FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

4-04
26 May 2004

DRAFT ASSESSMENT REPORT

APPLICATION A518

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

DEADLINE FOR PUBLIC SUBMISSIONS to FSANZ in relation to this matter:
21 July 2004
(See 'Invitation for Public Submissions' for details)

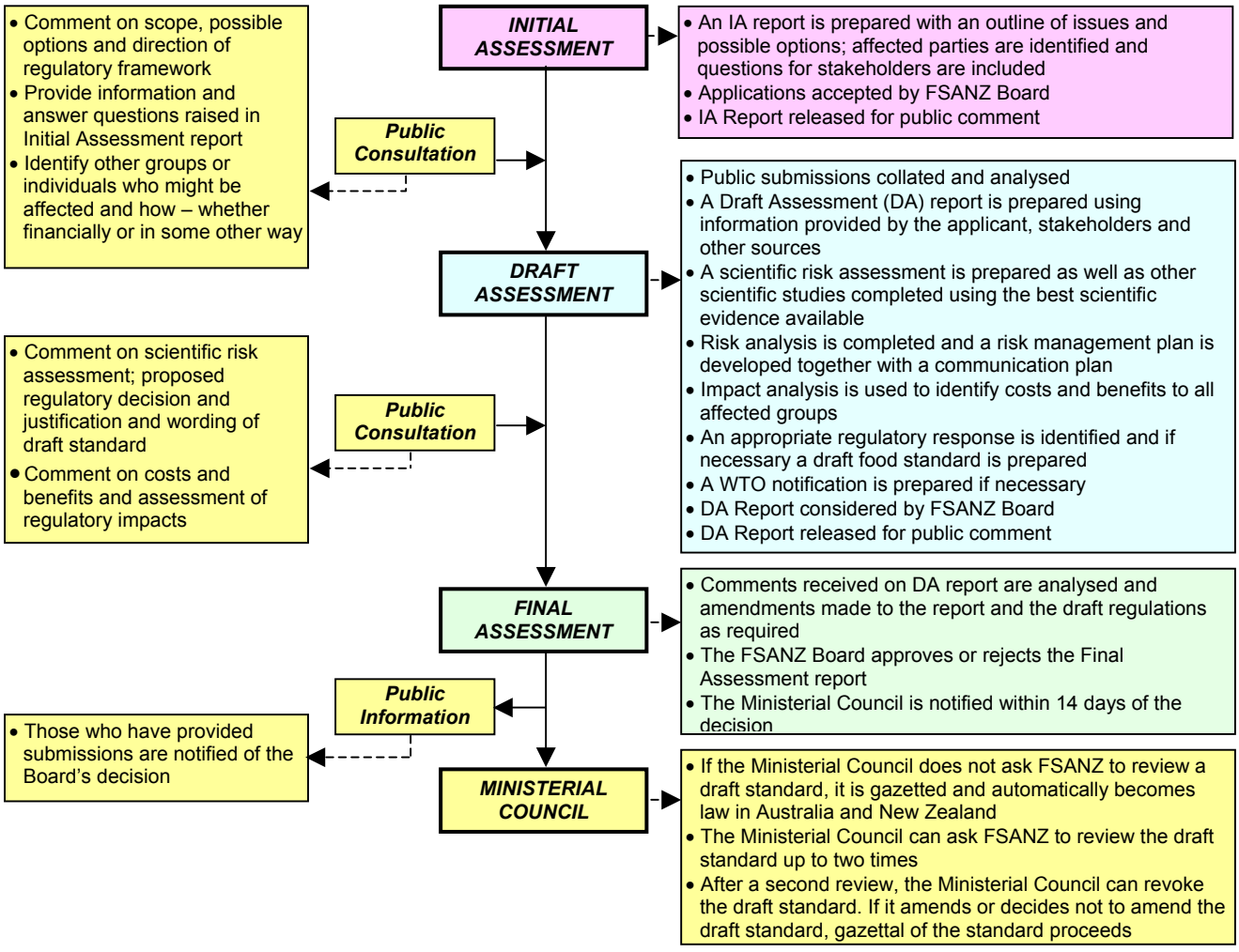
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

FSANZ's role is to protect the health and safety of people in Australia and New Zealand through the maintenance of a safe food supply. FSANZ is a partnership between ten Governments: the Commonwealth; Australian States and Territories; and New Zealand. It is a statutory authority under Commonwealth law and is an independent, expert body.

FSANZ is responsible for developing, varying and reviewing standards and for developing codes of conduct with industry for food available in Australia and New Zealand covering labelling, composition and contaminants. In Australia, FSANZ also develops food standards for food safety, maximum residue limits, primary production and processing and a range of other functions including the coordination of national food surveillance and recall systems, conducting research and assessing policies about imported food.

The FSANZ Board approves new standards or variations to food standards in accordance with policy guidelines set by the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) made up of Commonwealth, State and Territory and New Zealand Health Ministers as lead Ministers, with representation from other portfolios. Approved standards are then notified to the Ministerial Council. The Ministerial Council may then request that FSANZ review a proposed or existing standard. If the Ministerial Council does not request that FSANZ review the draft standard, or amends a draft standard, the standard is adopted by reference under the food laws of the Commonwealth, States, Territories and New Zealand. The Ministerial Council can, independently of a notification from FSANZ, request that FSANZ review a standard.

The process for amending the *Australia New Zealand Food Standards Code* is prescribed in the *Food Standards Australia New Zealand Act 1991* (FSANZ Act). The diagram below represents the different stages in the process including when periods of public consultation occur. This process varies for matters that are urgent or minor in significance or complexity.



INVITATION FOR PUBLIC SUBMISSIONS

FSANZ has prepared a Draft Assessment Report of Application A518; and prepared a draft variation to the *Australia New Zealand Food Standards Code* (the Code).

FSANZ invites public comment on this Draft Assessment Report based on regulation impact principles and the draft variation to the Code for the purpose of preparing an amendment to the Code for approval by the FSANZ Board.

Written submissions are invited from interested individuals and organisations to assist FSANZ in preparing the Final Assessment for this Application. Submissions should, where possible, address the objectives of FSANZ as set out in section 10 of the FSANZ Act. Information providing details of potential costs and benefits of the proposed change to the Code from stakeholders is highly desirable. Claims made in submissions should be supported wherever possible by referencing or including relevant studies, research findings, trials, surveys etc. Technical information should be in sufficient detail to allow independent scientific assessment.

The processes of FSANZ are open to public scrutiny, and any submissions received will ordinarily be placed on the public register of FSANZ and made available for inspection. If you wish any information contained in a submission to remain confidential to FSANZ, you should clearly identify the sensitive information and provide justification for treating it as commercial-in-confidence. Section 39 of the FSANZ Act requires FSANZ to treat in-confidence, trade secrets relating to food and any other information relating to food, the commercial value of which would be, or could reasonably be expected to be, destroyed or diminished by disclosure.

Submissions must be made in writing and should clearly be marked with the word 'Submission' and quote the correct project number and name. Submissions may be sent to one of the following addresses:

Food Standards Australia New Zealand
PO Box 7186
Canberra BC ACT 2610
AUSTRALIA
Tel (02) 6271 2222
www.foodstandards.gov.au

Food Standards Australia New Zealand
PO Box 10559
The Terrace WELLINGTON 6036
NEW ZEALAND
Tel (04) 473 9942
www.foodstandards.govt.nz

Submissions should be received by FSANZ **by 21 July 2004**.

Submissions received after this date may not be considered, unless the Project Manager has given prior agreement for an extension.

While FSANZ accepts submissions in hard copy to our offices, it is more convenient and quicker to receive submissions electronically through the FSANZ website using the Standards Development tab and then through Documents for Public Comment. Questions relating to making submissions or the application process can be directed to the Standards Management Officer at the above address or by emailing slo@foodstandards.gov.au.

Assessment reports are available for viewing and downloading from the FSANZ website. Alternatively, requests for paper copies of reports or other general inquiries can be directed to FSANZ's Information Officer at either of the above addresses or by emailing info@foodstandards.gov.au.

CONTENTS

EXECUTIVE SUMMARY AND STATEMENT OF REASONS 6

SAFETY ASSESSMENT 6
LABELLING 6
IMPACT OF REGULATORY OPTIONS 7
CONSULTATION 7
STATEMENT OF REASONS 7

1. INTRODUCTION 8

2. REGULATORY PROBLEM 8

3. OBJECTIVE 8

4. BACKGROUND 9

5. RELEVANT ISSUES 10

5.1 SAFETY ASSESSMENT OF FOOD FROM COTTON LINE MXB-13 10
5.2 LABELLING 10
5.3 ISSUES ARISING FROM PUBLIC SUBMISSIONS 10

6. REGULATORY OPTIONS 11

OPTION 1 – PROHIBIT FOOD FROM INSECT-PROTECTED, GLUFOSINATE AMMONIUM-TOLERANT COTTON LINE MXB-13 11
OPTION 2 – APPROVE FOOD FROM INSECT-PROTECTED, GLUFOSINATE AMMONIUM-TOLERANT COTTON LINE MXB-13 11

7. IMPACT ANALYSIS 11

7.1 AFFECTED PARTIES 11
7.2 IMPACT ANALYSIS 12

8. CONSULTATION 13

8.2 WORLD TRADE ORGANIZATION (WTO) 14

9. CONCLUSION AND RECOMMENDATION 14

10. IMPLEMENTATION AND REVIEW 14

ATTACHMENT 1: DRAFT VARIATION TO THE *AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE* 16

ATTACHMENT 2: DRAFT SAFETY ASSESSMENT REPORT 17

ATTACHMENT 3: SUMMARY OF PUBLIC SUBMISSIONS 55

Executive Summary and Statement of Reasons

An Application has been received from Dow AgroSciences Australia Pty. Ltd. to amend the *Australia New Zealand Food Standards Code* (the Code) to approve food derived from a genetically modified (GM) insect-protected, glufosinate ammonium-tolerant cotton, cotton line MXB-13 (cotton line MXB-13). Standard 1.5.2 – Food Produced using Gene Technology – requires that GM foods undergo a pre-market safety assessment before they may be sold in Australia and New Zealand. This is a cost recovered application.

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.

There is currently no approval for the sale and use of food (oil and linters) from cotton line MXB-13. If this Application is successful, FSANZ will amend the Code and insert a permission to use oil and linters from cotton line MXB-13 in the Table to clause 2 of Standard 1.5.2.

Cotton line MXB-13 has been developed for cultivation in North America and Australia. Therefore, if approved, food derived from cotton line MXB-13 could enter the food supply in Australia and New Zealand via domestically produced and imported products.

Public submissions are now invited on this Draft Assessment Report. Comments are specifically requested on the scientific aspects of this Application, in particular, information relevant to the safety assessment of food from cotton line MXB-13.

Safety assessment

FSANZ has completed a comprehensive safety assessment of food derived from cotton line MXB-13 as required under the *Food Standards Australia New Zealand Act 1999* (the Act). The assessment included consideration of: (i) the genetic modification to the plant; (ii) the safety of any transferred antibiotic resistance genes; (iii) the potential toxicity and allergenicity of any new proteins; and (iv) the composition and nutritional adequacy of the food, including whether there had been any unintended changes.

No potential public health and safety concerns were identified in the assessment of food derived from cotton line MXB-13. Therefore, on the basis of all the available evidence, including detailed studies provided by the Applicant, it has been concluded that food, namely oil and linters, derived from cotton line MXB-13 is as safe and wholesome as food derived from other cotton varieties.

Labelling

If approved, food derived from cotton line MXB-13 will require labelling if novel DNA and/or protein (refer to Standard 1.5.2 for the definition of novel DNA/protein) are present in the final food. The only food products derived from cotton are cottonseed oil and linters, neither of which contain DNA or protein.

Labelling addresses the requirement of section 10(1)(b) of the Act; provision of adequate information relating to food to enable consumers to make informed choices.

Impact of regulatory options

Two regulatory options were considered in the assessment: either (1) no approval; or (2) approval of oil and linters derived from cotton line MXB-13 based on the conclusions of the safety assessment. Following cost and benefit analysis of the potential impact of each of the options on the affected parties (consumers, the food industry and government), Option 2 is the preferred option as it potentially offers significant benefits to all sectors with very little associated cost. The proposed amendment to the Code, giving approval to food from cotton line MXB-13, is therefore considered of net benefit to both food producers and consumers.

Consultation

FSANZ made an Initial Assessment on this application and called for submissions on 17 December 2003. The closing date for submissions was 1 March 2004. Five submissions were received. A summary of submissions is at attachment 3. None of the submitters objected to the approval of food derived from cotton line MXB-13, however, one reserved comments until the release of the Draft Assessment Report.

Statement of Reasons

An amendment to the Code to give approval to the sale and use of food, namely oil and linters, derived from cotton line MXB-13 in Australia and New Zealand is recommended on the basis of the available scientific evidence for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce cotton line MXB-13;

- food derived from cotton line MXB-13 is equivalent to food from other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;

- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the amendment to the Code is of net benefit to both food producers and consumers; and

- the proposed draft variation to the Code is consistent with the section 10 objectives of the *Food Standards Australia New Zealand Act 1991* (FSANZ Act) and the regulatory impact assessment.

It is proposed that the draft variation come into effect on the date of gazettal.

1. Introduction

An Application was received from Dow AgroSciences Australia Pty Ltd on 6 November 2003 seeking approval for food derived from insect-protected, glufosinate ammonium-tolerant cotton line MXB-13 (cotton line MXB-13) under Standard 1.5.2 - Food Produced Using Gene Technology - in the *Australia New Zealand Food Standards Code* (the Code).

The genetic modification involved the transfer of the following bacterial genes into the cotton plant:

- the *cry1Ac* and *cry1F* genes from *Bacillus thuringiensis* subspecies *kurstaki* and *aizawai* respectively, which express the insect-specific protein δ endotoxins Cry1Ac and Cry1F; and

- the phosphinothricin-acetyltransferase gene, *pat,* from *Streptomyces viridochromogenes*, expressing the enzyme phosphinothricin-acetyltransferase (PAT) which confers tolerance to the herbicide glufosinate ammonium.

Draft Assessment of the Application, including a detailed safety assessment of food derived from cotton line MXB-13, has been completed and FSANZ has prepared a draft variation to Standard 1.5.2 of the Code (see attachment 1). Public comment is now being sought to assist in the Final Assessment of the Application.

2. Regulatory Problem

Standard 1.5.2 requires that a GM food undergo a pre-market safety assessment before it may be sold in Australia and New Zealand. Foods that have been assessed under the Act, once fully approved, are listed in the Table to clause 2 of Standard 1.5.2.

Dow AgroSciences Australia Pty Ltd has developed a new GM variety of cotton, known as cotton line MXB-13. Before food derived cotton line MXB-13 can enter the food supply in Australia and New Zealand it must first be assessed for safety and an amendment to the Code must be approved by the FSANZ Board, and subsequently be notified to the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council). An amendment to the Code may only be gazetted, once the Ministerial Council process has been finalised.

Dow AgroSciences Australia Pty Ltd has therefore applied to have Standard 1.5.2 amended to include oil and linters derived from cotton line MXB-13.

3. Objective

The objective of this Application is to determine whether it is appropriate to amend the Code to approve the use of food derived from cotton line MXB-13. In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives, which are set out in section 10 of the FSANZ Act. These are:

- the protection of public health and safety;
- the provision of adequate information relating to food to enable consumers to make informed choices; and

- the prevention of misleading or deceptive conduct.

In developing and varying standards, FSANZ must also have regard to:

- the need for standards to be based on risk analysis using the best available scientific evidence;
- the promotion of consistency between domestic and international food standards;
- the desirability of an efficient and internationally competitive food industry;
- the promotion of fair trading in food; and
- any written policy guidelines formulated by the Ministerial Council.

4. Background

Cotton line MXB-13 contains two insecticidal genes (cry1Ac and cry1F), 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 MXB9) and these two traits were subsequently combined by crossing the two GM cotton lines to produce cotton line MXB-13.

The main purpose of the genetic modification is to confer protection against the cotton bollworm (*Heliothis zea*), tobacco budworm (*H. virescens*) and pink bollworm (*Pectinophora gossypiella*). Using two *Bacillus thuringiensis* derived insecticidal proteins 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, cotton line MXB-13 contains two copies of a selectable marker gene (*pat*) from the bacterium *Streptomyces viridochromogenes*, which produces an enzyme (phosphinothricin acetyltransferase, (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.

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

Cotton line MXB-13 is being developed for cultivation in Australia and the USA. The Applicant has applied to the Office of the Gene Technology Regulator (OGTR) for a licence for field trials of this cotton (DIR 40/2003). This application is currently being assessed by the OGTR. It is intended that none of the plants produced during the field trials will enter the human food chain.

In addition, an application to permit the use of cotton line MXB-13 for food and feed use in the United States has been submitted to the USDA, the US EPA and the FDA.

5. Relevant Issues

5.1 Safety assessment of food from cotton line MXB-13

Food from cotton line MXB-13 has been evaluated according to the safety assessment guidelines prepared by FSANZ[1]. The safety assessment included the following:

- a detailed characterisation of the genetic modification to the plant;
- a consideration of the safety of any transferred antibiotic resistance genes;
- characterisation of any novel proteins, including their potential toxicity and allergenicity;
- a consideration of the composition and nutritional adequacy of the food, including whether there had been any unintended changes to the food.

The Applicant submitted a comprehensive data package in support of their application and provided studies on the molecular characterisation of cotton line MXB-13, the potential toxicity and allergenicity of Cry1Ac, Cry1F and PAT, and compositional analyses of cottonseed from cotton line MXB-13. In addition to information supplied by the applicant, the evaluation also had regard to other available information and evidence, including from the scientific literature, general technical information, other regulatory agencies and international bodies.

No potential public health and safety concerns were identified in the assessment of food (oil and linters) derived from cotton line MXB-13. Therefore, on the basis of all the available evidence, including detailed studies provided by the Applicant, it has been concluded that food derived from cotton line MXB-13 is as safe and wholesome as food derived from other cotton varieties. The full safety assessment report is at **Attachment 2** to this document.

5.2 Labelling

Under Standard 1.5.2, GM food or ingredients must be labelled if novel DNA and/or protein are present in the final food and also where the food has altered characteristics.

No novel protein is present in the refined cottonseed oil and linters, the only two food products derived from cotton. It is unlikely that novel DNA would be present either. If this is the case, then cottonseed oil and linters will not be required to be labelled as containing GM ingredients.

5.3 Issues arising from public submissions

In addition to the specific issues addressed below, FSANZ has also developed a Fact Sheet: *Frequently Asked Questions on Genetically Modified Foods – August 2002*, which responds to many of the general issues raised in connection with GM foods. The Fact Sheet may be obtained from the FSANZ website[2].

[1] http://www.foodstandards.gov.au/_srcfiles/ACF6A6.pdf
[2] www.foodstandards.gov.au/mediareleasespublications/factsheets/factsheets2002/index.cfm

5.3.1 Impact on Government resources / enforcement of mandatory labelling requirements

Queensland Health queried the impact analysis for option 2 where it is stated that there would be no direct impact on government and that a decision to take option 2 would be unlikely to impact on monitoring resources. It considered that costs incurred in GM testing is expensive and each new GM food approval adds to this cost as reference laboratories need to purchase marker genes for the new product and test accordingly.

The Department of Agriculture, Fisheries and Forestry mentioned that regulatory agencies have experienced difficulties related to the enforcement of the mandatory labelling requirements of Standard 1.5.2

5.3.1.1 Response

Under Standard 1.5.2, GM food must be labelled if novel DNA and/or protein are present in the final food and where the food has altered characteristics. Cottonseed oil and linters, the only food products derived from cotton that are consumed in Australia and New Zealand, are highly refined products and have not been found to contain either novel DNA or protein. Food derived from cotton line MXB-13 is not likely to require labelling and therefore there are no additional costs anticipated for government.

6. Regulatory Options

Option 1 – prohibit food from insect-protected, glufosinate ammonium-tolerant cotton line MXB-13

Maintain the *status quo* by not amending the Code to approve the sale and use in food of oil and linters derived from cotton line MXB-13.

Option 2 – approve food from insect-protected, glufosinate ammonium-tolerant cotton line MXB-13

Amend the Code to permit the sale and use in food of oil and linters derived from cotton line MXB-13, with or without listing special conditions in the Table to clause 2 of Standard 1.5.2.

7. Impact Analysis

7.1 Affected parties

- consumers, particularly those who have concerns about biotechnology;
- food importers and distributors of wholesale ingredients;
- The manufacturing and retail sectors of the food industry; and
- Government generally, where a regulatory decision may impact on trade or WTO obligations and enforcement agencies in particular who will need to ensure that any approved products are correctly labelled.

The cultivation of cotton line MXB-13 may have an impact on the environment, which would need to be assessed by the Office of the Gene Technology Regulator (OGTR) before cultivation in Australia could be permitted. The Applicant has indicated that they do intend to undertake field trials of cotton line MXB-13 in Australia in the future and have applied for a license from the OGTR to do so.

Cotton is not grown in New Zealand, However, if planting in New Zealand ever became likely, a comprehensive environmental risk analysis would be required by various New Zealand government agencies including as the Environmental Risk Management Authority (ERMA) and the Ministry of Agriculture and Fisheries (MAF) in New Zealand.

7.2 Impact Analysis

In the course of developing food regulatory measures suitable for adoption in Australia and New Zealand, FSANZ is required to consider the impact of all options on all sectors of the community, including consumers, the food industry and governments in both countries. The regulatory impact assessment identifies and evaluates, though is not limited to, the costs and benefits of the regulation, and its health, economic and social impacts.

The following is a draft assessment by FSANZ of the costs and benefits of the two regulatory options identified so far. This is based on information supplied by the applicant and experience FSANZ has gained from consideration of previous applications relating to GM foods. Your comments are also invited on the costs and benefits identified for the options below.

7.2.1 Option 1

Consumers: Cost in terms of a possible reduction in the availability of certain food products (loss of potential new products).

Cost associated with higher retail prices for segregated foods.

No impact on consumers wishing to avoid GM foods, as food derived from cotton line MXB-13 is not currently permitted in the food supply.

Government: No immediate impact.

Potential impact if considered inconsistent with WTO obligations but impact would be in terms of trade policy rather than in government revenue.

Industry: Cost in terms of restricting innovation in food/crop production for both growers and other sectors of the food industry. Cost to the food industry to source either segregated or non-GM supplies.

Potential longer-term impact - any successful WTO challenge has the potential to impact adversely on food industry.

7.2.2 Option 2

Consumers: Possible benefit of lower prices, to the extent that savings from production efficiencies are passed on.

Benefit of access to a greater range of products including imported food products containing ingredients derived from cotton line MXB-13.

Cost to consumers wishing to avoid GM food by a potential restriction of choice of products, or increased prices for non-GM food.

Government: No direct impact.

This decision is unlikely to impact on monitoring resources.

Industry: Possible benefit to growers in lower production costs and reduced exposure to agricultural chemicals used to manage insect pests.

Benefit to importers and distributors of overseas food products as the product range is extended.

Benefit for food manufacturers in that the choice of raw ingredients is extended.

Benefit to food retailers in an increased product range.

7.2.3 Discussion

Option 1 would impose significant costs, particularly on consumers and the food industry sector, without offering any commensurate health benefit. This option is also likely to be inconsistent with Australia's and New Zealand's obligations under the WTO. This option would also offer very little benefit to those consumers wishing to avoid GM foods, as food from other GM cotton varieties is already permitted in the food supply.

Option 2 is the preferred option as it potentially offers significant benefits to all sectors with very little associated negative impact.

The proposed amendment to the Code, giving approval to food derived from cotton line MXB-13, is therefore considered necessary, cost effective and of net benefit to both food producers and consumers.

8. Consultation

The Initial Assessment of this Application was advertised for public comment between 17 December 2003 and 1 March 2004. A total of 5 submissions were received during this period and a summary of these is included in **Attachment 3** to this Report.

FSANZ carried out an assessment of the Application, including a safety assessment of the food, taking into account the comments received in the first round of consultation. These issues have been addressed in section 5.3 above. No specific issues relating to the food safety of cotton line MXB-13 were raised in the public submissions.

8.2 World Trade Organization (WTO)

As members of the World Trade Organization (WTO), Australia and New Zealand are obligated to notify WTO member nations where proposed mandatory regulatory measures are inconsistent with any existing or imminent international standards and the proposed measure may have a significant effect on trade.

There are no relevant international standards for GM foods, however, the proposed amendment to the Code to allow food derived from cotton line MXB-13 may be of interest to other WTO member nations because it pertains to the safety of GM food and is likely to have a liberalising effect on international trade.

For these reasons, FSANZ will be recommending to the agencies responsible that the WTO be notified under the Sanitary and Phytosanitary Measure (SPS) Agreements, in order to enable other member nations to comment on the proposed changes to standards that may have a significant impact on them

9. Conclusion and Recommendation

An amendment to the Code to give approval to the sale and use of food, namely oil and linters, from cotton line MXB-13 in Australia and New Zealand is recommended on the basis of the available scientific information for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce cotton line MXB-13;
- food derived from cotton line MXB-13 is equivalent to food from other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the amendment to the Code is necessary, cost effective and of net benefit to both food producers and consumers; and
- the proposed draft amendment to the Code is consistent with the section 10 objectives of the FSANZ Act and the regulatory impact assessment.

The proposed draft variation is provided in **Attachment 1**.

10. Implementation and review

It is proposed that the draft variation come into effect on the date of gazettal.

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Draft safety assessment report
3. Submission summary

ATTACHMENT 1

DRAFT VARIATION TO THE *AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE*

To commence: on gazettal

[1] ***Standard 1.5.2*** *of the Australia New Zealand Food Standards Code is varied by inserting into* Column 1 *of the* Table to clause 2 –

Oil and linters derived from insect-protected, glufosinate ammonium-tolerant cotton line MXB-13

ATTACHMENT 2

DRAFT SAFETY ASSESSMENT REPORT

APPLICATION A518 – FOOD DERIVED FROM INSECT-PROTECTED, GLUFOSINATE AMMONIUM-TOLERANT COTTON LINE MXB-13.

SUMMARY AND CONCLUSIONS

Background

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. Therefore, if approved, food derived from cotton line MXB-13 may enter the Australian and New Zealand food supply as imported food products or from cotton grown in Australia (if a licence is granted for commercial release in 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. Cottonseed oil, the major product of cottonseed, has been consumed by humans for decades. Cottonseed oil is considered to be a premium quality oil, valued for its high unsaturated fatty acid content. The other food use of cottonseed is the linters, which are composed of greater than 99% cellulose. Cottonseed itself and the meal fraction are not 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 phosphinothrycin 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 data provided in the present application, and other available information, food produced from cotton line MXB-13 can be considered as safe and as wholesome as food produced from other cotton varieties.

1. INTRODUCTION

Dow AgroSciences Pty. Ltd. has submitted an application to Food Standards Australia New Zealand (FSANZ) to vary Standard 1.5.2 – Food Produced Using Gene Technology – in the Australian New Zealand Food Standards Code, to include food from a new genetically modified (GM) cotton variety. The GM cotton variety is known commercially as MXB-13 or 'Widestrike cotton'.

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) and these two traits were subsequently 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 acetyltransferase, 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.

Cotton line MXB-13 is being developed for cultivation in Australia and the United States. The Office of the Gene Technology Regulator (OGTR) has granted the applicant a licence for field trials of this cotton in Australian (DIR40/2003). None of the plants produced during the field trials will enter the human food chain. Cotton is not grown in New Zealand.

In addition, applications to permit the use of cotton line MXB-13 for food and feed use in the US have been submitted to the Department of Agriculture, the Environment Protection Agency and the Food and Drug Administration. If approved, food from cotton line MXB-13 may therefore enter the Australian and New Zealand food supply as imported food products or from cotton grown in Australia.

2. HISTORY OF USE

2.1 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.

2.2 Host Organism

Gossypium hirsutum L.

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.

3. DESCRIPTION OF THE GENETIC MODIFICATION

3.1 Method used in the genetic modification

Studies submitted

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

3.2 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 gene based on the native *cry*1F gene. It is the coding sequence for a Cry1F-based synthetic protoxin, referred to as Cry1F (synpro). This chimeric full length δ-endotoxin is comprised of sequences encoding the first 604 amino acids of the insecticidal protein Cry1Fa2 from *B. thuringiensis* var. *aizwai*, and 544 amino acids from the non-toxic portions of Cry1Ca3 (amino acids 605-640) and Cry1Ab1 (amino acids 641-1148) proteins. 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 pTi5955.

*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 1-1844 of the coding sequence encode the toxic portion of Cry1Ac1. Nucleotides 1845-1951, and 1952-3481 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 pTi16966.

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)	3.48	Synthetic, plant optimised, full-length version of *cry*1F from *B.t.* Nucleotides 1-1844 of the coding sequence encode the toxic portion of Cry1Ac1. Nucleotides 1845-1951 encode a portion of the Cry1C protoxin. Nucleotides 1952-3481 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
ORF25 polyA (2 copies)	0.72	Bidirectional terminator from *Agrobacterium tumefaciens*.
pat (2 copies)	0.55	The synthetic plant optimized glufosinate ammonium resistance gene, based on a phosphinothrycin acetlytransferase 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.

3.3 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 submitted:

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

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).

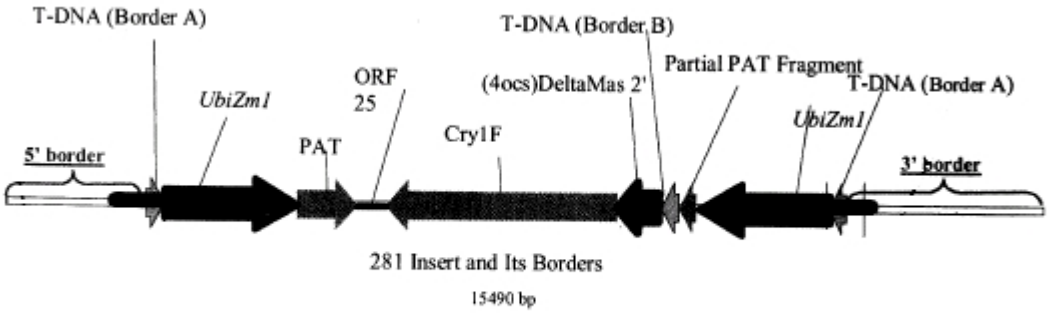


Figure 1: The complete insert and flanking genomic regions of event 281-24-236 in cotton line MXB-13.

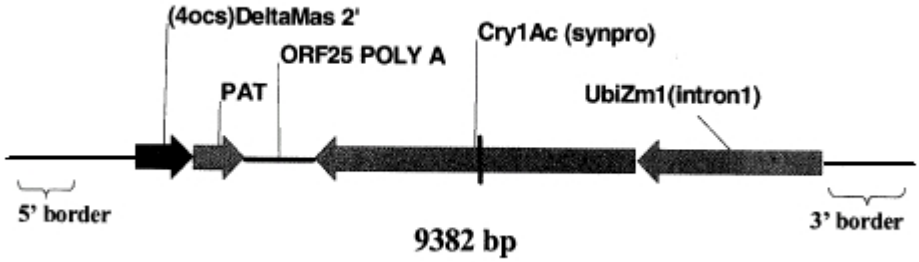


Figure 2: The complete insert and flanking genomic regions of event 3006-210-23 in cotton line MXB-13.

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 back-crossing, 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 submitted:
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

3.5 Antibiotic resistance genes

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

4. CHARACTERISATION OF NOVEL PROTEINS

4.1 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 insectidal 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 acetyltransferase (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).

4.2 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 submitted:
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 is such cases as there is less opportunity for negative interaction between the two inserts. The Applicant reports that expression of the two *cry* genes was 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.

4.3 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 in previous applications (A341, A372, A375, A380, A386, A436, A446 and A481) and found to be safe for human consumption. In addition, the applicant submitted three further acute oral toxicity studies in mice to further support the safety of these proteins.

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 submitted:

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.

One male mouse had a mechanical injury to the left hind limb, which was thought to be due to the implantation of a transponder and was not treatment related. One female mouse had a moderate increase in reactivity to handling on day 3. Because this was an isolated occurrence (one animal only on one day), it was considered to be unrelated to treatment. No other clinical signs were observed throughout the remainder of the study.

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 externum 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 (applications A372, A375, A386 and A481) 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 Submitted:
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[3] 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.

[3] 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.

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 E of <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 has E 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.

4.4 Potential allergenicity of novel proteins

A possible 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 submitted:
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 submitted

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 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 submitted:
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 LLC, Indianapolis, Indiana. Study Id. 010069

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

4.5 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.

5. 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.

5.1 Nutrient analysis

Study submitted

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 was 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 (2003; 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

5.2 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.

5.3 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.

6. 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.

REFERENCES

An, A.Q., McDowell, J.M., Huang, S., McKinney, E.C., Chambliss, S., Meagher, R.B. (1996) Strong constitutive expression of the Arabidopsis ACT2/ACT8 actin subclass in vegetative tissue. *Plant J.* 10 (1): 107-121.

Astwood, J.D. and Fuchs, R.L. (1996). Allergenicity of foods derived from transgenic plants. *In* Ortolani, C. and Wuthrich, B. (eds.) *Highlights in food allergy. Monographs in Allergy*, 32: 105-120.

Berardi, L.C. and Goldblatt, L.A. (1980) In *Toxic Constituents of Plant Foodstuffs*, 2nd Ed.; Liener, I.I., Ed.; Academic Press: New York, pp 211-266.

Berberich, S.A., Ream, J.E., Jackson, T.L., Wood, R., Stipanovic, R., Harvey, P., Patzer, S. and Fuch, R.L. (1996), The Composition of Insect-Protected Cottonseed is Equivalent to That of Conventional Cottonseed. J. Agric. Food Chem. 44, 365-371.

Betz, F.S., Hammond, B.G. and Fuchs, R.L. (2000) Safety and Advantages of *Bacillus thuringiensis*-Protected Plants to Control Insect Pests. Regulatory Toxicology and Pharmacology, 32, 156-173.

Bevan, M., Barnes, W.M., Chilton, M.D. (1983) Structure and transposition of the nopaline synthase gene region of T-DNA. *Nucleic Acids Res.* 11:369-385.

Bogosian, G. and Kane, J.F. (1991). Fate of recombinant Escherichia coli K-12 strains in the environment. In: *Advances in Applied Microbiology*, Volume 36, Neidleman, S. and Laskin, A. (eds). Academic Press, San Diego, pp 87 – 131.

Chambers, J.A., Jelen, A., Pearce Gilbert, M., Jany, C.S., Johnson, T.B. and Gawron-Burke, C. (1991). Isolation and Characterisation of a Novel Insecticidal Crystal Protein Gene from *Bacillus thuringiensis* subsp. *aizawai*. Journal of Bacteriology 173(13), 3966-3976.

CODEX (2001) Codex *Ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology, 2001. Report of the Working Group on Allergenicity. Vancouver Canada.

CODEX (2001) Codex Standard for Named Vegetable oils. CX-STAN 210 – 1999. Codex Alimentarius, Vol 8, http://www.mvo.nl/voeding-en-gezondheid/spvet/download/named-vegetable-oils.pdf.

Cotton Research and Development Corporation (CRDC). 2001. http://www.crdc.com.au/

Cottonseed Oil, National Cottonseed Products Association: Memphis, TN, 1990.

Cottonseed Feed Product Guide, National Cottonseed Products Association: Memphis, TN.

Ensminger, A.H., Ensminger, M.E., Konlande, J.E. and Robson, J.R.K. (1994). *Foods and Nutrition Encyclopedia,* 2nd edition. Ann Harbour, MI 1: 497-507.

EPA. 1998. RED Facts: *Bacillus thuringiensis* EPA 738-F-98-001.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., Koziel, M.G. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc. Natl. Acad. Sci. USA* 93:5389-5394

FAO (1996). *Biotechnology and food safety*. A report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper 61, Food and Agriculture Organization of the United Nations, Rome.

Feitelson, J.S., Payne, J. and Kim, L. (1992). *Bacillus thuringiensis*: insects and beyond. *Biotechnology* **10:** 271 – 275.

Feitelson, J.S. (1993). The *Bacillus thuringiensis* family tree. In: *Advanced Engineered Pesticides*, Kim, L. (ed). Marcel Dekker, Inc., New York, pp 63 – 83.

Forster, L.A. and Calhoun, M.C. (1995) Nutrient Values for Cottonseed Products Deserve New Look. Feedstuffs, Vol 67 No 44.

Franck, A.W. (1989). Food uses of cottonseed protein. In: *Development in Food Proteins – 5*. New York, pp31-80.

Hofte, H. and Whitely, H.R. 1989 Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* 53: 242-255.

IPCS WHO International Programme on Chemical Safety (2000). *Bacillus thuringiensis*. Environmental Health Criteria of the International Programme on Chemical Safety, No 217. http://www.who.int/pcs/ehc/summaries/ehc_217.html.

Jones, D.D. and Maryanski, J.H. (1991). Safety considerations in the evaluation of transgenic plants for human food. In: Levin MA and Strauss HS (eds) Risk assessment in genetic engineering. New York: McGraw-Hill.

Jones, L. and King, C. (eds). (1990). *Cottonseed Oil*. National Cottonseed Products Associations, Inc. and The Cotton Foundation, Memphis, TN, USA.

Jones, L. and King, C. (eds). (1993). *Cottonseed Oil*. National Cottonseed Products Associations, Inc. and The Cotton Foundation, Memphis, TN, USA.

Kaster, K.R., Burgett, S.G., Rao, R.N., Ingolia, T.D. (1983) Analysis of an bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. *Nucleic Acids Res.* 11(19), 6895-6911.

Kimber, I., Kerkvliet, N.I., Taylor, S.L., Astwood, J.D., Sarlo, K. and Dearman, R.J. (1999). Toxicology of protein allergenicity: prediction and characterisation. *Toxicological Sciences* 48: 157-162.

Lehrer, S.B. and Reese, G. (1998). Food allergens: implications for biotechnology. In: Thomas JA (ed.) Biotechnology and safety assessment. Taylor and Francis, Philadelphia.

McClintock, JT, CR Schaffer and RD Sjoblad. (1995). A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. 45:95-105.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. and Fuchs, R.L. (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* 36(S): S165-S186.

Murray, E.E., Lotzer, J., Eberle, M. (1989) Codon usage in plant genes. *Nucl Acid Res*. 17:477-498.

Nester, E.W., Thomashow, L.S., Metz, M. and Gordon, M. (2002). 100 years of *Bacillus thuringiensis*: A critical scientific assessment. A report from the American Academy of Microbiology based on a colloquium held on 16-18 November, 2001, in Ithaca, New York.

Nikokyris, P., Kandylis, K., Deligiannis, K. and Liamadis, D. 1991 Effects of gossypol content of cottonseed cake in the blood constituents in growing-fattening lambs. *J Dairy Sci* 74: 4305-4313

Norris, S.R., Meyer, S.E., Callis, J. (1993) The intron of Arabidopsis thaliana poly-unbiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. *Plant Molecular Biol*. 21 (5):895-906.

OGTR (2002) The biology and ecology of cotton (*Gossypium hirsutum*) in Australia. http://www.ogtr.gov.au/rtf/ir/biologycotton.rtf.

Poore, M. and Rogers, G.M. 1998 Potential for gossypol toxicity when feeding whole cottonseed. Department of Animal Science, North Carolina State University, USA. http://www.cals.ncsu.edu/an_sci/extension/animal/nutr/mhp95-1.htm

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. 1998 *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62: 775-806

Sjoblad, R.D., McClintock, J.T. and Engler, R. (1992) Toxicological considerations for protein components of biological pesticide products. *Regulatory Toxicol. Pharmacol.* 15: 3-9.

Tinland, B. and Hohn, B. (1995) Recombination between Procaryotic and Eukaryotic DNA: Tinegration of *Agrobacterium tumefaciens* T-DNA into the plant genome. *Genetic Engineering*. 17:209-229.

Waldron, C. (1997) United States Patent No. 5,668,298. Selectable market for development of vectors and transformation systems in plants.

WHO 1993 Health aspects of marker genes in genetically modified plants. Report of a WHO Workshop. World Health Organization, Geneva, 32 pp.

WHO (2000). *Safety aspects of genetically modified foods of plant origin*. Report of a Joint FAO/WHO Expert Consultation, World Health Organization, Geneva.

Attachment 3

Summary of Public Submissions

Submitter	Preferred Option	Comments
1. Australian Food and Grocery Council	2	-
2. Department of Agriculture Fisheries and Forestry	2	Regulatory agencies have reported difficulties related to enforcement of the mandatory labelling requirements of Standard 1.5.2
3. Food Technology Association Vic	2	-
4. New Zealand Food Safety Authority	-	Will provide comments on the safety assessment report at the draft assessment stage
5. Queensland Health	-	Comments on the added cost to government for each new GM approval as reference laboratories need to purchase marker genes for the new product and test accordingly.