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

17 December 2008
[20-08]

FINAL ASSESSMENT REPORT

APPLICATION A615

FOOD DERIVED FROM INSECT-PROTECTED COTTON LINE COT67B

For Information on matters relating to this Assessment Report or the assessment process generally, please refer to http://www.foodstandards.gov.au/standardsdevelopment/

Executive Summary

Food Standards Australia New Zealand (FSANZ) received a paid Application from Syngenta Seeds Pty Ltd (the Applicant) on 27 September 2007. The Applicant has requested an amendment to the *Australia New Zealand Food Standards Code* (the Code), specifically to Standard 1.5.2 – Food produced using Gene Technology, to permit the sale and use of food derived from a new genetically modified (GM) variety of cotton, COT67B. Standard 1.5.2 prohibits a food produced using gene technology from being sold or used as an ingredient or component of any food unless it is listed in the Table to clause 2 of that Standard.

COT67B cotton has been genetically modified to be protected against feeding damage caused by the larvae of certain insect pest species. Protection is achieved through the expression in the plant of an insecticidal protein derived from *Bacillus thuringiensis*, a common soil bacterium.

COT67B cotton is intended initially for cultivation in the United States of America but may also be grown in Australia at a later date. Food from COT67B cotton is therefore expected to initially enter the Australian and New Zealand food supply via imported products.

Safety Assessment

FSANZ has completed a comprehensive safety assessment of food derived from insect-protected cotton line COT67B. The assessment included consideration of (i) the genetic modification to the plant; (ii) the potential toxicity and allergenicity of the novel proteins; and (iii) the composition of COT67B cotton compared with that of conventional cotton varieties.

No public health and safety concerns were identified as a result of the safety assessment. On the basis of the available evidence, including detailed studies provided by the Applicant, food derived from insect-protected cotton line COT67B is considered as safe and wholesome as food derived from other commercial cotton varieties.

Labelling

If approved, food derived from insect-protected cotton line COT67B will be required to be labelled as genetically modified if novel DNA and/or novel protein is present in the final food. Studies undertaken by the Applicant indicate detectable levels of novel protein in linters and cottonseed meal, but not in refined cottonseed oil.

Labelling addresses the requirement of section 18(1)(b) of the *Food Standards Australia New Zealand Act 1991* (FSANZ 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: (1) no approval, or (2) approval of food derived from insect-protected cotton line COT67B based on the conclusions of the safety assessment.

Following analysis of the potential costs and benefits of each option on affected parties (consumers, the food industry and government), approval of this application is the preferred option as the potential benefits to all sectors outweigh the costs associated with the approval.

Purpose

The Applicant seeks amendment to Standard 1.5.2, to include food derived from insect-protected cotton line COT67B in the Table to clause 2.

Decision

Amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from insect-protected cotton line COT67B in the Table to clause 2.

Reasons for Decision

An amendment to the Code approving food derived from insect-protected cotton line COT67B in Australia and New Zealand is approved 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 insect-protected cotton line COT67B;
- food derived from insect-protected cotton line COT67B is equivalent to food from the conventional counterpart and other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain food commodities derived from insect-protected cotton line COT67B will be required if novel DNA and/or protein is present in the final food; and
- 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 preferred option is option 2, an amendment to the Code.

Consultation

The Initial Assessment was advertised for public comment between 12 December 2007 and 6 February 2008; eleven submissions being received. The Draft Assessment was advertised for public comment between 6 August 2008 and 17 September 2008; eighty-two submissions were received. A summary of these is provided in **Attachment 3** to this Report. The majority of second round submissions were campaign notices calling for process-labelling of all GM foods.

FSANZ has taken submitters' comments into account in preparing the Final Assessment Report. Specific issues relating to insect-protected cotton line COT67B have been addressed in the Report.

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INTRODUCTION

An Application was received from Syngenta Seeds Pty Ltd on 27 September 2007 seeking an amendment to Standard 1.5.2 – Food produced using Gene Technology, in the *Australia New Zealand Food Standards Code* (the Code), to approve food derived from insect-protected cotton line COT67B.

The genetic modification involved the transfer of the *cry1Ab* gene into cotton. This gene is derived from a common soil bacterium called *Bacillus thuringiensis* and encodes an insecticidal protein (a Cry protein) which protects the plant against feeding damage caused by certain insect pest larvae.

A Final Assessment of the Application has been completed, including a comprehensive safety assessment and consideration of issues raised in public consultation.

1. Background

1.1 Current Standard

Standard 1.5.2 prohibits a food produced using gene technology from being sold or used as an ingredient or component of any food unless it is listed in the Table to clause 2 of that Standard.

1.2 Description and Purpose of the Genetic Modification

The genetic modification in insect-protected cotton line COT67B involves the introduction of the *cry1Ab* gene derived from *B. thuringiensis* subspecies *kurstaki* HD-1. This gene encodes the Cry1Ab insecticidal protein which provides protection against two key cotton pests: *Helicoverpa zea*, cotton bollworm; and *Heliothis virescens*, tobacco budworm.

Cry proteins exert their effect on the target insects by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the insect. The lysis of the midgut epithelial cells is mediated by the binding of the activated Cry protein to specialised receptors on these cells.

Cotton lines containing the COT67B transformation event are intended initially for cultivation in the United States of America but may also be grown in Australia at a later date. Food from COT67B cotton is therefore expected to initially enter the Australian and New Zealand food supply via imported products.

1.3 Overseas Approvals

A permanent tolerance exemption for Cry1Ab in all crops was established by the US Environment Protection Agency on August 2, 1996 and is published in the Code of Federal Regulations (40 CFR 180.1173).

The Applicant submitted a request to the US Department of Agriculture (USDA) on 18 April 2007 for a determination of nonregulated status for COT67B, any progeny derived from crosses between COT67B and conventional cotton varieties and any progeny derived from crosses of COT67B with other genetically modified cotton that has also been granted nonregulated status under 7 CFR part 340.6. A draft Letter of Completeness for the COT67B Petition was issued by the USDA on June 19 2007.

The Applicant also made a submission to the US Food and Drug Administration (US FDA) on 3 July 2007. A decision by the US FDA is expected in January 2009.

Regulatory submissions for import and production clearances will be made to Japan, Canada, and Mexico.

2. The Issue / Problem

The Applicant has developed a GM cotton line called COT67B that is protected from feeding damage caused by lepidopteran insect pest larvae. Before food derived from insect-protected cotton line COT67B can enter the Australian and New Zealand food supply, it must first be assessed for safety and an amendment to the Code must be approved by the FSANZ Board, and the decision subsequently 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.

Syngenta Seeds Pty Ltd therefore applied to have Standard 1.5.2 amended to include food derived from cotton line COT67B.

3. Objectives

The purpose of this assessment is to determine whether it would be appropriate to vary the Code to approve the sale and use of food derived from cotton line COT67B under Standard 1.5.2.

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives which are set out in section 18(1) 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.

Section 18(2) requires FSANZ to 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. Key Assessment Questions

Based on information provided by the Applicant on the nature of the genetic modification, the molecular characterisation, the characterisation of the novel proteins, the compositional analysis and any nutritional issues, is food derived from cotton line COT67B comparable to food derived from conventional varieties of cotton in terms of its safety for human consumption?

Is other information available, including from the scientific literature, general technical information, independent scientists, other regulatory agencies and international bodies, and the general community, that needs to be considered?

Are there any other considerations that would influence the outcome of this assessment?

RISK ASSESSMENT

Food from insect-protected cotton line COT67B has been evaluated according to the FSANZ Guidance Document on the Safety Assessment of Genetically Modified Foods[1]. The summary and conclusions from the safety assessment report (at **Attachment 2**) are presented below. In addition to information supplied by the Applicant, other available resource material including published scientific literature and general technical information was used for the assessment.

5. Risk Assessment Summary

5.1 Safety Assessment Process

The safety assessment applied to food from cotton line COT67B addresses only food safety and nutritional issues. It therefore does not address: environmental risks related to the environmental release of genetically modified (GM) plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants; or the safety of food derived from the non-GM (conventional) plant.

In conducting a safety assessment of food derived from insect-protected cotton line COT67B, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the cotton genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

[1] http://www.foodstandards.gov.au/_srcfiles/GM%20FINAL%20Sept%2007L%20_2_.pdf

5.2 Outcomes of the Safety Assessment

COT67B cotton was generated through the transfer of the full length *cry1Ab* gene (*flcry1Ab*) to the conventional cotton line Coker 312. Detailed molecular analyses indicate that one intact copy of the *flcry1Ab* gene has been inserted at a single site in the plant genome, and is stably inherited and expressed from one generation to the next. No antibiotic resistance marker genes are present in COT67B cotton.

COT67B cotton expresses one novel protein, FLCry1Ab. This protein is expressed at moderately low levels in cottonseed (25.17 µg/g dry weight), with quantifiable levels also being detected in linters (9.65 µg/g dry weight), and cottonseed meal (47.50 µg/g dry weight). No FLCry1Ab was detected in refined cottonseed oil.

A large number of studies have been done with FLCry1Ab to confirm its identity and physicochemical and functional properties as well as to determine its potential toxicity and allergenicity. These studies have demonstrated that FLCry1Ab expressed in COT67B conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation, and exhibits the expected insecticidal activity.

In relation to its potential toxicity and allergenicity, it is well established that Cry proteins from *B. thuringiensis* are inherently non-toxic to mammals and have exhibited little potential to be allergenic to humans over their long history of use. In addition, bioinformatic studies with FLCry1Ab have confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that FLCry1Ab would be rapidly degraded in the stomach following ingestion. FLCry1Ab is also heat labile at temperatures of 65°C or greater. Acute oral toxicity studies in mice with FLCry1Ab have also confirmed the absence of toxicity in animals. Taken together, the evidence indicates that FLCry1Ab is unlikely to be toxic or allergenic to humans.

Compositional analyses were done to establish the nutritional adequacy of COT67B cotton, and to compare it to conventional cotton varieties. No differences of biological significance were observed between COT67B cotton and its conventional counterpart. Some minor differences in some of the key constituents were noted, however the differences observed were minor and the levels observed were within the range of values measured for conventional cotton varieties. Such differences most likely reflect normal biological variability. Food from COT67B cotton is therefore considered to be compositionally equivalent to food from conventional cotton varieties and its introduction into the food supply would therefore be expected to have little nutritional impact.

5.3 Conclusions

No potential public health and safety concerns have been identified in the assessment of food derived from insect-protected COT67B cotton. On the basis of the data provided in the present application, and other available information, food derived from insect-protected COT67B cotton is considered as safe and wholesome as food derived from conventional cotton varieties.

RISK MANAGEMENT

6. Options

There are no non-regulatory options for this Application. The two regulatory options available for this Application are:

6.1 Option 1 – Do not approve food from cotton line COT67B

Maintain the *status quo* by not varying Standard 1.5.2 to approve food derived from insect-protected cotton line COT67B.

6.2 Option 2 – Approve food from cotton line COT67B

Vary Standard 1.5.2 to permit the sale and use of food derived from food derived from insect-protected cotton line COT67B, with or without specified conditions in the Table to clause 2 of the Standard.

7. Impact Analysis

FSANZ is required to consider the impact of various regulatory (and non-regulatory) options on all sectors of the community in Australia and New Zealand.

7.1 Affected Parties

The affected parties may include the following:

- 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.

7.2 Benefit Cost 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 an assessment by FSANZ of the costs and benefits of the two regulatory options. This is based on information supplied by the Applicant, issues raised in public submissions on the Application and experience FSANZ has gained from consideration of previous applications relating to GM foods.

7.2.1 Option 1 – continue to prohibit food from cotton line COT67B

Consumers: Possible restriction in the availability of certain imported food products if they are found to contain ingredients derived from COT67B cotton.

No impact on consumers wishing to avoid GM foods, as food from COT67B cotton is not currently permitted in the food supply.

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

Likely to be increased costs associated with monitoring required for unapproved GM material derived from COT67B cotton which may inadvertently be incorporated into imported food products. Costs incurred relate to the use of detection methodology including: increased labour and reagent costs; methodology validation; and methodology consistency and competency maintenance.

Industry: Possible restriction on certain imported foods once COT67B cotton is commercialised overseas.

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

7.2.2 Option 2 – approve food from cotton line COT67B

Consumers: No restriction on imported food products if containing ingredients derived from COT67B cotton.

Potential benefit of lower prices, to the extent that savings from any increased agricultural production efficiencies are passed on to manufacturing and retail sectors.

Potential impact on consumers wishing to avoid GM cotton by a possible restriction of choice of products, or increased prices for non-GM food products.

Government: Benefit in that there would be no potential for trade disruption as a result of the detection of unapproved COT67B cotton in imported food products.

Approval of COT67B cotton would ensure no conflict with WTO obligations.

Likely to be increased costs associated with the additional monitoring required to ensure compliance with the labelling provisions of the Code.
Costs incurred relate to the use of detection methodology including: increased labour and reagent costs; methodology validation; and methodology consistency and competency maintenance.

Industry: Primary producers may benefit from an increased choice of crop lines with potentially lower production costs and higher yields.

Broader market access and increased choice in raw materials for food manufacturing.

Benefit to importers of processed foods containing cottonseed oil and linters derived from COT67B as an ingredient as these foods would be compliant with the Code.

Possible cost to food industry as some food ingredients derived from COT67B cotton may be required to be labelled as genetically modified.

7.3 Comparison of Options

As food from insect-protected cotton line COT67B has been found to be as safe as food from conventional varieties of cotton, option 1 is likely to be inconsistent with Australia's and New Zealand's WTO obligations. Option 1 would also offer little benefit to consumers wishing to avoid GM foods: a number of GM cottons are already approved for food use in Australia and New Zealand. Maintaining a prohibition on food from COT67B could potentially limit the availability of imported food products in Australia and New Zealand due to the approval of COT67B cotton by other countries.

Under Option 2, primary producers would benefit from an increased choice of crop lines with potentially lower production costs and higher yields. These potential savings could flow on to other sectors including food manufacturers and consumers in Australia and New Zealand as lower food prices. Government will also benefit in that potential disruption to trade will be avoided. While there will be costs to government associated with the additional monitoring required to ensure compliance with the Code, similar costs are also likely to be associated with Option 1. The overall impact on monitoring resources is therefore expected to be cost neutral. There is unlikely to be any additional impacts on consumers wishing to avoid GM foods, as a number of GM cottons are already approved for food use in Australia and New Zealand.

As food derived from COT67B cotton has been found to be safe for human consumption and the potential benefits outweigh the potential costs, Option 2, an amendment to Standard 1.5.2 giving approval to food derived from insect-protected cotton line COT67B, is therefore the preferred option.

COMMUNICATION AND CONSULTATION STRATEGY

8. Communication

FSANZ has applied a communication strategy to this Application that involves advertising the availability of assessment reports for public comment in the national press and placing the reports on the FSANZ website. In addition, FSANZ will issue a media release drawing journalists' attention to the matter.

As normally applies to all GM food assessments, the Final Assessment Report for this Application will be available to the public on the FSANZ website and distributed to major stakeholders.

9. Consultation

9.1 Public Consultation

The Draft Assessment was advertised for public comment between 6 August 2008 and 17 September 2008. Eighty-two submissions were received during this period and a summary of these is included in **Attachment 3** to this Report.

FSANZ has taken the submitters' comments into account in preparing the Final Assessment of this Application. Responses to general issues regarding GM food (e.g. labelling) are available from the FSANZ website[2]. Specific issues relating to food derived from cotton line COT67B have been addressed in the report. The major issues raised are discussed here.

9.1.1 The safety of ingested recombinant DNA

Queensland Health states that further consideration should be given to the fate of ingested DNA in the human gastrointestinal tract, citing studies published in 1994, 1997, 1998 and 2004.

9.1.1.1 Response

FSANZ has recently thoroughly reviewed the scientific literature relating to the fate of ingested DNA, both recombinant and non-recombinant DNA, present in food. In general, there are no inherent safety concerns with ingestion of recombinant DNA, as it is chemically identical to non-recombinant DNA. DNA from all sources is a normal part of human diets and is largely degraded by food processing, cooking, storage and finally digestion.

In terms of this Application, the inserted DNA sequences have been fully characterised as part of the safety assessment. Both the gene and the regulatory elements have been used previously in other approved GM crops. Humans have likely been exposed to these DNA sequences naturally as they are derived from common plants and soil bacteria. Moreover, no antibiotic resistance marker genes are present in this line of cotton, so there is no possibility, however remote, of conferring antibiotic resistance to microorganisms in the human gastrointestinal tract.
Based on these facts, there are no valid scientific grounds for concern regarding the safety of recombinant DNA from ingestion of foods derived from cotton line COT67B.

As a reflection of the level of scientific consideration FSANZ has applied to this issue, FSANZ has recently posted a Factsheet '*Safety of ingested recombinant DNA'* on the website, to broaden public understanding of this and other safety issues addressed during the course of an assessment.

9.1.2 Levels of phytic acid in cotton line COT67B

The New Zealand Food Safety Authority noted that the compositional analysis did not report the level of phytic acid in cotton line COT67B compared to the non-transgenic parental line.

[2] http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm

9.1.2.1 Response

Phytic acid is not a significant antinutrient in cotton. The compositional analyses of cotton line COT67B focussed on the key toxicant and antinutrients in cotton, namely gossypol and the cyclopropenoid fatty acids (sterculic, malvalic and dihydrosterculic acids), in accordance with the Consensus Document on Compositional Considerations for New Varieties of Cotton (OECD 2004[3]).

Cotton contains a number of terpenoid phytoalexins that are particularly toxic to non-ruminant animals. Gossypol is the most notable of the terpenoid phytoalexins and was first isolated from the pigment glands in cottonseed. Free, not bound, gossypol is the toxic compound and maximum limits apply to cottonseed meal used as feed for dairy cows.

Cotton also contains several cyclopropenoid fatty acids that are associated with the oil. Malvalic, sterculic and dihydrosterculic acids elevate the melting point of fats in animals fed whole cottonseed and cottonseed meal. These compounds appear to inhibit the desaturation of saturated fatty acids. In chickens, egg yolk discoloration and reduced hatchability are two detrimental effects, and consequently, industry limits the use of cottonseed meal and cottonseed oil in poultry diets (OECD 2004).

As reported in the safety assessment of cotton line COT87B, the levels of total and free gossypol as well as all cyclopropenoid fatty acids measured at four geographical locations were well within the reported reference ranges, and do not represent a food safety concern.

9.1.3 Process labelling of GM foods

The majority of submissions received in the second consultation period called for labelling of GM foods based on the method of production, irrespective of any physical or compositional differences between the food derived from a GM crop or a conventional (non-GM) crop.

On the other hand, a small number of submissions in favour of the application, claim that broader labelling requirements should not be applied on the grounds that this would not address a demonstrated consumer safety concern, would increase costs, and would be inconsistent with Ministerial Council policy.

9.1.3.1 Response

Health Ministers comprising the former Australia New Zealand Food Standards Council (ANZFSC) resolved in July 2000 to require labelling of GM foods with the words 'genetically modified' where novel DNA and/or protein from an approved GM variety is present in the final food, or where the GM food has altered nutritional characteristics. The Ministers resolved that highly refined food, such as oils, sugars and starches that have undergone refining processes that have the effect of removing DNA and/or protein, would be exempt from these requirements. The labelling provisions of Division 2 of Standard 1.5.2 came into effect in December 2001. At that time, Ministers acknowledged that these broad labelling requirements were primarily to satisfy consumer information issues and were not based on any safety concerns.

[3] OECD (2004) Consensus Document on Compositional Considerations for New Varieties of Cotton (Gossypium hirsutum and Gossypium barbadense): Key Food and Feed Nutrients and Anti-Nutrients. ENV/JM/MONO(2004)16.

GM labelling was reviewed by FSANZ in 2003 in the *Review of Labelling of Genetically Modified (GM) Foods* (available from the FSANZ website at http://www.foodstandards.gov.au/newsroom/publications/gmlabellingreviewrep2460.cfm). The Review found that the labelling requirements for GM foods prescribed in Standard 1.5.2 were rigorous and remain among the most comprehensive, both in scope and breadth of capture, of any country in the world.

The safety of a GM food is thoroughly assessed prior to approval, and only foods found to be as safe as their conventional counterpart are permitted onto the market. The purpose of current labelling requirements for GM foods is to provide information to consumers, allowing them to purchase or avoid a food where it contains a derivative of the GM process, however small this may be, in accordance with their views or beliefs. Where processing or refining of a GM food effectively means that it is indistinguishable from its non-GM counterpart irrespective of its source or method of production, there is no basis for testing for enforcement purposes. Labelling of GM foods under the current requirements therefore represents a balance between the desire to provide information to consumers and the ability of government agencies to enforce such requirements.

9.1.4 Use of company data

Queensland Health commented that FSANZ has relied mostly on data provided by the Applicant.

9.1.4.1 Response

It is entirely appropriate for the Applicant to be responsible for generating the scientific information required to support the safety of food derived from COT67B cotton. These responsibilities are also recognised by FSANZ formally in acknowledgement of an Exclusive Commercial Capturable Benefit (ECCB) in the commercialisation of the product. Only the Applicant would be expected to provide the necessary financial and other resources needed to ensure quality controlled studies sufficient to meet the regulatory requirements in a number of countries, including Australia and New Zealand.

This situation also applies to any sector within the food industry who seek regulatory approval for a new food additive, processing aid or novel food ingredient, or to pharmaceutical companies seeking authorisation of a new drug. The level of detail required in data packages and the costs involved in generating the data mean that the imperative is upon the Applicant to provide the information necessary to support an assessment of any potential risks to public health and safety.

Generally, safety data consist of studies conducted in-house by the Applicant, additional studies conducted in specialised independent laboratories commissioned by the Applicant, and in academic institutions. In addition to these types of studies, FSANZ routinely supplements its knowledge base with information obtained from other sources (the internet, published reference material, other Applications) to capture the full extent of data and information relevant to the assessment. FSANZ will also consult other regulatory agencies, both domestic and international, as well as other government departments where it is considered that information-sharing will inform the assessment.

Further discussion of this issue is available on the FSANZ website at www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm.

9.2 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.

Gazettal of the draft variation to the Code would have a trade enabling effect as it would permit food derived from COT67B cotton to be imported into Australia and New Zealand and sold, where currently it is prohibited. Accordingly, it was not considered necessary to notify this Application under the WTO Sanitary and Phytosanitary Measure (SPS) Agreement.

CONCLUSION

10. Conclusion and Decision

Decision

Amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from insect-protected cotton line COT67B in the Table to clause 2.

10.1 Reasons for Decision

An amendment to the Code permitting the sale and use of food derived from cotton line COT67B in Australia and New Zealand is approved 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 insect-protected cotton line COT67B;
- food derived from insect-protected cotton line COT67B is equivalent to food from the conventional counterpart and other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain food products derived from insect-protected cotton line COT67B will be required if novel DNA and/or protein is present in the final food; and
- 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 preferred option is option 2, an amendment to the Code.

11. Implementation and review

The proposed draft variation to the Code is expected to come into effect on gazettal, subject to any request from the Ministerial Council for a review of the Board's decision at Final Assessment.

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Final safety assessment report
3. Summary of submissions

Attachment 1

Draft variation to the *Australia New Zealand Food Standards Code*

Standards or variations to standards are considered to be legislative instruments for the purposes of the Legislative Instruments Act (2003) and are not subject to disallowance or sunsetting.

To commence: on gazettal

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

Food derived from insect-protected cotton line COT67B	

Attachment 2

Draft Safety Assessment

Food Derived from Insect Protected Cotton Line COT67B

Summary and Conclusion

Background

Insect-protected COT67B cotton has been genetically modified (GM) for protection against feeding damage caused by larvae of a number of insect pest species. Protection is conferred by expression in the plant of an insecticidal Cry protein, derived from *Bacillus thuringiensis*, a common soil bacterium. The Cry proteins exert their effect on the insect by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the insect.

COT67B cotton lines are intended initially for cultivation in the United States of America but may also be grown in Australia at a later date. Food from COT67B cotton will therefore initially enter the Australian and New Zealand food supply via imported products.

In conducting a safety assessment of food derived from insect-protected COT67B cotton, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the corn genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic in humans.

This safety assessment report addresses only food safety and nutritional issues. It therefore does not address: environmental risks related to the environmental release of GM plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants; or the safety of food derived from the non-GM (conventional) plant.

History of use

Cotton is one of the oldest cultivated crops, providing over 40% of the total fibre used in the world. Only the cottonseed is used as a source of human food, it being processed into four major by-products: oil, meal, hulls and linters. Typically, only the oil and linters are used as human food. 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. Cotton linters are short fibres removed from the cottonseed during processing and are a major source of cellulose for use as thickeners in ice cream, salad dressings and toothpaste.

B. thuringiensis, the microorganism from which the Cry proteins are derived, has been extensively studied and commercially exploited for over 40 years as the active ingredient in a number of insecticide products used in agriculture as well as home gardens. *B. thuringiensis* therefore has a long history of safe use and the Cry proteins it produces are not known to be toxic to any vertebrates, including humans and other mammals.

Molecular Characterisation

COT67B cotton was generated through the transfer of the full length *cry1Ab* gene (*flcry1Ab*) to the conventional cotton line Coker 312. The *flcry1Ab* gene, derived from *B. thuringiensis* subspecies *kurstaki* HD-1, has been modified to restore a 26 amino acid motif in the C-terminus of the protein which had been lost during a natural recombination event which generated the wild type *cry1Ab* gene. The *flcry1Ab* gene encodes FLCry1Ab, a full-length Cry protein consisting of 1181 amino acids with a molecular mass of ca. 133.5 kDa. The protein is identical to the native Cry1Ab protein produced by *B. thuringiensis* subsp. *kurstaki* HD-1, except for the additional 26 amino acids.

Detailed molecular analyses indicate that one intact copy of the *flcry1Ab* gene has been inserted at a single site in the plant genome, and is stably inherited and expressed from one generation to the next. No antibiotic resistance marker genes are present in COT67B cotton.

Characterisation of Novel Protein

COT67B cotton expresses one novel protein, FLCry1Ab. This protein is expressed at moderately low levels in cottonseed (25.17 µg/g dry weight), with quantifiable levels also being detected in linters (9.65 µg/g dry weight), and cottonseed meal (47.50 µg/g dry weight). No FLCry1Ab was detected in refined cottonseed oil.

A large number of studies have been done with FLCry1Ab to confirm its identity and physicochemical and functional properties as well as to determine its potential toxicity and allergenicity. These studies have demonstrated that FLCry1Ab conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation and exhibits the expected insecticidal activity.

In relation to its potential toxicity and allergenicity, it is worth noting that Cry proteins from *B. thuringiensis* are inherently non-toxic to mammals and have exhibited little potential to be allergenic to humans over their long history of use. In addition, bioinformatic studies with FLCry1Ab have confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that FLCry1Ab would be rapidly degraded in the stomach following ingestion. FLCry1Ab is also heat labile at temperatures of 65°C or greater. Acute oral toxicity studies in mice with FLCry1Ab have also confirmed the absence of toxicity in animals. Taken together, the evidence indicates that FLCry1Ab is unlikely to be toxic or allergenic to humans.

Compositional Analyses

Compositional analyses were done to establish the nutritional adequacy of COT67B cotton, and to compare it to conventional cotton varieties. The components analysed were proximates, fatty acids, amino acids, vitamin E, minerals, gossypol and the cyclopropenoid fatty acids.

No differences of biological significance were observed between COT67B cotton and its conventional counterpart. Some minor differences in some of the key constituents were noted, however the differences observed were minor and the levels observed were within the range of values measured for conventional cotton varieties.

Such differences most likely reflect normal biological variability. Food from COT67B cotton is therefore considered to be compositionally equivalent to food from conventional cotton varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of food derived from insect-protected COT67B cotton. The introduction of COT67B cotton into the food supply would therefore be expected to have little nutritional impact.

Conclusion

No potential public health and safety concerns have been identified in the assessment of food derived from insect-protected COT67B cotton. On the basis of the data provided in the present application, and other available information, food derived from insect-protected COT67B cotton is considered as safe and wholesome as food derived from conventional cotton varieties.

1. BACKGROUND

A safety assessment has been conducted on food derived from cotton that has been genetically modified (GM) to be protected against feeding damage caused by certain insect pest larvae. The GM cotton is referred to as COT67B cotton (OECD Unique Identifier: SYN-IR67B-1).

COT67B cotton is protected against two key cotton pests: *Helicoverpa zea*, cotton bollworm; and *Heliothis virescens*, tobacco budworm. Protection against these pests is achieved through expression in the plant of an insecticidal Cry protein, Cry1Ab, encoded by the full-length *cry1Ab* gene derived from *Bacillus thuringiensis* subspecies *kurstaki* HD-1.

Cry proteins exert their effect on the target insect by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the larvae. The lysis of the midgut epithelial cells is mediated by the binding of the activated Cry protein to specialised receptors on these cells.

Cotton lines containing the COT67B transformation event are intended initially for cultivation in the United States of America but may also be grown in Australia at a later date. Food from COT67B cotton will therefore initially enter the Australian and New Zealand food supply via imported products.

2. HISTORY OF USE

2.1 Host Organism

The host organism is cultivated cotton (*Gossypium hirsutum* L.).
Cotton is one of the oldest cultivated crops and is grown primarily as a fibre crop, providing over 40% of the total fibre used in the world (OECD 2004). Only the cotton boll, which develops from the plant ovary, is used for either textile fibre or food/feed. The cotton boll, once harvested, is processed (‘ginned’) to separate the cottonseed from the cotton fibre.

Only the cottonseed is used for human food, it being processed into four major by-products: oil, meal, hulls and linters. Only the oil and linters are typically used as human food. Food products from cottonseed are limited to highly processed products due to the presence of the natural toxicants (gossypol) and anti-nutrients (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 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 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 by-products – meal and hulls – 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.

Australia crushes around 150-200,000 tonnes of cottonseed annually, producing about 30-40,000 tonnes of oil. Cotton is not grown in New Zealand. Cottonseed oil makes up around 15% of the total domestic fat and oil supply and is primarily used in the food service/food manufacturing sector.

The cotton variety, Coker 312, was used as the parental variety for the transformation. Coker 312 is a United States Protected Variety of SeedCo Corporation, initially released in 1974, which has been shown to respond favourably to tissue culture and transformation techniques. Although no longer widely grown, Coker 312 is still considered a commercially acceptable cultivar.

2.2 Donor Organism

The Cry protein expressed in COT67B cotton is derived from *B. thuringiensis*.
The effect of *B. thuringiensis* products on human health and the environment was the subject of a critical review by the WHO International Programme on Chemical Safety (WHO 1999).

The review concluded that '*B. thuringiensis* products are unlikely to pose any hazard to humans or other vertebrates or the great majority of non-target invertebrates provided that they are free from non-*B. thuringiensis* microorganisms and biologically active products other than the insecticidal proteins'.

B. thuringiensis is a facultative anaerobic, gram-positive spore-forming bacterium that produces characteristic insecticidal proteins, as parasporal crystals, during the sporulation phase. These crystals are predominantly comprised of one or more Crystal (Cry) and Cytolytic (Cyt) toxins, also called δ-endotoxins. These toxins are highly specific to their target insect species, are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo et al 2007).

Over 60 subspecies of *B. thuringiensis* have been described. *B. thuringiensis* subspecies can synthesise more than one type of Cry protein, which are specifically toxic to the insect orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera, and also to nematodes. The Cyt toxins are mostly found in *B. thuringiensis* strains that are active against Diptera.

A number of different commercial *B. thuringiensis* formulations have been registered worldwide for use as an insecticide to be applied to foliage, soil, and water or food storage facilities. While the *B. thuringiensis* spores or vegetative cells may persist in the environment for weeks, months or years, the Cry proteins become inactive within hours or days.

Studies on mammals, particularly laboratory animals, demonstrate that *B. thuringiensis* is mostly non-pathogenic and non-toxic. *B. thuringiensis* has been demonstrated to be highly specific in its insecticidal activity and has demonstrated little, if any, direct toxicity to non-target insects.

The use of *B. thuringiensis* products in the field can result in considerable aerosol and dermal exposure in humans. With the exception of case reports on ocular and dermal irritation, no adverse health effects have been documented after occupational exposure to *B. thuringiensis* products (McClintock et al 1995). Studies with human volunteers who ingested and inhaled large quantities of a Btk formulation (*B. thuringiensis* subspecies *kurstaki*) did not reveal any adverse effects (Fisher & Rosner 1959). Similarly, *B. thuringiensis* present in drinking water or food has not been reported to cause adverse effects on human health (WHO 1999).

3. MOLECULAR CHARACTERISATION

Submitted studies:
Harper, B. (2007). Molecular characterization of the transgenic DNA in event COT67B cotton for Japan, Amended Report No. 1. Syngenta Biotechnology, Inc., Report No. SSB-131-07 A1.

Pence, K. (2006). Stability of Cry1Ab protein expression across multiple generations of event COT67B cotton. Syngenta Biotechnology, Inc., Report No. SSB-048-06 (Protocol No. CRYCOT-05-01).

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects: the transformation method together with a detailed description of the DNA sequences introduced to the host genome; a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation; and the genetic stability of the inserted DNA and any accompanying expressed traits.

3.1 Description of the gene construct

Two transformation vectors were used for the transformation process, each containing a single T-DNA – pNOV4641 (Figure 1 and 2) and pNOV1914 (Figure 3 and 4). pNOV4641 contains a synthetic full-length *cry1Ab* gene – designated *flcry1Ab* – originally derived from *B. thuringiensis*, subspecies *kurstaki* HD-1, which expresses the insecticidal FLCry1Ab protein and pNOV1914 contains the *aph4* gene derived from *Escherichia coli*, which expresses the enzyme hygromycin-B phosphotransferase for hygromycin resistance. The *aph4* gene is used as a selectable marker for the transformation process.

The native or wild-type *cry1Ab* gene from *B. thuringiensis* subsp. *kurstaki* HD-1 is a product of natural genetic recombination between the related *cry1Aa* and *cry1Ac* genes, also from the *kurstaki* HD-1 strain. During the recombination event between the *cry1Aa* and *cry1Ac* genes, which generated the *cry1Ab* gene, a segment of coding sequence corresponding to 78 base pairs, was deleted (Geiser et al 1986), resulting in a diminished capacity for the native *cry1Ab* gene to express the Cry1Ab protein in fermentative cultures of *B. thuringiensis*. To rectify this problem, the *cry1Ab* gene introduced into COT67B has had this 78 bp segment of DNA (encoding 26 additional amino acids) restored using coding sequence derived from the *cry1Aa* gene (Geiser & Moser, 1991). The *flcry1Ab* gene is therefore identical to the native cry1Ab gene in *B. thuringiensis* subsp. *kurstaki* HD-1, except for an additional 78 bps in the C-terminal portion of the coding sequence.

The *flcry1Ab* gene is under control of the actin-2 promoter from *Arabidopsis thaliana* and the *aph4* gene is under the control of the ubiquitin-3 promoter, also from *A. thaliana*. Both genes are terminated with the NOS 3' terminator sequence from the nopaline synthase gene from *A. tumefaciens*. The *flcry1Ab* coding sequence has been optimised for expression in plants (Koziel et al 1997). A description of all the genetic elements in each of the transformation vectors is provided in Table 1 and 2.

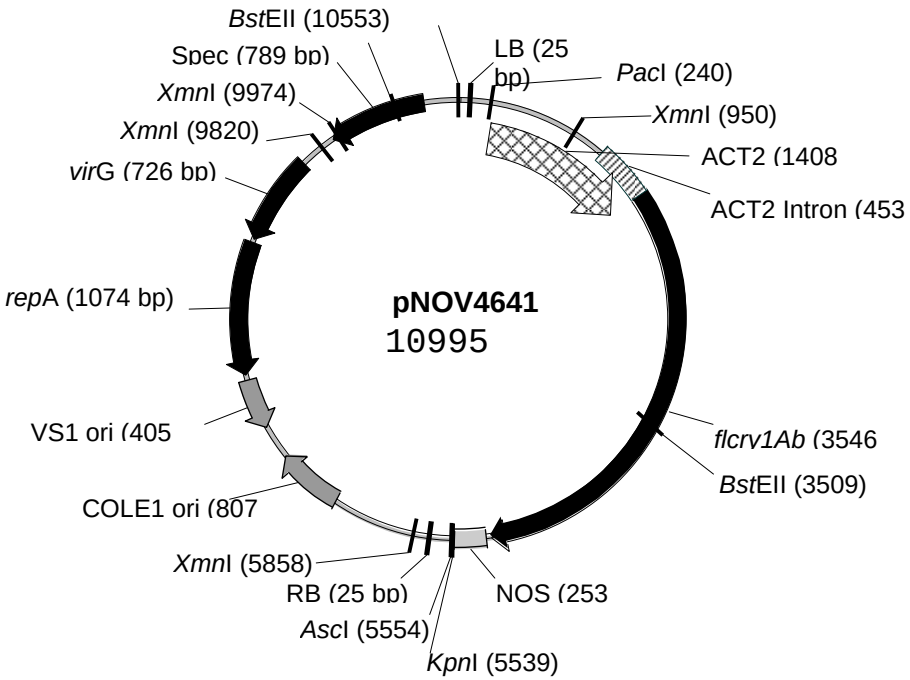


Figure 1: Plasmid map of pNOV4641 indicating the restriction enzyme sites used for Southern analyses

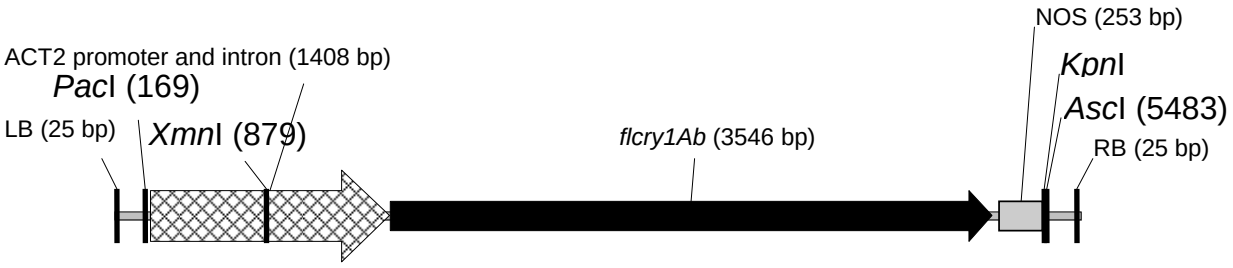


Figure 2: Schematic representation of the flcry1Ab insert in COT67B cotton

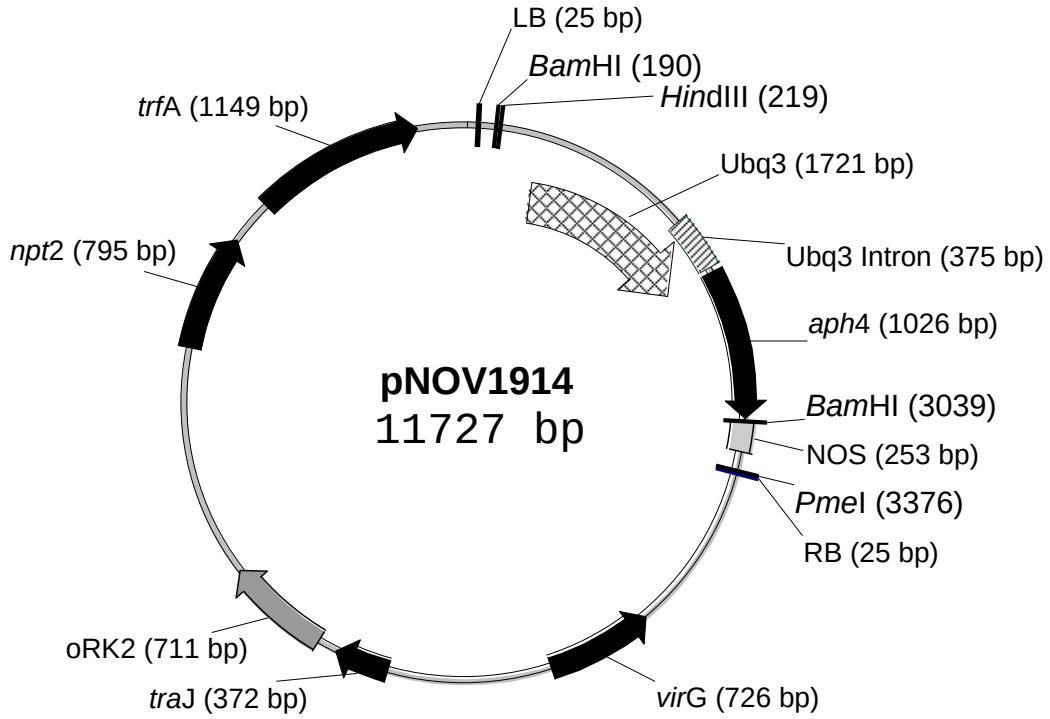


Figure 3: Plasmid map of pNOV1914 indicating the restriction enzyme sites used for Southern analyses

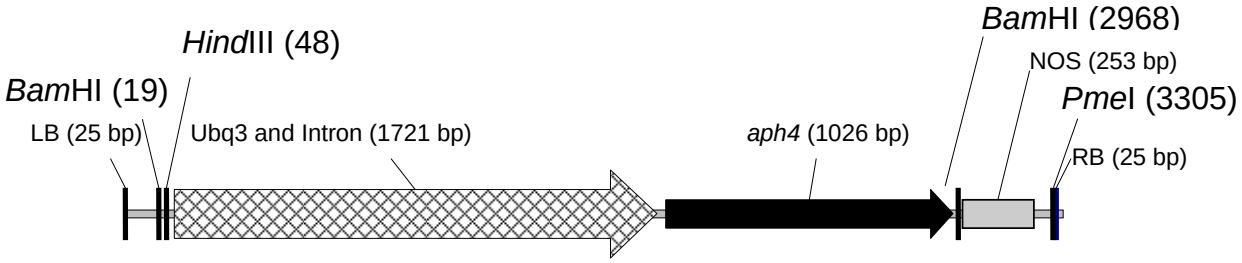


Figure 4: Schematic representation of the Ubq3-aph4 insert

Table 1: Description of Genetic Elements in Plasmid pNOV4641

Genetic Element	Location in pNOV4641 (bp)	Size (bp)	Function
GENE CASSETTE			
Act2	267 - 1674	1408	Promoter region from the actin-2 gene of *Arabidopsis thaliana* and its intron. It controls expression of the *flcry1Ab* gene.
flcry1Ab	1684 - 5230	3546	The full-length *cry1Ab* gene encodes a FLCry1Ab protein identical to Cry1Ab protein produced by *B. thuringiensis* subsp. *kurstaki* strain HD-1, except for an additional 26 amino acids in the C-terminal portion of the protein. Its nucleotide sequence has been codon optimised. Cry1Ab is insecticidal against certain lepidopteran species.
NOS	5273 - 5525	253	Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (Entrez Accession Number V00087 (NCBI, 2007)). Its function is to provide a polyadenylation site.
PLASMID BACKBONE COMPONENTS			
LB (left border)	71 - 95	25	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Entrez Accession Number J01825 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell.
spec	9928 - 10716	789	Streptomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (Entrez Accession Number X03043 (NCBI, 2007)). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker.
virG	8903 - 9628	726	VirGN54D (*A. tumefaciens*) from pAD1289 (similar to Entrez Accession Number AF242881 (NCBI, 2007)). The N54D substitution results in a constitutive virG phenotype. virG is part of the two-component regulatory system for the vir regulon in *A. tumefaciens*.
repA	7800 - 8873	1074	pVS1 replication protein from *Pseudomonas aeruginosa*, which is a part of the minimal pVS1 replicon that is functional in gram-negative plant associated bacteria (Entrez Accession Number AF133831 NCBI, 2007).
VS1ori	7353 - 7757	405	Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *P. aeruginosa* (similar to Entrez Accession Number U10487 (NCBI, 2007)). Serves as origin of replication in *A. tumefaciens* host.
ColE1ori	5869 - 6675	807	Origin of replication that permits replication of plasmid in *E. coli*. (similar to Entrez Accession Number V00268 (NCBI, 2007)).
RB (right border)	5732 - 5756	25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01826 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell.

Table 2: Description of Genetic Elements in Plasmid pNOV1914

Genetic Element	Location in pNOV1914 (bp)	Size (bp)	Function
SELECTABLE MARKER CASSETTE			
Ubq3	245 - 1965	1721	Promoter region plus the first intron from the ubiquitin 3 (ubi3) of *A. thaliana*.
aph4	1997 - 3022	1026	The *aph4* gene encodes a synthetic phosphotransferase enzyme (hygromycin B phosphotransferase; an aminocyclitol phosphotransferase from E. *coli*) that catalyzes the phosphorylation of hygromycin and some related aminoglycosides. The *aph4* gene, when transformed into some plant cells, enables the transformed cells to grow in the presence of the selection agent hygromycin.
NOS	3056 - 3308	253	Terminator sequence from the nopaline synthase gene of *A. tumefaciens* (Entrez Accession Number V00087 (NCBI, 2007)). Its function is to provide a polyadenylation site.
PLASMID BACKBONE COMPONENTS			
LB (left border)	71 - 95	25	Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01825, (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell.
trfA	10262 - 11410	1149	Encodes the replication initiation protein (*P. aeruginosa*) essential for plasmid replication.
npt2	9169 - 9963	795	5' region from the *E. coli* gene encoding the 3'5'-aminoglycoside phosphotransferase type III conferring kanamycin resistance.
oRK2	6880 – 7590	711	Region covering the origin of replication oriV of plasmid RK2 from *E. coli*.
*tra*J	6378 – 6749	372	Encodes the relaxosome protein (*E. coli*) for plasmid replication.
virG	4543 - 5268	726	VirGN54D (*A. tumefaciens*) from pAD1289 (similar to Entrez Accession Number AF242881 (NCBI, 2007)). The N54D substitution results in a constitutive virG phenotype. virG is part of the two-component regulatory system for the vir regulon in *Agrobacterium*.
RB (right border)	3390 - 3414	25	Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (Entrez Accession number J01826 (NCBI, 2007)). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell.

3.2 Transformation method

COT67B was developed through *Agrobacterium*-mediated transformation of the parental cotton line Coker 312, using the transformation vectors, pNOV4641 and pNOV1914, each carrying one T-DNA.

The use of a two T-DNA delivery system enabled the *flcry1Ab* gene to be segregated away from the *aph4* gene during self-pollination of the T_0 plant, once the initial selection had been completed, thus producing plants that are free of the original selectable marker gene.

Petioles[4] from 3 – 5 week old plants were used for the transformation. Excised petioles were co-cultured with *A. tumefaciens* harbouring pNOV4641 and pNOV1914 for 48 hours then transferred to culture medium containing cefotaxime (to eliminate the *Agrobacterium*) and hygromycin (to select cells containing the *aph4* gene). The explants were maintained in culture until callus tissue formed. The calli were dissected away from the original explant material and transferred to fresh growth medium containing cefotaxime and hygromycin. Following several more rounds of subculture, the callus tissue was broken up and put into liquid culture until embryonic tissue began to form. The embryogenic suspension culture cells were plated onto solid growth medium and cultured until true leaves had formed. Plantlets were maintained in tissue culture until large enough to be transferred to the glasshouse.

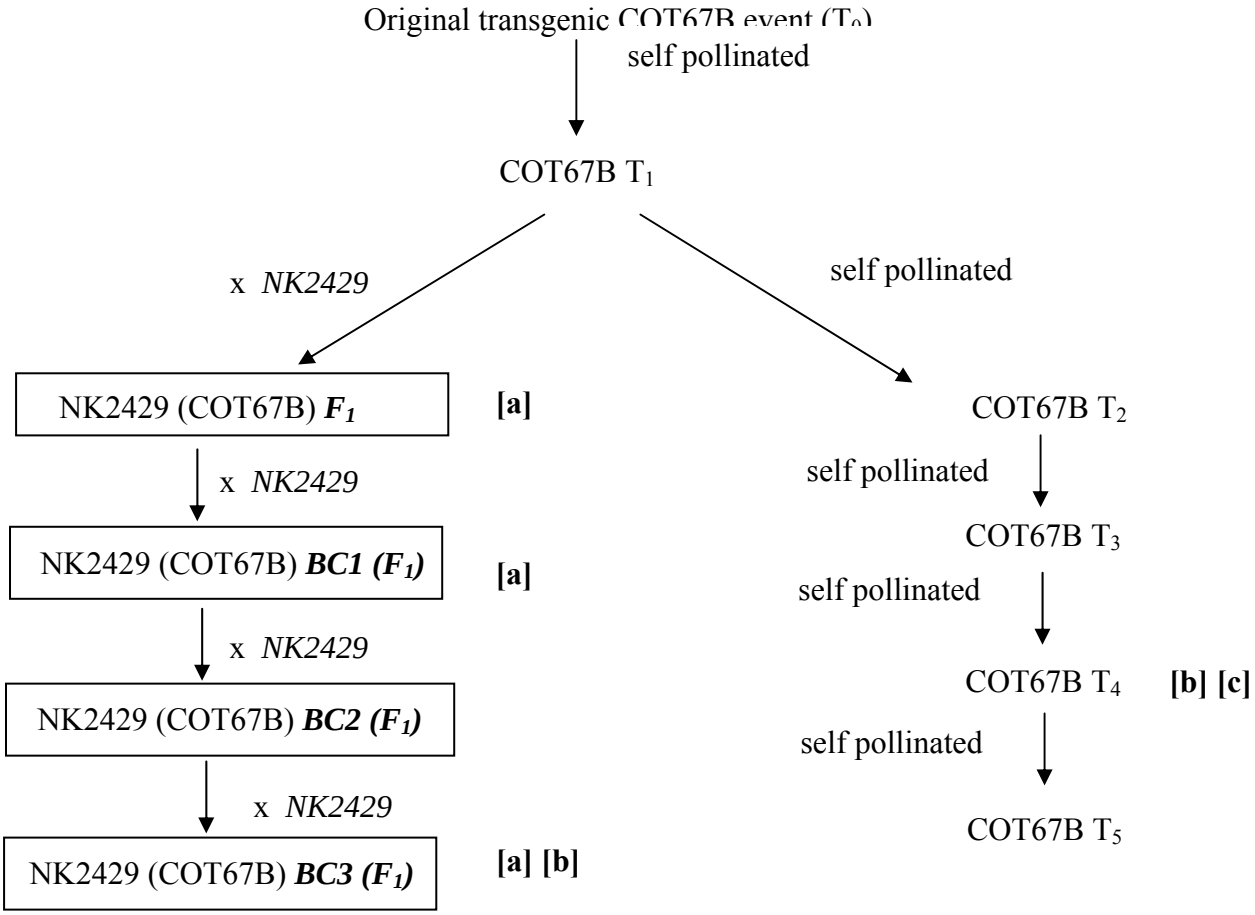
3.3 Breeding process

Primary transformants (T_0 plants) were assayed for insecticidal activity and the copy number of the *flcry1Ab* and *aph4* genes determined. Only those plants displaying insecticidal activity and a single copy of both genes were chosen for further study. These plants were self-pollinated to produce T_1 seed. Approximately 100 T_1 seedlings were screened to identify plants homozygous for the *flcry1Ab* gene and negative for the *aph4* gene. T_1 plants containing only the *flcry1Ab* gene were evaluated in the field, where the COT67B event was selected as the leading commercial candidate. This plant was then self-pollinated to produce T_2 seed. Successive self-pollinations were undertaken to produce the T_5 generation, which was used for the compositional analyses.

Homozygous plants from T_1 generation were also crossed with the elite cotton variety NK2429, creating the F_1 generation. NK2429 (COT67B) plants from this F_1 generation were then crossed into NK2429 again creating the BC1(F_1) (backcross 1) generation. This process was carried out two more times yielding the BC2(F_1) and BC3(F_1) generations. Material from the F_1, BC1(F_1) and BC3(F_1) generations was used for the Mendelian inheritance study.

A diagram of the breeding process used for COT67B is provided in Figure 5.

[4] The petiole is the part of the leaf attaching the leaf blade to the stem of the plant.



[a] Material used for Mendelian inheritance and generational stability Southern analyses
[b] Material used for Southern analyses and sequencing
[c] Material used for compositional analyses and processing study

Figure 5: Breeding tree for COT67B

3.4 Characterisation of the genes in the plant

A number of molecular analyses were done to characterise the inserted DNA in COT67B. The plants used for the molecular analyses are described in Table 3 (refer also to Figure 5).

Table 3: Test material used for the molecular analyses

COT67B Test Material	**Abbreviation**
First self pollinated generation	T_1
First COT67B generation crossed into elite cotton variety NK2429	F_1
First backcrossed generation between COT67B and NK2429	$BC1F_1$
Third backcrossed generation between COT67B and NK2429	$BC3F_1$
Null segregant of the third backcrossed generation between COT67B and NK2429	$BC3F_1$ Non-transgenic
Fourth self pollinated generation	T_4
Non-transgenic cotton (negative control)	Coker 312

All plants used for the molecular analyses were individually screened to confirm the presence/absence of the *flcry1Ab*, *aph4* and *spec* genes. Based upon these results, 10 *flcry1Ab* positive plants from the F_1, $BC1F_1$, $BC3F_1$, T_1 and T_4 generations were selected for the molecular analyses. For the analysis of genetic stability, 10 non-transgenic Coker 312 plants were pooled for use as a negative control. For all other Southern analyses, 10 negative $BC3F_1$ COT67B segregant plants were pooled for use as the negative control.

3.4.1 Southern analyses

Isolated genomic DNA from COT67B was analysed using Southern blot analysis to determine the: number of T-DNA insertions; copy number of each genetic element at each insertion site; presence or absence of plasmid backbone sequences; and presence or absence of the *aph4* selectable marker gene and associated promoter sequences.

Plant genomic DNA was digested with appropriate restriction enzymes, separated on agarose gels, and then transferred to a nylon membrane for Southern analysis. A negative control (DNA isolated from the non-transformed parental line) was included for all analyses, and positive controls consisted of DNA from the relevant transformation vector (either pNOV4641 or pNOV1914), which was spiked into the negative control DNA. Blots were probed with DNA that hybridized to the *flcry1Ab* gene, the associated actin 2 promoter and NOS terminator sequences, plasmid backbone from each of the vectors, the *aph4* gene and the associated ubiquitin 3 promoter.

These analyses demonstrated that COT67B contains a single copy of *flcry1Ab* gene. The hybridising bands were all of the expected size indicating the *flcry1Ab* coding sequence and its associated regulatory sequences (the actin 2 promoter and the NOS terminator) are all intact. No hybridisation signal was obtained when blots were probed with any of the other sequences indicating that COT67B does not contain any plasmid backbone sequences, and also does not contain the *aph4* gene.

3.4.2 DNA sequence analysis

The nucleotide sequence of the entire T-DNA insert in COT67B was determined to confirm the results of the Southern analyses and to demonstrate the overall integrity of the inserted DNA as well as the contiguousness of the individual genetic elements.

The COT67B insert was amplified by polymerase chain reaction (PCR) using genomic DNA isolated from the $BC3F_1$ generation (Table 3 and Figure 5). Two overlapping PCR fragments were produced, each of which was individually cloned, with three separate clones for each PCR product being identified and subsequently sequenced. The sequence data from the six individual clones were combined to generate a consensus sequence of the COT67B insert.

The sequence data demonstrated that the left border and the adjacent 13 base pairs of the insert, along with 24 bp of the right border, were deleted during the transformation process but that the remainder of the T-DNA was intact. There were no changes to the nucleotide sequence of the *flcry1Ab* coding sequence, or it associated regulatory elements.

3.4.3 Flanking sequence analyses

Approximately 1 kb of genomic DNA flanking the 5' and 3' regions of the COT67B insert was sequenced to determine: if any endogenous genes had been disrupted as a result of the insertion; and if any unexpected open reading frames (ORFs) had been created which may potentially be expressed in the plant.

BLAST analysis was used to screen for homology with sequences found in public databases, namely the National Centre for Biotechnology Information (NCBI) nr (non-redundant) database, which contains all sequences from GenBank®[5], RefSeq Nucleotides, EMBL[6], DDBJ[7] and PDB[8] databases.

BLAST analysis of the 5' flanking region showed homology (79% over 86 bp) to sequence defined as genomic microsatellite locus from *G. hirsutum* (Entrez Accession Number DQ908605). BLAST analysis of the 3' flanking region also showed homology to two sequences defined as genomic microsatellite loci from *G. hirsutum* (Entrez Accession Numbers DQ908555 and AF351368). Both DQ908555 and AF351368 have homology in two areas of the 3' region (81% over 292 bp and 84% over 160 bp; and 80% over 169 bp and 84% over 166 bp, respectively). The 3' region also showed homology (80% over 160 bp) to an unknown chloroplast sequence (Entrez Accession Number AF497429) that is located 190 bp from the COT67B T-DNA insert. Sequences homologous to chloroplast DNA have previously been found in nuclear DNA from other plants (Ayliffe et al 1988), therefore this does not represent an unusual finding.

These results indicate that the COT67B T-DNA has inserted into a region of the genome containing highly repetitive DNA. The Applicant states that such microsatellite regions are not typically associated with coding regions, suggesting the T-DNA has not disrupted any endogenous genes.

The 5' and 3' junction regions between genomic DNA and the T-DNA insertion were also analysed for the presence of any ORFs in all six reading frames using a bioinformatic program. For the analysis, an ORF was defined as a region corresponding to at least 50 amino acids, initiated with an ATG codon and terminated with any of the three stop codons: TAA; TAG; or TGA. No ORFs of 50 amino acids or greater were identified in the analysis.

3.4.4 Conclusion

Detailed molecular analyses indicate that one copy of T-DNA, containing one copy of the *flcry1Ab* gene expression cassette, has been inserted at a single genomic locus in COT67B. The coding region of the *flcry1Ab* gene and its associated regulatory elements are intact, with only small deletions (<25 bp) occurring at the left and right borders of the T-DNA. Such small deletions are not expected to impact on the function of the *flcry1Ab* gene cassette. The molecular analyses also confirmed the absence of plasmid backbone sequences and the *aph4* gene, which had been segregated away during the initial breeding process. The T-DNA does not appear to have disrupted any endogenous genes when it became inserted into the genomic DNA and its insertion also does not appear to have generated any unexpected ORFs.

[5] the National Institutes of Health genetic sequence database
[6] European Molecular Biology Laboratory
[7] the DNA Database of Japan
[8] the three-dimensional structure database

3.5 Stability of genetic changes

A number of analyses were done to demonstrate the stability of the genetic changes in COT67B cotton. This included examining the inheritance pattern of the T-DNA insert as well as its stability across multiple generations. The stability of Cry1Ab expression across multiple generations was also examined.

3.5.1 Mendelian inheritance

The inheritance pattern of the T-DNA insert in COT67B was examined using genomic DNA isolated from the F_1, $BC1F_1$ and $B3F_1$ generations (see Figure 5). Individual plants from each generation were assayed using PCR to confirm the presence or absence of the *flcry1Ab* gene. The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait in BC1 and BC3 populations is 1:1.All progeny of the F_1 generation are expected to be positive for the *flcry1Ab* gene. Genotype data were used to assess the goodness-of-fit of the observed genotypic ratio to the expected genotypic ratio using Chi-Square (χ^2) analysis with Yates correction factor. The results of the analysis are presented in Table 4 below.

Table 4: Observed (O) vs. Expected (E) genotype for multiple COT67B generations

Trait	F_1		$BC1(F_1)$		$BC3(F_1)$	
	O	**E**	**O**	**E**	**O**	**E**
Positive	23	23	25	27.5	20	22.5
Negative	0	0	30	27.5	25	22.5
Total	23	23	55	55	45	45
χ^2 value	All plants positive for *flcry1Ab*		0.291		0.356	

The critical value to reject the hypothesis that the *flcry1Ab* gene in COT67B is segregating in a Mendelian fashion at the 5% level of probability using a Chi-Square analysis is 3.84.
As the Chi-Square values for the $BC1(F_1)$ and $BC3(F_1)$ generations are less than 3.84, the hypothesis that the gene is segregating in a Mendelian fashion is accepted. As expected, all plants in the F_1 generation were positive for the *flcry1Ab* gene.

3.5.2 Generational stability

Southern analysis was used to demonstrate the stability of the inserted T-DNA across multiple generations. Genomic DNA isolated from the F_1, $BC1(F_1)$ and $BC3(F_1)$ generations was digested with a restriction enzyme (*Xmn*I) that cuts once within the T-DNA (see Figure 2) and then probed with a probe specific to the *flcry1Ab* gene. The transformation vector, pNOV4641 was used as a positive control and a negative segregant of $BC3(F_1)$ was used as a negative control. The results of the Southern analysis are provided in Table 5 below.

Table 5: Expected vs. Observed Hybridisation Bands

DNA source	Expected Bands	Expected band Size (kb)	Observed Band Size (kb)
F1	1	>4.8	6.1
BC1(F1)	1	>4.8	6.1
BC3(F1)	1	>4.8	6.1
BC3(F1) negative segregant	0	None	None
pNOV4641	1	4.9	4.9

The results presented in Table 5 confirm that the presence of identical hybridisation patterns for the F_1, BC1(F1) and BC3(F1) generations, and indicate the T-DNA insert is stable from one generation to the next.

Enzyme linked immunosorbent assay (ELISA) was used to determine the stability of Cry1Ab expression across multiple generations. Hemizygous plants from the F_1, BC1(F_1) and BC4(F_1)[9] generations were used for the analyses. Leaf samples for the ELISA were taken from five plants from each generation at equivalent developmental stages (3-5 open bolls). Identical plant tissue from two negative segregant plants from the BC1(F_1) and BC4(F_1) generations were sampled at the same time to serve as negative controls. The limit of quantitation (LOQ) for the ELISA was 0.024 µg/g dry weight, and the limit of detection (LOD) was 0.002 µg/gdw. The negative control samples all had mean Cry1Ab concentrations below the LOD. The results of the protein analyses are provided in Table 6 below.

Table 6: Cry1Ab concentration in leaf tissue from multiple generations of COT67B

Generation	Mean µg Cry1Ab/gdw ± SD (range)	Mean µg Cry1Ab/gfw ± SD (range)
F1	68.17 ± 9.64 (54.90 – 81.62)	19.68 ± 2.23 (17.28 – 22.42)
BC1(F1)	66.27 ± 16.88 (41.01 – 81.44)	17.62 ± 1.76 (14.84 – 19.63)
BC4(F1)	54.16 ± 12.08 (39.36 – 67.39)	16.60 ± 2.29 (14.35 – 20.31)

The results presented in Table 6 indicate that Cry1Ab concentrations are comparable across the three generations tested and suggest the expression of the insect protection trait will be stable across multiple generations.

3.5.3 Conclusion

The results of the segregation analysis are consistent with a single site of insertion for the *flcry1Ab* gene and confirm the results of the molecular characterisation. Southern and protein expression indicates that the inserted *flcry1Ab* gene is stably transformed, inherited and expressed from one generation to the next.

[9] This generation was produced by backcrossing the BC3 (F_1) generation (see Figure 5 and Table 3) with the recurrent parent NK2429.

4. CHARACTERISATION OF NOVEL PROTEIN

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g. because they are allergens or anti-nutrients. As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

4.1 Description and function of the novel protein

COT67B cotton expresses a single Cry protein, Cry1Ab. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity of Cry1Ab. Because the expression of proteins *in planta* is usually too low to allow purification of sufficient quantities for safety assessment studies, a bacterial expression system was used to generate large quantities of the Cry1Ab protein. The Cry1Ab protein was produced in *E. coli* and engineered so its amino acid sequence matched that of the plant-produced Cry1Ab. The equivalence of the bacterial-produced protein to the plant-produced protein was determined as part of the protein characterisation.

4.1.1 Mode of action of Cry proteins

The general mechanism of insecticidal activity of Cry proteins is well understood (Gill et al 1992, Schnepf et al 1998, Zhuang & Gill 2003, Bravo et al 2007), with the mode of action being characterised principally in lepidopteran insects. The Cry proteins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins, which after undergoing conformational change, are able to insert into, or translocate across, the cell membranes of their host. There are two main groups of PFT: (i) the α-helical toxins in which the α-helix regions form the trans-membrane pore; and (ii) the β-barrel toxins, that insert into the membrane by forming a β-barrel composed of β-sheet hairpins from each monomer (Parker & Feil 2005). The Cry proteins belong to the α-helical group of PFT, along with other toxins such as exotoxin A (from *Pseudomonas aeruginosa*) and diphtheria toxin.

The primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells, which subsequently leads to ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilised inactive protoxins are cleaved by midgut proteases yielding 60-70 kDa protease resistant proteins (Bravo et al 2005). Toxin activation involves the proteolytic removal of an N-terminal peptide (25-30 amino acids for Cry1 toxins, 58 residues for the Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (de Maagd et al 2001, Bravo et al 2005) before inserting into the membrane.

Toxin insertion leads to formation of lytic pores in microvilli apical membranes (Aronson & Shai 2001, Bravo et al 2005). Subsequently cell lysis and disruption of the midgut epithelium releases the cell contents providing spores a germinating medium leading to a severe septicaemia and insect death.

4.1.2 Description of the FLCry1Ab protein

FLCry1Ab is a full-length Cry protein consisting of 1181 amino acids with a molecular mass of ca. 133.5 kDa. The protein is identical to the native Cry1Ab protein produced by *B. thuringiensis* subsp. *kurstaki* HD-1, except for an additional 26 amino acids in the C-terminal portion of the protein. The Applicant refers to this stretch of 26 amino acids as the 'Geiser motif'. The Geiser motif was incorporated into the protein to restore a stretch of amino acids that had been deleted during a natural recombination event between the related *cry1Aa* and *cry1Ac* genes, which generated the native *cry1Ab* gene. The Geiser motif is stated to have no apparent functionality in plants and is not contained in the region of the Cry1Ab protein which is responsible for insecticidal activity (Geiser & Moser 1991, Koziel et al 1997), but appears to be important for efficient production of Cry1Ab in fermentative cultures of *B. thuringiensis*. Although the Geiser motif is absent from the native Cry1Ab protein, this motif as well as highly homologous sequences are present in virtually all other native full-length Cry1 proteins, in which the C-terminal portion of the protein is highly conserved.

4.2 Protein characterisation

Submitted studies:
Graser, G. (2005). Characterisation of Cry1Ab test substance FLCRY1AB-0103 and certificate of analysis. Syngenta Biotechnology, Inc., Study No. CRYCOT-04-02 (Report No. SSB-001-05).

Graser, G. & Li, X. (2006). Characterisation of the Cry1Ab protein produced in event COT67B-derived cotton plants and comparison with Cry1Ab protein produced in recombinant Escherichia coli. Syngenta Biotechnology, Inc., Study No. CRYCOT-06-02, Report No. SSB-016-06.

A range of biochemical techniques was used to determine the identity as well as the physicochemical and functional properties of the plant-produced FLCry1Ab protein isolated from COT67B and to compare it to *E. coli*-produced proteins. These techniques included Western blot analysis, peptide mass mapping analysis, glycosylation analysis, and insect bioactivity assays.

For these analyses, the FLCry1Ab protein was purified from a recombinant *E. coli* over-expression system and its identity, purity, stability and insecticidal activity separately determined before being used for comparative analyses with FLCry1Ab extracted from COT67B plants. The FLCry1Ab protein extract from the *E. coli* expression system was determined to contain 94.7% protein, with Cry1Ab representing 90.4% of the total protein. The overall purity was therefore determined to be 85.6% Cry1Ab. Western blot analysis indicated a dominant immunoreactive band corresponding to the predicted molecular mass of 133.5 kDa. The *E. coli*-produced FLCry1Ab was found to be bioactive against European corn borer, with an LC_{50} of 3.7 ng Cry1Ab/cm^2. N-terminal sequence analysis confirmed the *E. coli*-produced FLCry1Ab protein to have the same N-terminal amino acid sequence as the predicted sequence: MDNNPNINECIP.

4.2.1 Protein identity

Protein was extracted from leaf samples obtained from 7-12 week old greenhouse-grown COT67B plants. The extract was used to produce two test substances: one which represented a crude extract; and the other which was immunoaffinity purified using rabbit anti-Cry1Ab antibodies. A control extract was prepared from leaf samples obtained from the non-transgenic parental line Coker 312.

1. Western blot analysis – the identity and integrity of the plant-produced FLCry1Ab protein was determined by Western blot analysis using rabbit anti-Cry1Ab antibodies. Equivalent amounts of the plant-produced and *E. coli*-produced FLCry1Ab proteins were loaded onto the same gel, along with the negative control. A major immunoreactive band corresponding to the predicted molecular weight of 133.5 kDa was observed in the *E. coli*-produced and crude COT67B plant extracts, along with a number of minor immunoreactive bands of lower molecular weight. The immunoaffinity purified plant extract exhibited mainly immunoreactive bands of molecular weights lower than 133.5 kDa. The Applicant believes the lower molecular weight bands represent degradation products of the full length Cry1Ab protein as a result of the purification process. No immunoreactive bands were observed in the control plant extract.

2. Peptide mass mapping analysis – the *E. coli* and plant-produced immunoaffinity purified extracts were separated on a polyacrylamide gel, stained and the protein band corresponding to the molecular weight of 133.5 kDa was excised from the gel for peptide mass analysis, using a time-of-flight mass spectrometer. Peptide masses were identified by matching the detected peptide masses to a protein database. For the plant-produced immunoaffinity purified Cry1Ab extract, the mapping identified 14 peptides, corresponding to a total of 16% of the total predicted full length Cry1Ab amino acid sequence. For the *E. coli*-produced Cry1Ab extract, the mapping identified 18 peptides, corresponding to 30% of the total Cry1Ab amino acid sequence. For both proteins, the identified peptides mapped to regions throughout the entire sequence of the protein, including close to the N- and C- termini of the protein. The Applicant states this strongly supports the identity and integrity of the purified proteins from both sources.

4.2.2 Glycosylation analysis

To assess whether post-translational glycosylation of the plant-produced Cry1Ab protein occurred, the immunoaffinity purified protein sample was subjected to glycosylation analysis. As prokaryotic organisms lack the capacity for protein glycosylation, the *E. coli*-produced Cry1Ab protein was used as the negative control, along with creatinase, a non-glycosylated enzyme. Transferrin, which is known to contain 5% glycan moieties by weight, corresponding to about 25 glucose equivalents per molecule, was used as a positive control. Samples were separated on a polyacrylamide gel, transferred to a PVDF membrane, where any glycan moieties were oxidised using periodate, labelled with digoxigenin, and then detected using an anti-digoxigenin antibody coupled to alkaline phosphatase. No bands, representing glycosylated Cry1Ab were visible in the *E. coli*-produced extract, as expected. A very weak smear, corresponding approximately to the predicted molecular weight of the Cry1Ab protein, was observed in the plant-produced immunoaffinity purified sample.

The Applicant claims this smear is not indicative of glycosylation and is most likely an artefact from the plant matrix as a positive reaction would have resulted in a much stronger signal on the gel.

4.2.3 Insect bioassays

The insecticidal activity of the *E. coli* and plant-produced Cry1Ab proteins was determined by an insect feeding assay using freshly hatched first-instar *Ostrinia nubilalis* (European corn borer) larvae. The plant-produced Cry1Ab protein extract was freshly produced for the assay. The bioassays were conducted in Petri dishes containing insect diet overlaid with the Cry1Ab test substances, with final concentrations ranging from 0.11-100 ng Cry1Ab/cm^2 diet surface. Each treatment consisted of three replicates, with 10 larvae per Petri dish. The negative controls were deionised water only, buffer only and protein extract derived from the non-transgenic parental control line (Coker 312). Mortality was assessed after 72 hours.

Both test Cry1Ab extracts were found to be highly active against the insect larvae, with the plant-produced Cry1Ab protein extract having a higher bioactivity in comparison to the *E. coli*-produced Cry1Ab protein; the estimated LC_{50} after 72 hours being 1.3 ng Cry1Ab/cm^2 and 5.2 ng Cry1Ab/cm^2, respectively. No mortality was observed with any of the negative controls.

4.2.4 Conclusion

A number of studies have been done on the Cry1Ab protein to confirm its identity and functional properties as well as to determine its equivalence to the *E. coli*-produced Cry1Ab protein. These studies have demonstrated that the FLCry1Ab protein expressed in COT67B cotton conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation and also exhibits the expected insecticidal activity. The *E. coli*-produced protein was also shown to be equivalent to the plant produced protein in terms of its size, amino acid sequence, physicochemical properties and insecticidal activity. The *E. coli*-produced protein is therefore suitable to act as a substitute for the plant-produced protein for safety assessment purposes.

4.3 Protein expression levels

Submitted studies:
Hill, K. (2006). Quantification of Cry1Ab protein in event COT67B cotton tissues and whole plants. Syngenta Biotechnology, Inc., Report No. SSB-022-06 (Protocol No. CRYCOT-04-01).

De Fontes, J. & Hill, K. (2006). Analysis for the presence of Cry1Ab protein in linters, toasted cottonseed meal and once-refined cottonseed oil from processed seed of event COT67B cotton expressing a full-length Cry1Ab protein. Syngenta Biotechnology, Inc., Study No. CRYCOT-06-05, Report No. SSB-027-06.

4.3.1 Levels in plant tissue

ELISA was used to determine the concentration of Cry1Ab in several plant tissues as well as whole plants at various growth stages (Table 7), the plant being grown at four different locations[10] in the United States in 2004. The non-transgenic parental control line, Coker 312, was grown and analysed in parallel with the COT67B plants.

[10] Newport, Arkansas (NAR); Quitman, Georgia (QGA); Winnsboro, Louisiana (WLA); Leland, Mississippi (LMS).

Table 7: Cotton plant parts retained and analysed at each developmental stage

Tissue	Stage				
	Squaring	1st White Bloom	Peak Bloom	1st Open Boll	Pre-Harvest
Young Leaves	x	x	x	X	
Roots	x	x	x	X	
Old Leaves	x	x	x	X	
Bolls				X	
Seed					x
Fibre					x
Pollen			x		
Flowers			x		
Nectar			x		
Whole Plants					x

At each location, five plants from the COT67B line, plus five plants from the Coker 312 parental control line, were harvested at each of the five developmental time points (as indicated in Table 7). Young leaf samples from all five COT67B plants were pooled to make one composite sample per location per time point. This sampling procedure was also followed for the control plants.

For the other tissue samples, one control plant was used with the other four being discarded. At least three replicates of each composite sample was analysed for Cry1Ab. Only one replicate was analysed for the control samples. For the pollen, nectar and fibre samples, only one location was sampled.

The tissue extracts were quantitatively analysed for Cry1Ab by ELISA, with ELISA values being corrected for extraction efficiency. Limits of quantitation (LOQ) and detection (LOD) for Cry1Ab were determined for each of the tissues analysed at the various developmental stages analysed.

Quantifiable concentrations of Cry1Ab protein were detected in all COT67B plant tissues except fibre and nectar (See Tables 8 & 9). In the parental control line, Cry1Ab concentrations were either below the LOD or below the LOQ. The Cry1Ab concentrations were generally similar between the four locations. Across all growth stages, mean Cry1Ab concentrations measured in young leaves, old leaves and roots ranged from 87.70-323.84 µg/g dry weight, 194.02-255.74 µg/g dry weight, and 12.61-56.56 µg/g dry weight, respectively. The mean Cry1Ab concentration across the four locations in bolls was 45.24 µg/g dry weight, in whole plants was 42.87 µg/g dry weight, and in seed was 25.17 µg/g dry weight. The mean Cry1Ab concentration in flowers was 161.74 µg/g dry weight and in pollen was 5.45 µg/g dry weight.

4.3.2 Levels in processed fractions

In addition to determining the Cry1Ab concentration in plant tissues from COT67B, the concentration of Cry1Ab in various processed fractions was also determined using ELISA. ELISA values were corrected for extraction efficiency. The fractions analysed were cottonseed linters, defatted toasted cottonseed meal, and once refined cottonseed oil. The concentration of Cry1Ab in the fuzzy seed used to produce the processed fractions was also determined.

Cottonseed from COT67B and Coker 312 were individually pooled from field grown plants during the 2004 planting season from Leland, Mississippi, USA and fuzzy cottonseed samples provided to the Food Protein Research and Development Center, Texas A&M University for processing.

Quantifiable levels of Cry1Ab were found in the fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B but were not detectable in any of the Coker 312 samples or in the once refined cottonseed oil from COT67B (LOQ = 0.05 µg Cry1Ab/ml; LOD = 0.003 µg Cry1Ab/ml). The results are presented in Table 10 below.

Table 8: Cry1Ab concentrations in leaves and roots of COT67B cotton plants

Tissue	Location	Developmental Stage			
		Squaring	1st White Bloom	Peak Bloom	1st Open Boll
		Mean µg Cry1Ab/g dry weight ± SD (range)			
Young Leaves	NAR	360.75 ± 18.91 [a] (345.99-387.69)	280.93 ± 9.38 [a] (272.38-289.79)	216.07 [b]	108.05 [b]
	QGA	- [c]	-	150.57 [b]	NS [d]
	WLA	291.91 ± 13.55 [e] (268.96-309.71)	-	106.42 [b]	-
	LMS	318.87 ± 25.67 [a] (289.27-351.33)	X [f]	172.91 [b]	67.35 [b]
Average Across Locations ± SD		**323.84 ± 34.69**	**280.93 ± 0.00**	**161.49 ± 45.69**	**87.70 ± 28.78**
Old Leaves	NAR	304.56 ± 16.80 [g] (278.15-322.91)	279.52 ± 40.74 [g] (244.23-349.82)	341.41 ± 15.57 (323.44-350.81)	304.70 ± 20.99 (292.50-328.94)
	QGA	137.46 ± 9.89 [g] (122.72-150.04)	250.56 ± 26.49 [g] (224.69-285.52)	276.26 ± 5.48 (270.78-281.74)	NS
	WLA	210.51 ± 6.18 [h] (201.54-223.31)	237.14 ± 23.39 [g] (212.27-274.90)	160.19 ± 8.61 (155.10-170.13)	174.77 ± 12.22 (166.98-188.86)
	LMS	263.92 ± 20.48 [g] (224.15-293.53)	X	206.26 ± 22.26 (187.68-231.54)	102.59 ± 14.00 (92.69-118.61)
Average Across Locations ± SD		**229.11 ± 72.23**	**255.74 ± 21.66**	**246.03 ± 79.50**	**194.02 ± 102.42**
Roots	NAR	83.93 ± 7.16 (78.77-92.10)	29.79 ± 3.68 (25.67-32.72)	25.20 ± 2.60 (23.57-28.20)	16.86 ± 2.03 (14.52-18.11)
	QGA	29.08 ± 4.82 (23.55-32.33)	31.71 ± 1.39 (30.33-33.11)	37.03 ± 1.50 (35.49-38.49)	NS
	WLA	48.54 ± 11.74 [g] (37.20-63.12)	26.31 ± 5.54 (20.96-32.02)	16.79 ± 0.90 (15.91-17.70)	13.55 ± 0.85 (12.57-14.09)
	LMS	64.70 ± 7.43 57.80-72.56)	X	18.70 ± 0.85 (17.71-19.20)	7.43 ± 1.55 (6.18-9.16)
Average Across Locations ± SD		**56.56 ± 23.34**	**29.27 ± 2.74**	**24.43 ± 9.14**	**12.61 ± 4.79**

N = 3 replicate samples used to determine mean and standard deviations, unless otherwise noted.
[a] 4 replicate samples used.
[b] One sample analysed, therefore no mean or standard deviation was determined.
[c] No tissue available for analysis.
[d] NS = no samples analysed due to incorrect storage.
[e] 7 replicate samples used.
[f] X = PCR tests confirmed the plants received were not COT67B.
[g] 6 replicate samples used.
[h] 9 replicate samples used.

Table 9: Cry1Ab concentration in whole plants, seeds, flowers and bolls of COT67B cotton plants

Tissue	Location	Mean µg Cry1Ab/g dry weight ± SD (range)
Whole Plant (Pre-Harvest)	**NAR**	52.89 ± 5.40 (47.98-58.56)
	QGA	63.48 ± 12.01 (54.72-77.17)
	WLA	24.14 ± 2.87 (22.41-27.46)
	LMS	30.59 ± 1.60 (29.21-32.34)
Average Across Locations ± SD		**42.87 ± 18.50**
Seed (Pre-Harvest)	**NAR** *	29.35 ± 2.81 (26.14-31.33)
	QGA	27.75 ± 1.83 (25.63-28.82)
	WLA [a]	31.28 ±1.89 (29.37-34.29)
	LMS	12.33 ± 0.38 (11.90-12.60)
Average Across Locations ± SD		**25.15 ± 8.68**
Bolls (1st Open Boll)	**NAR**	53.17 ± 7.10 (48.26-61.31)
	QGA	NS [b]
	WLA	51.99 ± 4.26 (48.80-56.83)
	LMS	30.56 ± 3.34 (26.79-33.18)
Average Across Locations ± SD		**45.24 ± 12.73**
Flowers (Peak Bloom)	**WLA** [a]	161.74 ± 22.87 (137.30-189.52)
Pollen (Peak Bloom)	**WLA**	5.45

N = 3 unless otherwise noted
* Pooled seed sample from NAR consists of three plants, not five
[a] 6 replicate samples were used
[b] NS = no samples analysed due to incorrect storage

Table 10: Cry1Ab concentrations in COT67B fuzzy seed and processed fractions[1]

Fuzzy Seed and Processed Cottonseed Products	Mean µg Cry1Ab/g ± SD (range)
COT67B	
Fuzzy Seed	25.05 ± 1.92 (23.04 – 26.85))
Linters [2]	9.65 ± 6.03 (3.84 – 15.90)
Defatted Toasted Meal	47.50 ± 4.47 (42.69 – 51.52)
Once-Refined Oil	<LOD [3]
Coker 312	
Fuzzy Seed	<LOD
Linters	<LOD
Defatted Toasted Meal	<LOD
Once-Refined Oil	<LOD

[1] Except where indicated, three replicate samples were used to determine means and standard deviations
[2] six samples were used
[3] <LOD = all values for the sample were below the LOD

4.4 Potential toxicity of novel proteins

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein.

The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a concern.

Submitted studies:
Harper, B. (2006). Full length Cry1Ab: Assessment of amino acid sequence homology with known toxins. Syngenta Biotechnology, Inc., Report No. SSB-124-06.

Barnes, E. (2005). FLCRY1AB-0103: Singles dose oral toxicity study in the mouse. Central Toxicology Laboratory, Report No. AM7516-REG.

4.4.1 History of use

The Cry1Ab protein expressed in COT67B is virtually identical to the Cry1Ab protein contained in microbial formulations that have been safely used to control plant pests for over 30 years (Milner 1994). There is no evidence from its long history of use as a plant pesticide of any associated toxicity to humans.

4.4.2 Specificity

The Cry proteins are a diverse group of proteins which have a defined spectrum of insecticidal activity within particular insect orders (Lepidoptera, Diptera, Coleoptera, and Hymenoptera). The Cry proteins are not known to be toxic to any vertebrates, including humans and other mammals. This high degree of specificity is determined by a number of different factors, including (i) activation of the protein by specific proteolytic enzymes in the insect midgut; (ii) binding of the activated protein to specific midgut receptors; and (iii) changes in protein configuration, which enable the protein to insert into and form pores in the insect midgut membrane. As a consequence, only insects with specific receptors are affected, and no toxicity is observed in species that lack these receptors, including other insects. For example, the Cry1Ab protein is active against lepidopteran but not coleopteran insects.

4.4.3 Similarities with known protein toxins

Bioinformatic analyses were done to assess the FLCry1Ab protein for any amino acid sequence similarity with known protein toxins. The FLCry1Ab amino acid sequence was compared to the latest posting of the National Centre for Biotechnology Information (NCBI) Entrez Protein Database (NCBI 2006) using the BLASTP[11] search program (Altschul et al 1997). Using FLCry1Ab as the query sequence, all database sequences with an Expect (E) value of 10 or lower were identified. Typically, comparisons between highly homologous proteins yield E values approaching zero, indicating a low probability the matches would occur by chance. Control BLASTP searches were also done using five different shuffled versions of FLCry1Ab as the query sequence. The shuffling maintained the overall amino acid composition of the protein but randomly rearranged the primary amino acid sequence. The E value obtained for the five shuffled FLCry1Ab sequences therefore represented the background control incidence of random matches that could be expected based on the amino acids contained within FLCry1Ab, irrespective of their specific sequence. BLASTP searches using the five shuffled versions of the FLCry1Ab protein produced E values ranging from 0.27 to 8.8. For BLASTP searches using the unshuffled FLCry1Ab, matches returning an E value of <0.27 were therefore considered to represent proteins with significant amino acid homology. All proteins with significant amino acid homology with FLCry1Ab were evaluated for their protein identity and function (if known), in order to determine the biological significance of the match.

The database search returned 275 matches with E values below 0.27. Of these, 264 were to know or putative Cry proteins from four bacterial species or from synthetic gene constructs, having E values ranging from 0.0 to 0.014.

[11] BLASTP version 2.2.6.

Three additional matches with E values ranging from 3 $x10^{-6}$ to 0.001 were identified as hypothetical proteins of unspecified function from the source organisms *Methanosarcina acetivorans* (one match) and *Dictyostelium discoideum* (two matches). One further match (E value 2 x 10^{-8}) was similar to a twin arginine translocation pathway signal from *Ralstonia eutropha*. None of the matching proteins are known as or have been identified as putative toxins, other than the Cry proteins.

Seven of the matches to Cry proteins or hypothetical proteins from *B. thuringiensis* are described in the database as being part of the parasporin family of proteins or having high homology to known parasporins. The parasporins are not insecticidal but instead are known to have *in vitro* cytocidal activity against human leukaemia T cells and certain other human cancer cell lines, and low or no toxicity against other human cell lines (Mizuki et al 2000, Katayama et al 2005, Yamashita et al 2005). The overall sequence identity of these proteins to FLCry1Ab however was found to be no greater than 14.9% with no more than eight contiguous identical amino acids, which would not be regarded as biologically significant sequence similarity (Doolittle 1990).

4.4.4 Digestibility

See section 4.5.

4.4.5 Acute oral toxicity study

Acute oral toxicity studies using Alpk:Ap$_f$CD-1 mice were conducted to examine the potential toxicity of the FLCry1Ab protein. For these studies, *E. coli*-produced FLCry1Ab was used as the test substance because it was not possible to purify sufficient quantities of the FLCry1Ab from plant material. The equivalence of the *E. coli*- and COT67B cotton-produced protein was established using a range of methods including Western blot analysis, mass spectrometry, glycosylation analysis and insect bioassay (see Section 4.2).

E. coli-produced FLCry1Ab was administered by gavage to five male and five female 8-12 week old mice at a single dose of 1830 mg FLCry1Ab/kg bodyweight. The vehicle for the test substance was de-ionised water. A separate group of five male and five female mice were administered vehicle only as a control.

After dosing, all mice were observed for signs of systemic toxicity, twice on the day of dosing (day 0) and then daily until the end of the study (day 15). Body weight was measured four hours prior to dosing (to determine correct dose volume), at the time of dosing and daily thereafter. Food consumption was recorded daily for each cage of mice and calculated as a mean value for each cage (g food/mouse/day). All mice were killed at the study termination (day 15) and subjected to gross necropsy, haematology, blood clinical chemistry, full histopathology and microscopic examination of all processed tissues.

No treatment-related effects on clinical condition, bodyweight, food consumption, clinical pathology, organ weights, macroscopic or microscopic pathology were observed.

4.5 Potential allergenicity of novel proteins

The potential allergenicity of novel proteins 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 sequence similarity between the novel protein and known allergens; the structural properties of the novel protein, including susceptibility to digestion, heat stability and/or enzymatic treatment; and specific serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity with a known allergen. Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

Studies submitted:
Harper, B. (2006). Full-length Cry1Ab as expressed in event COT67B cotton: assessment of amino acid sequence homology with known allergens. Syngenta Biotechnology, Inc., Report No. SSB-137-06.

Graser, G. (2006). In vitro digestibility of full-length Cry1Ab protein (test substances FLCRY1AB-0103 and IAPCOT67B-0106) under simulated mammalian gastric conditions. Syngenta Biotechnology, Inc., Study No. CRYCOT-0603, Report No. SSB-026-06.

Graser, G. & Mims, G. (2006). Effect of temperature on the stability of full-length Cry1Ab protein. Syngenta Biotechnology, Inc., Study No. CRYCOT-06-06, Report No. SSB-035-06.

4.5.1 Source of the novel protein

The FLCry1Ab protein is derived from *B. thuringiensis* subsp. *kurstaki*. *B. thuringiensis* has been used as the active ingredient in insecticidal sprays for the last 40 years and during that period has not been associated with any reported allergic reactions associated with its use. Humans using the insecticidal sprays have been shown to develop antibodies to the expressed Cry proteins, but in no case has the presence of these antibodies been linked with any clinical, including allergic, symptoms (Nester et al 2002).

4.5.2 Similarity to known allergens

Bioinformatic analyses were done to assess the FLCry1Ab protein for any amino acid sequence similarity with known or putative allergenic proteins. The FLCry1Ab amino acid sequence was compared to a database compiled by the Applicant (SBI Allergen Database) and which is updated annually.

The database contains the amino acid sequences of known and putative allergens, including gliadins, and was compiled from the following sources: the GenPept, PIR or SWISS-PROT protein databases; the SWISS-PROT Allergen database (SWISS-PROT 2001); the List of Allergens database (International Union of Immunological Societies, 2001); the FARRP Protein Allergen database (Food Allergy Research and Resource Program, 2001): and putative allergens, identified from the scientific literature, which are not found in public databases. The version of the SBI Allergen database (Version 5.0) used for the comparison contained a total of 1735 entries and was updated in July 2006.

Two different searches against the SBI Allergen Database were performed.

Firstly, the database was searched for overall linear sequence identity with the FLCry1Ab sequence. Related protein sequences are considered to be potentially cross-reactive if linear identity is 35% or greater in a segment of 80 or more amino acids (FAO/WHO 2001). Secondly, searches were also done to identify short amino acid sequences that may represent linear IgE binding epitopes. For this analysis, searches were done for matches of 8 contiguous amino acids or more.

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No significant sequence similarity between the FLCry1Ab protein and known or putative allergens in the SBI Allergen Database was identified in the comparison. Additionally, no short peptide matches, representing putative IgE binding epitopes, were shared between the FLCry1Ab protein and proteins in the database.

4.5.3 Digestibility

Resistance to hydrolysis by digestive proteases has been observed in several food allergens (Astwood et al 1996), therefore a correlation is believed to exist between resistance to digestion by pepsin and allergenic potential. As a consequence, one of the criteria for assessing potential allergenicity is to determine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response.

The *in vitro* digestibility of the FLCry1Ab protein was assessed in simulated mammalian gastric fluid (SGF) using SDS-PAGE and Western blot analysis. Both the plant-produced and *E. coli*-produced FLCry1Ab proteins (described in Section 4.2) were used for the study. The limit of detection for SDS-PAGE analysis was 20 ng FLCry1Ab, and for the Western blot analysis was 1 ng FLCry1Ab.

SGF containing pepsin was incubated at 37°C with either the plant-produced or *E. coli*-produced FLCry1Ab protein to give a final ratio of about 10 pepsin activity units per µg FLCry1Ab. Aliquots of the SGF/protein mixture were removed at selected time points (0, 1, 2, 5, 10, 30 and 60 minutes), and the reaction stopped. For a negative control, the FLCry1Ab proteins were added to SGF without pepsin, with samples being taken after 0 and 60 minutes. The concentration of the plant-produced FLCry1Ab protein was not high enough to be visualised via Coomassie Blue staining after SDS-PAGE, therefore this was only done with the *E. coli*-produced FLCry1Ab.

No intact *E. coli*- or plant-produced FLCry1Ab (133.5 kDa) was detectable by either Coomassie blue staining or Western blot following one minute exposure to SGF. A lower molecular weight band of about 60 kDa was evident with both Coomassie blue staining and Western blot analysis, which the Applicant claims is most likely a degradation product of FLCry1Ab. This band became evident following one minute exposure to SGF and gradually disappeared as the time course proceeded. The 60 kDa band from the *E. coli*-produced FLCry1Ab was no longer evident after 30 minutes incubation, and for the plant-produced FLCry1Ab was no longer evident after 10 minutes incubation. Other lower molecular weight bands (< 45 kDa) are also evident with the plant-produced FLCry1Ab protein, but these too also degrade rapidly and are no longer evident following 10 minutes incubation. No significant digestion of FLCry1Ab occurred in SGF without pepsin over the 60-minute time course.

4.5.4 Heat stability

The effect of temperature on the stability of FLCry1Ab was determined by incubating the *E. coli*-produced protein at 4 (baseline control), 25, 37, 65 and 95°C for 30 minutes followed by a bioassay against European corn borer larvae. FLCry1Ab was tested at concentrations of 0.33 – 200 ng FLCry1Ab/cm^2 diet for the 4, 25 and 37°C incubated samples, and at 2.05 – 1250 ng FLCry1Ab/cm^2 diet for the 65 and 95°C incubated samples.

The results of the bioassay are presented in Figure 6. At 25 and 37°C there was no significant effect on the bioactivity of FLCry1Ab. Weak bioactivity was retained by the protein when incubated at 65°C, but its activity was completely destroyed following incubation at 95°C.

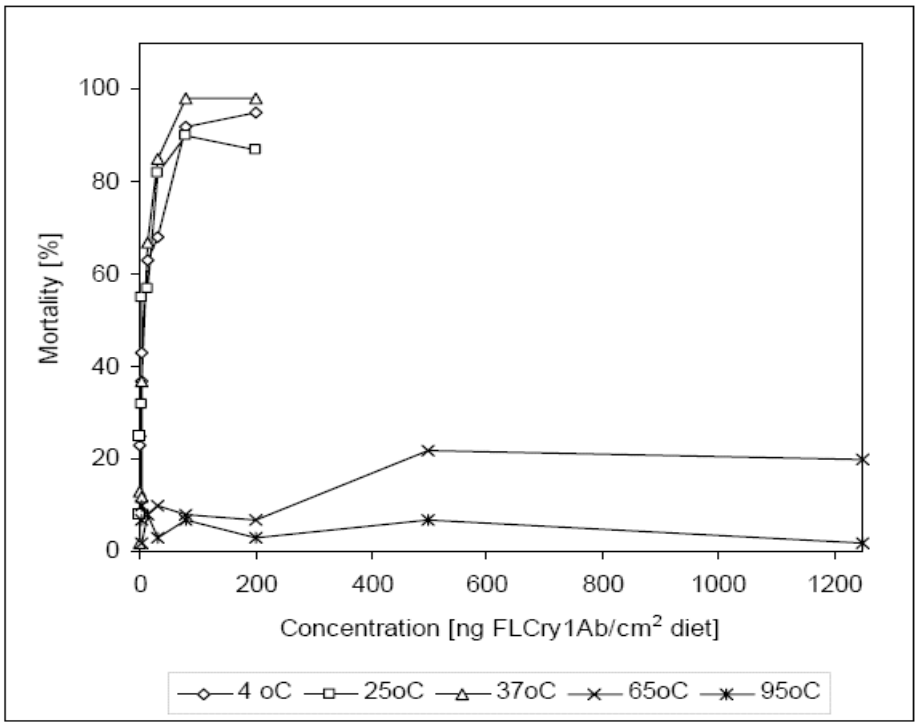

Figure 6: Effect of temperature on insecticidal activity of FLCry1Ab

4.6 Conclusion

COT67B cotton expresses one novel protein, FLCry1Ab. This protein is expressed at moderately low levels in cottonseed (25.17 µg/g dry weight), with quantifiable levels also being detected in linters (9.65 µg/g dry weight), and cottonseed meal (47.50 µg/g dry weight). No FLCry1Ab was detected in once refined cottonseed oil.

A large number of studies have been done with FLCry1Ab to confirm its identity and physicochemical and functional properties as well as to determine its potential toxicity and allergenicity. These studies have demonstrated that FLCry1Ab conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation and exhibits the expected insecticidal activity.

In relation to its potential toxicity and allergenicity, it is worth noting that Cry proteins from *B. thuringiensis* are inherently non-toxic to mammals and have exhibited little potential to be allergenic to humans over their long history of use.

In addition, bioinformatic studies with FLCry1Ab have confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that FLCry1Ab would be rapidly degraded in the stomach following ingestion. FLCry1Ab is also heat labile at temperatures of 65°C or greater. Acute oral toxicity studies in mice with FLCry1Ab have also confirmed the absence of toxicity in animals. Taken together, the evidence indicates that FLCry1Ab is unlikely to be toxic or allergenic to humans.

5. COMPOSITIONAL ANALYSES

The main purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of food.

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (e.g. solanine in potatoes).

In the case of cotton, the key components that should be considered in the comparison include proximates (cottonseed only), fatty acids, tocopherol, the natural toxicant gossypol and the anti-nutrients cyclopropenoid fatty acids (OECD 2004).

Studies submitted:
Cramer, C.K. (2006). Compositional analysis of cottonseed from event COT67B cotton plants. Syngenta Biotechnology, Inc., Study No. CRYCOT-04-101, Report No. SSB-015-06.

Study design and conduct

To determine whether unexpected changes have occurred in the composition of cottonseed from COT67B cotton as a result of the modification, and to assess its nutritional adequacy, compositional analyses were done on cottonseed collected from COT67B, and the non-transformed parental control line Coker 312, grown under field conditions.

The original COT67B transformant (T_0) was subjected to four rounds of self pollination to generate the T_4 seed which was the material used for the field trials to generate the compositional data for this study (see Figure 5).

Field trials were conducted in the United States in 2004 at four locations representing agricultural regions where cotton would normally be grown. At each location, four replicate plots for the COT67B and Coker 312 lines were planted in randomised complete blocks. Cottonseed was harvested from each plot and ginned.

A sample of whole (fuzzy) cottonseed from each plot was ground and shipped on dry ice to Covance Laboratories (Wisconsin) for analysis.

Following compositional analysis, the results for each analyte measured were statistically evaluated using an analysis of variance across locations. The statistical significance of any difference in concentration of an analyte measured between COT67B and Coker 312 was determined using an F-test at the 5% level of significance ($p \leq 0.05$). Results were also compared to the International Life Sciences Institute (ILSI) Crop Composition Database (ILSI-CCD 2006), and literature ranges reported by the OECD (2004). An F-test was also used to assess the significance of the location x genotype interaction. Where the F-test indicated that the effect of genotype was not consistent across locations, suggesting a comparison of genotypes across locations may not be valid, individual location means were analysed.

The constituents analysed in the cottonseed were: proximates (protein, carbohydrates, fat, ash, fibre, total dietary fibre), minerals (calcium and phosphorous), vitamin E, amino acids, fatty acids, gossypol, and the cyclopropenoid fatty acids (malvalic, sterculic and dihydrosterculic acid). Gossypol and the cyclopropenoid fatty acids were also measured in toasted cottonseed meal and refined cottonseed oil. The compositional analyses were done according to internationally recognised methods of analysis (e.g., AOAC International methods) or other published methods. The results of the comparisons are presented in Tables 11-17.

5.1 Nutrients

The primary food product derived from cotton is oil; therefore from a human nutrition perspective the key nutrients are the fatty acids and vitamin E. However, because other cottonseed products may be used to some extent in human food, proximate analysis is also considered important. The Applicant has also undertaken additional analyses of the mineral and amino acid content, which may be more relevant from an animal feed perspective, where cottonseed meal is often incorporated into livestock feeds; the results of these analyses are included for information.

5.1.1 Proximates

No statistically significant differences in proximate levels were observed between COT67B and Coker 312 (Table 11), with mean levels being within the range of natural variation for conventional varieties.

5.1.2 Minerals

No statistically significant differences in the level of phosphorous were observed between COT67B and Coker 312 (Table 12).
COT67B had statistically significant increased levels of calcium compared to Coker 312, however the magnitude of the observed increase was small and the value reported was within the range of natural variation for conventional cotton varieties.

5.1.3 Amino acids

No statistically significant differences in amino acid levels were observed between COT67B and Coker 312 (Table 13), with mean levels being within the range of natural variation for conventional varieties.

5.1.4 Fatty acids

The fatty acids measured were: 14:0 myristic; 16:0 palmitic; 16:1 palmitoleic; 18:0 stearic; 18:1 oleic; 18:2 linoleic; 18:3 linolenic (Table 14). All other fatty acids were below the LOQ. Statistically significant differences were observed in the levels of palmitic, stearic and oleic acids between COT67B and Coker 312. For palmitic acid, the levels were slightly decreased compared to Coker 312, and for stearic and oleic acid, the levels were slightly increased. The levels of all measurable fatty acids were within the range of natural variation for conventional cotton varieties.

5.1.5 Vitamin E

Statistically significant differences in vitamin E levels were observed between COT67B and Coker 312 at three of the four locations (Table 15). At two locations, COT67B exhibited statistically significantly higher vitamin E levels, whereas in one other location the level of vitamin E in COT67B was statistically significantly lower. In all locations, the levels of vitamin E measured in COT67B were within the range of natural variation for conventional cotton varieties.

5.3 Natural toxicants

Cotton contains the natural toxicant gossypol, a terpenoid phytoalexin that is present in the seeds, foliage and roots of cotton plants. Its purpose in the plant is to provide protection against insect damage and disease. Gossypol occurs in both a free and bound form but is toxic only in the free form. Gossypol is primarily toxic to non-ruminants but may also cause toxicity in immature ruminants.

No statistically significant differences in total and free gossypol levels were observed between COT67B and Coker 312 (Table 16) in both cottonseed as well as the processed fractions (cottonseed meal and refined oil).

The mean levels for total and free gossypol measured in cottonseed, total gossypol in refined oil and free gossypol in cottonseed meal were within the range of natural variation for conventional varieties. The levels of total gossypol in cottonseed meal for both COT67B and Coker 312 were however slightly higher than the upper range reported by the OECD (2004). The Applicant states that this result is likely an artefact of the processing of the seed into cottonseed meal as the compositional analyses of the seed, from which the meal was derived, showed levels well within the range of natural variation.

5.2 Anti-nutrients

Cotton contains several cyclopropenoid fatty acids associated with the oil. Those most commonly analysed are malvalic, sterculic and dihydrosterculic acid.

Cyclopropenoid fatty acids are considered to be anti-nutritional compounds as they are known to inhibit the desaturation of saturated fatty acids, resulting in alterations to membrane permeability and an increase in the melting point of fats in animals fed whole cottonseed and cottonseed meal (OECD 2004).

In cottonseed, a statistically significant difference between COT67B and Coker 312 was observed only in the levels of dihydrosterculic acid, where its level was slightly decreased compared to the control (Table 17). The levels of all three cyclopropenoid fatty acids were within the range of natural variation for conventional varieties. In cottonseed oil, the level of malvalic acid was slightly increased in COT67B compared to the control, although all levels were within the range of natural variation for cottonseed oil from conventional varieties (Table 17).

5.3 Conclusion

Compositional analyses were done to establish the nutritional adequacy of COT67B cotton, and to compare it to conventional cotton varieties. The components analysed were proximates, fatty acids, amino acids, vitamin E, minerals, gossypol and the cyclopropenoid fatty acids.

No differences of biological significance were observed between COT67B cotton and its conventional counterpart. Some minor differences in some of the key constituents were noted, however the differences observed were minor and the levels observed were within the range of values measured for conventional cotton varieties. Such differences most likely reflect normal biological variability. Food from COT67B cotton is therefore considered to be compositionally equivalent to food from conventional cotton varieties.

Table 11: Proximate composition of whole (fuzzy) cotton seed from COT67B (% dry weight)

	Moisture (% fresh weight)	Protein	Fat	Ash	Carbohydrates	Acid Detergent Fibre	Neutral Detergent Fibre	Total Dietary Fibre
Coker 312 (range)	4.9 (4.0 – 6.3)	18.7 (11.7 – 26.2)	17.0 (10.6 – 20.4)	4.3 (3.7 – 4.9)	60.1 (52.2 – 67.9)	52.6 (39.4 – 66.6)	56.4 (45.4 – 71.4)	49.8 (41.5 – 70.5)
COT67B (range)	5.1 (4.2 – 6.3)	19.5 (12.7 – 27.4)	18.5 (15.1 – 21.7)	4.3 (3.7 – 5.0)	57.7 (52.6 – 64.6)	52.2 (38.7 – 61.2)	58.0 (47.0 – 71.8)	48.4 (41.5 – 59.4)
F-test Probability (p≤0.05)	0.333	0.550	0.069	0.613	0.208	0.882	0.530	0.434
ILSI range	7.3 – 11.8	11.7 – 28.3	9.2 – 24.6	3.2 – 6.2	47.4 – 74.4	31.5 – 66.9	38.1 – 71.4	41.5 – 74.5
OECD range	4.0 – 9.9	21.8 – 34.2	15.4 – 36.3	3.8 – 5.0	NA*	29.0 – 40.1	40.0 – 54.8	NA

* NA = not available

Table 12: Calcium and phosphorous composition of whole (fuzzy) cottonseed from COT67B (mg/kg dry weight)

	Calcium	Phosphorous
Coker 312 (range)	1447 (1110 – 1740)	6376 (4040 – 7770)
COT67B (range)	1522 (1250 – 1830)	6347 (3950 – 8120)
F-test Probability (p≤0.05)	**0.042**	0.913
ILSI range	877.6 – 1857.9	3089.5 – 8342.5
OECD range	1050 - 3300	5600 – 8600

Table 13: Amino acid composition of whole (fuzzy) cottonseed from COT67B (mg/g dry weight)

	ASP	THR	SER	GLU	PRO	GLY	ALA	CYS	VAL
Coker 312 (range)	14.78 (10.6 – 18.8)	4.97 (3.51 – 6.29)	7.50 (5.34 – 9.26)	30.4 (21.8 – 38.6)	6.04 (4.55 – 7.56)	6.70 (4.99 – 8.38)	6.40 (4.73 – 8.09)	2.64 (1.73 – 3.51)	6.83 (5.07 – 9.10)
COT67B (range)	15.01 (9.39 – 20.3)	4.85 (3.34 – 6.59)	7.79 (5.08 – 10.4)	30.7 (18.4 – 41.9)	6.10 (3.81 – 8.33)	6.77 (4.31 – 9.01)	6.47 (4.15 – 8.79)	2.53 (1.69 – 3.29)	7.03 (4.40 – 9.64)
F-test Probability (p≤0.05)	0.786	0.734	0.464	0.856	0.857	0.843	0.847	0.440	0.608
ILSI range	10.01 – 23.25	3.37 – 8.11	5.03 – 10.70	19.58 – 49.89	4.10 – 9.39	4.44 – 10.42	4.19 – 9.57	1.59 – 4.26	4.52 – 11.29
	MET	**ILE**	**LEU**	**TYR**	**PHE**	**HIS**	**LYS**	**ARG**	**TRP**
Coker 312 (range)	2.39 (1.57 – 3.15)	5.03 (3.76 – 6.69)	9.39 (6.74 – 12.1)	4.40 (3.23 – 5.66)	8.17 (5.74 – 10.7)	4.57 (3.32 – 5.78)	7.51 (5.54 – 9.38)	16.56 (11.7 – 22.2)	1.86 (1.41 – 2.62)
COT67B (range)	2.33 (1.75 – 2.93)	5.21 (3.30 – 7.30)	9.58 (6.10 – 13.3)	4.41 (2.94 – 5.99)	8.32 (5.09 – 11.7)	4.65 (2.92 – 6.28)	7.69 (5.15 – 10.0)	16.65 (9.84 – 22.7)	1.85 (1.03 – 2.43)
F-test Probability (p≤0.05)	0.674	0.570	0.734	0.956	0.773	0.781	0.677	0.931	0.935
ILSI range	1.52 – 3.87	3.45 – 7.90	6.27 – 14.40	3.16 – 6.69	5.36 – 13.05	3.09 – 6.99	5.24 – 10.96	10.51 – 27.88	1.04 – 4.08

Table 14: Fatty acid composition[1] of whole (fuzzy) cottonseed from COT67B (% total fatty acids)

	14:0 Myristic	16:0 Palmitic	16:1 Palmitoleic	18:0 Stearic	18:1 Oleic	18:2 Linoleic	18:3 Linolenic
Coker 312 (range)	0.70 (0.58 – 0.89)	24.23 (23.18 – 24.90)	0.56 (0.46 – 0.66)	2.32 (2.19 – 2.40)	15.17 (14.47 – 15.75)	56.35 (54.88 – 57.70)	0.22 (0.16 – 0.36)
COT67B (range)	0.68 (0.56 – 0.83)	23.84 (22.81 – 24.67)	0.54 (0.44 – 0.64)	2.39 (2.29 – 2.48)	15.59 (14.82 – 16.13)	56.27 (55.02 – 57.52)	0.23 (0.17 – 0.36)
F-test Probability (p≤0.05)	0.226	**<0.001**	0.131	**0.002**	**<0.001**	0.498	0.566
ILSI range	0.53 – 0.99	21.1 – 26.9	0.46 – 0.89	2.15 – 3.32	13.4 – 20.0	48.9 – 60.7	0.16 – 0.62

[1] All other fatty acids were < LOQ (0.01 – 0.02 % total fatty acids)

Table 15: Vitamin E levels in whole (fuzzy) cottonseed from COT67B at four locations (mg/g dry weight)

Location	Plant line	Vitamin E (α-tocopherol)
Bossier City, Louisiana	Coker 312	0.159
	COT67B	0.162
Winnsboro, Louisiana	Coker 312	0.093
	COT67B	0.146
Leland, Missouri	Coker 312	0.143
	COT67B	0.121
Newport, Arizona	Coker 312	0.162
	COT67B	0.195
All locations	Coker 312	0.139
	COT67B	0.156
F-test Probability for genotype (p≤0.05)		**0.016**
F-test Probability for location x genotype interaction (p≤0.05)		**0.004**
ILSI range		0.0821 – 0.2252

Table 16: Gossypol content of whole (fuzzy) cottonseed and processed fractions from COT67B

	Plant Line	Total Gossypol	Free Gossypol
Whole seed (% dry weight)	**Coker 312 (range)**	0.579 (0.350 – 0.791)	0.492 (0.298 – 0.669)
	COT67B (range)	0.642 (0.393 – 0.828)	0.536 (0.328 – 0.737)
	F-test Probability (p≤0.05)	0.183	0.339
	ILSI range	0.23 – 1.39	0.46 – 1.99
	OECD range	0.51 – 1.43	0.47 – 0.70
Toasted cottonseed meal (% dry weight)	**Coker 312**	1.47	0.329
	COT67B	1.62	0.430
	OECD range	0.93 – 1.43	0.02 – 1.77
Refined cottonseed oil (%)	**Coker 312**	<0.002	-
	COT67B	<0.002	-
	OECD range	0.00 – 0.09	-

Table 17: Cyclopropenoid fatty acid content in whole (fuzzy) cottonseed and refined cottonseed oil from COT67B (% total fatty acids)

	Plant Line	Sterculic Acid	Malvalic Acid	Dihydrosterculic Acid
Whole seed	**Coker 312 (range)**	0.297 (0.251 – 0.350)	0.490 (0.413 – 0.589)	0.174 (0.146 – 0.202)
	COT67B (range)	0.297 (0.257 – 0.322)	0.470 (0.422 – 0.525)	0.151 (0.139 – 0.163)
	F-test Probability (p≤0.05)	0.984	0.095	**<0.001**
	ILSI range	0.17 – 0.66	0.18 – 0.66	0.11 – 0.24
	OECD range	0.13 – 0.70	0.17 – 0.61	0.11 – 0.50
Refined oil	**Coker 312**	0.252	0.760	0.163
	COT67B	0.241	0.512	0.162
	OECD range	0.08 – 0.58	0.22 – 1.44	0.00 – 0.22

6. NUTRITIONAL IMPACT

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

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (OECD 2003).

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. This assessment should include consideration of the bioavailability of the modified nutrient.

In this case, COT67B cotton is the result of a simple genetic modification to confer insect protection with no intention to significantly alter nutritional parameters in the food. In addition, extensive compositional analyses have been undertaken to demonstrate the nutritional adequacy of COT67B cotton and these indicate it is equivalent in composition to conventional cotton varieties. The introduction of COT67B cotton into the food supply is therefore expected to have little nutritional impact.

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Attachment 3

Summary of second round public submissions

Submitter	Comments
Colette Connolly, Elisa Clarke, Laurel Buxton, Tessa Reimers, Victoria Lane, Jennifer Raco, Melissa Quigley, Michelle Parker, Jane Barrett, John Watson, Kim Healy, Glynis Gilkes, Helen Bell, Merrilyn and Garry Craig, Mary Gazzo, Pam Gladstones, Allison Rocher, Trevor Archibald, Danielle Romaine, Janne Asano, Lynne Turnbull, Jenni Bourke, Tobias Koberle, Adam Breasley, M. Rucco, Carol and Keith Keller, D. Hellale, Lesley Irving, Christine and Peter Villiers, Arthur Vasiliou, Jenifer Sharp, Mary Rainsbury, Jason Blake, Leony Malak, Janet Riches, Francine Moore, Diana Cole, Anna Rosenburg, Helen Ericson, J.S. Roszkowski, Margaret Wynne, Amy Jennings, Sarah Aitken, Michele Smith, Kelly Madden, Russell Langfield, Don and Liz Bretherton, Bryan Sait, Berenice Jamieson, Gaye de Lisle, Neville and Jeannette Dowling, Herbert Gerig, James Harris, Nick Maas, Meredith Stanton, Michael Dunne, Sarah Burnell, John Richards, Wendy Adamis, Rebecca Clark, Aram Joukadjian, Yeliz Surucu, Matthew Boyes, Rosslyn Evans, Richard Bouwmeester.	Comments received as a campaign letter: ▪ Opposed to the approval of all GM foods until such time that foods derived from GM cottons are labelled as being derived from a GM source.
Dr Anna Lavelle, CEO AusBiotech Ltd represents approximately 3000 members in the health, medical device, environmental and agricultural sectors.	▪ Supports approval of food derived from insect protected cotton line COT67B on the grounds that the safety assessment found no cause for concerns. ▪ Approximately 90% of Australian cotton is genetically modified. ▪ The TGA and APVMA assess company data in conjunction with published studies. The public process undertaken by FSANZ is a strong peer-review and critiquing process and concerns expressed about this are not justified. ▪ One way of controlling costs associated with testing would be to require 'GM-free' claims to be verified by testing, 'non-GM' and 'contains GM' claims to be justified by paper audits. This would provide information to consumers while allowing producer and market choice. ▪ Current labelling requirements are satisfactory to allow choice in the market place.
Madeleine Love representative for Mothers Are Demystifying Genetic Engineering (MADGE)	▪ Particularly concerned with the allergenicity of novel proteins and strongly believes that increased food allergies are tied to the introduction of GM foods. ▪ The FSANZ assessment shows the novel protein was not fully characterised and the information provided by Syngenta Seeds is completely inadequate.

Submitter	Comments
Ivan Jeray	▪ Strongly opposed to the approval of cotton line COT67B on the grounds that GM foods are not safe, have caused deaths and disabilities. ▪ GM food will induce antibiotic resistance. ▪ Animal testing is inadequate and human testing is not done. ▪ Too many foods escape GM food labelling, and labelling is not policed. ▪ FSANZ Notification Circular did not state that A615 is a GM food application.
Christine Bennett	▪ Opposed to the Application because cross-breeding a eukaryotic plant with a prokaryotic bacterium is particularly disturbing and presents untested dangers. ▪ Fair trading has nothing to do with exploitative agri-giants forcing GM upon an unwilling market. ▪ Labelling should clearly cite all GM substances in products marketed for human or animal consumption. ▪ The Applicant's GM food is an aberration of forced genetic transmutation. Humans cannot thrive on aberrant substances; only food produced by nature is biologically balanced. ▪ GM foods contravene social expectations of restorative and regenerative health benefits from food. ▪ Most Australians do not want GM foods. There was no referendum to ask the public if GM foods are wanted. ▪ The application process should be disseminated in all forms of media and be highly visible. ▪ Where are the peer-reviewed [safety] studies? ▪ Every person has the moral right to protection from material interests resulting from any scientific production: GM foods are unethical. ▪ Aberrant GM substances potentially endanger mental health. ▪ There are major documented adverse health effects including organ deformity, unnatural immune responses, nerve damage, skin problems, airway and lung inflammation and fatalities caused by ingesting GM substances. ▪ There is evidence that GM substances are teratogenic.
New Zealand Food Safety Authority (NZFSA)	▪ The Institute of Environmental Science and Research Limited (ESR) reviewed the FSANZ safety assessment for the NZFSA. Only one issue was noted: data on the levels of phytic acid in cotton line COT67B compared to the non-GM counterpart were not presented.

Submitter	Comments
The Food Technology Association of Australia	▪ Supports approval of this Application. ▪ GM labelling should be opened to debate and review; GM ethical considerations should be part of the review.
Sally Wylie representative for Consumers Against GM (WA)	▪ More comprehensive GM food labelling based on the production process is needed to protect consumer choice, as applies in Europe.
Queensland Health	▪ Advances in science and technology should not be impeded if shown to be safe for humans and the environment. ▪ The use of GM crops and GM foods is considered on a case-by-case basis with particular consideration and relevance to human health. ▪ Updates on the regulatory status of COT67B in other countries should be provided in the FAR. ▪ Further consideration should be given to the fate of ingested recombinant DNA. ▪ FSANZ has used mostly data provided by the Applicant.
Australian Food and Grocery Council	▪ Supports approval of cotton line COT67B to allow commercial decisions to be made on the use of the product. ▪ Notes concerns of jurisdictions on the costs of enforcement. Suggests an auditing system for products to ensure that manufacturers have assessed whether they are using GM products and are labelling them correctly. ▪ Strongly rejects suggestions that broader GM labelling is required because this would not address a demonstrated consumer safety concern, would increase costs, and would be inconsistent with the Ministerial Council's policies.
Rhys Brown representing family (WA)	▪ Oil from GM cotton lines is not safe and should not be used in foods. Long term safety studies have not been undertaken. ▪ GM processes are not the same as plant breeding.
Joan Kennedy	▪ Opposed to the Application. All foods derived from a GM source should be labelled to give consumers the right to decide whether to buy the GM product.
Paul Elwell- Sutton	▪ Opposed to this Application for a number of reasons including that it could be considered unethical for New Zealand to expect other countries to grow GM crops at the expense of their environment. ▪ Approval of this Application could pose a risk to organic farming methods because of the possibility of accelerating insect resistance. ▪ Consumers are denied informed food choices without labelling on the GM process itself and therefore cannot support any GM foods.