a	0.630	
OXY47	2	b	0.635	0.643[2]
Stoneville 474	1	a	0.520	
Stoneville 474	1	b	0.680	
Stoneville 474	2	a	0.580	
Stoneville 474	2	b	0.650	0.608

[1] The values are the percentage of free gossypol in seed and are the average of two replicate samples analysed in duplicate.

[2] There is no significant difference between the means at the 95% confidence level.

Conclusion

Data from field trials performed in the United States in 1991 and 1993, and in Spain in 1997 demonstrate that the transformation and line selection process have not caused gossypol levels to be increased in BXN cotton – the gossypol levels of the BXN cotton lines are equivalent to those of the non-transformed control line as well as current commercial varieties of cotton and also fall within the published ranges expected for cotton. The spraying of BXN cotton with a bromoxynil-containing herbicide does not result in significant increases in the levels of gossypol in the seed of BXN cotton.

Cyclopropenoid fatty acids

Cyclopropenoid fatty acid levels were determined for homozygous BXN cotton lines derived from transformation events 10211 and 10222. Cottonseed samples were collected from replicated field trials in the United States and South Africa in 1993 and in Spain in 1997. Oil extracted from the cottonseed samples was analysed for the cyclopropenoid fatty acids (dihydrosterculic, sterculic and malvalic) using a colourimetric reaction (modified Halphen reaction) based on the Association of Official Analytical Chemists (AOAC) International Method 974.19 and Bailey *et al* (1965). The values obtained for BXN cotton were compared to those obtained for the non-transformed control line and also with commercial cotton varieties. The BXN cotton grown in Spain was sprayed with Buctril® at the rate of 563 g ai/hectare.

1993 USA field trial data

The cotton plants were grown in three locations in the United States. Four separate replicated field plots, planted in a randomised complete block design, were harvested from each genotype at each location. The Engineering Biosciences Research Centre at the Texas A&M University performed small scale processing of the cottonseed samples under the United States Environmental Protection Agency Good Laboratory Practice protocols. This is a bench-top laboratory scale processing facility that is designed to produce oil (and meal) fractions comparable to what would be produced by large scale

commercial processing. Data on cyclopropenoid fatty acid levels are presented in Table 20.

Table 20: Level of cyclopropenoid fatty acids[1] in oil extracted from cottonseed from BXN cotton and commercial varieties of cotton grown in the United States in 1993

Line	Mississippi	South Carolina	Arizona	Overall mean
10211-1	0.73 abc[2]	0.63 ab	0.81 a	0.73 a
10211-20	0.74 abc	0.60 a	0.78 a	0.72 a
10222-1	0.71 ab	0.59 a	0.81 a	0.71 a
Coker 315 control	0.67 a	0.70 abc	0.73 a	0.72 a
DP 5415	0.66 a	0.56 a	0.86 a	0.68 ab
LA 887	0.82 c	0.80 c	0.88 a	0.79 c
Stoneville 453	0.81 bc	0.75 bc	0.80 a	0.76 bc

[1] values presented are the percentage of cyclopropenoid fatty acids in oil and are means from four replicates analysed in duplicate
[2] lines within the same location containing the same letter are not significantly different at a 95% confidence level.

Significant differences were observed between locations, sample runs and lines, however none of the BXN cotton lines differed significantly in cyclopropenoid fatty acid levels compared to the parental control line Coker 315, grown at the same location. Two of the commercial varieties, LA 887 and Stoneville 453 were found to have the highest levels of cyclopropenoid fatty acids overall.

1993 South African field trial data

Cyclopropenoid fatty acid levels were determined in crude oil that had been produced from cottonseed collected from a homozygous line of OXY cotton, derived from transformation event 10222, and three Coker 315 control lines grown in the field in South Africa in 1993. The OXY cotton had not been sprayed with bromoxynil. The values obtained were compared to those obtained for refined corn and cottonseed oils. The results are presented in Table 21.

Table 21: Cyclopropenoid fatty acid levels in oil extracted from BXN cotton and non-transformed control line grown in South Africa in 1993

Sample	Type of oil	Absorbance A_{547nm}
10222	Crude	0.69
Coker 315	Crude	0.65
Coker 315	Crude	0.70
Coker 315	Crude	0.73
Commercial corn oil	Refined	0.0
Commercial cotton oil	Refined	0.10

1997 Spanish field trial data

Cyclopropenoid fatty acid levels were determined in crude oil that had been produced from cottonseed taken from OXY 47, which is a BXN cotton variety developed from transformation event 10222 in a Stoneville 474 genetic background. These levels were compared to those obtained for crude oil produced from cottonseed taken from the

Stoneville 474 variety which had been grown at the same site. The BXN cotton had been sprayed with Buctril® at the rate of 563 g ai/hectare, which is representative of an agronomic dose. The plants were grown in two replicates planted in a randomised complete block design at two different locations. The data are presented in Tables 22a and 22b.

Table 22a: Cyclopropenoid fatty acid levels[1] in cottonseed oil extracts from BXN cotton sprayed with Buctril® and a commercial variety of cotton grown in Spain in 1997

Line	Rep. #	Location	Malvalic acid	Dihydrosterculic acid	Sterculic acid
OXY47	1	a	0.50	0.30	0.20
OXY47	1	b	0.50	0.30	0.20
OXY47	2	a	0.50	0.30	0.20
OXY47	2	b	0.50	0.30	0.25
Stoneville 474	1	a	0.50	0.30	0.20
Stoneville 474	1	b	0.50	0.30	0.30
Stoneville 474	2	a	0.50	0.30	0.30
Stoneville 474	2	b	0.40	0.20	0.20

[1] values presented are the percentage of cyclopropenoid fatty acids in oil and are the average of duplicate analyses

Table 22b: Comparison of means[1] for cyclopropenoid fatty acid levels

	OXY47		Stoneville 474		Literature range[3]
Malvalic acid	0.50	a	0.48	a	<0.1 – 1.9
Dihydrosterculic acid	0.30	a	0.28	a	0.2 – 0.8
Sterculic acid	0.21	a	0.25	b	0.3 – 0.7

[1] mean values across two field sites
[2] rows containing the same letter are not significantly different at a 95% confidence level.
[3] Wood 1986

The only significant difference is in relation to the levels of sterculic acid, which were found to be slightly decreased in BXN cotton compared to the isogenic control line. As the difference is minor, and both values are still within the published range for sterculic acid, this finding is not considered to have any biological or food safety significance.

Conclusion

In virtually all cases, the levels of cyclopropenoid fatty acids in oil produced from seeds of BXN cotton were lower or comparable to the levels in the controls. The levels reported are also within the literature reported ranges. It is therefore concluded that the transformation and line selection process has not resulted in an increase to the levels of cyclopropenoid fatty acids in oil from BXN cotton. The levels of cyclopropenoid fatty acids are unaffected by the spraying of the plants with a bromoxynil-containing herbicide.

Key anti-nutrients

In addition to its toxic effects the terpenoid gossypol, naturally occurring in cottonseed, has anti-nutritive characteristics through reducing the availability of lysine (Yannai and Bensai, 1983). The level of gossypol in events 10211 and 10222 are equivalent to levels found in the non-transformed controls and are comparable to levels found in

commercial varieties of cotton. Furthermore, refined cottonseed oil is essentially free of gossypol.

Naturally-occurring allergenic proteins

Some common foods, e.g. cow's milk, soybeans and tree nuts, are known to elicit an allergic response in susceptible individuals. This response is primarily due to an immune reaction to a particular protein component of the food, whereas the components of fats or oils (such as fatty acids etc) are not generally associated with such reactions. Moreover, refined cottonseed oil and cellulose from linters are devoid of protein therefore their consumption is unlikely to result in an allergic reaction.

There have been reported incidences of allergic reaction in humans in response to consumption of foods containing cottonseed protein (Atkins *et al* 1988, Malanin and Kalimo 1988). However, whole cottonseed, cottonseed meal and cottonseed flour are not used for human consumption in Australia and New Zealand.

NUTRITIONAL IMPACT

In assessing the safety of food produced using gene technology, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further re-assurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

The compositional and other data presented are considered adequate for establishing the ability of oil and linters from BXN cotton to support typical growth and well-being. Additional studies are therefore not required.

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