FOOD DERIVED FROM INSECT-PROTECTED, GLUFOSINATE AMMONIUM-TOLERANT COTTON LINE MXB-13

A SAFETY ASSESSMENT

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SUMMARY

Food derived from genetically modified (GM) cotton line MXB-13 has been assessed for its safety for human consumption. This cotton line has been genetically modified to be resistant to insect attack and has been developed for cultivation in North America and Australia. A number of criteria have been addressed in the safety assessment including: a characterisation of the transferred genes, their origin, function and stability; changes at the DNA, protein and whole food levels; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic to humans.

History of Use

Cotton is grown primarily for the value of its fibre, with cottonseed and its processed products being a by-product of the crop. Humans have consumed cottonseed oil, the major product of cottonseed, for decades. Cottonseed oil is considered to be premium quality oil, valued for its high unsaturated-fatty acid content. The other food use of cottonseed is the linters, which are composed of greater than 99% cellulose. Cottonseed itself and the meal fraction are not used in Australia and New Zealand as a food for human consumption because they contain naturally occurring toxic substances. These toxins are essentially removed in the production of oil and linters, making them fit for human consumption. The types of food products likely to contain cottonseed oil are frying oils, mayonnaise, salad dressing, shortening, and margarine. After processing, linters may be used as high fibre dietary products and thickeners in ice cream and salad dressings.

Description of the Genetic Modification

Cotton line MXB-13 contains two novel genes encoding the insecticidal proteins Cry1Ac and Cry1F. These two genes were derived from the soil bacterium *Bacillus thuringiensis* and are selectively toxic to certain insect pests of cotton. Cotton line MXB-13 also contains two copies of the *pat* gene, which confers tolerance to the herbicide phosphinothricin acetyl transferase (PAT) and was used as a selectable marker in the early stages of plant development.

Detailed molecular and genetic analyses of cotton line MXB-13 indicate that the transferred *cry*1Ac, *cry*1F and *pat* genes are stably integrated into the plant genome at two independent insertion sites and are stably inherited from one generation to the next.

Characterisation of Novel Protein

Cotton line MXB-13 expresses 3 novel proteins – Cry1Ac, Cry1F, and PAT. In the plant tissues, the average expression levels of Cry1Ac ranged from not detectable (ND) to
1.83 ng/mg dry weight. The average expression levels of Cry1F ranged from ND to 22.8 ng/mg dry weight. The average expression levels of PAT across all matrices ranged from ND to 0.54 ng/mg dry weight.

No novel protein was detected in refined oil. Linters are composed of greater than

99% cellulose and are therefore unlikely to contain substantial levels of protein. Therefore exposure to the novel protein through consumption of oil and linters derived from cotton line MXB-13 would be very low to negligible.

Acute oral toxicity studies have been conducted on the Cry1Ac, Cry1F, and PAT proteins – there was no evidence of toxicity in all cases. Potential allergenicity was assessed by sequence comparison to known allergens, and by determining thermolability – these data did not indicate any potential for allergenicity.

Comparative Analyses

Compositional analyses were done to establish the nutritional adequacy of cotton line MXB-13, and to compare it to a non-transgenic control line and commercial varieties of cotton. The constituents measured were protein, fat, carbohydrate, ash, moisture, fibre, fatty acids, amino acids, minerals and the naturally occurring toxicants gossypol, and cyclopropenoid fatty acids. The levels of aflatoxins were also investigated.

No differences of biological significance were observed between the transgenic cotton line and its non-GM counterpart. Several minor differences in key nutrients and other constituents were noted however the levels observed represented very small differences and do not indicate an overall pattern of change that would warrant further investigation. On the whole, it was concluded that food from cotton line MXB-13 is equivalent in composition to that from other commercial cotton varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of the food and indicate that food derived from cotton line MXB-13 is equivalent in composition to food from non-GM cotton varieties. The introduction of food produced from cotton line MXB-13 into the food supply is therefore expected to have minimal nutritional impact.

Conclusion

No potential public health and safety concerns have been identified in the assessment of food produced from cotton line MXB-13. On the basis of the available information, food produced from cotton line MXB-13 can be considered as safe and as wholesome as food produced from other cotton varieties.

BACKGROUND

A safety assessment has been conducted on food derived from cotton that has been genetically modified to be protected from insect attack and tolerant to the herbicide glufosinate ammonium. The GM cotton is referred to as cotton line MXB-13 but is known commercially as 'WideStrike TM'.

Cotton line MXB-13 has been genetically modified for protection against the cotton bollworm (*Heliothis zea*), tobacco budworm (*H. virescens*) and pink bollworm (*Pectinophora gossypiella*), significant pests of cotton crops in Australia. Protection is conferred by the expression in the plant of bacterially derived protein toxins (*Bt*-δ-endotoxins) that are specific for these insects. Cotton line MXB-13 also contains two copies of a gene encoding resistance to the herbicide glufosinate ammonium.

Cotton line MXB-13 contains two insecticidal genes (*cry*1Ac and *cry*1F), derived from the common soil bacterium *Bacillus thuringiensis* (*Bt*). These genes express insecticidal proteins (Cry1Ac and Cry1F) that are toxic to specific lepidopteran caterpillar insects, including the major pests of cotton. The insecticidal genes were introduced separately into two cotton lines (MXB-7 and MXB-9). Subsequently the two traits were combined by crossing the two GM cotton lines using conventional breeding to produce cotton line MXB-13.

Using two *B. thuringiensis* derived insecticidal proteins, rather than one, in the same plant improves the spectrum of control, the seasonal efficacy and significantly reduces the chances of selecting insects resistant to the toxins. *Bt* formulations are widely used as biopesticides on a variety of cereal and vegetable crops grown organically or under conventional agricultural conditions.

In addition to the two *cry* genes, cotton line MXB-13 contains two copies of a selectable marker gene (*pat*) from the bacterium *Streptomyces viridochromogenes*, which produces an enzyme (phosphinothricin acetyl transferase, PAT) that detoxifies the herbicide glufosinate ammonium. PAT functions as a selectable marker in the initial laboratory stages of plant cell selection and thus cotton line MXB-13 is also tolerant to the herbicide glufosinate ammonium, however, this trait is not used in commercial production of cotton line MXB-13.

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

HISTORY OF USE

Host Organism

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

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

Cottonseed oil is regarded as premium quality oil and has a long history of safe food use. It is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine and packing oil. It is considered to be a healthy oil as it contains predominantly unsaturated fatty acids.

Cottonseed oil has been in common use since the middle of the nineteenth century (Jones and King 1990, 1993) and achieved GRAS (Generally Recognised As Safe) status under the United States Federal Food Drug and Cosmetic Act because of its common use prior to 1958. In the US, it ranks third in volume behind soybean and corn oil, representing about 5-6% of the total domestic fat and oil supply.

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

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

In Australia, cotton was planted on 484 000 hectares in 2000-2001 season (CRDC, 2001). Cotton is not grown in New Zealand.

Donor Organisms

Bacillus thuringiensis

The source of the *cry*1F and *cry*1Ac genes used in this GM cotton is the ubiquitous soil and plant bacterium *Bacillus thuringiensis* (*Bt*). The *cry*1Fa2 gene was isolated from the *Bt* subspecies *aizawai* and the *cry*1Ac gene from the *Bt* subspecies *kurstaki*. The WHO International Program on Chemical Safety (IPCS) report on environmental health criteria for *Bt* concludes that '*Bt* has not been documented to cause any adverse effects on human health when present in drinking water or food' (IPCS, 2000).

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

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

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

Agrobacterium tumefaciens

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

Agrobacterium naturally contains a plasmid (the *Ti* plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally therefore, *Agrobacterium* is a plant pathogen causing root deformation mainly with

sugar beets, pome fruit and viniculture crops. However, adaptation of this natural process has now resulted in the ability to transform a broad range of plant species without causing adverse effects in the host plant.

Streptomyces viridochromogenes

Streptomyces viridochromogenes is a ubiquitous soil fungus and was the source of the PAT encoding gene that was used in the gene constructs of both the *cry*1F and *cry*1Ac genes as a selectable marker. *S. viridochromogenes* is a gram-positive sporulating soil bacteria. Few *Streptomyces* have been isolated from animal or human sources and pathogenicity is not a typical property of these organisms. *S. viridochromogenes* is itself not known to be a human pathogen and nor has it been associated with other properties (e.g. production of toxins) known to affect human health.

Zea mays

Zea mays (maize) is the source of the regulatory element ZmUbi1 (ubiquitin 1 promoter plus exon 1 and intron 1), which was used to control the transcription of the *pat* gene. Thousands of food, feed and industrial products depend on maize based ingredients. Maize and products processed from maize have a long history of safe use and do not pose a health risk to humans.

DESCRIPTION OF THE GENETIC MODIFICATION

Method used in the genetic modification

Studies evaluated:
Narva, K.A., Palta, A., Pellow, J.W. (2001a) Product characterisation data for *Bacillus thuringiensis* var. *aizawai* Cry1F (synpro) insect control protein as expressed in cotton. Study ID: GC-C 5304. Dow AgroSciences LLC, San Diego, California.

Narva, K.A., Palta, A., Pellow, J.W. (2001b) Product characterisation data for *Bacillus thuringiensis* var. *kurstaki* Cry1Ac (synpro) insect control protein as expressed in cotton. Study ID: GC-C 5303. Dow AgroSciences LLC, San Diego, California.

Cotton line MXB-13 was produced via conventional breeding between two GM cotton lines, MXB-7 and MXB-9. Cotton lines MXB-7 and MXB-9 were both produced by *Agrobacterium*-mediated transformation of *Gossypium hirsutum* L. GC510, using the transformation vectors pMYC3006 and pAGM281 respectively. The plasmid pMYC3006 contains the *cry*1Ac and *pat* genes, and the plasmid pAGM281 contains the *cry*1F and *pat* genes (see Table 1).

In both transformations, cotyledon segments were isolated from 7-10 day old *in vitro* germinated seedlings of the cotton genotype GC510. The segments were co-cultivated with disarmed *Agrobacterium tumefaciens* containing the one of the two plasmids described above. The disarmed *Agrobacterium* strain LBA4404 carrying the binary vector was used in these experiments.

Following co-cultivation, treated segments were transferred to callus induction medium containing glufosinate ammonium as the selection agent. Putative transformed calli formed at the cut ends of cotyledon segments growing on selection medium.

Each callus was isolated from the cotyledon segments and cultured on fresh selection medium. Subsequently the callus was transferred to embryo induction medium. Once the somatic embryos were regenerated, these were advanced for embryo development and plant regeneration.

Following transformation and selection, Southern analysis of the transgenic plants confirmed the presence of the *cry*1Ac and *pat* genes (event 3006-210-23), or the *cry*1F and *pat* genes (event 281-24-236). The cotton lines carrying these two separate events were developed through a series of back crosses and self pollination (see Table 2) and finally crossed together by conventional breeding to give the ‘stacked’ GM cotton line MXB-13. In this case, gene ‘stacking’ refers to two

separate DNA inserts in two separate cotton lines being combined by conventional breeding so that the progeny contains both inserts.

Table 1 Plasmids, genes, event and product code for the three transgenic cotton lines

Transforming plasmid	Gene	Transformation Event number	Field code/line	Commercial trademark
pMYC3006	*cry*1Ac, *pat*	3006-210-23	MXB-7	
pAGM281	*cry*1F, *pat*	281-24-236	MXB-9	
	*cry*1Ac, *cry*1F, *pat*		MXB-13	WideStrike™

Table 2: Breeding chart outlining the creation of the stacked cotton line containing *cry*1F and *cry*1Ac plus *pat* genes. At each stage plants segregating for non-expression of the traits were removed

Event 281-24-236 (cry1F)	**Generation**	**Event 3006-210-23 (cry1Ac)**	
*cry*1F and *pat* genes inserted in cotton variety GC510		*cry*1Ac and *pat* genes inserted in cotton variety GC510	
event 281-24-236 crossed with PSC355 (a high quality commercial cotton variety)	F_1	event 3006-210-23 crossed with PSC355	
F_1 above backcrossed to PSC355	BC_1F_1	F_1 above backcrossed to PSC355	
BC_1F_1 above backcrossed to PSC355	BC_2F_1	BC_1F_1 above backcrossed to PSC355	
BC_2F_1 above backcrossed to PSC355	BC_3F_1	BC_2F_1 above backcrossed to PSC355	
	Crossed	The BC_3F_1 of both events crossed	
Possible genotypes -/- *cry*1F/- (+/-) -/ *cry*1Ac (-/+) *cry*1F/ *cry*1Ac (+/+)	F_1	BC_3F_1 of both events were crossed to produce the stacked line MXB-13. Plants identified with both transformation events were self-pollinated. Plants identified without either transformation event were also self pollinated and used as the null segregant	
	F_2	Self pollinated +/+	Self pollinated -/-
	F_3	Self pollinated +/+ *	Self pollinated -/-
	F_4	Self pollinated +/+	Self pollinated -/-**

* These plants were used as the stacked event for Southern blot analysis
** These plants were used as the null-segregant (control) for Southern blot analysis

Function and regulation of novel genes

Cotton line MXB-13 contains two inserts. One insert (transformation event 3006-210-23) contains the genes from plasmid pMYC3006 (*cry*1Ac and *pat*), and the other insert (transformation event 281-24-236) contains genes from plasmid pAGM281 (*cry*1F and a second *pat*). All the genes and their regulatory elements are described in Table 3.

*Cry*1F

The *cry*1F gene used in the transformation plasmid is a synthetic 3447 base pair gene based on the native *cry*1F gene. It is the coding sequence for a Cry1F-based synthetic protoxin, referred to as Cry1F (synpro). Nucleotides 1-1810 of the coding sequence encode the toxic portion of *cry*1Fa2 from *B. thuringiensis* var. *aizawai.* Nucleotides 1811-1917 encode a portion of the Cry1C protoxin. Nucleotides 1918 – 3447 encoded a portion of the Cry 1Ab protoxin.

These later sequences are removed by alkaline proteases during formation of the active core insecticidal protein within the insect gut. The DNA sequences encoding Cry1F (synpro) were modified for optimal plant codon usage. For the purpose of this assessment this synthetic gene is referred to as c*ry*1F and the protein as Cry1F.

Transcription of the *cry*1F gene is controlled by the mannopine synthase (δ -mas 2') promoter from *Agrobacterium tumefaciens* pTi15955 (Barker *et. al.*, 1983), and four copies of the octopine synthase enhancer (4OCS) from pTiAch5 (Ellis *et. al.*, 1987). Polyadenylation and termination sequences were derived from the bidirectional open reading frame-25 (ORF25) terminator from pTi15955.

*Cry*1Ac

The *cry*1Ac gene present in the transformation plasmid is a synthetic version of the native gene derived from *B. thuringiensis* subspecies *kurstaki*. It is the coding sequence for a Cry1Ac-based synthetic protoxin, referred to as Cry1Ac (synpro). Nucleotides 1-1834 of the coding sequence encode the toxic portion of Cry1Ac1. Nucleotides 1835-1941, and 1942-3471 encode portions of the Cry1C and Cry1Ab1 protoxins respectively. As with Cry1F, this C-terminal section of the protein is cleaved in the insect's midgut to produce the active toxin core. The DNA sequence encoding Cry1Ac (synpro) was modified for optimal plant codon usage. For the purpose of this assessment this synthetic gene is referred to as c*ry*1Ac and the protein as Cry1Ac.

Transcription of the *cry*1Ac gene is controlled by the maize (*Zea mays* L.) ubiquitin-1 promoter and terminated by the ORF25 polyadenylation sequence.

Pat

The *pat* gene encodes the PAT enzyme, which confers resistance to the herbicide glufosinate ammonium. This gene was introduced as a selectable marker for the identification of transformed plants. The *pat* gene was originally isolated from *Streptomyces viridochromogenes* Tu494, but in this construct has been modified in order to alter the guanosine and cytosine codon bias to a level more typical for plant

codons. The deduced amino acid sequence is identical to the native bacterial PAT enzyme.

Transcription of the *pat* gene is controlled with a regulatory element consisting of the maize ubiquitin 1 promoter plus exon 1 and intron 1 (ZmUbi1). As with the *cry*1F gene, polyadenylation and termination sequences were derived from the bi-directional ORF25 terminator from pTi15955.

There are two copies of the *pat* gene.

Table 3: Genetic elements in the inserts in cotton line MXB-13

Genetic Element	Size (Kb)	Details
*cry*1Ac (synpro)	3.47	Synthetic, plant optimised, full-length version of *cry*1Ac from *B.t.* Nucleotides 1-1834 of the coding sequence encode the toxic portion of Cry1Ac1. Nucleotides 1835-1941 encode a portion of the Cry1C protoxin. Nucleotides 1942-3471 encode a portion of the Cry1Ab1 protoxin.
*cry*1F (synpro)	3.45	Synthetic, plant optimised, full-length version of *cry*1F from *B.t.* Nucleotides 1-1810 of the coding sequence encode the toxic portion of Cry1Fa2. Nucleotides 1811-1917 encode a portion of the Cry1C protoxin. Nucleotides 1918-3447 encode a portion of the Cry1Ab protoxin.
(4OCS)mas 2' (2 copies)	0.61	Mannopine synthase promoter from pTi15955, including 4 copies of the octopine synthase (OCS) enhancer from pTiAch5 (Ellis *et. al.*, 1987).
ORF25 polyA (2 copies)	0.72	Bidirectional terminator from *Agrobacterium tumefaciens* pTi15955 (Barker et al, 1983).
pat (2 copies)	0.55	The synthetic plant optimized glufosinate ammonium resistance gene, based on a phosphinothricin acetyltransferase gene sequence from *Streptomyces viridochromogenes*.
UbiZm1 (2 copies)	1.99	*Zea mays* promoter plus *Zea mays* exon1 (untranslated enhancer) and intron1.

No other genes were transferred to cotton line MXB-13.

Characterisation of the genes in the plant

Traditional molecular techniques were used to analyse the inserted DNA in cotton line MXB-13. Southern blot analysis and DNA sequencing were used to demonstrate integration, copy number, and integrity of the *cry*1F, *cry*1Ac, and *pat* genes, and the regulatory elements controlling gene expression, and to assess whether vector backbone sequences were present in cotton line MXB-13.

Studies evaluated:
Green SB (2002) Molecular Characterisation of Cry1F (synpro)/Cry1Ac (synpro) stacked transgenic cotton line 281-24-236/3006-210-23. Dow AgroSciences LLC, Indianapolis, Indiana. Study Id. 010075

Song P (2002a) Cloning and Characterisation of DNA sequences in the insert and flanking border regions of *B.t.* Cry1Ac cotton 3006-24-236. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id GH-C 5522

Song P (2002b) Cloning and Characterisation of DNA sequences in the insert and flanking border regions of *B.t.* Cry1Ac cotton 281-24-236. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. GH-C 5529

Song P, Collins R, Hey T, Madduri K, Ni W, Schafer B, Xu U (2002) Expression of the Partial PAT Open Reading Frame in B.t. Cry1F Cotton Event 281-24-236. Dow AgroSciences LLC Indianapolis, Indiana. Study Id. GH-C 5573

Insert and copy number

The two parental cotton lines, MXB-7 and MXB-9, had been found to each contain one DNA insertion. To determine whether cotton line MXB-13 contained these same two insertions, Southern hybridization was used. Cotton line MXB-13 genomic DNA, non-transgenic cotton genomic DNA, pAGM281 DNA and pMYC3006 DNA were digested with restriction enzymes, processed by gel electrophoresis, transferred by blotting to nylon membranes, and probed with four different probes. Three of the probes were specific to the *Cry*1Ac gene, the *Cry*1F gene and the *pat* gene, all of which reside within the T-DNA region of the plasmids. A fourth probe was specific to the erythromycin resistance gene, which resides outside of the T-DNA region of the two plasmids.

In general, only the T-DNA region is transferred during the transformation, therefore sequences residing outside of the T-DNA region should not be present in the plant genome. The erythromycin resistance gene probe did not hybridise with the MXB-13 cotton genomic DNA, indicating that this gene had not been transferred.

The three probes specific to sequences within the T-DNA region (the *Cry*1Ac gene, the *Cry*1F gene and the *pat* gene probes) all hybridised with the MXB-13 genomic DNA, indicating that all these elements were present as expected. The banding patterns for each of the restriction enzyme and probe combinations gave the expected results based on the Southern blot analysis of the two parental lines, MXB-7 and MXB-9, and showed that only two insertion sites were present as expected (insertion events 281-24-236 and 3006-210-23).

PCR and sequence analysis

The entire insert region, plus flanking sequences, from each of the two parental lines, MXB-7 and MXB-9, were cloned using standard and inverse PCR techniques in order to determine the nucleotide sequence.

Sequence analysis of insert 3006-210-23 in cotton line MXB-7 indicates the presence of the intact T-DNA containing the *cry*1Ac and *pat* genes. Sequence analysis of insert 281-24-236 in cotton line MXB-9 also indicates an intact T-DNA, with the exception of 2 nucleotide changes within the UbiZm1 promoter region. In addition, sequencing results also indicate the presence of a partial *pat* gene expression cassette including the entire UbiZm1 promoter and a 231 base pair truncation of the PAT coding sequence. The inserts are shown in figures 1 and 2. As

the partial *pat* gene represents an unexpected open reading frame (ORF) it was further characterised (described in the following section).

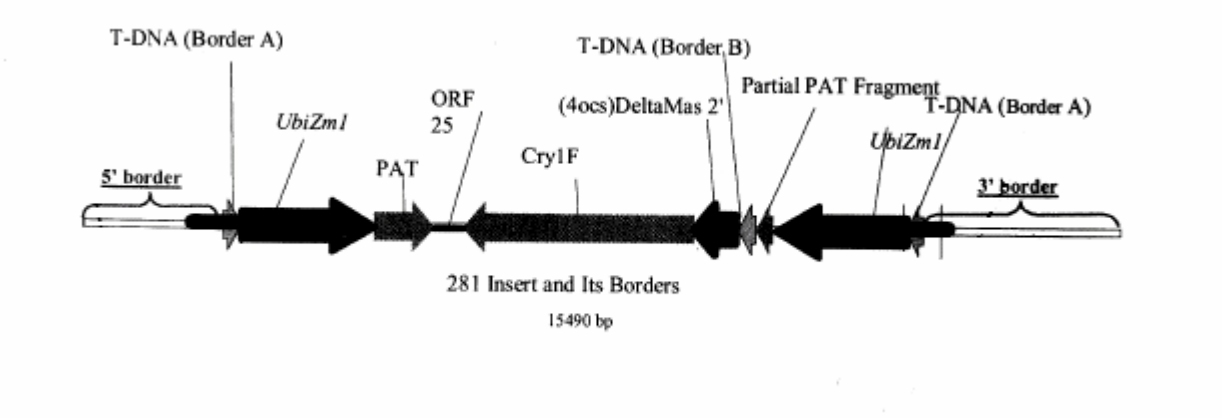

Figure 1: The complete insert and flanking genomic regions of event 281-24-236 in cotton line MXB-13.

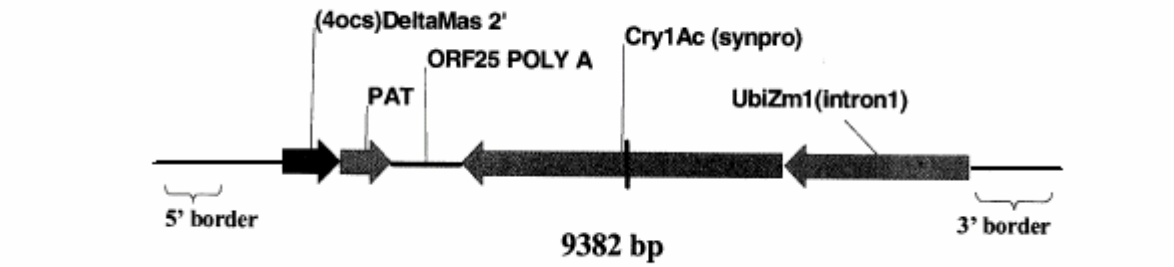

Figure 2: The complete insert and flanking genomic regions of event 3006-210-23 in cotton line MXB-13.

Flanking regions and putative Open Reading Frame analysis

The 5' and 3' flanking border regions of insert 281-24-236 were sequenced (2071 base pairs and 2902 base pairs respectively). PCR and sequencing analyses using primers from these genomic DNA regions confirmed that they were present in the untransformed cotton genome, however 53 base pairs from the original locus were deleted at the insertion site, possibly in the process of T-DNA integration. No ORFs (>450 bp) were identified in the cotton genomic region of the original locus nor did a BLAST search using these cotton DNA sequences against the GenBank database produce any significant homologies.

The DNA sequence of insert 281-24-236 and flanking regions was screened in all 6 reading frames to identify any novel ORFs starting with ATG and extending more than 150 amino acids. There were no novel ORFs identified which met these criteria either within the insert or at the junction regions. The 231 base pair partial *pat* plus 24 base pairs of sequence from the adjacent 3' T-DNA border region constitutes a 255 base pair ORF (85 amino acids). The potential amino acid sequence of the partial PAT ORF is 90% identical to PAT, consisting of 77 amino acids from the

amino terminus of PAT with an 8 amino acid carboxyl terminal tail. This pPAT ORF was the subject of further analysis (described in following sections).

The 5' and 3' flanking regions of insert 3006-210-23 were also sequenced (534 bp and 481 bp respectively). PCR and sequencing analyses using primers from these genomic DNA regions confirmed that they were present in the untransformed cotton genome, however, 16 base pairs from the original locus were deleted at the insertion site, again thought to have occurred in the process of T-DNA integration. No ORFs (>450 bp) were identified in the cotton genomic region of the original locus nor did a BLAST search using these cotton DNA sequences against the GenBank database produce any significant homologies. There were no unexpected ORFs (>450 bp) associated with the whole insert and flanking regions.

RT-PCR analysis of the partial pat ORF

As the partial *pat* gene in insert 281-24-236 has the same promoter (maize *ubi*-1) as the full-length *pat* expression cassette, the partial *pat* is expected to be transcribed into RNA. Transcription of the partial *pat* in the MXB-9 cotton was investigated. Reverse transcription PCR (RT-PCR) was performed on messenger RNA (mRNA) extracted from cotyledons from cotton lines MXB-9, MXB-7 (as a positive control for the full-length *pat* transcripts) and non-transgenic cotton, using *pat* and partial *pat* specific primers.

Analysis of the RT-PCR results showed that the partial *pat* gene was transcribed into mRNA in MXB-9 cotyledons at levels at least 16-fold less than the full-length *pat* gene. Protein expression analysis was also performed and is described in Section 4.2.

Conclusion

Detailed molecular analyses have been performed on cotton line MXB-13 to characterise the novel genes present in the genome. Results indicate that there are two insertion sites. One of these (3006 210-23) contains one copy of the T-DNA from plasmid pMYC3006 (with the intact *cry*1Ac and *pat* expression cassettes) at a single locus in the cotton genomic DNA. The other (281-24-236) contains one full-length copy of the T-DNA from plasmid pAGM281 (with the intact *cry*1F and *pat* expression cassettes) and a fragment of the *pat* expression cassette at a second, single locus.

The *cry*1Ac, *cry*1F, and two of the three *pat* genes are intact. A partial *pat* expression cassette is also present. No novel ORFs (>450 bp) were created by the insertion of the novel genes and nor were any existing ORFs destroyed.

3.4 Stability of the genetic changes

Breeding process

The cotton lines carrying single events of *cry*1F and *cry*1Ac were developed through a series of backcrosses and self-pollination. The cotton variety GC510 was used in the initial transformation for each of the two transformation events, as it is a cotton type amenable to transformation. The original transformed lines were then crossed

to the cotton variety PSC355, which is a high quality commercial cotton variety. The F_1 of this cross was repeatedly backcrossed to PSC355. At each backcross generation, in addition to further backcrossing, the plants were also self-pollinated to obtain the F_2 generation (i.e. BC_2F_1 -> BC_2F_2). The lines and generations of the individual and stacked events used in the various studies are listed in Table 2.

Segregation analysis

Studies evaluated:
Green, SB (2003a) Stability with a generation of the *cry1Ac* and *pat* genes in transgenic cotton event 3006-210-23. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 020017

Green, SB (2003b) Stability with a generation of the *cry1F* (synpro) and *pat* genes in transgenic cotton event 281-24-236. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 020016

Narva, KA, Palta A, Pellow JW (2001a) Product Characterisation data for *Bacillus thuringiensis* var *aizawai* Cry1F (synpro) insect control protein as expressed in cotton. Dow AgroSciences, LLC, San Diego, California. Study Id. GC-C 5304

Narva KA, Palta A, Pellow JW (2001b) Product Characterisation data for *Bacillus thuringiensis* var *kurstaki* Cry1Ac (synpro) insect control protein as expressed in cotton. Dow AgroSciences, LLC, San Diego, California. Study Id.GH-C 5303

Cotton lines MXB-7 and MXB-9 were analysed separately to determine insert stability and segregation patterns prior to the two lines being crossed to produce the stacked cotton line MXB-13.

The BC_2F_2 progeny of each of the two lines, MXB-7 and MXB-9 (produced by self pollination of the hemizygous BC_2F_1 generation), were tested by Southern blotting for the presence of the *cry*1Ac and *cry*1F genes. The transgene in both lines segregated as expected for a single insertion consistent with a Mendelian pattern of inheritance (Table 4). There was no statistically significant difference between the observed and expected values based on a binomial proportions test (P>0.05).

Table 4: Segregation Analysis

Segregation analysis	Generation	Number of plants tested	Expected*		Observed*	
			+ve	-ve	+ve	-ve
MXB-7 (*cry*1Ac)	BC_2F_2	56	42	14	47	9
MXB-9 (*cry*1F)	BC_2F_2	71	53	18	54	17

*+ve or –ve for presence of the transgene

In addition to the segregation analysis carried out on the two parental lines, the stacked cotton line was also analysed. The F_1 generation following crossing of the two parental lines and the F_2 generation (produced by self-pollination of the F_1 containing both events) were analysed for segregation using qualitative ELISA strips specific for the Cry1Ac and Cry1F proteins. The results of the studies are shown in Table 5. For the F_1 generation with two independently segregating genes, it is expected to have a 1:1:1:1 ratio (Cry1F+/Cry1Ac+ : Cry1F+/Cry1Ac- : Cry1F-/Cry1Ac+ : Cry1F-/Cry1Ac-). Likewise, in the F_2 generation with two independently segregating genes it is expected to obtain a 9:3:3:1 ratio (Cry1F+/Cry1Ac+ :

Cry1F+/Cry1Ac- : Cry1F-/Cry1Ac+ : Cry1F-/Cry1Ac -). In both generations for both events, Chi square values indicated no significant difference to expected ratios.

Table 5: Mendelian segregation of MXB-13 based on qualitative Cry1F and Cry1Ac protein detection

Generation	No of plants	Observed ratio	Expected ratio	chi-square	p-value	Significant Difference?
F_1	112	32:29:22:29	28:28:28:28	1.929	0.587	No
F_2	326	203:53:52:18	183.4:61.1:61.1:20.4	4.819	0.186	No

Antibiotic resistance genes

No antibiotic resistance marker genes are present in cotton line MXB-13.

CHARACTERISATION OF NOVEL PROTEINS

Biochemical function and phenotypic effects

The only novel proteins in cotton line MXB-13 are Cry1Ac, Cry1F and PAT.

Cry1Ac and Cry1F

The Cry1Ac and Cry1F proteins are insecticidal δ-endotoxins derived from *B. thuringiensis*. During sporulation, *B. thuringiensis* produces cytoplasmic inclusions containing one or more of the insecticidal crystal proteins.

Most crystal proteins are synthesised intracellularly as inactive protoxins that spontaneously form small crystals, approximately 1 µm in size. Upon ingestion by susceptible insects, the highly alkaline pH of the midgut promotes solubilisation of the protoxin-containing crystals. The protoxin is then activated by trypsin-like gut proteases, which cleave off domains from the carboxy- and amino- termini, leaving a protease resistant core, which is the active toxin. The active toxin binds to a highly specific glycoprotein receptor on the surface of midgut epithelial cells in the insect. Aggregation of the core toxins results in the formation of a pore through the cell membrane. These cells eventually swell and burst causing loss of gut integrity and resulting in larval death within 1 to 2 days (Hofte and Whitely, 1989; Schnepf et al, 1998)

From intensive study of Bt species, four major classes of insecticidal protein genes (*cry1*, *cry2*, *cry3* and *cry4*) have been identified that are useful for the control of pest species among certain of the insect orders. This includes proteins that encode lepidoptera-specific (Cry1), lepidoptera- and diptera-specific (Cry2), coleoptera-specific (Cry3) and diptera-specific (Cry4) proteins respectively (Chambers *et al*., 1991).

The Cry1Ac protein produced in cotton line MXB-13 is a chimeric full-length δ-endotoxin comprised of the core toxin of Cry1Ac1 and the non-toxic portions of Cry1Ca3 and Cry1Ab1 proteins. Together, the portions of the Cry1Ca3 and Cry1Ab1 proteins comprise the C-terminal domain and are removed by alkaline proteases

during the formation of the Cry1Ac core toxin. The full length Cry1Ac is approximately 131 kDa and 1156 amino acids, however this is digested by plant enzymes into the insecticidally active 65 kDa core toxin.

The Cry1F protoxin (1149 amino acids in length) in cotton line MXB-13 is a chimeric, full-length δ-endotoxin comprised of the core toxin of Cry1Fa2 and the non-toxic portions of Cry1Ca3 and Cry1Ab1 proteins. Together the portions of Cry1Ca3 and Cry1Ab1 that comprise the chimeric C-terminal domain are approximately those removed by alkaline proteases during the formation of the active Cry1Fa2 core toxin. The expressed protoxin (Cry1F synpro) is truncated to an active core toxin of approximately 65kDa.

PAT

The herbicide tolerant trait, which was used as a selectable marker following transformation, is conferred by the expression of the introduced *pat* gene, which encodes the phosphinothricin acetyl transferase (PAT) protein. PAT functions by detoxifying phosphinothricin (PPT), the active constituent of glufosinate ammonium herbicides. PPT acts by inhibiting the endogenous enzyme glutamine synthetase, an enzyme involved in amino acid biosynthesis in plant cells. By inhibiting this enzyme, PPT causes rapid accumulation of ammonia in the plant cell, leading to plant death. In transformed cotton plants, the introduced PAT enzyme chemically inactivates the PPT by acetylation of the free ammonia group, giving rise to herbicide tolerance in the whole plant.

The PAT protein consists of 183 amino acids, has a molecular weight of 22 kDa, and exhibits a high degree of enzyme specificity; recognising only one substrate, L-glufosinate in the acetylation reaction. This high substrate specificity was tested in the presence of each of 21 L-amino acids at substrate concentrations exceeding 50 times the K_M value for L-glufosinate. None of the tested amino acids substituted as an alternative substrate in the PAT catalysed reaction, but the enzyme reaction with L-glufosinate was not inhibited (Schulz, 1993).

Protein expression analysis

In cotton line MXB-13 the only novel proteins expected to be expressed are the Cry1Ac, Cry1F and PAT proteins (including the possibility of a truncated PAT protein). Expression levels of these proteins were determined using enzyme-linked immunosorbent assay (ELISA) and are reported below.

Studies evaluated:
Phillips AM, Collins RA (2002) Generation and Compositional Analysis of Cry1F/Cry1Ac Cottonseed meal for Regulatory Studies. Dow AgroScience, LLC, Indianapolis, Indiana. Study Id 020011

Phillips AM, Embrey SK, Shan G, Korjagin VA (2002). Field Expression of Cry1F (synpro), Cry1Ac (synpro) and phosphinothricin acetyltransferase (PAT) proteins in transgenic cotton plants, cottonseed and cottonseed processed products; and compositional analysis of cottonseed and cottonseed processed products. Dow AgroScience, LLC, Indianapolis, Indiana. Study Id 010015.02

Song P, Collins R, Hey T, Madduri K, Ni W, Schafer B, Xu U (2002) Expression of the Partial PAT Open Reading Frame in B.t. Cry1F Cotton Event 281-24-236. Dow AgroSciences LLC Indianapolis, Indiana. Study Id. GH-C 5573

Field trials of cotton line MXB-13 and control lines were conducted under USDA permit in 2001. The trials were at six sites representing diverse agronomic practises and environmental conditions located in major cotton producing regions of the US. Plants were sampled at various stages of development and protein levels were measured in a variety of matrices including young leaves, terminal leaves, squares, bolls, whole plant, root, pollen, nectar, cottonseed and cottonseed processed fractions consisting of kernels, hulls, meal, and oil. The soluble extractable Cry1Ac, Cry1F, and PAT proteins were measured using quantitative enzyme linked immunosorbent assay (ELISA) methods. Results are reported in ng protein/mg sample dry weight, with fresh weight used for cottonseed, pollen, nectar, and processed products (see Table 6).

The analytical method for both Cry1Ac and Cry1F has a validated limit of quantification of 0.001 to 0.2 ng protein/mg, depending on the matrix. The method for PAT has a limit of quantification of 0.002 to 0.4 ng protein/mg, also depending on the matrix.

All matrices except nectar, meal and oil, were found to express the Cry1F protein at measurable levels. Average expression levels of Cry1F ranged from not detectable (ND) to 22.8 ng/mg. Expression of Cry1Ac was observed in all matrices except the nectar, hulls, and oil. Average expression levels of Cry1Ac ranged from ND to 1.83 ng/mg The average expression levels of PAT across all matrices ranged from ND to 0.54 ng/mg.

Table 6: Summary of the expression of the novel proteins in line MXB-13

Cotton Tissue	**Mean Protein Expression (ng/mg dry weight*)**		
	Cry1F	**Cry1Ac**	**PAT**
Young leaf (3-6 weeks)	6.81	1.82	0.43
Terminal leaf	8.19	1.31	0.23
Flowers	5.44	1.83	0.35
Square	4.88	1.82	0.52
Boll (Early)	3.52	0.64	0.27
Whole plant (seedling)	14.1	1.37	0.35
Whole plant (pollination)	25.3	1.05	0.30
Whole plant (defoliation)	22.0	0.6	0.34
Root (seedling)	0.88	0.17	0.06**
Root (pollination)	0.54	0.07**	ND
Root (defoliation)	0.51	ND	0.05**
Pollen	0.06**	1.45	0.05**
Nectar	ND	ND	ND
Seed	4.13	0.55	0.54
Cotton processed fraction			
Cottonseed	3.1	0.46	0.53
Kernel	3.9	0.51	0.78
Hulls	0.16	ND	ND
Toasted meal	ND	ND	ND
Refined oil	ND	ND	ND

*Results are reported in ng protein/mg sample dry weight, with fresh weight used for cottonseed, pollen, nectar, and processed products
** Calculated concentration is less than the LOQ of the method

Another study was conducted comparing Cry1Ac and Cry1F levels in MXB-13 cottonseed and meal. The levels of Cry1F decreased from 6.2 ng/mg in the cottonseed to 0.21 ng/mg in the processed meal. Levels of Cry1Ac decreased from 0.64 ng/mg to 0.11 ng/mg upon processing.

Plant expression of the Cry1F protein is higher than that of the Cry1Ac protein. This is probably due to the use of different promoters for the two genes. The use of a different promoter for each gene is common in such cases as there is less opportunity for negative interaction between the two inserts. Expression of the two *cry* genes was reported to be sufficient to achieve good protection against the target pest species.

Partial PAT ORF Expression

As described in Section 3.3, insert 281-24-236 contains a partial *pat* ORF that is transcribed into mRNA at levels 16-fold less that the full-length *pat* gene. To determine if this results in expression of a truncated PAT protein in the cotton, the partial *pat* ORF was characterised and expression levels examined by Western blotting.

The pPAT sequence was cloned from MXB-9 cotton into a recombinant *E. coli* protein expression vector and pPAT protein was expressed in bacterial cells. The bacterial pPAT protein was characterised by N-terminal sequencing, MALDI-TOF, SDS-PAGE and Western blot analysis. A PAT specific polyclonal antibody was found to be highly immunoreactive with the pPAT protein, with detection of quantities less than 1 ng. Western blot analysis of cotton line MXB-9 using this antibody showed no detectable pPAT protein in any of the plant tissues analysed. The results demonstrate that while pPAT mRNA transcript is detected at a low level in MXB-9 cotton, no protein expression could be detected in cotton tissue.

Potential toxicity of novel proteins

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

The Cry1Ac, Cry1F and PAT proteins have been assessed by FSANZ a number of times in different genetically modified foods and found to be safe for human consumption. In addition, three further acute oral toxicity studies in mice were assessed.

As it is very difficult to extract and purify sufficient quantities of the subject protein from transgenic cotton plants for the acute oral toxicity studies, it has become standard practice to instead use equivalent proteins that have been produced using

bacterial expression systems. Prior to use, the bacterially produced proteins are compared to the proteins produced *in planta* in order to establish their equivalence. Cry1F and Cry1Ac proteins were produced in recombinant *Pseudomonas fluorescens* and the PAT protein was produced in recombinant *Escherichia coli*.

The molecular identity and biochemical characteristics of the proteins expressed *in planta* and in the bacterial-expression systems were examined using various biochemical methods such as N-terminal sequencing, molecular weight determination, immunoreactivity, glycosylation analysis, peptide mass fingerprinting and matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry. These studies established that bacterially produced Cry proteins were equivalent to those proteins produced in cotton line MXB-13, thus the bacterial proteins were used in the toxicity testing.

Studies evaluated:
Brooks KJ and Andrus AK (1999) Cry 1F microbial protein (FL): Acute oral toxicity study in CD-1 mice. The Dow Chemical Company, Midland, Michigan. Laboratory Report Code 991178

Brooks KJ and Yano BL, (2001a) Cry1Ac (synpro) microbial protein: Acute oral toxicity study in CD-1 mice. The Dow Chemical Company, Midland, Michigan. Laboratory Report Code 011126

Brooks KJ and Yano BL, (2001b) Cry1F (synpro) microbial protein + cry1Ac (synpro) microbial protein: acute oral toxicity study in CD-1 mice. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 011127

Gao Y (2002) Partial purification and Characterisation of Cry1Ac Delta Endotoxin from Transgenic Cotton Event 3006-210-23. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. GH-C 5509

Gao Y, Gilbert JR, Ni W, Xu X (2002a) Characterisation of Cry1Ac (synpro) Delta Endotoxin derived from Recombinant *Pseudomonas fluorescens*. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. GH-C 5508

Gao Y, Gilbert, JR, Schwedler DA, Xu X. (2002b) Characterisation of Cry1F protein derived from *Pseudomonas fluorescens* and transgenic cotton. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 010018

Gao Y, Gilbert JR, Ni W, Xu X (2002c) Purification and Characterisation of Cry1Ac Delta Endotoxin from transgenic cotton event 3006-210-23. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. GH-C 5548

Potential toxicity of Cry1F: Acute oral toxicity limit test in mice.

Test material	*Pseudomonas fluorescens* derived Cry1F protein (30% pure)
Vehicle control	0.5% methylcellulose
Test Species	Five male and five female CD-1 mice
Dose	2000 mg/kg body weight (600 mg Cry1F /kg bw), administration by 2 gavage doses, 1 hour apart
GLP/guidelines	OECD Guideline No. 401

Parameters evaluated during the two-week observation period included body weights, detailed clinical observations, and gross pathological changes. Animals were given one detailed clinical observation before the test material was administered for comparison with the observations recorded throughout the study.

Animals were observed twice on the day of treatment, including a detailed clinical observation. A detailed clinical observation was made on each day of the study. Individual body weights were measured on day –1, 1, 2, 8 and 15. There was a slight decrease in body weights in all mice on day 2, however, all animals gained weight over the course of the two-week observation period.

All mice survived to the end of the two-week observation period. A necropsy was performed on all animals. The eyes were examined *in situ* using a moistened glass microscope slide applied to the corneal surface. Following inspection of external features and body orifices, the nasal, cranial, oral, thoracic, and abdominal cavities were opened and the visceral organs were examined both *in situ* and following dissection. There were no gross pathologic lesions in any animal.

Under the conditions of this limit test, the acute oral LD_{50} of Cry1F microbial protein in male and female CD-1 mice was greater than 600 mg Cry1F/kg body weight.

Potential toxicity of Cry1Ac: Acute oral toxicity limit test in mice.

Test material	*Pseudomonas fluorescens* derived Cry1Ac protein (14% pure)
Vehicle control	0.5% methylcellulose
Test Species	Five male and five female CD-1 mice
Dose	5000 mg/kg body weight (700 mg Cry1Ac /kg bw), administration by 3 gavage doses, 1 hour apart
GLP/guidelines	OECD Guideline No. 401

Parameters evaluated during the two-week observation period included body weights, detailed clinical observations, and gross pathological changes as described in the study above.

All mice survived to the end of the two-week observation period. There were no clinical observations noted throughout the study. All male mice gained weight and all female mice maintained or slightly gained weight over the duration of the study. There were no gross pathological lesions for any animal on study.

Under the conditions of this limit test, the acute oral LD_{50} of Cry1Ac microbial protein in male and female CD-1 mice was greater than 700 mg Cry1Ac/kg body weight.

Potential toxicity of Cry1Ac and Cry1F mixture: Acute oral toxicity limit test in mice

Test material	50:50 mixture of Cry1F (15% pure) and Cry1Ac (14% pure) derived from *Pseudomonas fluorescens*
Vehicle control	0.5% methylcellulose
Test Species	Five male and five female CD-1 mice
Dose	5000 mg/kg body weight (375 mg Cry1F/kg bw and 350 mg Cry1Ac/kg bw), administration by 3 gavage doses, 1 hour apart
GLP/guidelines	OECD Guideline No. 401

Parameters evaluated during the two-week observation period included body weights, detailed clinical observations, and gross pathological changes as described in the Cry1F study above.

All mice survived to the end of the two-week observation period. There were no clinical observations noted throughout the study. All mice gained weight over the duration of the study. There were no gross pathological lesions for any animal on study.

Under the conditions of this limit test, the acute oral LD_{50} of the mixture of Cry1F and Cry1Ac microbial proteins in male and female CD-1 mice was greater than 375 mg and 350 mg/kg body weight respectively.

Potential toxicity of PAT

Extensive animal testing has shown that the PAT protein is non-toxic to humans and animals. The same gene has been expressed in other transgenic crops assessed by FSANZ and is considered to pose no risks to human health and safety.

Similarities with known protein toxins

A comparison of the amino acid sequence of an introduced protein for similarity to known protein toxins is one of the steps in a multilevel analytical process to assess potential toxicity (CODEX 2001). Bioinformatic analyses were done to assess the Cry1Ac (synpro), Cry1F (synpro) and PAT proteins for any similarity with known protein toxins.

Studies evaluated:
Song P (2002c) Comparison of the Amino Acid Sequence of the Phosphinothricin N-Acetyltransferase (PAT) as Expressed in Plants to Known Protein Toxins in the Public Sequence Database. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. GH-C 5535

Song P (2002d) Comparison of the Amino Acid Sequence of the Potential Partial Phosphinothricin N-Acetyltransferase (PAT) ORF in *B.t.* Cry1F Cotton Event 281-24-236 to Known Protein Toxins in the Public Sequence Database. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id GH-C 5554

Song P (2003a) Comparison of the Amino Acid Sequence of the *Bacillus thuringiensis* var. *aizawai* Cry1F (synpro) Insect Control Protein as Expressed in Cotton to Known Protein Toxins in the Public Protein Sequence Database. Dow AgroSciences LLC, Indianapolis, Indiana. Study Id. GH-C 5621

Song P (2003b) Comparison of the Amino Acid Sequence of the *Bacillus thuringiensis* var. *kurstaki* Strain HD73 Cry1Ac (synpro) Insect Control Protein as Expressed in Cotton to Known Protein Toxins in the Public Protein Sequence Database. Dow AgroSciences LLC, Indianapolis, Indiana. Study Id GH-C 5620

Sequence analysis was performed using the BLASTP tools available at the NCBI web site (http://www.ncbi.nlm.nih.gov/blast/) to compare the novel proteins with known protein toxins. The database contained more than one million sequences.

Cry1F

The Cry1F BLASTP analysis revealed a total of 190 proteins with an expectation value of less than one. Of those, all but one were Cry proteins and Cry protein C-

terminal fragments. The one non-Cry protein that showed significant homology was a hypothetical protein[1] from *Methanosarcina acetivorans*, which showed 28% identity with Cry1F over 124 residues. However, this hypothetical protein appears to be a newly identified Cry protein homolog. Thus there is no evidence that the Cry1F (synpro) protein sequence is related to known protein toxins other than the Cry delta endotoxins.

Cry1Ac

Similarly, the Cry1Ac BLASTP analysis revealed a total of 193 proteins with an expectation value less than one. Of these, only three were not Cry proteins or Cry protein C-terminal fragments. One of these was the hypothetical protein described above from *Methanosarcina acetivorans.* The other two were also hypothetical proteins, neither of which showed any sequence similarity with any known protein toxins. Thus there is no evidence that the Cry1Ac (synpro) protein sequence is related to known protein toxins other than the Cry delta endotoxins.

PAT

The PAT BLASTP search against the NCBI non-redundant protein database revealed 68 accessions with expectation values of less than one. However, 51 of the 68 accessions were identified as either phosphinothricin acetyltransferase or other acetyltransferases. The remaining 17 accessions were generally unidentified and putative or hypothetical proteins. Although 7 of these 17 had expectation values less than 0.01 when aligned with the PAT sequence, BLASTP searching with these proteins against the NCBI protein database generated statistically significant hits only associated with proteins like phosphinothricin acetyltransferase, other acetyltransferases and hypothetical proteins without assigned function. Thus no significant sequence homology was found between the PAT protein and a known protein toxin.

Partial PAT ORF

The potential amino acid sequence of the partial PAT ORF that is present in cotton line MXB-13 as part of transformation event 281-24-236 has been evaluated by a BLASTP search for similarity to known protein toxins. Forty-four accessions with an expectation value of less than 1 were identified. Twenty-eight of these were either phosphinothricin acetyltransferase or other acetyltransferases. The remaining 16 accessions were generally unidentified and putative proteins.

Although 7 of these had expectation values less than 0.01 when aligned with the pPAT protein, BLASTP searching with these proteins against the NCBI protein database generated statistically significant hits only associated with proteins like PAT, other acetyltransferases and hypothetical proteins with no assigned function. Thus, no significant sequence homology was found between the putative partial PAT ORF and any known or putative toxins.

[1] The definition of a hypothetical protein is an amino acid sequence translation that is derived from an automated gene model prediction. There is no evidence that the hypothetical protein is translated or expressed in vivo.

Potential allergenicity of novel proteins

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

The three novel proteins expressed in cotton line MXB-13 and the putative protein pPAT were assessed using these criteria for their potential allergenicity.

Similarity to known allergens

Studies evaluated:
Stelman SJ (2001a) Comparison of the Amino Acid Sequence of the *Bacillus thuringiensis* var. *aizawai* Cry1F (synpro) Insect Control Protein as Expressed in Cotton to Known Protein Allergens. Dow AgroSciences LLC, San Diego, California. Study Id. GH-C 5315

Stelman SJ (2001b) Comparison of the Amino Acid Sequence of the *Bacillus thuringiensis* var. *kurstaki* Cry1Ac (synpro) Insect Control Protein as Expressed in Cotton to Known Protein Allergens. Dow AgroSciences LLC, San Diego, California. Study Id.GH-C 5316

Stelman SJ (2001c) Comparison of the Amino Acid Sequence of the phosphinothricin acetyltransferase (PAT) Protein as Expressed in Cotton to Known Protein Allergens. Dow AgroSciences LLC, San Diego, California. Study Id. GH-C 5314

Stelman SJ (2002) Comparison of the Putative Amino Acid Sequence of the Partial Phosphinothricin Acetyltransferase (PAT) ORF in Cry1F Cotton Event 281-24-236 to Known Protein Allergens. Dow AgroSciences LLC, San Diego, California. Study Id. GH-C 5530

A sequence evaluation scheme was used to assess the similarity of the transgenic proteins to known protein allergens. An immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids. No immunologically significant sequence identity was detected for Cry1F, Cry1Ac, or PAT. In addition pPAT was also evaluated and based on the amino acid sequence it is predicted not to have allergenic potential.

In vitro digestibility

Studies evaluated:
Korjagin VA (2001a) *In Vitro* Simulated Gastric Fluid Digestibility Study of Microbially Derived Cry1Ac (synpro). Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 010026.

Korjagin VA (2001b) *In Vitro* Simulated Gastric Fluid Digestibility Study of Microbially Derived Cry1Ac (synpro). Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 010081.

Korjagin VA (2003) *In Vitro* Simulated Intestinal Fluid Digestibility Study of Recombinant Cry1Ac (synpro) Delta-Endotoxin. Dow AgroSciences. LLC, Indianapolis, Indiana. Study Id. 020094.

Korjagin VA and Embrey SK (2003) *In Vitro* Simulated Gastric Fluid Digestibility Study of Recombinant Cry1F (synpro) Delta Endotoxin. Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 020095.

Korjagin VA and Herman RA (2002) *In Vitro* Simulated Gastric Fluid Digestibility Study of Recombinant Phosphinothricin Acetyltransferase (PAT). Dow AgroSciences, LLC, Indianapolis, Indiana. Study Id. 020107

Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergic response (Kimber *et al.*, 1999; Astwood *et al.*, 1996; Metcalfe *et al.*, 1996). The Cry1Ac and Cry1F proteins were therefore investigated for their digestibility in simulated digestion models.

Samples of both Cry1Ac and Cry1F (produced in recombinant *P. fluorescens*) were incubated with simulated gastric fluid (SGF) at 37ºC to determine if these two proteins would be digested. The digestions were performed at time intervals of 1, 3, 6, 10, 15, 20, 30, and 60 minutes. Following digestion, the protein samples were analysed by SDS-PAGE and Western blotting. Both Cry1Ac and Cry1F were fully digested in SGF in under 1 minute.

The PAT protein was also assessed to determine if it would be digested in SGF and it was determined that > 98% of the protein was degraded within 30 seconds in SGF at 37ºC.

Samples of both Cry1Ac and Cry1F were also incubated with simulated intestinal fluid (SIF) at 37ºC. The digestions were performed for time intervals of approximately 0, 10 and 30 minutes and 1, 2, 3, and 4 hours.

Following digestion, the protein samples were analysed by SDS-PAGE and Western blotting. Both Cry1Ac and Cry1F were rapidly (less than 10 minutes) digested in SIF to their trypsin-resistant core toxins. The core toxins remained stable against further SIF digestion of the duration of the 4-hour assay.

Thermolability

Studies evaluated:
Herman RA and Gao Y (2001a) Thermolability of Cry1Ac (synpro) Delta-Endotoxin. Dow AgroSciences LLC, Indianapolis, Indiana. Study Id. 010085.

Herman RA and Gao Y (2001b) Thermolability of Cry1F (synpro) Delta-Endotoxin. Dow AgroSciences

Thermolability of Cry1F and Cry1Ac proteins (produced in recombinant *Pseudomonas fluorescens*) was investigated by incubating aqueous formulations of each protein at 60ºC, 75ºC, and 90ºC for 30 minutes. The samples were then cooled on ice and applied to the surface of artificial insect diet in bioassay trays. Neonate tobacco budworm, (*Heliothis virescens*), a susceptible insect, were grown in the trays and mortality data were collected after 6 days. As can be seen in Table 7 both proteins were totally inactivated after treatment at 90ºC, Cry1F was inactivated at 75ºC and Cry1Ac was almost entirely inactivated at this temperature too.

Table 7: Percentage mortality of tobacco budworm after 30 minutes heat treatment of microbially produced Cry1F and Cry1Ac proteins

Treatment	Cry1F[1]	Cry1Ac[2]
4ºC (negative control)	100%	100%
60ºC	100%	100%
75ºC	0%	7%
90ºC	0%	0%
buffer control	0%	0%

[1] Cry1F was at a concentration of 80 ng/cm^2 of diet
[2] Cry1Ac was at a concentration of 10 ng/cm^2 of diet

Conclusion regarding characterisation of the novel proteins

Cotton line MXB-13 expresses three novel proteins – Cry1Ac, Cry1F, and PAT, all expressed at low levels.

A number of studies have been done on the Cry1Ac, Cry1F, and PAT proteins to determine their potential toxicity and allergenicity. These studies demonstrate that the proteins are non-toxic to mammals, and have limited potential to be allergenic.

COMPARATIVE ANALYSES

Most crops, including oilseed crops, exhibit considerable variability in their nutrient composition. Environmental factors and the genotype of the plant have an enormous impact on composition. Thus, variation in these nutrient parameters is a natural phenomenon and is considered to be normal.

A comparative approach focussing on the determination of similarities and differences between the GM food and its conventional counterpart aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO 2000). The critical components to be measured are determined by identifying key nutrients, key toxicants and anti-nutrients for the food source in question (FAO 1996).

The key nutrients and toxicants/anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. These may be major constituents (e.g., fats, proteins, carbohydrates) or minor components (e.g., minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g., solanine in potatoes if the level is increased). The key components of cottonseed that have been considered in this comparison include proximates, amino acids, fatty acids, minerals, and the toxicants gossypol and cyclopropenoid fatty acids.

Nutrient analysis

Study evaluated:
Phillips, A.M., Embrey, S.K., Shan, G., Koragin, V.A. (2002) Field Expression of Cry1F (synpro), Cry1Ac (synpro) and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed and Cottonseed Processed Products and Compositional Analysis of Cottonseed and Cottonseed Processed Products. Study ID: 010015.02 Dow AgroSciences LLC, Indianapolis, Indiana.

Phillips, A.M., Herman, R.A., Embrey, S.K., Shan, G., Koragin, V.A. (2003) Field Expression of Cry1F (synpro), Cry1Ac (synpro) and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed and Cottonseed Processed Products and Compositional Analysis of Cottonseed and Cottonseed Processed Products. Study ID: 010015.03 Dow AgroSciences LLC, Indianapolis, Indiana.

To determine whether unexpected changes had occurred in the nutrient composition of cotton line MXB-13 as a result of the genetic modification, and to assess the nutritional adequacy of this line, compositional analysis was done on whole cottonseed and processed fractions from cotton line MXB-13 and from its non-transgenic counterpart. The non-transgenic counterpart used as a control was seed grown from the null plants from the F_1 segregating generation after stacking the *cry*1F and *cry*1Ac genes. A total of 69 components were analysed - these were proximate content (moisture, fat, protein, fibre, ash and carbohydrate), amino acids, fatty acids, minerals, gossypol, cyclopropenoid fatty acids, and aflatoxins.

Field trials were conducted at six sites located in the major cotton-producing regions of the U.S. (Arizona, California, Mississippi, North Carolina and 2 sites in Texas). These sites represent regions of diverse agronomic practices and environmental

conditions. Four lines of cotton were grown at each test site; control non-transgenic cotton, line MXB-9 (contains only event 281-24-236), line MXB-7 (contains only event 3006-210-23) and the stacked cotton line MXB-13 (which contains both events). However, only the data collected from the control cotton and cotton line MXB-13 were analysed in this safety assessment.

Cotton tissue samples were collected at various times during the development of the plants. Samples of terminal leaf, squares, de-linted cottonseed, and the processed fractions of cottonseed – kernel, toasted meal, refined oil and hulls were analysed for nutrient content using a variety of tests.

Statistical analysis was performed on the cottonseed compositional data since these samples were analysed in replicate. A single sample of the processed fractions (hulls, meal and oil) was analysed for each variable, therefore these values were not analysed statistically, but just compared to the literature range. Statistical treatment of the data in this study consisted of calculation of the means, standard deviations and regression analysis. Statistical differences in composition between the transgenic and non-transgenic cotton were determined using a mixed model (SAS Institute 1999). The transgenic cotton was compared to the control line using a t-test and again with the P-values adjusted using a Dunnett procedure to maintain the experiment-wide error rate at 0.05. Significant differences were declared at the 95% confidence level.

The result of the nutritional analysis for the cottonseed and processed fractions were also compared to values reported in literature (Berberich *et al*, 1996; Forster and Calhoun, 1995; Codex 2001; *Cottonseed Oil* 1990; and Cottonseed Feed Products Guide by the NCPA). Literature ranges from each of these references were listed and composite ranges were obtained.

Proximate analysis

No significant difference was found between the control cottonseed and MXB-13 cottonseed for any of the proximates other than the crude fibre. The crude fibre content in MXB-13 cottonseed was significantly lower than the control, but was similar to the value reported in the literature and differed from the control by less than 10% and is not considered to biologically significant.

All proximates were within or very similar to the literature ranges except for moisture. This was thought to be due to sampling and preparation as results were comparable between the control and the transgenic cottonseed. Results of the proximate analysis are shown in Tables 8 and 9.

Mineral Analysis

The minerals calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, sodium, sulphur, and zinc were analysed and compared between the transgenic cottonseed and the control cottonseed. A summary of the results is presented in Tables 10 and 11.

There were no significant differences between MXB-13 cottonseed and control

cottonseed in regard to mineral content.

Fatty Acid Analysis

Twenty-two fatty acids were analysed and compared between MXB-13 and control cottonseed. A summary of the results is shown in Table 12. Literature values have been reported for only a limited number of fatty acids in cottonseed.

Thirteen of the twenty-two fatty acids were present in both the control and transgenic cotton lines at levels below 0.02% dry weight. Of the other nine, there was only one statistically significant difference (stearic acid). Levels for both the transgenic and control cottonseed were below the literature range for stearic acid, however, the transgenic cottonseed was closer to the literature range than the control. Further, the difference was less than 6% and was not considered to be biologically significant.

The same 22 fatty acids were measured in refined cottonseed oil derived from cotton line MXB-13 and its control. Twelve of the fatty acids were present in the refined oil from both cotton lines at levels of less than 0.1 %. The other fatty acids (myristic, palmitic, palmitoleic, stearic, oleic, linoleic, gamma linolenic, linolenic, arachidic, and behenic) were present in very similar levels in both lines and in all cases were within the literature range for fatty acid content in refined cottonseed oil.

Amino Acid Analysis

Eighteen amino acids were analysed in MXB-13 and control cottonseed. The results are summarised in Tables 13 and 14. No statistically significant differences were observed between the control and transgenic lines.

For the cottonseed meal, cotton line MXB-13 had slightly higher levels of all the amino acids, which is not unexpected given the slightly higher level of protein in MXB-13 compared to the control (51% compared to 47% in Table 9). Values were comparable between lines and comparable to the literature range.

Table 8: Summary of the proximate analysis of MXB-13 cotton and control cottonseed from all sites (n = 6)

Component[1]	MXB-13[2]	Control[2]	Paired t-test P-Value	Dunnet Adjusted P-Value	Literature Reference Range[3]
Ash	**3.9** (3.5 - 4.1) 0.21	**4.0** (3.7 - 4.4) 0.28	0.238	0.489	4.1-4.9
Fat	**22.9** (20.9 – 23.7) 1.02	**22.6** (21.4 - 24.3) 1.15	0.657	0.941	16.1-26.7
Moisture	**3.5** (2.6 - 5.6) 1.09	**3.3** (2.5 – 4.2) 0.65	0.659	0.943	5.4-15.9
Protein	**27.9** (26.4 – 29.0) 0.95	**27.6** (26.1 – 29.3) 1.19	0.717	0.966	12-32
Carbohydrates	**45.4** (43.5 – 47.2) 1.34	**45.8** (42.1 – 48.1) 2.09	0.691	0.956	42.8-47.6
Calories (Kcalories/100 gm)	**499** (489 - 505) 5.32	**497** (491 - 504) 4.93	0.552	0.875	479-508
Crude Fibre	**15.9** (14.7 – 17.0) 0.79	**17.6** (16.6 – 18.6) 0.69	**0.003**	**0.009**	17.2
Acid Detergent Fibre	**25.2** (23.9 – 26.4) 0.96	**25.2** (23.1 – 27.2) 0.96	0.989	1.0	26
Neutral Detergent Fibre	**34.1** (30.7 – 36.9) 2.35	**35.9** (32.8 – 38.5) 1.92	0.316	0.613	37

[1] All values (mean and range) expressed as % dry weight.
[2] Values shown are the mean (bold) the range (in brackets) and the standard deviation.
[3] Combined literature range

Table 9: Proximate analysis of cottonseed processed fractions

Component	Kernels		
	MXB-13	Control	Literature Range
Moisture	6.9	7.6	NA
Component	**Hulls**		
	MXB-13	**Control**	**Literature Range**
Ash	2.8	3.0	2.39-3.97
Fat	2.0	3.0	1.0-3.3
Moisture	10.6	10.3	8.5-12.3
Protein	6.2	7.1	4.0-6.9
Carbohydrates	89.0	86.8	NA
Calories (Kcalories/100 gm)	399	403	NA
Component	**Toasted Meal**		
	MXB-13	**Control**	**Literature Range**
Ash	6.7	6.0	4.6-9.8
Fat	2.0	4.6	0.6-4.7
Moisture	9.2	2.2	9-13.3
Protein	51.3	47.2	43.0-52.4
Carbohydrates	40.0	42.1	NA
Calories (Kcalories/100 gm)	383	399	NA
Crude Fibre	9.3	12.4	8.4-15.3
Acid Detergent Fibre	14.1	18.5	12.2-23.9
Neutral Detergent Fibre	20.2	24.2	15.8-32.4
Component	**Refined Oil**		
	MXB-13	**Control**	**Literature Range**
Fat	100.1	100.2	NA
Moisture	<0.1	<0.1	NA
Protein	<0.1	<0.1	NA

All values are expressed as % dry weight except for the refined oil, which is % fresh weight
NA = not available

Table 10: Summary of the mineral analysis of MXB-13 cotton and control cottonseed from all sites (n = 6)

Component (mg/100g dry weight)	MXB-13[2]	Control[2]	Paired t-test P-Value	Dunnet Adjusted P-Value	Literature Reference Range[3]
Calcium	**160** (140 - 190) 18.25	**151** (129 - 185) 20.89	0.076	0.178	108-210
Copper	**0.93** (0.79 – 1.11) 0.11	**0.91** (0.83 – 1.03) 0.08	0.829	0.992	0.4-1.19
Iron	**5.59** (4.76 - 6.67) 0.71	**6.17** (4.95 – 7.65) 1.00	0.099	0.227	3.79-15.1
Magnesium	**417** (370 – 450) 35.14	**421** (377 – 461) 31.68	0.799	0.988	305-460
Manganese	**1.51** (1.35 – 1.66) 0.14	**1.42** (1.27 – 1.68) 0.15	0.149	0.328	1.0-2.0
Molybdenum	**<0.2** (<0.2) -	**<0.2** (<0.2) -	-	-	0.1-0.4
Phosphorus	**687** (590 – 769) 61.39	**699** (579 – 869) 107.72	0.763	0.980	447-750
Potassium	**1219** (1109 - 1324) 70.87	**1237** (1065 – 1371) 102.26	0.406	0.731	990-1280
Sodium	**26.5** (<10 – 40) 19.16	**15.6** (<10 – 24) 7.25	-	-	3-38
Zinc	**4.43** (4.09 – 4.82) 0.31	**4.23** (3.61 – 5.38) 0.62	0.247	0.502	2.49-4.2
Sulphur	**275** (226 – 315) 35.26	**276** (248 – 293) 16.65	0.857	0.996	144-260

[1] All values (mean and range) expressed as % dry weight.
[2] Values shown are the mean (bold) the range (in brackets) and the standard deviation.
[3] Combined literature range

Table 11: Mineral analysis of cottonseed processed fractions

Component (mg/100g dry wt.)	Hulls		
	MXB-13	Control	Literature Range
Calcium	150	146	100-250
Copper	0.36	0.33	0.3-1.3
Iron	2.14	2.97	1.8-13.1
Magnesium	183	181	120-230
Manganese	1.70	1.49	1.2-2.2
Molybdenum	<0.2	<0.2	0-0.15
Phosphorus	96	113	50-260
Potassium	1208	1215	870-1240
Sodium	12.9	16.1	5-20
Zinc	1.30	1.23	0.6-2.2
Sulphur	59	54	30-100
Component (mg/100g dry wt.)	Toasted Meal		
	MXB-13	Control	Literature Range
Calcium	203	191	160-360
Copper	1.74	1.41	0.7-2.2
Iron	9.98	11.35	7.5-22.8
Magnesium	718	628	440-820
Manganese	2.05	1.89	1.4-2.5
Molybdenum	<0.2	<0.2	0.13-0.51
Phosphorus	1388	1155	860-1540
Potassium	1696	1534	1280-1980
Sodium	<10	15.2	4-330
Zinc	8.07	7.10	4.9-8.3
Sulphur	506	443	280-500

NA = not available

Table 12: Summary of the fatty acid analysis of MXB-13 cotton and control cottonseed from all sites (n = 6)

Fatty Acids (% dry weight)	MXB-13[1]	Control[1]	Paired t-test P-Value	Dunnet Adjusted P-Value	Lit Reference Range[2]
8:0 Caprylic	**<0.0200**	**<0.0200**			
10:0 Capric	**<0.0200**	**<0.0200**			
12:0 Lauric	**<0.0200**	**<0.0200**			
14:0 Myristic	**0.198** (0.163 – 0.224) 0.03	**0.185** (0.165 – 0.208) 0.02	0.192	0.408	0.22-0.36
14:1 Myristoleic	**<0.0200**	**<0.0200**			
15:0 Pentadecanoic	**<0.0200**	**<0.0200**			0.11-0.20
15:1 Pentadecenoic	**<0.0200**	**<0.0200**			
16:0 Palmitic	**5.11** (4.86 – 5.38) 0.22	**5.03** (4.59 – 5.36) 0.31	0.621	0.922	8.31-9.31
16:1 Palmitoleic	**0.117** (0.106 – 0.125) 0.01	**0.113** (0.098 – 0.128) 0.01	0.389	0.709	0.16-0.24
17:0 Heptadecanoic	**<0.0200**	**<0.0200**			0.04-0.07
17:1 Heptadecenoic	**<0.0200**	**<0.0200**			
18:0 Stearic	**0.595** (0.549 – 0.643) 0.05	**0.563** (0.531 – 0.58) 0.02	**0.036**	0.088	0.78-1.09
18:1 Oleic	**3.66** (3.35 – 0.385) 0.23	**3.51** (3.13 – 3.89) 0.28	0.227	0.469	4.96-5.36
18:2 Linoleic	**11.6** (9.49 – 12.8) 1.14	**11.7** (10 – 12.9) 1.27	0.889	0.998	15.5-16.7
18:3 Gamma Linolenic	**<0.0200**	**<0.0200**			
18:3 Linolenic	**0.0900** (0.0813 – 0.0966) 0.01	**0.0888** (0.079 – 0.101) 0.01	0.742	0.974	0.04-0.10
20:0 Arachidic	**0.0668** (0.0596 – 0.0724) 0.01	**0.0638** (0.0563 – 0.0677) 0.01	0.298	0.584	0.09-0.10
20:1 Eicosenoic	**<0.0200**	**<0.0200**			
20:2 Eicosadienoic	**<0.0200**	**<0.0200**			

20:3 Eicosatrienoic	**<0.0200**	**<0.0200**			
20:4 Arachidonic	**<0.0200**	**<0.0200**			
22:0 Behenic	**0.0361** (0.0337 – 0.0398) 0.00	**0.0354** (0.0324 – 0.0423) 0.00	0.608	0.914	0.04-0.06

[1] Values shown are the mean (bold) the range (in brackets) and the standard deviation
[2] Literature range from Berberich *et al*, 1996

Table 13: Summary of the amino acid analysis of MXB-13 cotton and control cottonseed from all sites (n = 6)

Amino Acid (% dry weight)	MXB-13[1]	Control[1]	Paired t-test P-Value	Dunnet Adjusted P-Value	Literature Reference Range[2]
Aspartic acid	**2.60** (2.46 – 2.79) 0.12	**2.51** (2.37 – 2.69) 0.13	0.399	0.725	2.03-2.62
Threonine	**0.787** (0.743 - 0.95) 0.08	**0.766** (0.704 – 0.832) 0.05	0.622	0.924	0.65-0.92
Serine	**1.27** (1.21 – 1.33) 0.04	**1.22** (1.15 – 1.29) 0.06	0.300	0.590	0.90-1.25
Glutamic acid	**5.49** (5.36 – 5.86) 0.19	**5.41** (5.04 – 5.92) 0.35	0.749	0.977	4.74-5.28
Proline	**1.04** (0.992 – 1.131) 0.05	**1.03** (0.968 – 1.142) 0.07	0.829	0.993	0.72-1.14
Glycine	**1.15** (1.09 – 1.24) 0.05	**1.12** (1.04 – 1.19) 0.06	0.569	0.889	0.88-1.17
Alanine	**1.08** (1.03 – 1.18) 0.05	**1.05** (0.98 – 1.13) 0.06	0.508	0.840	0.83-1.11
Cysteine	**0.423** (0.387 – 0.457) 0.02	**0.404** (0.360 – 0.435) 0.0.3	0.264	0.533	0.43-0.79
Valine	**1.23** (1.14 – 1.30) 0.07	**1.19** (1.10 – 1.35) 0.10	0.562	0.885	0.99-1.22
Methionine	**0.391** (0.347 – 0.434) 0.03	**0.378** (0.331 – 0.407) 0.03	0.408	0.733	0.30-0.42
Isoleucine	**0.888** (0.827 – 0.939) 0.04	**0.867** (0.811 – 0.961) 0.06	0.614	0.919	0.69-0.88
Leucine	**1.60** (1.53 – 1.73) 0.07	**1.56** (1.46 – 1.68) 0.08	0.536	0.864	1.27-1.61
Tyrosine	**0.718** (0.665 – 0.784) 0.04	**0.691** (0.638 – 0.754) 0.04	0.437	0.769	0.65-0.79
Phenylalanine	**1.44** (1.35 – 1.53) 0.06	**1.40** (1.30 – 1.53) 0.08	0.619	0.922	1.16-1.44
Histidine	**0.734** (0.633 – 0.790) 0.06	**0.684** (0.638 – 0.728) 0.04	0.189	0.403	0.60-0.73

[1] Values shown are the mean (bold) the range (in brackets) and the standard deviation.
[2] Literature range from Berberich *et al*, 1996.

Table 13 continued: Summary of the amino acid analysis of MXB-13 cotton and control cottonseed from all sites (n = 6)

Amino Acid (% dry weight)	MXB-13[1]	Control[1]	Paired t-test P-Value	Dunnet Adjusted P-Value	Literature Reference Range[2]
Lysine	**1.16** (1.07 – 1.23) 0.07	**1.08** (0.97 – 1.18) 0.08	0.113	0.258	0.90-1.22
Arginine	**3.08** (2.88 - 3.4) 0.22	**2.91** (2.73 – 3.05) 0.13	0.307	0.600	2.52-3.02
Tryptophan	**0.275** (0.247 – 0.296) 0.02	**0.258** (0.24 – 0.266) 0.01	0.074	0.174	0.23-0.32

[1] Values shown are the mean (bold) the range (in brackets) and the standard deviation.
[2] Literature range from Berberich *et al*, 1996.

Table 14: Amino acid analysis of cottonseed meal

Component (mg/100g dry wt.)	Meal		
	MXB-13	Control	Literature Range[1]
Aspartic acid	4.70	4.15	3.72-4.27
Threonine	1.65	1.32	1.46-1.61
Serine	2.27	1.84	1.91-2.15
Glutamic acid	9.58	8.59	8.40-10.2
Proline	1.91	1.63	1.42-1.69
Glycine	2.15	1.88	1.80-2.12
Alanine	2.04	1.77	1.62-1.86
Cysteine	0.795	0.723	0.64-0.84
Valine	2.28	2.11	1.66-2.10
Methionine	0.760	0.683	0.58-0.79
Isoleucine	1.65	1.50	1.17-1.61
Leucine	3.02	2.65	2.45-2.63
Tyrosine	1.39	1.12	0.94-1.24
Phenylalanine	2.79	2.41	2.19-2.44
Histidine	1.51	1.31	1.21-1.51
Lysine	2.26	2.01	1.56-1.97
Arginine	5.86	5.00	4.35-5.03
Tryptophan	0.548	0.468	0.49-0.60

[1] Combined literature range

Tocopherol Analysis of Cottonseed Oil

Cottonseed oil was analyzed for various tocopherol isomers that act as naturally occurring antioxidants found in cottonseeds. The data summarised in Table 15 compared results for the control and MXB-13 cottonseed oil. Tocopherol results for the control and transgenic lines are very similar and fall within the combined literature ranges for occurrence of alpha, beta, gamma, and delta tocopherols in crude cottonseed oil.

Table 15: Tocopherol analysis of cottonseed oil

Component (mg/kg)	Refined Oil		
	MXB-13	Control	Literature Range[1]
Alpha Tocopherol	515	548	136 - 674
Beta Tocopherol	<60.0	<60.0	ND - 29
Gamma Tocopherol	372	372	138 – 746
Delta tocopherol	<60.0	<60.0	ND - 75

ND = not detected
[1] Combined literature range

Key toxicants

Cotton contains two naturally occurring toxic compounds – gossypol and cyclopropenoid fatty acids. These compounds have been analysed in cottonseed from line MXB-13 and compared with the non-transgenic control line (Tables 16).

Table 16: Summary of Gossypol and Cyclopropenoid fatty acids in cottonseed

Amino Acid (% dry weight)	MXB-13[1]	Control[1]	Paired t-test P-Value	Dunnet Adjusted P-Value	Literature Reference Range[2]
Gossypol % dry wt.	**0.791** (0.623 – 0.876) 0.09	**0.870** (0.715 – 1.034) 0.11	0.137	0.304	0.39 – 1.7
Sterculic (% of fatty acids)	**0.292** (0.26 – 0.325) 0.03	**0.321** (0.252 – 0.361) 0.04	**0.020**	0.050	0.48 – 0.70
Malvalic (% of fatty acids)	**0.344** (0.313 – 0.42) 0.04	**0.397** (0.33 – 0.463) 0.06	**0.022**	0.056	0.22 – 0.45
Dihydrosterculic (% of fatty acids)	**0.209** (0.187 – 0.243) 0.02	**0.220** (0.183 – 0.259) 0.03	0.167	0.361	0.29 – 0.50

[1] Values shown are the mean (bold) the range (in brackets) and the standard deviation.
[2] Literature range from Berberich *et al*, 1996.

There was no significant difference in the level of gossypol in the cottonseed between
MXB-13 and control cottonseed as can be seen in table 16. Levels of sterculic and

malvalic fatty acids in MXB-13 cottonseed were statistically significantly decreased in comparison to the control, but this is not a safety concern nor is it biologically relevant. All values were within or below the literature range.

Free and total gossypol were analysed in cotton kernels, meal and refined cottonseed oil and were comparable between MXB-13 cotton and the control, and within or below the literature range (where there was data available). The cyclopropenoid fatty acids were also analysed in refined cottonseed oil and levels were also found to be comparable between MXB-13 cotton and the control, and within or below the literature range.

Aflatoxins were measured in cottonseed and were below 1.00 parts per billion (ppb) dry weight in both MXB-13 and control cottonseed compared to the literature range of less than 20 ppb.

Conclusions of the comparative analysis

The comparative analyses do not indicate that there are any compositional differences of biological significance in cottonseed from transgenic cotton line MXB-13, compared to the non-GM control. Several minor differences in key nutrients and other constituents were noted, however, the levels observed were generally within the range of natural variation for commercial cotton lines and do not indicate an overall pattern of change that would warrant further investigation. On the whole, it can be concluded that MXB-13 cottonseeds are equivalent in composition to non-GM cottonseeds.

NUTRITIONAL IMPACT

In assessing the safety and suitability of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

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

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

In the case of cotton line MXB-13, the extent of the compositional and other available data is considered to be adequate to establish the nutritional adequacy of the food.

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