

**6-04**  
**4 August 2004**

## **FINAL ASSESSMENT REPORT**

### **APPLICATION A509**

### **FOOD DERIVED FROM INSECT-PROTECTED COTTON LINE COT102**

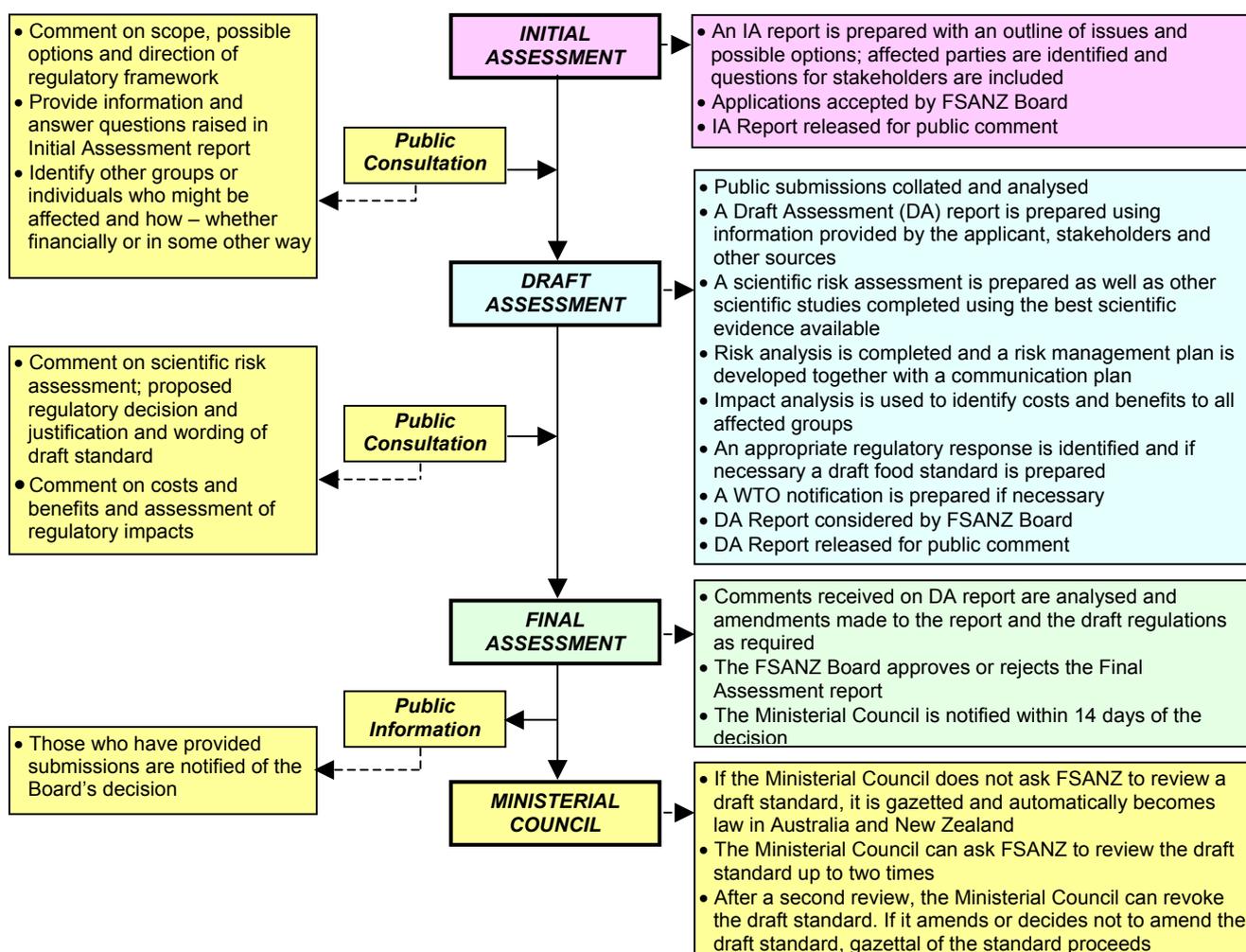
## FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

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

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

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

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



## **Final Assessment Stage**

FSANZ has now completed two stages of the assessment process and held two rounds of public consultation as part of its assessment of this Application. This Final Assessment Report and its recommendations have been approved by the FSANZ Board and notified to the Ministerial Council.

If the Ministerial Council does not request FSANZ to review the draft amendments to the Code, an amendment to the Code is published in the *Commonwealth Gazette* and the *New Zealand Gazette* and adopted by reference and without amendment under Australian State and Territory food law.

In New Zealand, the New Zealand Minister of Health gazettes the food standard under the New Zealand Food Act. Following gazettal, the standard takes effect 28 days later.

## **Further Information**

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Assessment reports are available for viewing and downloading from the FSANZ website [www.foodstandards.gov.au](http://www.foodstandards.gov.au) or alternatively paper copies of reports can be requested from FSANZ's Information Officer at [info@foodstandards.gov.au](mailto:info@foodstandards.gov.au) including other general enquiries and requests for information.

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## **Executive Summary and Statement of Reasons**

An Application has been received from Syngenta to amend the *Australia New Zealand Food Standards Code* (the Code) to approve food derived from a genetically modified (GM) insect-protected cotton, cotton line COT102. Standard 1.5.2 – Food Produced using Gene Technology – requires that GM foods undergo a pre-market safety assessment before they may be sold in Australia and New Zealand. This is a cost-recovered application.

Cotton line COT102 has been genetically modified for protection against cotton bollworm (*Helicoverpa armigera*) and native budworm (*H. punctigera*) two significant pests of cotton crops in Australia. Protection is conferred by the expression in the plant of a bacterially derived protein toxin (a *Bt*-toxin) that is specific for these two insects. Cotton line COT102 also contains a gene encoding resistance to the antibiotic hygromycin.

There is currently no approval for the sale and use of food from cotton line COT102. The only food products derived from cotton are cottonseed oil and linters. Approval will require an amendment to the Code, in the Table to clause 2 of Standard 1.5.2.

Cotton line COT102 has been developed for cultivation in North America and Australia. Therefore, if approved, food derived from cotton line COT102 may enter the food supply in Australia and New Zealand via both domestically produced (if approved by the Office of the Gene Technology Regulator) and imported products.

### **Safety assessment**

FSANZ has completed a comprehensive safety assessment of food derived from cotton line COT102. The assessment included consideration of: (i) the genetic modification to the plant; (ii) the safety of any transferred antibiotic resistance genes; (iii) the potential toxicity and allergenicity of any new proteins; and (iv) the composition and nutritional adequacy of the food, including whether there had been any unintended changes.

No potential public health and safety concerns were identified in the assessment of food derived from cotton line COT102. Therefore, on the basis of all the available evidence, including detailed studies provided by the applicant, it has been concluded that food derived from cotton line COT102 is as safe and wholesome as food derived from other cotton varieties.

### **Labelling**

Food from cotton line COT102 will require labelling if novel DNA and/or protein (refer to Standard 1.5.2 for the definition of novel DNA/protein) are present in the final food. The only food products derived from cotton are cottonseed oil and linters, neither of which contain DNA or protein. Therefore, food products containing cottonseed oil or linters derived from cotton line COT102 will not be required to be labelled as containing GM ingredients. Labelling addresses the requirement of section 10 (1)(b) of the Act, namely, provision of adequate information relating to food to enable consumers to make informed choices.

## **Impact of regulatory options**

Two regulatory options were considered in the assessment: either (1) no approval; or (2) approval of food from cotton line COT102 based on the conclusions of the safety assessment. Following an assessment of the potential impact of each of the options on the affected parties (consumers, the food industry and government), option 2 is the preferred option as it potentially offers significant benefits to all sectors with very little associated cost. The proposed amendment to the Code, giving approval to food from cotton line COT102, is therefore considered of net benefit to both food producers and consumers.

## **Consultation**

FSANZ undertook two rounds of public consultation in relation to this Application. In response, three submissions were received during the first round, and four submissions were received in the second round. Two of the first-round submitters expressed support for the application, contingent on a satisfactory safety assessment, and the remaining submitter informed FSANZ that it would comment once the Draft Assessment Report was available. After the second round of consultation, one of the submissions supported the application. The other three expressed no opinion either way. Other specific comments made in the submissions are discussed in section 5.3.

As this application involves a novel gene and protein that FSANZ has not assessed before, comments on the safety assessment were sought from two external reviewers. In general, the reviewers agreed with the conclusions of the safety assessment of COT102. Specific comments have been addressed in the safety assessment report or in this report.

## **Statement of Reasons**

An amendment to the Code to give approval to the sale and use of food derived from cotton line COT102 in Australia and New Zealand without any special conditions, is agreed on the basis of the available scientific evidence for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce cotton line COT102;
- food derived from cotton line COT102 is equivalent to food from other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the amendment to the Code is of net benefit to both food producers and consumers;
- the proposed draft amendment to the Code is consistent with the section 10 objectives of the *Food Standards Australia New Zealand Act 1991* (FSANZ Act) and the regulatory impact assessment.

## 1. Introduction

An Application was received from Syngenta Seeds Pty Ltd on 15 July 2003 seeking approval for food derived from insect-protected cotton line COT102 under Standard 1.5.2 – Food Produced Using Gene Technology.

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

- the *vip3a* gene from *Bacillus thuringiensis* subspecies *kurstaki*, which expresses an insect-specific protein toxin called VIP3A (vegetative insecticidal protein 3A); and
- the hygromycin resistance gene, *hph*, from *Escherichia coli*, expressing the enzyme hygromycin B phosphotransferase (APH4) which confers resistance to the antibiotic hygromycin.

## 2. Regulatory Problem

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

Syngenta Seeds Pty Ltd has developed a new GM variety of insect protected cotton, known as line COT102. Before food derived from cotton line COT102 can enter the food supply in Australia and New Zealand, it must first be assessed for safety and an amendment to the Code must be approved by the FSANZ Board, and subsequently be notified to the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council). An amendment to the Code may only be gazetted if the Ministerial Council raises no objections within 60 days of this notification.

Syngenta Seeds Pty Ltd has therefore applied to have Standard 1.5.2 amended to include food derived from insect protected cotton line COT102.

## 3. Objective

The objective of this Application is to determine whether it is appropriate to amend the Code to approve the use of food derived from cotton line COT102. In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives, which are set out in section 10 of the FSANZ Act. These are:

- the protection of public health and safety;
- the provision of adequate information relating to food to enable consumers to make informed choices; and
- the prevention of misleading or deceptive conduct.

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

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

In addressing the issue of approving the sale and use of food from cotton line COT102, the key objectives are the protection of public health and safety and the provision of adequate information to consumers.

#### **4. Background**

Cotton line COT102 has been genetically modified to produce an insecticidal protein that is selectively toxic to certain Coleopteran insects. The insecticidal protein (VIP3A) is produced by the soil bacterium *Bacillus thuringiensis* (also known as *Bt*). VIP3A is derived from the subspecies *kurstaki*. *Bt* formulations are widely used as biopesticides on a variety of cereal and vegetable crops grown organically or under conventional agricultural conditions.

The main purpose of the genetic modification is to confer protection against the cotton bollworm (*Helicoverpa armigera*) and the native budworm (*Helicoverpa punctigera*). As the VIP3A protein targets a different receptor in sensitive species than the Cry proteins (another type of *Bt* protein used to produce insect resistant crops), it represents a potentially useful tool in the management of pest resistance to Cry proteins.

Cotton line COT102 is also resistant to hygromycin through the expression of the enzyme hygromycin B phosphotransferase (APH4), which catalyzes the phosphorylation of hygromycin. APH4 functions as a dominant selectable marker in the initial laboratory stages of plant cell selection as cells expressing APH4 are able to survive and grow in the presence of hygromycin, which would normally kill them. Hygromycin is not used in clinical medicine and the APH4 protein has no role in the final cotton crop.

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

Cotton line COT102 is being developed for cultivation in Australia. It is intended that cotton line COT102 will be used in conventional breeding programs to produce cotton hybrids tolerant to cotton bollworm and native budworm.

The Applicant has been issued with licenses for limited and controlled release of cotton line COT102 from the Office of the Gene Technology Regulator (OGTR license numbers DIR 017/2002 and DIR 025/2002) to carry out small-scale field trials. There have been no reports of adverse effects on human health or the environment resulting from either of these releases.

More recently, the Applicant has been issued with licences by the OGTR for larger field trials (licence numbers DIR034/2003 and DIR 36/2003). None of the plants produced during the field trials will enter the human food chain.

In addition, an application to permit the use of cotton line COT102 for food and feed use in the United States has been submitted to US EPA and the FDA. If approved, food from cotton line COT102 may therefore enter the Australian and New Zealand food supply as either domestic or imported food products.

## **5. Relevant Issues**

### **5.1 Safety assessment of food from cotton line COT102**

Food from cotton line COT102 has been evaluated according to the safety assessment guidelines prepared by FSANZ<sup>1</sup>. The safety assessment included the following:

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

The Applicant submitted a comprehensive data package in support of their application and provided studies on the molecular characterisation of line COT102, the potential toxicity and allergenicity of VIP3A and APH4, and compositional analyses of cottonseed from line COT102. In addition to information supplied by the Applicant, the evaluation also had regard to other available information and evidence, including from the scientific literature, general technical information, other regulatory agencies and international bodies.

No potential public health and safety concerns were identified in the assessment of food from cotton line COT102. Therefore, on the basis of all the available evidence, including detailed studies provided by the Applicant, it has been concluded that food derived from cotton line COT102 is as safe and wholesome as food derived from other cotton varieties. The full safety assessment report is at **Attachment 2** to this document.

### **5.2 Labelling**

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

Food derived from cotton line COT102 does not have altered characteristics. Nor do the two food products derived from cotton, cottonseed oil and linters, contain any novel DNA or protein. Therefore, cottonseed oil and linters derived from cotton line COT102 will not be required to be labelled as containing GM ingredients.

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<sup>1</sup> FSANZ (2001) Information for Applicants – Amending Standard A18/Standard 1.5.2 – Food Produced Using Gene Technology.

### 5.3 Issues arising from public submissions

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

Specific issues relating to this application raised in the public submissions are addressed below.

#### 5.3.1 *Potential for novel proteins to be present in food derived from animals fed cotton line COT102*

The Dietitians Association of Australia have raised the issue that although the novel proteins in cotton line COT102 have limited potential as food allergens, this has not been studied in humans. They are concerned that although the oil and linters are essentially free of protein, the meal and hulls, which are used in animal feeds, are not. They request that foods derived from animals raised on these by-products be monitored for the presence of COT102 proteins and that the potential allergenicity of these proteins in humans be further investigated.

##### 5.3.1.1 Response

The meal and hulls of cotton line COT102 do contain the novel proteins, and may be used as animal feed. However, like any other protein in the diet, VIP3A and APH4 are expected to be broken down in the digestive system and are not absorbed by the animal as intact proteins. In fact, both VIP3A and APH4 have been shown to be degraded in simulated gastric fluid *in vitro* and are expected to be as readily digested as conventional dietary protein *in vivo*.

#### 5.3.2 *Safety assessment of conventionally bred cotton lines developed from cotton line COT102*

The New Zealand Food Safety Authority (NZFSA) has expressed concern that the applicant intends to use cotton line COT102 in conventional plant breeding programs to produce agronomically improved cotton lines. The NZFSA states that each individual product of conventional breeding needs to be assessed for safety as there is the potential for genetic rearrangements leading to the generation of novel unintended proteins in the plant. Furthermore, the NZFSA believes that food derived from products of conventional breeding with cotton line COT102 should individually be tested for the presence of novel DNA and protein.

##### 5.3.2.1 Response

While it is recognised that genetic rearrangements may occur through conventional breeding techniques, products of conventional breeding are not individually tested for safety as these techniques are considered to have a long history of safe use. Every time a conventional plant breeder develops a new line, it is possible that genetic rearrangements have occurred.

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<sup>2</sup> [www.foodstandards.gov.au/mediareleasespublications/factsheets/factsheets2002/index.cfm](http://www.foodstandards.gov.au/mediareleasespublications/factsheets/factsheets2002/index.cfm)

However, there is no reason to suppose that inserted DNA is less stable and more prone to genetic rearrangements than the endogenous DNA of the plant. Stable integration of the inserted DNA can be demonstrated both with phenotypic and genotypic evidence. This is recognised within Standard 1.5.2 as any new GM line approved in the Table to clause 2, can be used in conventional breeding programs to develop agronomically improved varieties.

In regard to the issue of testing food derived from conventionally produced hybrids of cotton line COT102 for the presence of novel DNA or protein, the absence of DNA and protein in food derived from cotton line COT102 is due to the refined nature of the foods, and not based on the levels of novel DNA or protein present in the cotton plant. For example, if less refined cottonseed oil for human consumption was found to contain novel proteins, it would be required to be labelled as GM, regardless of whether it was sourced from the progenitor COT102 plant or from any progeny produced through conventional breeding.

### *5.3.3 Stability of the insert in cotton line COT102*

NZFSA has raised the issue of the stability of the insert from one generation to the next and argues that ELISA results which show expression of VIP3A is stably maintained over five progeny generations is not enough evidence to support the conclusion that the insert is stably inherited.

#### 5.3.3.1 Response

The safety assessment involved an analysis of ELISAs of the VIP3A protein over five generations. This demonstrated that the plants were phenotypically stable over multiple generations. A Chi squared statistical analysis demonstrated no significant difference between the expected number of offspring expressing the VIP3A protein and those that did not express VIP3A over the same five generations, which demonstrates that the segregation of the insect-protection trait is consistent with a single insertion site and supports the conclusion that no genetic rearrangements have occurred at the DNA level. As mentioned in section 5.3.2, there is no evidence that inserted DNA, once incorporated into the plant genome, is less stable than endogenous DNA.

### *5.3.4 Institute for Science in Society (ISIS) article on the VIP3A protein*

Queensland Health requested advice on information contained in a article titled “New GM Toxin Looms over Our Food” issues by ISIS in a press release dated 2 December 2003.

#### 5.3.4.1 Response

In relation to the issues raised in the ISIS article about the safety of the VIP3A protein, FSANZ was able to offer the following advice.

The ISIS article implies that because VIP3A exerts its effect by binding to a receptor protein in the epithelium of susceptible insects that has homology to other proteins involved in apoptosis, it is inherently dangerous. However, the insect VIP3A receptor is unique among programmed cell death receptors and the binding of VIP3A to that receptor is a highly specific reaction. Results from experimental studies demonstrate the toxicity of VIP3A is highly selective and does not extend beyond the larvae of certain Lepidopteran species.

Furthermore, the VIP3A protein requires the conditions of the insect gut to activate it to its functional toxin-core, and no adverse effects were observed in mice receiving doses of up to 3675 mg VIP3A protein/kg body weight. The VIP3A protein has been shown to be absent from cottonseed oil, the major food product derived from COT102.

The ISIS article also questions the equivalence of plant produced and bacterially produced VIP3A used in toxicity studies. The use of bacterially-produced proteins instead of the plant-produced protein in toxicity studies is a well accepted practice and is recognised in the Codex Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants. The practice is considered acceptable provided it is demonstrated that the bacterially-expressed protein is biochemically, structurally and functionally equivalent to that produced in the GM plant.

The VIP3A protein is expressed in cotton line COT102 at very low levels, the highest level recorded being 3.23 µg VIP3A protein/g dry weight (or 3 parts in one million). Therefore, it is extremely difficult to extract enough VIP3A protein from cotton line COT102 to do meaningful toxicity studies, which require large excesses of the test substance. For this reason, VIP3A protein expressed at very high levels in bacteria is used in the toxicity studies. Three different bacterial expression systems were used to produce VIP3A protein for the toxicity studies. One of the bacterial VIP3A proteins was identical in amino acid sequence to that encoded by the synthetic *vip3A* gene in line COT102 cotton, the other two bacterial VIP3A genes varied by one or two amino acids only. In addition, contrary to information provided in the ISIS report, a mammalian acute toxicity study was conducted using plant produced VIP3A protein purified from a GM corn containing the same synthetic *vip3A* gene as COT102 cotton. These studies are summarised in the Draft Safety Assessment Report at attachment 2.

#### *5.3.5 Issue of the exemption from the requirement for a tolerance for the VIP3A protein*

Queensland Health requested advice as to how the issue of a tolerance for residues of the VIP3A protein, granted by the US Environmental Protection Agency would be addressed in Australia.

##### 5.3.5.1 Response

In Australia, if producers wished to use a pesticide product containing the VIP3A protein, the product would have to be approved by the Australian Pesticides and Veterinary Medicines Authority. In regard to cotton line COT102, the levels of the VIP3A protein in cotton line COT102 are very low, and the protein is absent from the cottonseed oil. This, in conjunction with the absence of any toxic effects in mice fed high levels of VIP3A, indicates there are no human health or safety concerns associated with the use of the VIP3A protein in cotton line COT102.

## **5.4 External review of safety assessment**

Draft safety assessment reports for several GM food applications have undergone external review by individuals with scientific expertise in relevant disciplines. The safety assessment report (at **Attachment 2**) for this application was submitted to two external reviewers, both with particular expertise in the molecular biology of plants.

Both of the reviewers generally considered that the safety of food derived from cotton line COT102 was adequately demonstrated by the available information presented in the safety assessment report prepared by FSANZ. Specific comments raised by the reviewers were incorporated into the safety assessment report where appropriate.

## **6. Regulatory Options**

### **6.1 Option 1 – not approve food from cotton line COT102**

Maintain the *status quo* by not amending the Code to approve the sale of food derived from cotton line COT102.

### **6.2 Option 2 – approve food from cotton line COT102**

Amend the Code to permit the sale and use of food (specifically oils and linters) derived from cotton line COT102, with or without listing special conditions in the Table to clause 2 of Standard 1.5.2.

## **7. Impact Analysis**

### **7.1 Affected parties**

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

The cultivation of cotton line COT102 may have an impact on the environment, which would need to be assessed by the OGTR before cultivation in Australia could be permitted. The applicant has indicated that they do intend to undertake commercial cultivation of cotton line COT102 in Australia in the future. Field trials have already been carried out in Australia under licences issued by the OGTR. A commercial release of cotton line COT102 in Australia is anticipated by the applicant in 2006–2007, relying on the appropriate permissions being granted by FSANZ, OGTR, Australian Pesticides and Veterinary Medicines Authority (APVMA) and Environment Australia (EA).

If planting in New Zealand ever became likely, a comprehensive environmental risk analysis would be required by various New Zealand government agencies including as the Environmental Risk Management Authority (ERMA) and the Ministry of Agriculture and Fisheries (MAF) in New Zealand.

## 7.2 Impact analysis

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

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

### *Option 1: Maintain the status quo and not approve food derived from cotton line COT102*

There may be a cost to consumers in terms of a possible reduction in the availability of certain food products, if cotton line COT102 is given approval in other countries that do not require segregation of products according to GM status (loss of potential new products). Similarly, there may be a cost to consumers associated with the segregation of GM and non-GM cotton, where the necessity for segregation may contribute to higher retail prices to consumers in general. There would be no direct impact on those consumers who wish to avoid GM foods, as food derived from cotton line COT102 is not currently permitted in the food supply.

There may be a cost to industry in terms of restricting innovation in food/crop production for both growers and other sectors of the food industry. There may also be costs for industry to source either segregated or non-GM supplies.

There would be no immediate impact on government. However, if this option were to be considered inconsistent with WTO obligations, there would be a potential impact on government in terms of trade policy rather than to government revenue. In the longer term, any successful WTO challenge has the potential to also impact adversely on the food industry.

### *Option 2: Amend the Code to approve food derived from cotton line COT102*

Consumers may benefit from lower prices, to the extent that savings from production efficiencies are passed on. There may also be benefits in terms of access to a greater range of products including imported food products containing cotton line COT102. There may be a cost to consumers wishing to avoid GM food by a potential restriction of choice of products, or increased prices for non-GM foods however the totality of this impact will be minimal as there are a number of other GM cotton varieties already permitted in the food supply.

Food manufacturers would have an extended choice of raw ingredients and food retailers would have an increased product range.

There is no direct impact on government as this decision is unlikely to impact on monitoring resources.

### 7.2.1 Discussion

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

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

The proposed amendment to the Code, giving approval to food from cotton line COT102, is therefore considered necessary, cost-effective and of net benefit to both food producers and consumers.

## 8. Consultation

### 8.1 Public Consultation

The Initial Assessment of this Application was advertised for public comment between 8 October 2003 and 19 November 2003. A total of three submissions were received during this period and a summary of these is included in **Attachment 3** to this report.

Following the first round of consultation, FSANZ carried out an assessment of the Application, including a safety assessment of the food, taking into account the comments received in the first round of consultation. In assessing the safety of the food, specific issues relating to cotton line COT102 were addressed in the preparation of the Draft Assessment Report.

On completion of this report, further public comment was invited between 17 March and 10 May 2004. In response to the release of the Draft Assessment Report, FSANZ received four submissions that are summarised in **Attachment 3** to this Final Assessment Report.

In the second-round consultation, one submission supported the Application. The other three submissions expressed no opinion either way.

FSANZ has now completed the assessment of the Application, involving a safety evaluation of the food and consideration of comments received in two rounds of public consultation. FSANZ will notify the outcomes of this Final Assessment Report to the Ministerial Council.

### 8.3 World Trade Organization (WTO)

During the FSANZ assessment process, comments are also sought internationally from other Members of the World Trade Organization (WTO). As Members of the WTO, Australia and New Zealand are signatories to the agreements on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) and on Technical Barriers to Trade (TBT Agreements). In some circumstances, Australia and New Zealand have an obligation to notify the WTO of changes to food standards to enable other member countries of the WTO to make comment.

A WTO notification was considered necessary for this Application as there is significant international interest in the safety of GM foods, and the proposed amendments are likely to have a liberalising effect on international trade. No comments were received in response to the notification.

## **9. Conclusion**

An amendment to the Code to give approval to the sale and use of food from cotton line COT102 in Australia and New Zealand is agreed on the basis of the available scientific information for the following reasons:

- the safety assessment did not identify any public health and safety concerns associated with the genetic modification used to produce cotton line COT102;
- food derived from cotton line COT102 is equivalent to food from other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the amendment to the Code is necessary, cost effective and of net benefit to both food producers and consumers; and
- the proposed draft amendment to the Code is consistent with the section 10 objectives of the FSANZ Act and the regulatory impact assessment.

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

## **10. Implementation and review**

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

### **ATTACHMENTS**

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Safety Assessment Report
3. Summary of first and second round public submissions

## ATTACHMENT 1

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

To commence: On gazettal

[1] *Standard 1.5.2 of the Australia New Zealand Food Standards Code is varied by –*

[1.1] *inserting in Column 1 of the Table to clause 2 -*

Oil and linters derived from insect-protected cotton line COT102	
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### DRAFT SAFETY ASSESSMENT REPORT

#### APPLICATION A509 – FOOD DERIVED FROM INSECT PROTECTED COTTON LINE COT102.

#### SUMMARY AND CONCLUSIONS

##### Background

Food derived from genetically modified (GM) cotton line COT102 has been assessed for its safety for human consumption. This cotton line has been genetically modified to be resistant to insect attack and has been developed for cultivation in North America and Australia. Therefore, if approved, cotton line COT102 may be entering the Australian and New Zealand food supply as domestic or imported food products.

A number of criteria have been addressed in the safety assessment including: a characterisation of the transferred genes, their origin, function and stability; changes at the DNA, protein and whole food levels; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be either allergenic or toxic to humans.

##### History of Use

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

##### Description of the Genetic Modification

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

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

### **Characterisation of Novel Protein**

Cotton line COT102 expresses two novel proteins – VIP3A and APH4. Protein expression analyses indicate that VIP3A is expressed in COT102 cottonseed at low levels, the highest level recorded being 3.23 µg VIP3A protein/g dry weight. APH4 levels in COT102 cottonseed ranged from undetectable to 150 ng/g dry weight. Neither protein was detected in refined cottonseed oil or cotton fibres. Therefore exposure to the protein through consumption of oils and linters derived from cotton line COT102 would be unlikely and if it did occur the levels of protein would be extremely low.

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

### **Comparative Analyses**

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

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

### **Nutritional Impact**

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

### **Conclusion**

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

## 1. INTRODUCTION

Syngenta Seeds Pty Ltd has submitted an application to Food Standards Australia New Zealand (FSANZ) to vary Standard 1.5.2 – Food Produced Using Gene Technology in the Australian New Zealand Food Standards Code, to include food from a new genetically modified (GM) cotton variety. The GM cotton variety is known commercially as COT102.

Cotton line<sup>3</sup> COT102 has been genetically modified for protection against cotton bollworm (*Helicoverpa armigera*) and native budworm (*H. punctigera*), two significant pests of cotton crops in Australia. Protection is conferred by the expression in the plant of a bacterially derived protein toxin (a *Bt*-toxin) that is specific for these two insects. This protein is known as the vegetative<sup>4</sup> insecticidal protein 3A (VIP3A) and is encoded by the *vip3A* gene. The *vip3A* gene in COT102 is a synthetic version of the *vip3A* gene derived from *Bacillus thuringiensis* subspecies *kurstaki*. The VIP3A protein is an exotoxin and is structurally, functionally and biochemically distinct from the *Bt* delta endotoxins (or Cry proteins), which have been widely used in other insect protected crops.

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

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

Cotton line COT102 is being developed for cultivation in Australia. It is intended that cotton line COT102 will be used in conventional breeding programs to produce cotton hybrids tolerant to cotton bollworm and native budworm.

In addition, an application to permit the use of cotton line COT102 for food and feed use has been submitted in the United States. If approved, food from cotton line COT102 may therefore enter the Australian and New Zealand food supply as both domestic and imported food products.

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<sup>3</sup> For the purpose of this assessment ‘line’ denotes a plant (cotton) containing a particular genetic modification derived from a unique transformation event. The usage is intended to be inclusive of the introduction of the genetic modification into other plant (cotton) backgrounds by conventional breeding. For the legal definitions of ‘conventional breeding’, ‘line’ and ‘transformation event’ refer to the interpretation section of Standard 1.5.2 of the Food Standards Code.

<sup>4</sup> VIP3A Insecticidal proteins are expressed vegetatively, that is, in the vegetative stage of growth starting at mid-log phase, as well as during sporulation, establishing a clear distinction from the Cry proteins which are expressed only during sporulation

## HISTORY OF USE

### 2.1 Donor Organisms

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

More than 60 serotypes and hundreds of different subspecies of *B. thuringiensis* have been described. Several of these subspecies have been extensively studied and commercially exploited as the active ingredients in a number of different insecticide products for use on agricultural crops, harvested crops in storage, ornamentals, bodies of water and in home gardens. The majority of described *B. thuringiensis* strains have insecticidal activity, mediated via the Cry proteins, predominantly against Lepidopteran insects (moths and butterflies) although a few have activity against Dipteran (mosquitoes and flies), Coleopteran (beetles), and Hemipteran (bugs, leafhoppers etc) insects. Other Cry proteins with toxicity against nematodes, protozoans, flatworms and mites have also been reported (Feitelson et al 1992, Feitelson 1993). The subspecies that served as the source of the *vip3A* gene expressed in cotton COT102 is selectively active against the cotton bollworm (*Helicoverpa armigera*) and native budworm (*H. punctigera*), two significant pests of cotton in Australia (OGTR, 2002).

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

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

#### *Escherichia coli*

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

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

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

### *Agrobacterium tumefaciens*

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

*Agrobacterium* naturally contains a plasmid (the *Ti* plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally therefore, *Agrobacterium* is a plant pathogen causing root deformation mainly with sugar beets, pome fruit and viniculture crops. However, adaptation of this natural process has now resulted in the ability to transform a broad range of plant species without causing adverse effects in the host plant.

## **2.2 Host Organism**

### *Gossypium hirsutum* L.

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

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

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

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

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

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

### 3. DESCRIPTION OF THE GENETIC MODIFICATION

#### 3.1 Method used in the genetic modification

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

Transformation was carried out by incubating *Agrobacterium* cells, containing the transformation vector pCOT1, with cotton hypocotyl tissue and subsequent plating of the tissue onto synthetic culture medium containing hygromycin B. Plants were regenerated and individually analysed for the presence of the *vip3A* gene by polymerase chain reaction (PCR) techniques and for insecticidal bioactivity. The selected T<sub>0</sub> transformed plants were self-pollinated to produce T<sub>1</sub> seed, and a single homozygous plant designated line COT102 was selected from the T<sub>1</sub> generation for further breeding.

#### 3.2 Function and regulation of novel genes

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

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

Genetic Element	Size (kb)	Source	Function
Left border	0.025	<i>Agrobacterium tumefaciens</i> nopaline Ti plasmid	Required for transfer of T-DNA in to the plant cell. No function in the plant cell.
nos terminator (2 copies)	0.254	<i>Agrobacterium tumefaciens</i> nopaline Ti plasmid	Transcription terminator for <i>vip3A</i> and <i>hph</i> genes (Bevan <i>et al.</i> , 1983)

<i>hph</i> gene	1.025	<i>E. coli</i>	Antibiotic resistance marker (hygromycin) used to select for transformed plant cells. (Waldron, 1997 and Kaster <i>et al.</i> , 1983)
ubiquitin-3 promoter + first intron of the ubiquitin gene	1.720	<i>Arabidopsis thaliana</i>	Confers constitutive expression of the <i>hph</i> gene in the cotton plant. (Norris <i>et al.</i> , 1993)
actin-2 promoter	1.407	<i>Arabidopsis thaliana</i>	Confers constitutive expression of the <i>vip3A</i> gene in the cotton plant. (An <i>et al.</i> , 1996)
Synthetic <i>vip3A</i> gene	2.369	Synthetic version of gene from <i>B. thuringiensis</i> (Murray <i>et al.</i> , 1989)	Gene for production of the VIP3A protein which is toxic to certain insect pests of cotton. (Estruch <i>et al.</i> , 1996)
Right border	0.025		Required for transfer of T-DNA in to the plant cell. No function in the plant cell.

### *The vip3A gene*

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

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

The *vip3A* gene is under the regulatory control of the actin-2 promoter (An *et al.*, 1996) derived from *Arabidopsis thaliana*, which confers constitutive expression of the VIP3A protein throughout the plant. The nos terminator from *Agrobacterium tumefaciens* has been used to terminate transcription and to provide a polyadenylation site.

### *The hph gene*

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

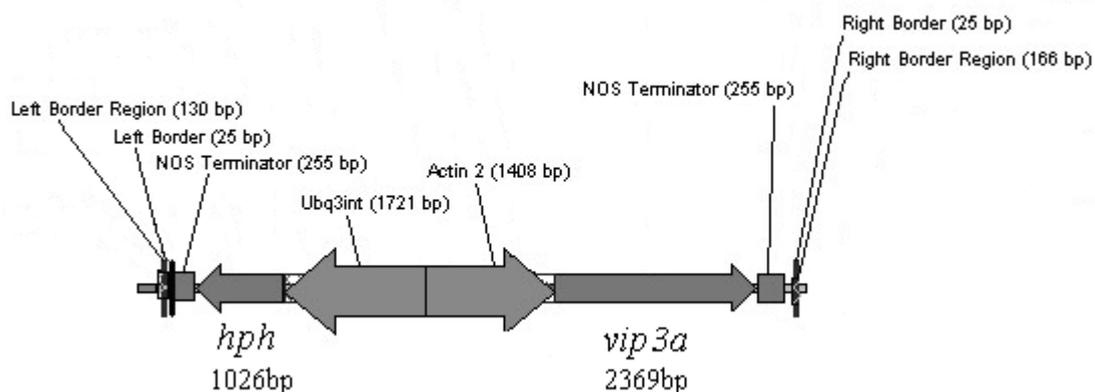
### 3.3 Characterisation of the genes in the plant

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

#### *Insert and copy number*

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

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



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

#### *PCR and sequence analysis*

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

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

#### *Flanking regions and putative Open Reading Frame analysis*

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

#### *Conclusion*

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

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

### **3.4 Stability of the genetic changes**

#### *Breeding process*

The transformed cotton cells were selected on hygromycin and regenerated into T<sub>0</sub> cotton plants. These plants were analysed phenotypically, and for expression of the *vip3A* protein. The T<sub>0</sub> plants were self pollinated to produce T<sub>1</sub> seed and a single homozygous plant designated COT102 was selected from the T<sub>1</sub> generation for seed increase and backcrossing into elite varieties. Coker 312 is not grown commercially, so the plants that have been assessed are the progeny from crosses between the genetically modified Coker 312 (COT102) and commercial cultivars. Table 2 indicates the genetic lineage of COT102 and analysis carried out at each generation.

**Table 2: Genetic Lineage and analysis of COT102**

<b>Generation</b>	<b>Method Produced</b>	<b>Analysis</b>
T <sub>0</sub>	Primary transformant in Coker 312 background	
T <sub>1</sub>	Result of self pollinated T <sub>0</sub>	Homozygous chosen and designated line COT102
T <sub>2</sub>	Result of self pollinated T <sub>1</sub>	Field evaluations - USA
T <sub>3</sub>	Result of self pollinated T <sub>2</sub>	Field evaluations - USA

T <sub>4</sub>	Result of self pollinated T <sub>3</sub>	Field evaluations; fibre quality analysis, cottonseed compositional analysis; molecular characterisation - USA
T <sub>5</sub>	Result of self pollinated T <sub>4</sub>	Field evaluations -USA
F <sub>1</sub>	Result of T <sub>1</sub> generation backcross to Coker 312	Mendelian inheritance analysis
BC <sub>1</sub> F <sub>1</sub>	Result of F <sub>1</sub> generation backcross to non transgenic commercial germplasm	
BC <sub>1</sub> F <sub>2</sub>	Result of self-pollinated BC <sub>1</sub> F <sub>1</sub>	Mendelian inheritance analysis
BC <sub>2</sub> F <sub>1</sub>	Result of BC <sub>1</sub> F <sub>1</sub> generation back cross to non-transgenic commercial germplasm	Mendelian inheritance analysis
BC <sub>2</sub> F <sub>2</sub>	Result of self pollinated BC <sub>2</sub> F <sub>1</sub>	Mendelian inheritance analysis
BC <sub>3</sub> F <sub>1</sub>	Result of BC <sub>2</sub> F <sub>1</sub> generation back cross to non-transgenic commercial germplasm	Mendelian inheritance analysis

### Segregation analysis

The stability of the insert in COT102 was analysed over five generations (F<sub>1</sub>, BC<sub>1</sub>F<sub>2</sub>, BC<sub>2</sub>F<sub>1</sub>, BC<sub>2</sub>F<sub>2</sub>, and BC<sub>3</sub>F<sub>1</sub>) by examining the expression of the VIP3A protein using an enzyme linked immunosorbent assay (ELISA).

Segregation data comparing the frequency of observed-to-expected numbers of progeny expressing the VIP3A protein were analysed statistically using Chi square analysis. As can be seen in Table 3, all generations segregated as expected for a single insertion site. The F<sub>1</sub> progeny were produced from a homozygous population and, as expected, all expressed the VIP3A protein. All four of the other generations tested produced the expected results with regard to expression of the VIP3A protein, consistent with a Mendelian pattern of inheritance.

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

Generation	Expected		Observed <sup>1</sup>		Chi square
	Positive	Negative	Positive	Negative	
F <sub>1</sub> (all +)	122	0	122	0	
BC <sub>1</sub> F <sub>2</sub> (3:1)	82.5	27.5	85	25	0.1939 <sup>ns</sup>
BC <sub>2</sub> F <sub>1</sub> (1:1)	54.5	54.4	47	62	1.7982 <sup>ns</sup>
BC <sub>2</sub> F <sub>2</sub> (3:1)	36	12	33	15	0.5277 <sup>ns</sup>
BC <sub>3</sub> F <sub>1</sub> (1:1)	24	24	26	22	0.1875 <sup>ns</sup>

<sup>1</sup> Number of plants that are positive or negative for VIP3A based on ELISA.

<sup>ns</sup> not significant at p = 0.05 (chi square = 3.84, 1 degree of freedom)

### Conclusion

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

### 3.5 Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the presence in the food of the antibiotic resistance gene DNA *per se* (WHO 1993). There have been concerns expressed however that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of some antibiotics. This section of the report will therefore concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from cotton line COT102 to microorganisms present in the human digestive tract.

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

The first issue that must be considered in relation to the presence of the *hph* gene in cotton line COT102 is the probability that this gene would be successfully transferred to and expressed in microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

1. a fragment of DNA, containing the coding region of the *hph* gene, would have to be released, probably as a linear fragment, from the DNA in the GM food;
2. the DNA fragment would then have to survive exposure to various nucleases excreted by the salivary glands, the pancreas and the intestine;
3. the DNA fragment would have to compete for uptake with dietary DNA and would have to be available at a time and place in which competent bacteria develop or reside;
4. the recipient bacteria would have to be competent for transformation;
5. the DNA fragment would have to be stably integrated into the bacterium, either as a self-replicating plasmid or through a rare recombination event with the bacterial chromosome;
6. the *hph* gene would have to be expressed, that is, would have to be integrated into the bacterial chromosome in close association with a promoter or would need to already be associated with a promoter that will function in the recipient bacterium;
7. the *hph* gene would have to be stably maintained by the bacterial population.

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

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

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

### *Conclusion*

It is extremely unlikely that the *hph* gene would transfer from cotton line COT102 to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. In the highly unlikely event that the *hph* gene were transferred the human health impacts would be negligible because the antibiotic to which it confers resistance has no clinical use in Australia and New Zealand.

## **4. CHARACTERISATION OF NOVEL PROTEINS**

### **4.1 Biochemical function and phenotypic effects**

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

#### **Studies submitted:**

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

#### *VIP3A*

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

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

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

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

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

The general symptomatology displayed by sensitive lepidopteran larvae following ingestion of VIP3A protein resembles that caused by *Bt*  $\delta$ -endotoxins, i.e., cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death (Yu *et al.*, 1997). Histopathological examination of intoxicated larvae reveals that VIP3A specifically impacts the midgut epithelium, which is also the target of the  $\delta$ -endotoxins. Following exposure of *Agrotis ipsilon* (black cutworm) larvae to 100-200 ng VIP3A/cm<sup>2</sup> diet cube for 24 hours, Yu *et al.* (1997) observed morphological changes in columnar and goblet cells. By 48 hours, the midgut lumen was filled with cellular debris, and by 72 hours, desquamation of the epithelial layer was complete and the larvae were dead. Similar histopathology was observed for *Spodoptera frugiperda* (fall armyworm), but not for *Ostrinia nubilalis* (European corn borer), which is relatively insensitive to VIP3A.

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

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

#### *APH4*

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

APH4 has a molecular weight of approximately 42kDa and catalyses the phosphorylation of hygromycin B, thereby inactivating it. The enzyme has a narrow range of substrates, in that it phosphorylates hygromycin B, hygromycin B<sub>2</sub>, and the closely-related antibiotics destomycin A and destomycin B, but does not phosphorylate other aminocyclitol or aminoglycoside antibiotics.

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

## 4.2 Protein expression analysis

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

### Studies submitted:

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

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

### *VIP3A and APH4 protein expression levels*

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

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

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

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

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

**Table 4: VIP3A Levels in Processed Cottonseed Products**

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

<sup>1</sup> ELISA analysis completed on May 9, 2002.

<sup>2</sup> ELISA analysis completed on November 15, 2002.

Nd = not detected (the mean absorbance generated during ELISA did not exceed that of the controls). The limit of detection was from 40 ng/g – 270 ng/g fresh weight, depending on the tissue and developmental stage.

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

Four leaf stage, *ca.* 2 weeks post emergence  
 Squaring, *ca.* 4 weeks post emergence  
 First White Bloom, *ca.* 9 weeks post emergence  
 Peak Bloom, *ca.* 13 weeks post emergence  
 First Open Boll, *ca.* 15 weeks post emergence  
 Pre-harvest, *ca.* 22 weeks post emergence  
 (Sampling time varied depending on environmental conditions)

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

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

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

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

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

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

As APH4 was not detected or detected at very low levels in COT102 seeds, and given the absence of any detectable protein of any sort in the refined oil, human exposure to the APH4 protein through consumption of oil and linters derived from cotton line COT102 would be unlikely and if it did occur the levels of protein would be extremely low.

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

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

<sup>1</sup> Values were determined by ELISA and were not corrected for extraction efficiency. Values for all control plants corresponded to 0 ng VIP3A or APH4/g fresh or dry weight.

<sup>2</sup> Where traces of APH4 were found, but could not be quantitated, the value is indicated as less than the lower limit of quantification.

<sup>3</sup> 'nd' VIP3A or APH4 was considered not detectable because the mean absorbance generated during ELISA did not exceed that of the controls.

<sup>4</sup> Only dry weight values for cotton fibre were calculated.

### 4.3 Potential toxicity of novel protein

#### Studies submitted:

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

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

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

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

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

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

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

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

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

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

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

Comparisons of the biological activity of *E. coli*-expressed and cotton-expressed VIP3A protein in larval diet bioassays with several lepidopteran species demonstrated very similar activities and rank order of VIP3A sensitivity among the four species surveyed.

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

#### *Potential toxicity of VIP3A*

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

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

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

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

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

**VIP3A-0100;** VIP3A protein produced in an *E. coli* expression system. The expression vector contains the same synthetic *vip3A* gene that was used to produce cotton line COT102 and Pacha corn. Test substance VIP3A-0100 was estimated to contain 73.5% VIP3A by weight and 81% of the total protein as measured by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation and ELISA.

**LPPACHA-0199**; VIP3A-enriched protein extracted from corn leaves. Test substance IAPPACHA-0100, which was used in the N-terminal sequencing and mass spectrometry described above, is also a VIP3A-enriched protein sample extracted from corn line Pacha leaves.

Test substance VIP3A-0199 was estimated to contain 0.36% VIP3A by weight and 0.39% of the total protein as measured by densitometric analysis of a Coomassie blue-stained SDS-PAGE preparation and ELISA.

**Acute Oral Toxicity Study with Test Substance VIP3A-0196 Protein in Mice. Study Director: Janice Kuhn. Stillmeadow Inc. Report no. 2989-96. 17 April 1997**

Test material	VIP3A-0196 (32% VIP3A protein by weight)
Vehicle control	2% w/v carboxymethylcellulose
Test Species	6 female and 6 male HSD:ICR mice
Dose	5050 mg/kg body weight (1616 mg VIP3A/kg bw) divided into two gavage doses administered one hour apart
GLP/guidelines	US EPA Test Guideline 81-1

Food was provided *ad libitum*, except during the 16 hours prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study. Observations for mortality and clinical/behavioural signs of toxicity were made at least three times on the day of dosing, and at least once daily thereafter for 14 days. Clinical observations included the skin, fur, eyes and mucous membranes, somatomotor activity, and behaviour pattern, and also noted any presence of tremors, convulsions, salivation, diarrhoea, lethargy, sleep or coma. Individual body weights were recorded just prior to dosing and 7 and 14 days post-dosing, or at the time of discovery after death. Surviving animals were sacrificed 14 days post dosing and subjected to gross necropsy, which included gross observations of external surfaces; all orifices; and thoracic, abdominal and pelvic cavities. For the animals sacrificed after 14 days, the following organs were weighed: brain, liver, kidneys, and adrenal glands.

With the exception of one control group male, all test and control animals survived to the scheduled sacrifice time. The control male that died prior to the scheduled sacrifice time was subjected to a necropsy examination. All animals in both groups appeared normal throughout the study with the exception of the one control male, in which decreased activity, piloerection and a swollen leg were noted. Body weight gain was unaffected by the administration of test substance VIP3A-0196, with the exception of one female that failed to gain weight during the first week of the study. Organ weights, organ-to-body weight ratios and organ-to-brain weight ratios were similar between the control and treated groups. Gross necropsy in the control animal that died revealed a torn oesophagus and a small amount of material in the chest cavity, indicative of a dosing injury. No other abnormalities were observed among the other animals.

As part of a separate analysis of the test animals, the gastrointestinal tract (glandular and non-glandular stomach, duodenum, jejunum, ileum, colon and caecum) and the pancreas were preserved and submitted to Experimental Pathology Laboratories, Inc. (EPL; Research Triangle Park, NC), where the tissues were sectioned in paraffin, stained with hematoxylin and eosin, and examined microscopically. There were no histopathological changes associated with test substance administration in any of the tissues examined.

In summary, no toxicity was observed in mice given an acute oral gavage dose of 5050 mg VIP3A-0196/kg body weight, representing *ca.* 1616 mg VIP3A/kg body weight. Based on the results of this study, the estimated LD<sub>50</sub> value for pure VIP3A protein in male and female mice is >1616 mg/kg body weight.

**Acute Oral Toxicity Study with Test Substance VIP3A-0199 Protein in Mice. Study Director: Stephen Glaza. Covance Laboratories Inc. Report no. 7012-100 7 November 2000**

Test material	VIP3A-0199 (54% VIP3A protein by weight)
Vehicle control	0.5% carboxymethyl-cellulose
Test Species	12 male and 12 female CD-1®(ICR)BR mice; 6 – 8-weeks old
Dose	5000 mg/kg body weight (2700 mg VIP3A/kg bw) in a single gavage dose
GLP/guidelines	US EPA Test Guideline OPPTS 870.1100

Food was provided *ad libitum*, except during the 4 hours prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study. Scheduled interim sacrifices occurred 6 hours post-dosing (4 or 5 mice/sex/group) and at 24 hours post-dosing (3 or 5 mice/sex/group). The final sacrifice occurred at 14 days post-dosing (5 mice/sex/group). Animals were observed twice daily (a.m. and p.m.) for mortality until the day of sacrifice. Animals were observed for clinical signs of toxicity pre-dose and at *ca.* 1, 2.5, 4 and 6 hours after test or control material administration on Day 1 and once daily thereafter until their designated day of sacrifice. Detailed clinical observations were made for each animal at each observation time. These observations included, but were not limited to, changes in skin, fur, eyes, and mucous membranes; occurrences of secretions and excretions; and autonomic activity (*e.g.*, lacrimation, piloerection, pupil size, unusual respiratory pattern). Animals were also assessed for changes in posture and reactivity to handling and the presence of clonic or tonic movements, stereotypes (*e.g.*, excessive grooming, circling) or bizarre behaviour (*e.g.*, self-mutilation, walking backwards) and, if present, abnormalities were recorded. Changes in gait were assessed by allowing the animal to walk freely to permit evaluation.

Body weights were recorded for each animal for randomization purposes, before dose administration on the day of treatment (Day 1, fasted body weight), on Day 8 for the animals scheduled to be sacrificed at study termination, and at the respective animal's scheduled sacrifice time (6 hours, 24 hours or 14 days post-dose). For the control group, five animals/sex were sacrificed at each of the three sacrifice times (6 hours, 24 hours and 14 days post-dose). For the test group, four animals/sex were sacrificed 6 hours post-dose, three animals/sex were sacrificed 24 hours post-dose, and five animals/sex were sacrificed 14-day post-dose. At each scheduled sacrifice time, the respective animals were weighed, anaesthetised, exsanguinated, and subjected to macroscopic necropsy examination. The necropsy included examination of the external features of the carcass; external body orifices; the abdominal, thoracic and cranial cavities; organs; and tissues. The brain, kidneys, stomach and liver with drained gallbladder were weighed. Organ-to-body weight and organ-to-brain weight ratios were calculated.

The following tissues were collected from each animal, preserved, sectioned in paraffin, stained with hematoxylin and eosin, and examined microscopically: brain, gallbladder, heart, intestines (caecum, colon, duodenum, ileum, jejunum, rectum), kidneys, liver, lung, stomach (glandular and non-glandular) and any macroscopic lesions observed.

All test and control animals survived to the scheduled sacrifice time. All animals in both groups appeared normal throughout the study with the exception of one female in the test material-treated group, which exhibited few faeces on Days 6 – 8, and one male in the control group, which exhibited a rough hair coat on Days 7 – 15. These are considered to be incidental findings. There were no statistically significant differences in body weights between test and control groups. The only statistically significant difference ( $p < 0.05$ ) in body weight gain was observed at the 6-hour sacrifice time where the mean values for the males and females in the test group were significantly greater than the controls. This difference was not considered to be biologically important, and similar differences were not observed at the 24-hour sacrifice, or for the weight measurements taken 7 and 14 days after dosing. There were no statistically significant differences considered treatment-related in the organ weights between control and treated animals sacrificed after 6 hours, 24 hours, or 14 days post-dose. Liver-to-body weight percentages were statistically significantly lower in VIP3A-treated males sacrificed 24 hours post-dose, but this finding had no microscopic correlates and there were no significant differences in liver-to-brain weight ratios. Therefore, the observed difference in liver-to-body weight percentage was not considered biologically meaningful. No other statistically significant differences in organ-to-body weight percentages and no differences in organ-to-brain weight ratios were observed between the treated and control groups at any sacrifice time. There were few grossly observed macroscopic alterations among the test and control groups, and none was suggestive of any relation to the test material. A similar frequency and severity of commonly seen spontaneous lesions and incidental findings were noted in control and VIP3A-treated mice of both sexes. No test material-related histomorphological findings were observed at any sacrifice time (6 hours, 24 hours or 14 days post-dose).

In summary, no toxicity was observed in mice given an acute oral gavage dose of 5000 mg VIP3A-0199/kg body weight, representing *ca.* 2700 mg VIP3A/kg body weight. Based on the results of this study, the estimated LD<sub>50</sub> value for pure VIP3A protein in male and female mice is >2700 mg/kg body weight.

**Acute Oral Toxicity Study with Test Substance VIP3A-0100 Protein in Mice. Study Director: Stephen Glaza. Covance Laboratories Inc. Report no. 7012-103 15 February 2002**

Test material	VIP3A-0100 (73.5% VIP3A protein by weight)
Vehicle control	0.5% carboxymethyl-cellulose
Test Species	8 male and 8 female Crl-1®(ICR)BR mice; 4 – 5-weeks old
Dose	5000 mg/kg body weight (3675 mg VIP3A/kg bw) in a single gavage dose
GLP/guidelines	US EPA Test GuideLine OPPTS 870.1100

Food was provided *ad libitum*, except during the 4 hours prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study. Animals were observed twice daily (a.m. and p.m.) for mortality until the day of sacrifice.

Animals were observed for clinical signs of toxicity pre-dose and at *ca.* 1, 2.5, 4 and 6 hours after test or control material administration on Day 1 and once daily thereafter until their designated day of sacrifice. Detailed clinical observations, as detailed in the study above (Glaza, 2000), were made for each animal at each observation time.

Body weights were recorded for each animal for randomisation purposes, before dose administration on the day of treatment (Day 1, pre-fasted and fasted body weight), and on Days 8 and 15 for the animals scheduled to be sacrificed on Day 15. For the test and control groups, three animals/sex/group were sacrificed 6 hours post-dose, and five animals/sex/group were sacrificed 14 days post-dose. At each scheduled sacrifice time, the respective animals were weighed, anaesthetised, exsanguinated, and subjected to macroscopic necropsy examination as described in the previous study.

All test and control animals survived to the scheduled sacrifice time. All animals in both treatment groups appeared normal throughout the study. There were no statistically significant differences in body weights between test and control groups. Although all animals exhibited weight gains throughout the study (as compared to the Day 1 fasted weights), the males treated with VIP3A-0100 had statistically significantly ( $p < 0.05$ ) lower mean weight gains on Day 8, but not on Day 14, as compared to the control males. There were no statistically significant differences in absolute organ weights, organ-to-body weight percentages or organ-to-brain weight percentages between the animals treated with VIP3A-0100 and the control animals sacrificed 14 days post-treatment. There were few grossly observed macroscopic alterations among the test and control groups, and none was suggestive of any relation to the test material. A similar frequency and severity of commonly seen spontaneous lesions and incidental findings were noted in control and VIP3A-treated mice of both sexes. No test material-related histomorphologic findings were observed at either sacrifice time (6 hours or 14 days post-dose).

In summary, no toxicity was observed in mice given an acute oral gavage dose of 5000 mg VIP3A-0100/kg body weight. The dose of 5000 mg VIP3A-0100/kg body weight represents *ca.* 3675 mg VIP3A/kg body weight. Based on the results of this study, the estimated LD<sub>50</sub> value for pure VIP3A protein in male and female mice is >3675 mg/kg body weight.

**Acute Oral Toxicity Study with Test Substance LPPACHA-0199 Protein in Mice. Study Director: Stephen Glaza. Covance Laboratories Inc. Report no. 7012-102 15 February 2002**

Test material	LPPACHA-0199 (0.36% VIP3A by weight)
Vehicle control	0.5% carboxymethyl-cellulose
Test Species	5 male and 5 female CD-1®(ICR)BR mice; 4 – 6-weeks old
Dose	5000 mg/kg body weight (18 mg VIP3A protein/kg bw) in a single gavage dose
GLP/guidelines	US EPA Test GuideLine OPPTS 870.1100

Food was provided *ad libitum*, except during the 4 hours prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study.

Animals were observed twice daily (a.m. and p.m.) for mortality until the day of sacrifice. Animals were observed for clinical signs of toxicity pre-dose and at *ca.* 1, 2.5, 4 and 6 hours after test or control material administration on Day 1 and once daily thereafter until their scheduled sacrifice 14 days later. Detailed clinical observations, as detailed in the study described above (Glaza, 2000), were made for each animal at each observation time. At sacrifice time, the animals were anaesthetised, exsanguinated, and subjected to macroscopic necropsy examination as described in the previous study.

All test and control animals survived to the scheduled sacrifice time. All animals in both groups appeared normal throughout the study. There were no statistically significant differences in body weights between test and control groups, and all animals gained weight during the study. The only statistically significant difference ( $p < 0.05$ ) in body weight gain was observed at Day 8; the mean value for the females in the test group was significantly lower than the female controls. This difference was not considered to be biologically important, and similar differences were not observed at the 14-day sacrifice. There were no visible lesions observed at the macroscopic necropsy examination.

In summary, no toxicity was observed in mice administered an acute oral gavage dose of 5000 mg test substance LPPACHA-0199/kg body weight, representing *ca.* 18 mg VIP3A/kg body weight. Similarly, no toxicity was observed in mice administered 5000 mg control substance LPPACHA-0199C/kg body weight. Based on the results of this study, the estimated LD<sub>50</sub> value for pure VIP3A protein in male and female mice is  $>18$  mg/kg body weight. Although the dose tested was significantly lower than that achieved by testing VIP3A produced in recombinant *E. coli* (see studies described above), it represented the highest practical dose that could be administered using VIP3A extracted from plants.

#### Overall LD<sub>50</sub> Estimate for VIP3A Protein

Among the four acute oral toxicity studies summarized above, the highest dose tested was *ca.* 3675 mg VIP3A/kg body weight. Because toxicity was not observed at this dose, it can be concluded that the LD<sub>50</sub> for pure VIP3A is  $>3675$  mg/kg body weight.

#### *Potential toxicity of APH4*

##### **Study submitted:**

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

In most tissues of COT102 plants, APH4 has not been detected or the levels have been too low to quantify by ELISA ( $<150$  ng/g dry weight), so it was not possible to extract sufficient APH4 protein from COT102 transformed plants for toxicology studies. Instead, APH4 protein was expressed from the inducible over-expression pET3a vector in *E. Coli* BL21DE3pLysS cells. The APH4 protein encoded by this vector was identical in amino acid sequence to that encoded by the plant transformation vector pCOT1, except for an additional 11 amino acids at the C-terminus from the T7 tag and three amino acids at the N-terminus from the vector polylinker. Following purification from *E. coli* the resulting sample, designated Test Substance APH4-0102, was estimated by ELISA to contain *ca.* 42.6% APH4 protein by weight. The material was confirmed to be enzymatically active.

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

**Acute Oral Toxicity Study with APH4 Protein in Mice. Study Director: I.R. Johnson. Syngenta Central Toxicology Laboratory. Report no. CTL/AM7143. 6 November 2002**

Test material	APH4-0102 (42.6% APH4 by weight)
Vehicle control	1% methylcellulose
Test Species	5 male and 5 female AP <sub>1</sub> CD-1 mice
Dose	1828 mg/kg body weight (779 mg APH4 /kg bw) in two gavage doses, two hours apart
GLP/guidelines	US EPA Test GuideLine OPPTS 870.1100

Observations for mortality and clinical/behavioural signs of toxicity were made at least twice on the day of dosing, and at least once daily thereafter for 14 days. Detailed clinical observations were made for each animal at each observation time. Body weights were recorded daily and food consumption was recorded weekly. No mortalities occurred during the study. The animals were sacrificed 14 days post dosing and subjected to gross necropsy. Organ weights (brain, liver with gall bladder, kidneys and spleen) were recorded and principal tissues were processed for microscopic examination. No clinical signs of toxicity were observed in either the test or control groups. There were no treatment-related effects on body weight, food consumption or organ weights, nor were there any treatment-related effects observed following macroscopic or microscopic examination.

APH4 is not acutely toxic to mice. There is no evidence of toxicity of the test substance at 1828 mg/kg body weight, representing 799 mg APH4 protein/kg body weight. The estimated LD<sub>50</sub> value for pure APH4 protein in male and female mice is >779 mg/kg body weight, the single dose tested.

*Similarities with known protein toxins*

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

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

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

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

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

#### **4.4 Potential allergenicity of novel proteins**

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

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

##### **Studies submitted:**

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

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

##### *Similarity to known allergens*

##### VIP3A

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

## APH4

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

### *In vitro* digestibility

#### **Studies submitted**

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

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

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

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

## VIP3A

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

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

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

#### APH4

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

#### 4.5 Conclusion

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

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

### 5. COMPARATIVE ANALYSES

A comparative approach focussing on the determination of similarities and differences between the GM food and its conventional counterpart aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO 2000). The critical components to be measured are determined by identifying key nutrients, key toxicants and anti-nutrients for the food source in question (FAO 1996). The key nutrients and toxicants/anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. These may be major constituents (e.g., fats, proteins, carbohydrates) or minor components (e.g., minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g., solanine in potatoes if the level is increased). The key components of cottonseed that have been considered in this comparison include proximates, amino acids, fatty acids, minerals, and the toxicants gossypol and cyclopropenoid fatty acids.

## 5.1 Nutrient analysis

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

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

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

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

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

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

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

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

### Proximate analysis

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

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

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

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

### Mineral Analysis

The minerals iron, phosphorus, potassium, calcium, zinc, copper, sodium, magnesium, manganese, and chromium were analysed and compared between the transgenic cottonseed and the control cottonseed. A summary of the mineral analyses is shown in Tables 10, 11 and 12. No statistically significant differences were observed in any of the minerals measured in 2001.

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

### Fatty Acid Analysis

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

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

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

### Amino Acid Analysis

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

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

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

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

<sup>1</sup> All values (mean and range) expressed as % dry weight except moisture which is % fresh weight.

<sup>2</sup> SD = Standard deviation

<sup>3</sup> C of V = Coefficient of variation

<sup>4</sup> Range includes data from four commercially available non-transgenic cotton varieties.

<sup>5</sup> Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

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

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

<sup>1</sup> All values (mean and range) expressed as % dry weight except moisture which is % fresh weight.

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

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

<sup>1</sup> All values (mean and range) expressed as % dry weight except moisture which is % fresh weight.

<sup>2</sup> SD = Standard Deviation

<sup>3</sup> C. of V. = Coefficient of Variation

<sup>4</sup> F-test probability for genotype

<sup>5</sup> F-test probability for interaction

<sup>6</sup> Range includes data from four commercially available non-transgenic cotton varieties.

<sup>7</sup> Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

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

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

<sup>1</sup> mean and range.

<sup>2</sup> SD = Standard deviation

<sup>3</sup> C of V = Coefficient of variation

<sup>4</sup>Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

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

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

<sup>1</sup> All values (mean and range) expressed as mg/100g.

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

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

<sup>1</sup> Mean across locations.

<sup>2</sup> SD = Standard deviation

<sup>3</sup> C of V = Coefficient of variation

<sup>4</sup> F-test probability for genotype

<sup>5</sup> F-test probability for genotype x location interaction

<sup>6</sup>Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

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

Component <sup>1</sup> (g/100g)	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test prob
<b>14:0 myristic</b>	0.837 (0.59-0.99)	0.813 (0.54-0.96)	0.022	2.6%	31.7%
<b>16:0 palmitic</b>	24.84 (22.81-25.87)	24.27 (22.59-25.64)	0.20	0.8%	15.2%
<b>16:1 palmitoleic</b>	0.587 (0.57-0.62)	0.570 (0.55-0.59)	0.022	3.7%	44.4%
<b>18:0 stearic</b>	2.51 (2.39-2.58)	2.51 (2.41-2.58)	0.01	0.3%	42.3%
<b>18:1 oleic</b>	15.25 (13.53-16.14)	15.51 (13.94-16.73)	0.34	2.2	44%
<b>18:2 linoleic</b>	55.04 (52.97-59.14)	55.94 (52.59-58.14)	0.27	0.5%	70.5%
<b>18:3 linolenic</b>	0.393 (0.27-0.53)	0.513 (0.48-0.58)	0.058	12.8%	12.6%
<b>20:0 arachidic</b>	0.240 (0.21-0.26)	0.237 (0.20-0.27)	0.008	3.4%	66.7%
<b>22:0 behenic</b>	0.120 (0.1-0.13)	0.123 (0.11-0.14)	0.008	6.7%	66.7%

<sup>1</sup> mean and range.

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

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

<sup>1</sup> All values (mean and range) expressed as g/100g.



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

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

<sup>1</sup> All values (mean and range) expressed as g/100 gm cottonseed.

<sup>2</sup> SD = Standard Deviation

<sup>3</sup> C. of V. = Coefficient of Variation

<sup>4</sup> p-value Genotype

<sup>5</sup> p-value interaction

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

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

<sup>1</sup> mean and range.

<sup>2</sup> SD = Standard deviation

<sup>3</sup> C of V = Coefficient of variation

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

Location	Visalia, CA					Leland, MS				
	Component <sup>1</sup>	COT102	Coker 312	Standard Deviation	Coefficient of Variance	F-test probability	COT102	Coker 312	Standard Deviation	Coefficient of Variance
<b>Asp</b>	2525 (2250-2710)	2605 (2460-2730)	83	3.2	26.5%	2480 (2280-2610)	2488 (2280-2600)	51	2.1	85%
<b>Thr</b>	785 (730-840)	805 (760-840)	17	2.2	20.1%	773 (700-820)	745 (700-770)	20	2.6	14%
<b>Ser</b>	1113 (1050-1180)	1170 (1080-1250)	29	2.6	6.8%	1098 (980-1210)	1190 (1050-1250)	51	4.5	8.4%
<b>Glu</b>	5740 (5160-6180)	5925 (5410-6230)	201	3.5	28.4%	5780 (5300-6140)	5883 (5650-6050)	157	2.7	42.5%
<b>Pro</b>	1085 (940-1160)	1078 (1000-1140)	32	3.0	76.5%	1025 (950-1070)	1083 (1120-1180)	36	3.4	11.1%
<b>Gly</b>	1155 (1060-1220)	1168 (1090-1220)	30	2.6	60%	1128 (1050-1180)	1155 (1120-1180)	38	3.3	37.7%
<b>Ala</b>	1068 (980-1130)	1085 (1020-1130)	29	2.7	44.9%	1063 (980-1110)	1095 (1060-1120)	39	3.6	32.7%
<b>Cys</b>	443 (420-460)	470 (450-520)	35	7.7	35.1%	468 (450-510)	463 (450-480)	14	2.9	63.8%
<b>Val</b>	1313 (1170-1400)	1325 (1220-1380)	35	2.7	65.1%	1288 (1200-1380)	1323 (1280-1370)	42	3.2	32.6%
<b>Met</b>	388 (380-400)	390 (360-430)	24	6.2	89.3%	413 (390-440)	405 (400-410)	12	3.0	44.4%
<b>Ile</b>	920 (830-980)	925 (850-970)	22	2.4	76.9%	905 (840-970)	925 (890-950)	33	3.6	45.6%

<sup>1</sup> All values (mean and range) expressed as mg/100g.

**Table 17: continued**

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

<sup>1</sup> All values (mean and range) expressed as mg/100g.

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

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

<sup>1</sup> Mean expressed as mg/100g cottonseed.

<sup>2</sup> F-test probability for Genotype

<sup>3</sup> F-test probability for Genotype x Location interaction

## 5.2 Key toxicants

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

### Gossypol

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

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

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

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

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

### Cyclopropenoid fatty acids

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

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

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

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

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

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

<sup>1</sup> mean and range.

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

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

<sup>1</sup> All values (mean and range) expressed as g/100g of cottonseed.

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

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

<sup>1</sup> All values (mean and range) expressed as g/100g cottonseed.

<sup>2</sup> SD = Standard Deviation

<sup>3</sup> C. of V. = Coefficient of Variation

<sup>4</sup> F-test probability for Genotype

<sup>5</sup> F-test probability for interaction between genotype and location

### 5.3 Conclusion

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

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

### NUTRITIONAL IMPACT

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

To date, all approved GM plants with modified agronomic production traits (e.g. herbicide tolerance) have been shown to be compositionally equivalent to their conventional

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

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

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

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

## Acknowledgements

FSANZ gratefully acknowledges the expert comments on the safety assessment of food derived from insect-protected cotton line COT102 provided by Professor Peter Langridge, CEO and Director, Australian Centre for Plant Functional Genomics, The University of Adelaide, Waite Campus, Glen Osmond, South Australia, and Dr Alan Neale, Senior Lecturer, School of Biological Sciences, Monash University, Victoria.

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## ATTACHMENT 3

### SUMMARY OF PUBLIC SUBMISSIONS

#### FIRST ROUND

Submitter	Position	Comments
1. Australian Food and Grocery Council	Supports Option 2	<ul style="list-style-type: none"><li>• Supports approval of oil and linters from cotton line COT102 contingent on them being found to be safe by FSANZ.</li></ul>
2. Food Technology Association of Victoria	Supports Option 2	<ul style="list-style-type: none"><li>• No further comments.</li></ul>
3. New Zealand Food Safety Authority	-	<ul style="list-style-type: none"><li>• Will comment once the Draft Assessment Report is available.</li></ul>

#### SECOND ROUND

Submitter	Position	Comments
1. Dietitians Association of Australia	Supports Option 2	<ul style="list-style-type: none"><li>• Supports approval of oil and linters from cotton line COT102.</li><li>• Concerned that the VIP3A protein might be present in products from animals fed COT102 and asks for these products to be monitored for this protein.</li><li>• Supports full labelling of all food products derived from GMOs</li></ul>
2. Food Technology Association of Victoria Inc	-	<ul style="list-style-type: none"><li>• No comment on this application.</li></ul>
3. New Zealand Food Safety Authority	-	<ul style="list-style-type: none"><li>• Believes that other cotton varieties produced from cotton line COT102 by conventional breeding need to be assessed individually for safety</li><li>• Comments that DNA rearrangements may occur in progeny generations and that these should be assessed by molecular characterisation to determine that the insert is stably inherited over a number of generations</li></ul>
4. Queensland Health	-	<ul style="list-style-type: none"><li>• Requested information on an article by the Institute for Science in Society regarding the VIP3A protein and on an EPA notice of an exemption from the requirement for a tolerance for VIP3A protein as a pesticide. Information was provided in a letter to Queensland Health on 28 May 2004</li></ul>