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

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FINAL ASSESSMENT REPORT

APPLICATION A614

FOOD DERIVED FROM GLYPHOSATE-TOLERANT COTTON LINE GHB614

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

Executive Summary

On 27 September 2007, Food Standards Australia New Zealand (FSANZ) received a paid Application from Bayer CropScience Pty Ltd (the Applicant) seeking approval for food derived from genetically modified (GM) cotton, line GHB614 under Standard 1.5.2 – Food produced using Gene Technology in the *Australia New Zealand Food Standards Code* (the Code). Standard 1.5.2 prohibits a food produced using gene technology from being sold or used as an ingredient or component of any food unless it is listed in the Table to clause 2 of that Standard. To be approved under Standard 1.5.2, FSANZ conducts a pre-market safety assessment on all GM foods before they may be sold in Australia and New Zealand.

The genetic modification in cotton line GHB614 consists of a single herbicide tolerance trait introduced by the transfer of a gene encoding a modified form of the enzyme 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS). This enzyme catalyses a key step in the shikimate pathway for biosynthesis of aromatic amino acids in plants, and is normally inhibited by glyphosate which ultimately leads to the death of the plant. Two simple mutations were introduced into the wild type e*psps* gene derived from corn, using site-directed mutagenesis. The mutations introduced into the 2mEPSPS enzyme significantly reduce its sensitivity to glyphosate, allowing continued function in the presence of the herbicide. Plants expressing 2mEPSPS are therefore able to tolerate treatment with glyphosate-containing herbicides.

Cotton line GHB614 has been developed for cultivation in major cotton producing countries worldwide, including eventually in Australia. Cotton derivatives, such as cottonseed oil and linters, are used in many food products and may enter the Australian and New Zealand food supply via locally produced and imported processed products. Currently, there is no approval to grow cotton line GHB614 in Australia or New Zealand.

Safety Assessment

FSANZ has completed a comprehensive safety assessment of food derived from glyphosate-tolerant cotton line GHB614, which included consideration of (i) the genetic modification introduced into the plant; (ii) the potential toxicity and allergenicity of the novel protein; and (iii) the composition of GHB614 cottonseed, compared with that from conventional cotton varieties.

No public health and safety concerns were identified in the safety assessment. On the basis of the available evidence, including detailed studies provided by the Applicant, food derived from glyphosate-tolerant cotton line GHB614 is considered as safe and wholesome as food derived from other commercial cotton varieties.

Labelling

If approved, food derived from glyphosate-tolerant cotton line GHB614 will be required to be labelled as genetically modified if there is novel DNA and/or novel protein present in the final food. Studies undertaken by the Applicant indicate detectable levels of the novel protein, 2mEPSPS, in cottonseed meal, but not in processed fractions including refined cottonseed oil and linters.

Labelling addresses the requirement of section 18(1)(b) of the FSANZ Act, namely the provision of adequate information relating to food to enable consumers to make informed choices.

Impact of regulatory options

Two regulatory options were considered in the assessment: (1) not approving; or (2) approving food derived from glyphosate-tolerant cotton line GHB614, based on the conclusions of the safety assessment. Following analysis of the potential costs and benefits of each option on affected parties (consumers, the food industry and government), approval of this Application is the preferred option as the potential benefits to all sectors outweigh the costs associated with the approval.

Purpose

The Applicant seeks amendment to Standard 1.5.2 to include food derived from glyphosate-tolerant cotton line GHB614 in the Table to clause 2.

Preferred Approach

To amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from glyphosate-tolerant cotton line GHB614 in the Table to clause 2.

Reasons for Preferred Approach

An amendment to the Code approving food derived from glyphosate-tolerant cotton line GHB614 in Australia and New Zealand is proposed 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 glyphosate-tolerant cotton line GHB614;
- food derived from glyphosate-tolerant cotton line GHB614 is equivalent to food from the conventional counterpart and other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain food commodities derived from glyphosate-tolerant cotton line GHB614 will be required if novel DNA and/or protein is present in the final food; and
- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the preferred option is an amendment to the Code.

Consultation

The Initial Assessment was advertised for public comment between 12 December 2007 and 6 February 2008; thirteen submissions were received. The Draft Assessment Report was advertised for public comment between 6 August 2008 and 17 September 2008; eighty-two submissions were received.

A summary of these is provided in **Attachment 3** to this Report. The majority of second round submissions were campaign notices calling for process-labelling of all GM foods.

FSANZ has taken submitters' comments into account in preparing the Final Assessment Report. Specific issues relating to glyphosate-tolerant cotton line GHB614 have been addressed in this Report.

CONTENTS

INTRODUCTION

An Application was received from Bayer CropScience Pty Ltd on 27 September 2007 seeking approval in the Code for food derived from genetically modified (GM) cotton, line GHB614 (known commercially as GlyTol^{TM}), under Standard 1.5.2 – Food produced using Gene Technology. Cotton line GHB614 is tolerant to the broad leaf herbicide glyphosate.

A Final Assessment of the Application has been completed, including a comprehensive scientific evaluation of the food (oil and linters) derived from cotton line GHB614 according to FSANZ guidelines[1], and consideration of issues raised in two rounds of public consultation.

1. Background

1.1 Description and Purpose of the Genetic Modification

The genetic modification in cotton line GHB614 consists of a single herbicide tolerance trait introduced by the transfer of a modified 5-enol-pyruvylshikimate-3-phosphate synthase gene, *2mepsps*, derived from corn. The EPSPS protein is a key enzyme involved in the shikimate pathway for biosynthesis of aromatic amino acids in plants, and is normally inhibited by glyphosate, ultimately leading to the death of the plant. Two simple mutations were introduced into the wild type e*psps* gene from corn, using site-directed mutagenesis. The mutations introduced into the 2mEPSPS enzyme significantly reduce its sensitivity to glyphosate, allowing the enzyme to continue to function in the presence of the herbicide. Plants expressing 2mEPSPS are therefore able to tolerate treatment with herbicides containing glyphosate as the active ingredient.

The Applicant has developed glyphosate-tolerant cotton line GHB614 for cultivation in major cotton producing countries worldwide, including eventually in Australia. While cottonseed oil is used in a large number of food products consumed by humans, the main source in the Australian domestic market is from cotton cropped locally. It is expected therefore that if approved, oil derived from GHB614 cotton would be found mainly in imported foods and would be unlikely to be present in significant amounts in the Australian or New Zealand markets. To date, the Applicant has not made an application to the Office of the Gene Technology Regulator (OGTR) seeking approval for the commercial cultivation of glyphosate-tolerant cotton line GHB614 in Australia.

1.2 Regulatory status in other countries

The Applicant has sought approval for GHB614 cotton with the United States Food and Drug Administration and the United States Department of Agriculture, and FSANZ is advised that these are likely to be finalised in the near future. Food and feed approval for GHB614 cotton has been recently granted by Health Canada and the Canadian Food Inspection Agency respectively.

[1] FSANZ (2007) Guidance Document – Safety Assessment of Genetically Modified Foods

2. The Issue / Problem

Genetically modified (GM) foods must be approved in Standard 1.5.2 before they may be sold in Australia and New Zealand. If approved, the food is listed in the Table to clause 2 of the Standard. Approval is contingent upon the completion of a pre-market safety assessment undertaken by FSANZ.

Cotton line GHB614 is a new variety of GM cotton developed by Bayer CropScience Pty Ltd. The oil or linters derived from GHB614 cotton could be present as an ingredient or component of foods imported to Australia and/or New Zealand, and therefore the company has applied to FSANZ for a variation to Standard 1.5.2 to include food derived from glyphosate-tolerant cotton line GHB614. An amendment to the Code must be approved by the FSANZ Board. The Board's decision is subsequently notified to the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council). An amendment to the Code may only be gazetted once the Ministerial Council process has been finalised.

3. Objectives

The objective of this assessment is to determine whether it would be appropriate to amend the Code to approve the use of food derived from cotton line GHB614 under Standard 1.5.2. In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives, which are set out in section 18 of the FSANZ Act. These are:

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

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

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

4. Key assessment questions

The Initial Assessment of this Application identified the key questions:

- Is food derived from glyphosate-tolerant cotton line GHB614 as safe for human consumption as food from conventional varieties of cotton?

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

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

RISK ASSESSMENT

Food from glyphosate-tolerant cotton line GHB614 has been evaluated according to the FSANZ Guidance Document on the Safety Assessment of Genetically Modified Foods[2]. The summary and conclusions from the safety assessment report (at **Attachment 2**) are presented below. In addition to information supplied by the Applicant, other available resource material including published scientific literature and general technical information was used for the assessment.

5. Risk Assessment Summary

5.1 Safety Assessment Process

The safety assessment applied to food from cotton line GHB614 addresses only food safety and nutritional issues. It does not address: environmental risks related to the environmental release of genetically modified (GM) plants used in food production; the safety of animal feed or animals fed with feed derived from GM plants; or the safety of food derived from the non-GM (conventional) plant.

In conducting a safety assessment of food derived from glyphosate-tolerant cotton line GHB614, a number of criteria have been addressed including: a characterisation of the transferred genetic elements, their origin, function and stability in the cotton genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes, and the potential for the newly expressed protein to be either allergenic or toxic in humans. FSANZ has also reviewed previously held information relating to the safety of the 2mEPSPS protein which was assessed in 1999-2000 within Application A362.

5.2 Outcomes of the Safety Assessment

A modified version of the wildtype corn *epsps* gene, designated *2mepsps*, was inserted into the conventional cotton line Coker 312, generating cotton line GHB614. The combined results from the molecular characterization of cotton line GHB614 confirm the presence of one functional, intact copy of the *2mepsps* gene at a single site in the plant genome. The new genetic trait is stably incorporated into the cotton genome and is transferred to subsequent generations in a normal pattern of inheritance. No antibiotic resistance marker genes are present in cotton line GHB614.

The modified gene encodes the 47 kDa 2mEPSPS protein, characterized by two amino acid substitutions in the naturally occurring corn enzyme: one at position 102 and the other at position 106 of the protein.

[2] http://www.foodstandards.gov.au

These specific amino acid changes significantly reduce the binding of glyphosate, allowing the enzyme to function normally in the presence of the herbicide.

The 2mEPSPS protein is expressed at relatively low levels in cottonseed, corresponding to approximately 0.01% of the total crude protein. The 2mEPSPS protein was not detected in processed oil fractions or linters derived from seeds of GHB614 cotton plants, grown under normal field conditions and sprayed with glyphosate.

The potential toxicity and allergenicity of the 2mEPSPS protein has been assessed previously by FSANZ and no safety concerns were identified. The protein is more than 99% identical to the endogenous corn protein, which is a natural component of food. The results from a large number of studies confirm the identity and physicochemical and functional properties of the 2mEPSPS protein expressed in GHB614 cotton. No adverse effects were identified in acute toxicity studies in mice using purified 2mEPSPS protein. The 2mEPSPS protein does not exhibit sequence similarity with known protein toxins or allergens, and is degraded like other dietary proteins in conditions that mimic human digestion. Based on bioinformatic, biochemical and acute animal toxicity studies, 2mEPSPS is considered non-toxic to humans and is unlikely to be allergenic.

Compositional analyses were done to establish the nutritional adequacy of cotton line GHB614, and to compare it to the conventional counterpart when grown under typical agricultural conditions. The components analysed in cottonseed were protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, gossypol, cyclopropenoid fatty acids, and phytic acid.

No differences of biological significance were observed in the composition of cottonseed derived from cotton line GHB614 when compared with the conventional counterpart. A small number of minor differences were noted in some key constituents, however the magnitude of the differences was very small and the levels of all constituents were within the range of values measured for conventional cotton varieties. Cottonseed typically shows a wide natural variation in composition due to environmental factors and the differences observed in GHB614 cottonseed reflect this normal biological variability. Food derived from cotton line GHB614 is therefore considered to be compositionally equivalent to food from conventional varieties of cotton.

The results of a feeding study in broiler chickens indicate that GHB614 cotton is equivalent to conventional cotton varieties in its ability to support typical growth and well being in rapidly growing animals. On the basis of the comparative assessment, the introduction of cottonseed oil and linters from GHB614 cotton into the food supply would be expected to have negligible nutritional impact.

5.3 Conclusions

No potential public health and safety concerns have been identified in the comprehensive assessment of glyphosate-tolerant cotton line GHB614. On the basis of the data provided in the present Application, and other information available to FSANZ, food derived from glyphosate-tolerant cotton line GHB614 is as safe and wholesome as food derived from conventionally produced cotton varieties.

RISK MANAGEMENT

6. Options

There are no non-regulatory options that could apply to this Application. The two regulatory options available for this Application are:

6.1 Option 1: Do not approve food derived from cotton line GHB614

Maintain the *status quo* by not amending Standard 1.5.2 to approve food derived from glyphosate-tolerant cotton line GHB614.

6.2 Option 2: Approve food derived from cotton line GHB614

Vary Standard 1.5.2 to permit the sale and use of food (oil and linters) derived from cotton line GHB614, with or without specified conditions of use listed in the Table to clause 2 of the Standard.

7. Impact Analysis

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

7.1 Affected Parties

In considering this Application, affected parties include the following:

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

The cultivation of cotton line GHB614 in Australia or New Zealand could have an impact on the environment, which would need to be formally assessed by the Office of Gene Technology Regulator (OGTR) in Australia, and by various New Zealand Government agencies including the Environmental Risk Management Authority (ERMA) and Ministry of Agriculture and Fisheries (MAF) before growing in either country could be permitted. At this stage, no applications concerning cotton line GHB614 have been received by these agencies.

7.2 Benefit Cost Analysis

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

7.2.1 Option 1 – not approve food derived from cotton line GHB614

Consumers: Possible restriction in the availability of certain imported food products if they are found to contain ingredients derived from cotton line GHB614.
No impact on consumers wishing to avoid GM foods, as food from GHB614 cotton is not currently permitted in the food supply.

Government: Potential impact if considered inconsistent with WTO obligations but impact would be in terms of trade policy rather than in government revenue.
Potential costs include resources required for monitoring and ensuring compliance with the Code.

Industry: Possible restriction on certain imported foods once cotton line GHB614 is commercialised overseas.
Potential longer-term impact, particularly where a successful WTO challenge has the potential to impact adversely on food industry.

7.2.2 Option 2 – approve food from cotton line GHB614

Consumers: No restriction on imported food products if containing ingredients derived from GHB614 cotton.
Potential for lower food prices, to the extent that savings from increased or improved agricultural production efficiencies are passed on to other sectors.
Potential impact on consumers wishing to avoid GM foods by a possible restriction of choice of products, or increased prices for non-GM food products.

Government: Food products containing derivatives of GHB614 cotton would comply with the Code which would reduce the potential for trade disruption on regulatory grounds.
Approval of GHB614 cotton on the basis of the risk assessment would ensure no potential conflict with WTO obligations.

Potential costs associated with additional monitoring required for compliance with the labelling provisions of the Code. Costs incurred relate to the use of detection methodology including: labour and reagent costs, methodology validation, and maintenance of methodology consistency and competency.

Industry: Broader market access and increased choice in raw materials for food manufacturing.
Benefit to importers of processed foods containing cottonseed oil and linters as ingredients, as foods derived from GHB614 cotton would be compliant with the Code.
Possible cost to food industry as some food ingredients derived from GHB614 cotton may be required to be labelled as genetically modified.
If grown in Australia at a later date, primary producers may benefit from an increased choice of crop lines with potentially lower production costs and higher yields

7.3 Comparison of Options

As food from glyphosate-tolerant cotton line GHB614 has been found to be as safe as food from conventional varieties of cotton, option 1 is likely to be inconsistent with Australia's and New Zealand's WTO obligations. Option 1 would also offer little benefit to consumers wishing to avoid GM foods: a number of GM cottons are already approved for food use in Australia and New Zealand. Maintaining a prohibition on food from GHB614 cotton could potentially limit the availability of imported food products in Australia and New Zealand due to the approval of GHB614 cotton by other countries.

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

As food derived from cotton line GHB614 has been found to be safe for human consumption and the potential benefits outweigh the potential costs, Option 2, an amendment to Standard 1.5.2 giving approval to food derived from glyphosate-tolerant cotton line GHB614, is therefore the preferred option.

COMMUNICATION AND CONSULTATION STRATEGY

8. Communication

This Application seeks approval of a food under an existing standard. As a result, FSANZ has applied a basic communication strategy to the Application, which involves advertising the availability of assessment reports for public comment in the national press and making the finalised reports available on the FSANZ website. FSANZ will issue a media release drawing journalists' attention to the matter.

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

9. Consultation

9.1 Public consultation

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

FSANZ has given submitters' comments careful consideration in preparing the Final Assessment of this Application. Specific issues relating to food derived from cotton line GHB614 have been addressed in the Report. Responses to general issues regarding GM food (e.g. labelling), food allergies, and the safety of recombinant DNA in the food supply, are also available from the FSANZ website[3]. The major issues raised are discussed here.

9.1.1 Presentation of safety data

The New Zealand Food Safety Authority suggested that additional references be provided in the safety assessment of cotton line GHB614 to clarify some of the results, particularly in the statistical analysis of the compositional data and in the description of acute oral toxicity studies in mice.

9.1.1.1 Response

In terms of the compositional data, FSANZ acknowledges that the level of detail presented in the Draft Assessment Report was not reflective of the amount of data and information provided by the Applicant. Sections 5.1 to 5.5 of the safety assessment (**Attachment 2**) have been amended to include additional information on the compositional analyses of GHB614 cotton, including a more detailed description of the statistical results, to clarify the basis of the conclusion.

Additional references have been included in the relevant section of the safety assessment concerning acute oral toxicity studies conducted on the novel protein (see Attachment 2, Section 4.4.3).

FSANZ has assessed the potential toxicity of the 2mEPSPS protein in a previous application (Application A362) for glyphosate-tolerant corn line GA21 (a product of Monsanto), which was approved in Australia and New Zealand in 2000. At this time, the results of an acute oral toxicity study in mice were evaluated. A similar, smaller study was conducted by Bayer CropScience in 2006, to examine acute oral toxicity of the 2mEPSPS protein at a higher dose.

FSANZ included the results of both studies in the Draft Assessment Report for this Application, however, the source of the studies was not presented in the safety assessment of GHB614 cotton. This has now been rectified.

9.1.2 The safety of ingested recombinant DNA

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

9.1.2.1 Response

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

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

In terms of this Application, the inserted DNA sequences have been fully characterised as part of the safety assessment. The *2mepsps* gene and the regulatory elements have been used previously in other approved GM crops. Humans have likely been exposed to these DNA sequences naturally as they are derived from common plants such as corn and sunflower, and soil bacteria. Moreover, no antibiotic resistance marker genes are present in GHB614 cotton, so there is no possibility, however remote, of conferring antibiotic resistance to microorganisms in the human gastrointestinal tract. Based on these facts, there are no valid scientific grounds for concern regarding the safety of recombinant DNA from ingestion of foods derived from cotton line GHB614.

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

9.1.3 Previous use of the 2mepsps gene

The Food Technology Association of Australia supports approval of this Application, however, the Association expressed a strong objection to the use of the same gene in multiple unrelated plant species.

9.1.3.1 Response

FSANZ has assessed eleven separate GM crop lines (canola, corn, cotton, soybean and sugarbeet) in which glyphosate-tolerance has been introduced as a new genetic trait. The trait is conferred by the insertion of one of only a small number of suitable genes into the host plant. The genes used to date are derived from either a common soil bacterium (*Agrobacterium* strain CP4) or from other plants (e.g. corn).

In the latter case, small changes have been introduced into the naturally occurring plant gene, which then produces an enzyme with a reduced affinity for glyphosate, allowing the plant to grow normally in the presence of the herbicide. The bacterial gene has a naturally low affinity for glyphosate.

Plant breeding is an ongoing process for generating new stock lines with desirable characteristics of economic and agricultural value. As an agronomic characteristic of importance to primary producers, herbicide tolerance has been introduced into numerous crop species to produce a number of independent lines. Each line has a combination of other distinguishing characteristics that make it more suitable for growing in particular geographical locations, or under different environmental conditions. When developing new plant lines with the added characteristic of glyphosate tolerance, the use of one of the small number of previously characterised genes is scientifically preferred and often necessary because of the limited choice of genes available. The use of the *2mepsps* gene in numerous crops is therefore completely justified.

In this Application, glyphosate tolerance in cotton line GHB614 is conferred by the introduction of the modified corn gene, *2mepsps*. Although this gene has been used before, each new transformation event is unique and every new plant line must be assessed separately on a case-by-case basis to evaluate both the intended and any unintended changes in the plant as a result of the genetic modification. The purpose of noting previous uses of the gene in the assessment is to assist with establishing a history of safe use.

9.1.4 Assessment of novel proteins for potential allergenicity

One submission asserted that there could reasonably be more than one novel protein present in the final food due to incomplete and variable cleavage of the chloroplast transit peptide (CTP) from the CTP-2mEPSPS protein. That submission also claimed that FSANZ should request a full protein analysis of the seed products of the plant, as only the one novel protein, 2mEPSPS without the CTP, has been assessed. It asserted that the potential presence of variant novel proteins in this cotton variety could introduce new allergens into the food supply. It also claimed that the molecular characterisation did not go far enough to rule out the possibility of other novel proteins being produced.

The same submission claimed that the approach used to determine the likelihood that the intended novel protein could be allergenic was inadequate, and that FSANZ should adhere to the approach suggested in the 2001 FAO/WHO Expert Consultation where a 6 amino acid segment was proposed as immunologically relevant. In particular, it is claimed that protein glycosylation is irrelevant to a determination of potential allergenicity and therefore this information does not support the safety of the product. Furthermore, it is asserted that *in vitro* digestibility of a protein is not a reliable indicator of potential allergenicity, based on citation of a published article.

9.1.4.1 Response

Knowledge of cell biology indicates that variable post-translational processing of proteins is a natural occurrence.
Many proteins that are destined for subcellular localisation in organelles (e.g. mitochondria or chloroplasts) are synthesised as larger precursor proteins with so-called leader sequences that are often attached at the N-terminus of the protein. The purpose of the leader sequence is to direct the protein to the appropriate cellular compartment. Upon uptake into the organelle, cleavage of the pre-protein occurs, allowing the mature protein to enter the organelle where it is functionally active. The leader sequence has no residual function and is degraded in the cytoplasm of the cell by normal protein degradative mechanisms.

It is possible that variable cleavage of the CTP could occur with some low frequency. However, incomplete cleavage of the CTP would be likely to result in a protein that is enzymatically inactive. Given that the correct enzyme function is readily observed and measurable in GHB614 cotton, it is reasonable to conclude that post-translational processing to the mature form of the protein, correctly located in chloroplasts, is occurring most of the time.

From a food safety perspective, incorrect molecular processing occurring at a low frequency in the cell is not a concern. Indeed, as this is a known occurrence in all cells, it is therefore highly likely to occur in all food crops whether or not they have been genetically modified. There is no evidence to suggest that the frequency of incorrect processing is higher in GM plants. On the contrary, the CTP used in cotton line GHB614 was designed to be highly efficient at targeting the new protein to the intended sub-cellular compartment where it can exhibit maximum enzyme activity.

9.1.4.2 Potential allergenicity

The assessment of potential allergenicity of novel proteins has been under scientific scrutiny for more than a decade.

The consensus of expert opinion is that assessment of novel proteins must rely on a weight-of-evidence approach, since there is no single test that can be applied to the protein that will definitively predict its likely allergenicity. The conclusion of numerous reviews on the most appropriate methods for predicting allergenicity of novel proteins is that the application of a series of targeted bioinformatic, physicochemical and immunological tests provides reasonable assurance that the novel protein is not likely to become an allergen[4]. To date, there are no reliable animal models that can be used for this purpose.

The weight of evidence approach used by FSANZ considers the following key questions:

- Does the novel protein come from a known allergenic source?
- Does the novel protein share molecular sequence or structural similarity to known allergens?
- Is the novel protein stable to heat processing (thermal stability)?
- Is the novel protein degraded under conditions that mimic human digestion (resistance to proteolysis)?

Other information may be considered relevant to specific proteins and may be taken into consideration in the assessment process.

For example, N-glycosylation of a protein is reported to have a stabilising effect on protein structure[5] and may make a protein less susceptible to proteolysis. In addition, if a GM crop contains a gene from a known allergenic source, the novel protein expressed in the plant can be assessed with reasonable certainty by evaluating reactivity to the protein by sensitive individuals known to be allergic to the source, either using serum (*in vitro* assay) or skin-prick testing using volunteers, or both.

Other factors, such as the level of expression of the novel protein in the food are also considered likely to be important. This is based on the observation that allergies are usually elicited by food proteins where dietary exposure to that protein is comparatively high, such as in peanut and soybean allergies[6]. In the case of foods such as peanuts and soy however, the proteins are potent allergens, and occasionally exposure to relatively small amounts of protein in severely allergic individuals may be sufficient to elicit an adverse reaction.

True food allergies are relatively uncommon (approximately 4% of the population), and are largely accounted for by eight major foods or food groups that are widespread in human diets. Those foods are milk, eggs, fish, crustacea, peanuts, soybeans, tree nuts (e.g. almonds, walnuts, pecans, Brazil nuts, pistachios, cashew and macadamia nuts) and cereals (wheat). Although the human diet consists of many thousands of proteins, only a very small proportion of the proteins in foods are allergenic. FSANZ has provided further information on the issue of potential allergenicity under 'Frequently Asked Questions', on the website at www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm.

[4] S. L. Taylor (2006). Review of the development of methodology for evaluating the human allergenic potential of novel proteins. Mol. Nutr. Food Res. 50, 604-609.
[5] H. Breiteneder and E.N. Clare Mills (2005). Molecular properties of food allergens. J. Allergy Clin Immunol. 115(1), 14-23.
[6] S.L. Taylor and S.L. Hefle (2001). Food Allergies and Other Food Sensitivities. Food Technology 55(9), 68-83.

In the assessment of this Application, the approach outlined above has been applied to the 2mEPSPS protein. The available scientific evidence indicates that the mature 2mEPSPS protein is the single novel protein produced in GHB614 cotton in amounts considered of relevance to food safety. The identity of the expected novel protein produced *in planta* was confirmed by Western blot analysis, N-terminal peptide sequencing, SDS-PAGE, HPLC/Electrospray Mass Spectrometry and phenotypic analysis. Furthermore, biochemical and bioinformatic analyses clearly indicate that the 2mEPSPS protein is highly unlikely to act as an allergen if present in foods.

While it has been demonstrated that some edible plant oils may contain small amounts of plant proteins depending on the level of processing, this is primarily of concern where there is the possibility of the presence of an identified major food allergen, such as with soybean, peanut or other tree nut derived oils in the WHO group of eight. In contrast, although the EPSPS protein family is widespread in plant- and microbially-derived foods, these proteins are not associated with food allergies. Therefore, even if present in cottonseed oil in miniscule amounts, the 2mEPSPS protein does not represent a safety concern.

9.1.5 Process-labelling of GM foods

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

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

9.1.5.1 Response

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

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

The safety of a GM food is thoroughly assessed prior to approval, and only foods found to be as safe as their conventional counterpart are permitted onto the market.

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

9.1.6 Use of company data

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

9.1.5.1 Response

It is entirely appropriate for the Applicant to be responsible for generating the scientific information required to support the safety of food derived from GHB614 cotton. These responsibilities are also recognised by FSANZ formally in acknowledgement of an Exclusive Commercial Capturable Benefit (ECCB) in the commercialisation of the product. Only the Applicant would be expected to provide the necessary financial and other resources needed to ensure quality controlled studies sufficient to meet regulatory requirements in a number of countries, including Australia and New Zealand.

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

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

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

9.2 World Trade Organization (WTO)

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

Gazettal of the variation to the Code to allow food derived from glyphosate-tolerant cotton line GHB614 in Australia and New Zealand, is likely to have a liberalising effect on international trade, as currently the food is prohibited. FSANZ considered therefore that notification of this Application, under the Sanitary and Phytosanitary Measures (SPS) Agreement was not necessary.

CONCLUSION

10. Conclusion and Preferred Approach

Preferred Approach

Amend Standard 1.5.2 – Food produced using Gene Technology, to include food derived from glyphosate-tolerant cotton line GHB614 in the Table to clause 2.

10.1 Reasons for Preferred Approach

An amendment to the Code to give approval to the sale and use of food derived from cotton line GHB614 in Australia and New Zealand is proposed 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 glyphosate-tolerant cotton line GHB614;
- food derived from glyphosate-tolerant cotton line GHB614 is equivalent to food from the conventional counterpart and other commercially available cotton varieties in terms of its safety for human consumption and nutritional adequacy;
- labelling of certain food products derived from glyphosate-tolerant cotton line GHB614 will be required if novel DNA and/or protein is present in the final food; and
- a regulation impact assessment process has been undertaken that also fulfils the requirement in New Zealand for an assessment of compliance costs. The assessment concluded that the preferred option is option 2, an amendment to the Code.

11. Implementation and Review

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

ATTACHMENTS

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

Attachment 1

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

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

To commence: on gazettal

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

Food derived from glyphosate-tolerant cotton line GHB614	

Attachment 2

SAFETY ASSESSMENT: FOOD DERIVED FROM GLYPHOSATE-TOLERANT COTTON LINE GHB614

Summary and Conclusions

Background

Glyphosate normally exerts herbicide activity by binding and inactivating EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), an enzyme that is essential for the synthesis of proteins in plants. Cotton line GHB614 has been genetically modified (GM) for tolerance to glyphosate herbicides by expression in the plant of a modified *epsps* gene from corn, *2mepsps*, which introduces two amino acid changes in the enzyme. The amino acid changes in the 2mEPSPS protein significantly lower the sensitivity to glyphosate, allowing the enzyme to continue to function in the presence of the herbicide.

Although no plans currently exist to introduce cotton line GHB614 into the Australian cropping system, the Applicant intends that it will be approved for growing in major cotton producing countries overseas, and eventually in Australia. Once approved, food products, such as cottonseed oil and linters, could enter the market via imported foods or from locally produced crops.

In conducting a safety assessment of food derived from glyphosate-tolerant GHB614 cotton, a number of criteria have been addressed including: a characterisation of the transferred gene, its origin, function and stability in the cotton genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed protein to be either allergenic or toxic in humans.

The safety assessment addresses only food safety and nutritional issues; it does not address environmental risks related to the release of GM food crops into the environment, the safety of animal feed or food products derived from animals fed GM plants, or the safety of food derived from the non-GM (conventional) plant.

History of Use

Cotton is one of the oldest cultivated crops, providing over 40% of the total fibre used in the world. Cottonseed can be processed into oil, meal, hulls and linters. Only the oil and linters are typically used as human food due to the presence of natural toxicants in the seed, which may cause toxicity if consumed in sufficient amounts. These substances are removed or reduced by the processing of cottonseed into oil and linters.

Cottonseed oil has been in common use as food since the middle of the nineteenth century. It is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine and packing oil. Cotton linters are short fibres removed from the cottonseed during processing and are a major source of cellulose for both chemical and food uses. They are used in high fibre dietary products and as a viscosity enhancer (thickener) in ice cream, salad dressings and toothpaste.

The cotton variety, Coker 312, was used as the parental variety for the transformation. Although no longer widely grown, Coker 312 is still considered a commercially acceptable cultivar.

The modified *epsps* gene present in cotton line GHB614 was derived from corn, the world's third leading cereal crop behind wheat and rice. Corn-derived products are routinely used in a large number and diverse range of foods and have a long history of safe use.

Molecular Characterisation

A modified version of the wildtype corn *epsps* gene, designated *2mepsps*, was inserted into the conventional cotton line Coker 312, generating cotton line GHB614. The combined results from the molecular characterization of cotton line GHB614 confirm the presence of one functional, intact copy of the *2mepsps* gene inserted at a single site in the plant genome. The new genetic trait is stably incorporated into the cotton genome and is transferred to subsequent generations in a normal pattern of inheritance. No antibiotic resistance marker genes are present in cotton line GHB614.

Characterisation of Novel Protein

The modified gene encodes the 47 kDa 2mEPSPS protein, characterized by two amino acid substitutions in the naturally occurring corn enzyme: one at position 102 and the other at position 106 of the protein. These specific amino acid changes significantly reduce the binding of glyphosate, allowing the enzyme to function normally in the presence of the herbicide.

The 2mEPSPS protein is expressed at relatively low levels in cottonseed. The average level of the 2mEPSPS protein in fuzzy seed from GHB614 cotton plants, grown under normal field conditions including spraying with glyphosate, was approximately 21.2 µg/g, on a fresh weight basis, which corresponds to about 0.01% of the total crude protein. The 2mEPSPS protein was not detected in processed oil fractions or linters derived from GHB614 cottonseed.

The potential toxicity and allergenicity of the 2mEPSPS protein has been assessed previously by FSANZ and no safety concerns have been identified. The protein is more than 99% identical to the endogenous corn protein, which is a natural component of the food supply. The results from a large number of studies confirm the identity and physicochemical and functional properties of the 2mEPSPS protein expressed in GHB614 cotton. No adverse effects were identified in acute toxicity studies in mice using purified 2mEPSPS protein. The 2mEPSPS protein does not exhibit sequence similarity with known protein toxins or allergens, and is degraded in conditions that mimic human digestion, as other dietary proteins. Based on bioinformatic, biochemical and acute toxicity studies, 2mEPSPS is considered non-toxic to humans and is unlikely to be allergenic.

Compositional Analyses

Compositional analyses were done to establish the nutritional adequacy of cotton line GHB614, and to compare it to the conventional counterpart when grown under typical agricultural conditions.
The components analysed in cottonseed were protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals, gossypol, phytic acid and cyclopropenoid fatty acids.

No differences of biological significance were observed in the composition of cottonseed derived from cotton line GHB614 when compared with the conventional counterpart. A small number of minor differences were noted in some key constituents, however the magnitude of the differences was very small and the levels of all constituents were within the range of values measured for conventional cotton varieties. Cottonseed typically shows a wide natural variation in composition due to environmental factors and differences observed in GHB614 cotton most likely reflect normal biological variability. Food derived from cotton line GHB614 is therefore considered to be compositionally equivalent to food from conventional varieties of cotton.

Nutritional Impact

Detailed compositional studies are considered adequate to establish the nutritional adequacy of food derived from GHB614 cotton. The introduction of cottonseed oil and linters from GHB614 cotton into the food supply would therefore be expected to have little nutritional impact. This was also demonstrated in a feeding study in rapidly growing broiler chicks, which demonstrated that cottonseed from GHB614 cotton is equivalent to its conventional counterpart and cottonseed from other commercial varieties of cotton in its ability to support typical growth and well being.

Conclusion

No potential public health and safety concerns have been identified in the assessment of glyphosate-tolerant cotton line GHB614. On the basis of the data provided in the present Application, and other information available to FSANZ, food derived from glyphosate-tolerant cotton line GHB614 is as safe and wholesome as food derived from conventionally produced cotton varieties.

1. BACKGROUND

A safety assessment has been conducted on food (cottonseed oil and linters) derived from cotton that has been genetically modified (GM) for tolerance to herbicides containing glyphosate as the active ingredient. The GM cotton is referred to as line GHB614.

Glyphosate (N-phosphonomethylglycine) is a non-selective, broad spectrum herbicide. The mode of action of glyphosate is to specifically bind to, and block, the activity of a native plant enzyme, 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS is a key enzyme in the shikimate pathway in plants which links the metabolism of carbohydrates to the biosynthesis of ring-containing compounds including aromatic amino acids. Plant EPSPS enzymes are normally inactivated by glyphosate which leads to cellular deficiencies in certain amino acids resulting ultimately in the death of the plant.

In cotton line GHB614, tolerance to glyphosate is achieved through expression in the plant of a modified form of the EPSPS enzyme, 2mEPSPS, derived from corn. Two point (single nucleotide) mutations were introduced to the corn *epsps* gene to generate *2mepsps*, using site-directed mutagenesis. These changes significantly reduce the sensitivity of the 2mEPSPS enzyme to glyphosate, allowing it to continue to function in the presence of the herbicide.

Cotton line GHB614 has been developed for agriculture in major cotton producing countries worldwide, including Australia.

2. HISTORY OF USE

2.1 Host organism

The host organism is cotton (*Gossypium hirsutum* L.), grown extensively for its fibre. Cotton is one of the oldest cultivated crops, providing over 40% of the total fibre used in the world (OECD 2004). Only the cotton boll, which develops from the plant ovary, is used for either textile fibre or food/feed. The cotton boll, once harvested, is processed (ginned) to separate the cottonseed from the cotton fibre. Cottonseed can be processed into four major by-products: oil, meal, hulls and linters. Only the oil and linters are typically used as human food.

Food products from cottonseed are limited to highly processed products due to the presence of the natural toxicants, gossypol and cyclopropenoid fatty acids in the seed, which may cause toxicity if consumed in sufficient amounts. These substances are removed or reduced by the processing of the cottonseed into oil and linters.

Cottonseed oil is regarded as premium quality oil and has a long history of safe food use. It is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine and packing oil. It contains predominantly unsaturated fatty acids. Cottonseed oil has been in common use since the middle of the nineteenth century 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 by-products – meal and hulls – are used as stock feed. Cottonseed meal is not used for human consumption in Australia or New Zealand. Although it has permission to be used for human food (after processing) in the 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. Cottonseed flour is also permitted for human consumption in the USA, provided it meets certain specifications for gossypol content, although no products are currently being produced.

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

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

2.2 Donor Organisms

Corn, *Zea mays*, is the source of the *epsps* gene that was modified to produce the *2mepsps* gene in cotton line GHB614. Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries (OECD 2002). Also known as maize, corn has been grown in Mexico and Central America for some 8000 years and in Europe for 500 years and can thus be said to have a long history of safe use as a human food. The majority of corn that is grown however is destined for use as animal feed. In 2005, worldwide production of corn was over 700 million tonnes, with the United States and China being the major producers (FAOSTAT 2005).

The *epsps* gene was isolated from a cell suspension of Black Mexican Sweet (BMS) maize (Lebrun *et al.*1997). Black Mexican is a cultivar of New England (USA) sweet corn originally introduced to the food supply in 1864. Sweet corn is categorized as a vegetable and is mainly used for human consumption.

Corn was also the source of some of the regulatory gene elements. Other plants used as a source of regulatory elements include *Arabidopsis thaliana* and sunflower (*Helianthus annuus*). Arabidopsis is not consumed as food however is not considered to be harmful in humans or other animals. Sunflowers have a safe history of human consumption; both the whole seed and extracted oil are readily consumed.

3. MOLECULAR CHARACTERISATION

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

Studies submitted:

1. Description of vector pTEM2, M. Lecleir; completed July 2007. Report ID: BIO2-004_VectDescript_029
2. Detailed insert characterization of *Gossypium hirsutum* transformation event GHB614, V. Habex and M. Lecleir; completed October 2006. Study No: BBS06-001
3. Full DNA sequence of event insert and integration site of *Gossypium hirsutum* transformation event GHB614, V. Habex; completed September 2006. Study No: BBS06-004. Amendment completed January 2007; Report No: BBS06-004-F1
4. Demonstration of the nature of the flanking sequences of *Gossypium hirsutum* transformation event GHB614, V. Habex and M. Lecleir; completed October 2006. Study No: BBS06-005

3.1 Transformation method

Cotton line GHB614 was developed through *Agrobacterium*-mediated transformation of the cotton variety Coker 312, using the transformation vector pTEM2 (see following sections).

Cotton explants were exposed to a culture of disarmed *Agrobacterium tumefaciens* containing plasmid p-TEM2. After co-culture, the cotton cells were regenerated to whole plants using the appropriate regeneration media with 500 mg/L claforan to eliminate residual *Agrobacterium*, and then selected with glyphosate.

The shoots that developed were transferred to the greenhouse, further tested for tolerance to glyphosate, and allowed to flower and set seed.

The transformation was confirmed by 2mEPSPS enzyme activity assay, by glyphosate application to leaves, and by polymerase chain reaction (PCR) and Southern blot analyses.

3.2 Description of the breeding process

The primary transformant (R_0) was crossed with its isogenic non-transgenic parental line. The progeny of this backcross effectively becomes a transgenic parental line which can then be used in conventional breeding programs to cross with other non-transgenic cotton lines to develop a number of new glyphosate-tolerant cotton varieties. This process is depicted in Figure 1 below.

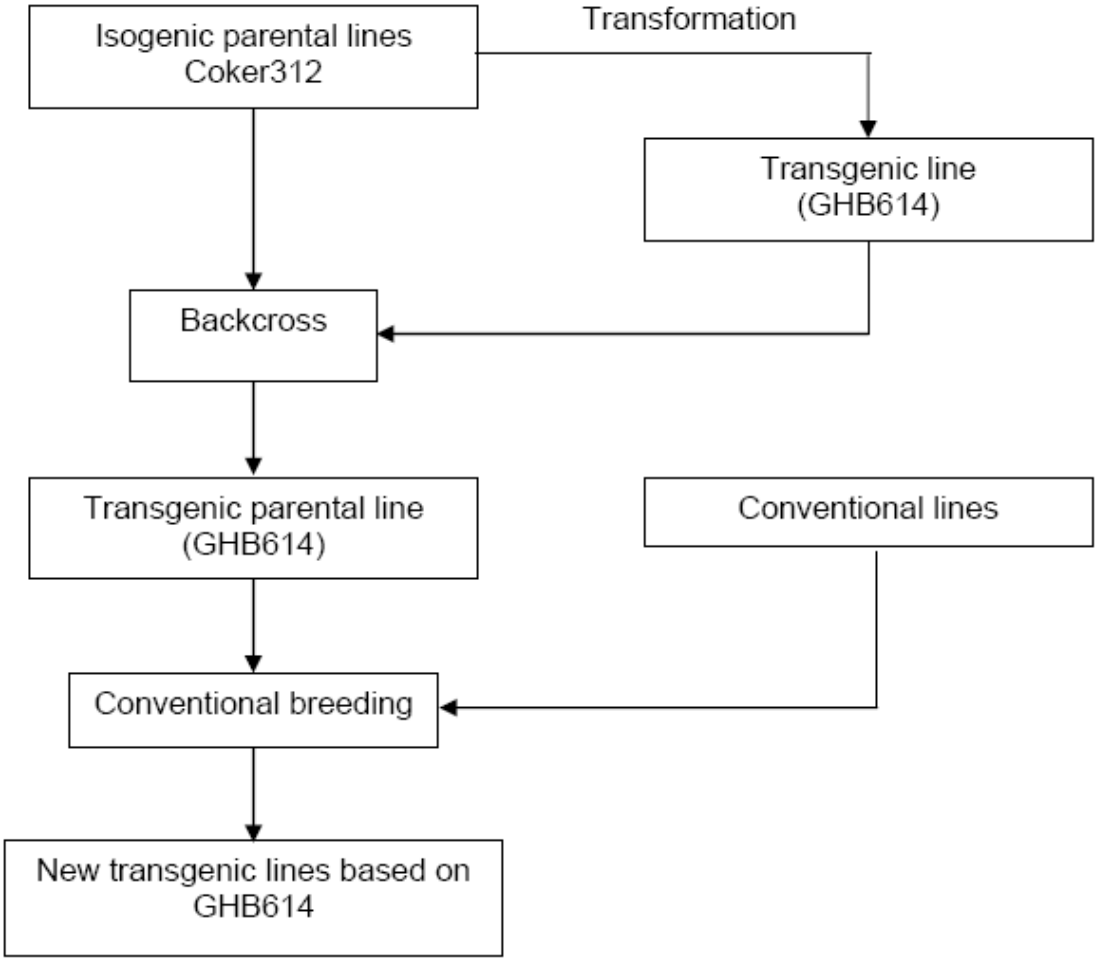

Figure 1: Schematic diagram of the breeding to develop new lines of cotton based on transformation event GHB614 cotton

3.3 Description of the gene construct

3.3.1 Coding gene

The transformation vector used to generate cotton line GHB614, p-TEM2, contains one gene expression cassette within the left and right border segments (T-DNA).

The sequence of the *2mepsps* gene is derived from the wildtype *epsps* gene from corn (*Zea mays*) with two single nucleotide mutations introduced by site directed mutagenesis. A methionine codon has been added to the N-terminal end of the 2mEPSPS protein sequence in order to restore the cleavage site of the optimized plastid transit peptide. The double mutant produces a 47 kDa protein with normal enzyme function and reduced affinity for glyphosate.

3.3.2 Other elements

The Ph4a748At promoter and h3At intron are regulatory elements used to control expression of the *2mepsps* gene in cotton and are derived from the histone H4 gene of the plant *Arabidopsis thaliana*. The use of these elements directs high level constitutive expression, particularly in rapidly growing plant tissues.

TPotp C, encodes the optimized transit peptide derived from genes of corn and sunflower and targets the mature protein to the plastids where it is normally located in the cell. The 3'histonAt terminator from *Arabidopsis thaliana* corresponds to the polyadenylation signal which is essential to end transcription of the introduced gene.

A full description of the genetic elements within the T-DNA of the transformation vector is provided below.

Symbol	Definition	Source	Size	Reference	Function
LB	Left border repeat	*Agrobacterium tumefaciens*	25	Zambrysky, 1988	Cis-acting element for T-DNA transfer
Ph4a748At	Promoter	*Arabidopsis thaliana*	1011	Chaboute *et al.*, 1987	High level constitutive expression
intron1 h3At	Intron	*Arabidopsis thaliana*	517	Chaubet *et al.*, 1992	High level constitutive expression
TPotp C	Optimized transit peptide	*Zea mays, Helianthus annuus*	373	Lebrun *et al.*, 1996	Targets the mature protein to the plastids, where the wild-type protein is located
2mepsps	Glyphosate tolerance gene	*Zea mays*	1338	Lebrun *et al.*, 1997	Herbicide tolerance and selectable marker
3'histonAt	Terminating signal	*Arabidopsis thaliana*	743	Chaboute *et al.*, 1987	Stop signal
RB	Right border repeat	*Agrobacterium tumefaciens*	25	Zambrysky, 1988	*Cis*-acting element for T-DNA transfer

3.4 Characterisation of the genes in the plant

A number of molecular analyses were conducted to determine the number of insertions and characterise the inserted DNA in GHB614 cotton. One copy of the introduced gene expression cassette is present in cotton line GHB614. A summary of each of the molecular analyses and the findings are given below.

3.4.1 Southern blot analyses

Genomic DNA from leaf tissue of GHB614 cotton plants (identity confirmed by PCR) was analysed using Southern blot analysis to determine the insert number, the copy number, the integrity of the inserted *2mepsps* gene cassette, and evaluate the presence or absence of plasmid backbone sequences. Conventional wildtype cotton variety Coker312 (used in the transformation) was used as the negative control for these analyses. The transformation vector, p-TEM2, and a wildtype control with 1 copy of digested pTEM2 were used as positive hybridisation controls. The latter reconstituted sample served to show that the hybridizations were performed under conditions allowing detection of target sequences.

Isolated genomic DNA samples from GHB614 cotton and conventional cotton were digested with nine different restriction enzymes, separated on agarose gels and then subjected to Southern blot analysis. To determine the insert and copy number of the introduced DNA, the separated DNA fragments were transferred to a membrane and sequentially hybridized with different radioactively labelled probes: four probes containing each single genetic element present in the p-TEM2 vector used for the transformation, and the complete T-DNA probe. The number of hybridising fragments detected indicates the number of inserts present in GHB614 cotton.

The hybridisation results obtained with the DNA positive and negative controls demonstrate that the Southern blot analysis was performed under conditions allowing hybridization of the specific probes with the target sequences. Based on a comparison of the size and pattern of observed fragments with the expected fragment sizes from digestion of genomic DNA, a single and unique site of insertion of the transgenic sequences is present in cotton line GHB614.

3.4.2 Polymerase chain reaction and DNA sequence analyses

The organisation of the genetic elements within the insert in GHB614 cotton was further characterised using PCR analysis by amplifying three overlapping regions of DNA spanning the entire length of the insert. The PCR products generated, following PCR of genomic DNA from GHB614 cotton, were all of the expected size.

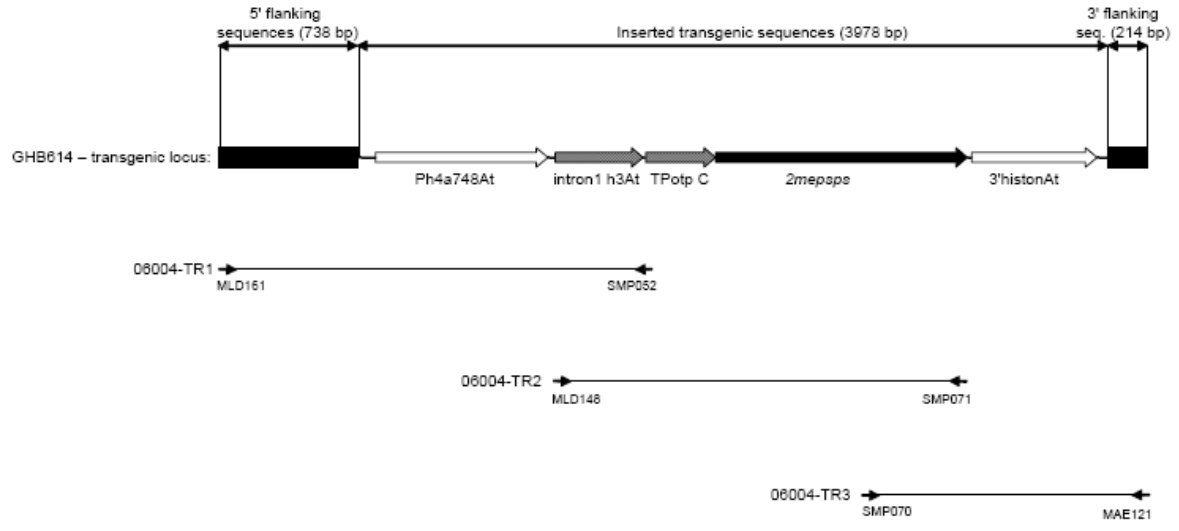

Figure 2: Overlapping PCR products generated across the insert in GHB614 cotton

The PCR products generated from GHB614 cotton genomic DNA were subject to DNA sequencing to further confirm the organisation of genetic elements within the insert, as well as to determine the 5' and 3' insert-to-genomic DNA junctions, and the complete DNA sequence of the inserted DNA and adjacent genomic DNA regions. A consensus sequence of the inserted DNA was generated by compiling the results of numerous sequencing reactions performed on each of the overlapping PCR products. This consensus sequence was then aligned to the DNA sequence of the corresponding T-DNA to determine if any changes had occurred during the transformation process.

The sequence determination indicated that the size of the inserted DNA in GHB614 cotton is 3978 base pairs (bp) and the arrangement of the genetic elements within the insert is identical to the corresponding transformation vector, pTEM2. In addition to the insert sequence, 214 bp of Right Border flanking sequence (3' end of insert) and 738 bp of Left Border flanking sequence (5' end of insert) were found to be completely identical to the cotton genomic sequences present at the integration site before transformation.

Determination of the wildtype target locus sequence was performed using DNA isolated from homozygous (BC_2F_5) transgenic and control cotton DNA (see Figure 3). A flanking DNA specific primer upstream of the T-DNA insert was used together with a flanking DNA specific primer downstream of the T-DNA insert to amplify the target (insertion) site in the non-transgenic cotton. A 947 bp segment was generated and sequenced. The obtained sequence was aligned with the 5' and 3' flanking sequences in cotton even GHB614. A fragment of 17 bp is present in the non-transgenic cotton but not in GHB614 at the transgene locus. Therefore, this short fragment was deleted at the insertion site upon integration of the T-DNA from pTEM2.

3.4.3 Bioinformatic analysis of the 5' and 3' junction regions

Bioinformatic analyses were performed on the junction regions between plant genomic and inserted DNA in GHB614 cotton, to ascertain whether any known cotton genes were interrupted by insertion of the transgene and whether putative polypeptides encoded by the 5' and 3' junction regions were likely to be expressed. These analyses were entirely theoretical, but were conducted to exclude the possibility that chimaeric proteins would be produced in GHB614 cotton as a result of the transformation.

Studies submitted:

1. Bioinformatics analysis of newly created ORFs from GlyTol cotton transformation event GHB614, N. Vandermarliere and K. De Pestel; completed August 2007.
Report No: 2006-GHB614-EPC-018
2. Bioinformatics analysis of the pre-insertion locus of *Gossypium hirsutum* transformation event GHB614, V. Habex and S. Tanghe; completed May 2007.
Report No: 2006-GHB614-NAC004
3. GlyTol Cotton Elite Event GHB614 (Glyphosate-tolerant cotton) *In silico* analysis of putative Open Reading Frame (ORF) sequences for identifying potential homologies to known toxins and allergens, Junguo Zhou and C. Herouet-Guicheney; completed November 2006.

To identify the presence of endogenous genes located near the 5' and 3' junction regions in GHB614 cotton, a BLASTn similarity search was performed (version 2.0, National Centre for Biotechnology Information, NCIB). The BLASTn similarity search compares a specific query nucleotide sequence with sequences in nucleotide databases. The results show no homology of the flanking DNA regions in GHB614 cotton with known cotton genes, mRNA, cDNA or ESTs present in the databases used.

Open reading frame (ORF) analysis and gene search tools were applied to predict the presence of any newly created coding sequences in the 5' flanking genomic/insert DNA junction region and in the 3' flanking insert/genomic DNA junction region. The ORFs were defined as regions between start (ATG) and stop (TAA, TAG, TGA) translation codons with a minimum size of eight amino acids (corresponding to twenty-four nucleotides, not including stop codon). In all cases, all six reading frames were examined.

Two ORFs were found across the 5' region. Several bioinformatics tools were applied to look for regulatory elements such as core promoters, polyadenylation (polyA) signals and ribosome binding sites (RBS) to gauge whether these identified ORFs could be putatively active. No ORFs or genes were found across the 3' region.

The findings relating to the deduced ORFs at the 5' junction region were[7]:

- ORF-1 (sense strand): a CAAT-box, a potential polyA signal and RBS were found. No homology was found with a TATA-box.

- ORF-2 (anti-sense strand): homology was found with a CAAT-box and a polyA signal. No homology was found with the TATA-box and RBS.

The Applicant claims that the sequence similarities with certain regulatory elements are not sufficient indication of newly created, functional ORFs. The absence of a TATA-box in ORFs 1 and 2 and no ribosome binding site in ORF-2 would indicate that these ORFs are not active either at the level of transcription or translation and therefore the probability of expression of a newly created peptide due to insertion of the transgene is remote.

The putative polypeptides from each ORF identified in GHB614 cotton were subjected to detailed *in silico* analysis to evaluate homology with known toxins or allergens contained in a number of large, publicly available databases, including the updated Uniprot-Swissprot, Uniprot-trEMBL, PIR, DAD, Nrl-3d, GenPept and Allergen databases, using FindPatterns or BLASTP algorithms. The overall structural similarity of the putative polypeptides to sequences in each database was assessed. The extent of structural relatedness was evaluated using visual inspection of the aligned sequences, the calculated percent identity over a linear contiguous eight amino acid segment, and *E* (expectation) score. The *E* score is a statistical measure of the likelihood that the observed similarity could have occurred by chance. A larger *E* score indicates a lower degree of similarity. Typically, alignments between two sequences will need to have an *E* score of less than 1×10^{-5} to be considered to have significant homology. Based on this global analysis, the putative ORF-1 and ORF-2 amino acid sequences showed no biologically relevant identities with known toxins or allergens.

3.4.4 Conclusion

Detailed molecular analyses indicate that one functional copy of the *2mepsps* gene expression cassette has been inserted at a single genomic locus in GHB614 cotton. The regulatory elements and coding region of the inserted gene are intact and no additions, deletions or gene rearrangements within the T-DNA are present in GHB614 cotton.

[7] The transcription complex recognises the CAAT-box and initiation of transcription starts at the TATA-box. The presence of a polyA signal sequence at the 3' end of an ORF results in the addition of a polyA tail which protects the mRNA from degradation. Ribosome binding at the RBS is necessary for initiation of translation.

3.5 Stability of the genetic changes

A number of analyses were done to demonstrate the stability of the genetic changes in GHB614 cotton. Segregation analysis over multiple generations was done to determine the heritability and stability of the new trait (the *2mepsps* gene) and Southern blot analysis over multiple generations was done to determine the stability of the inserted DNA. Polymerase chain reaction (PCR) testing was used to verify the event.

3.5.1 Segregation analyses

Following transformation, T_1 seed harvested from self-pollinated T_0 plants surviving a glyphosate herbicide screen were planted in the greenhouse for seed increase and evaluation. Resistance screenings were done on subsequent generations in the greenhouse using glyphosate at the 1X rate, to identify segregating seed lots. PCR based analysis was also performed as a secondary means of identifying homozygous plants with the *2mepsps* trait. Selfed T_3 homogyzous seed was used to produce homozygous T_4 seed which was the source of the lines used in early event-specific agronomic and stability studies.

For breeding purposes and further evaluation of inheritance, various backcrosses were performed and evaluated in the greenhouse for segregation and glyphosate resistance. Mendelian inheritance for a single gene locus would predict one resistant plant for every one susceptible plant within BC_2F_1 progenies. Furthermore, BC_2F_2 progeny would be expected to show three resistant plants for every one susceptible plant (see Figure 3 and Table 1).

For the segregation analysis, data from a Chi-square test of inheritance were used to determine the heritability and stability of the new trait. The Chi-square test is based on testing the observed segregation ratio of glyphosate resistant plants to the ratio that is expected according to Mendelian principles. All Chi-square values indicate no significant differences between observed and expected genetic ratios across all tested generations of GHB614 cotton. These results are consistent with a single site of insertion for the *2mepsps* gene expression cassette.

Table 1: Segregation Analysis of GHB614 Cotton

Parents and zygosity for the *2mepsps* locus	Generation	Ratio	Observed		Expected		χ^2 calculated[a]
		R:S	R	S	R	S	
Hemizygous "F_1" plant crossed with conventional line B (*2mepsps*/-)x(-/-)	BC_1F_1	1:1	9	12	10.5	10.5	0.43
Hemizygous BC_1F_1 plant crossed with conventional line B (*2mepsps*/-)x(-/-)	BC_2F_1	1:1	11	6	8.5	8.5	1.47
Hemizygous BC_2F_1 plant (conventional line A), self-pollinated (*2mepsps*/-)x(*2mepsps*/-)	BC_2F_2	3:1	28[b]	8	27	9	0.15
Hemizygous BC_2F_2 plant crossed with conventional line B (*2mepsps*/-)x(-/-)	"F_1" population[c]	1:1	7	9	8	8	0.25
Self-pollinated hemizygous "F_1" plants (*2mepsps*/-)x(*2mepsps*/-)	"F_2" populations (pooled)	3:1	113	43	117	39	0.60

[a] assumes a one locus model. There was no significant difference for the χ square goodness-of-fit test for the hypothesis of one locus. To reject the null hypothesis, the χ square value must be greater than 3.84, with one degree of freedom.
[b] tested by homozygosity PCR (19 heterozygous and 9 homozygous plants).
[c] all F1 population material was generated using a hemizygous transgene donor source (BC2F1).
S = susceptible; R = resistant to glyphosate

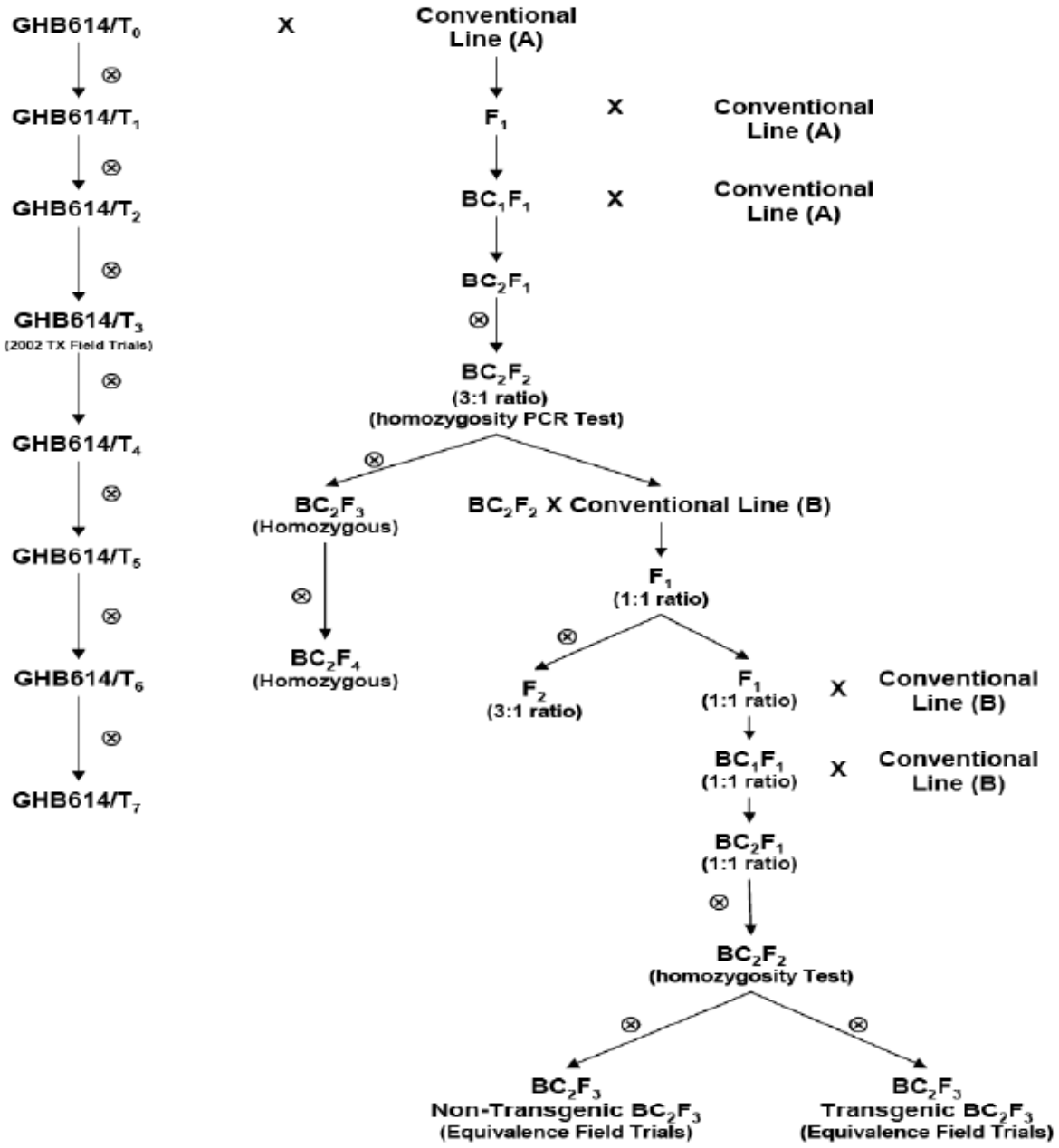

Figure 3: Breeding tree for the development and evaluation of GHB614 cotton

At each generation, plants were sprayed with glyphosate to eliminate those not expressing the 2mepsps gene

⊗ = self cross

Generation BC_2F_4 (homozygous) was used for detailed insert characterization and protein expression levels.

Generations T_3, T_4, T_5, T_6 and BC_2F_2 were used for molecular stability analysis.

Generation T_5 was used for seed composition analysis.

Generations T_5 and BC_2F_3 were used for replicated agronomic field tests.

Generation T_7 was used for analyses on absence/presence of vector backbone sequences.

3.5.2 Stability of the inserted DNA

Study submitted:
1. Structural stability analysis of *Gossypium hirsutum* transformation event GHB614, V. Habex; completed October 2006. Report No: 2006-GHB614-NAC005

To determine the stability of the inserted DNA, Southern blot analyses were done using genomic DNA isolated from multiple generations of GHB614 cotton (T3, T4, T5 and T6, see Figure 3 for the specific generations used). For these analyses, DNA samples from leaf tissue representing each generation were digested and probed to detect two integration fragments corresponding to ~4850 bp and ~9100 bp. In all tested samples, the expected 5' and 3' integration fragments were present. These results are consistent with bands detected in other Southern analyses of GHB614 cotton and confirm the stability of the insert across multiple generations of breeding.

3.5.3 Conclusion

The results of the segregation analysis are consistent with a single site of insertion for the *2mepsps* gene expression cassette and the results of the molecular characterization studies. Phenotypic and molecular analyses of breeding lines over multiple generations indicate that the inserted DNA is stably transformed and inherited as a single locus from one generation to the next.

3.6 Presence of antibiotic resistance genes

No genes that encode resistance to antibiotics are present in the genome of GHB614 cotton. The molecular characterisation confirmed the absence of both the *aad* gene and *nptI* fragment, which were present in the plasmid backbone outside of the T-DNA (region between the Left and Right border sequences).

4. CHARACTERISATION OF THE NOVEL PROTEIN

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

4.1 Description and function of novel protein

Studies submitted:
1. The double mutant 5-enolpyruvylshikimate-3-phosphate synthase gene product: 2mEPSPS Description and Characterisation, R-J. van der Klis, K. Hendrickx, C. Herouet-Guicheney and D. Rouan; completed September 2006. Report ID: 2006-2mEPSPS-EPC002

Cotton line GHB614 expresses one novel protein, 2mEPSPS, a modified form of the EPSPS protein naturally occurring in corn. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity of the modified protein. Because the expression of a novel protein *in planta* is usually too low to allow purification of sufficient quantities for use in safety assessment studies, a bacterial expression system was used to generate larger quantities of the 2MEPSPS protein for safety assessment. The 2mEPSPS protein produced in ***E. coli*** was engineered so its amino acid sequence matched that of the plant-produced 2mEPSPS protein. The equivalence of the bacterial-produced protein to the plant-produced protein was determined as part of the protein characterisation.

4.1.1 Mode of action of glyphosate on EPSPS proteins

Glyphosate acts as a herbicide by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This endogenous enzyme is involved in the shikimate pathway for aromatic amino acid biosynthesis which occurs exclusively in plants and microorganisms, including fungi. Inhibition of the wildtype EPSPS enzyme by glyphosate leads to deficiencies in aromatic amino acids in plant cells and eventually to the death of the whole plant. The shikimate biochemical pathway is not present in animals. For this reason, enzymes of the shikimate pathway have been considered as potential targets for essentially non-toxic herbicides (such as glyphosate) and antimicrobial compounds.

Naturally occurring EPSPS proteins are widespread in nature and have been extensively studied over a period of more than thirty years. The *epsps* gene from maize has been completely sequenced and it encodes a 47 kDa protein consisting of 445 amino acids. The modified 2mEPSPS protein present in GHB614 cotton differs from the wildtype maize enzyme by two amino acid substitutions – threonine replaced by isoleucine at position 102, and proline replaced by serine at position 106. These two amino acid changes result in a protein with greater than 99.5% identity to the native maize EPSPS protein, however the modified protein is highly tolerant to glyphosate. Plants expressing the modified maize enzyme therefore are able to continue to function adequately in the presence of the herbicide.

4.1.2 2mEPSPS activity

For the purposes of conferring tolerance to glyphosate, variants of the naturally occurring EPSPS enzyme would ideally exhibit no alteration in affinity for natural substrates (K_{cat} and K_m unchanged) but would have at least 10-fold enhancement of the K_i for glyphosate. That is, the aim was to identify a modification in the enzyme that would result in a significantly reduced affinity for glyphosate (an amino acid analogue) while retaining affinity for the cellular substrates (shikimate-3-phosphate and phosphoenolpyruvate, PEP). In addition, to obtain adequate tolerance levels to glyphosate in plants, the modified protein should be targeted to the chloroplasts where the shikimate pathway is normally functional.

Site directed mutagenesis of the wildtype *epsps* gene from maize produced the double mutant enzyme 2mEPSPS which carries two amino acid changes. When fused to a chimaeric optimized chloroplast transit peptide, the 2mEPSPS enzyme is reported to generate optimal glyphosate tolerance in crops (Lebrun *et al.* 1997a). A methionine codon was added to the amino-terminal end of the mature 2mEPSPS protein sequence to restore the cleavage site of the transit peptide. With the addition of the methionine residue, the mutations are at positions 103 (Thr to Ile) and 107 (Pro to Ser) of the mature protein (445 amino acids).

Kinetic and enzyme activity analyses indicate that the 2mEPSPS enzyme interacts with the normal EPSPS substrates, shikimate-3-phosphate and phosphoenolpyruvate, similarly to the wildtype corn EPSPS enzyme. Biochemical analyses comparing the 2mEPSPS and wildtype EPSPS enzymes also show that:

(i) the specific activities of the enzymes at 25º C and pH 7 are 5.2 and 11.8 U/mg respectively;
(ii) optimal pH activities of the enzymes are between pH 5.5 to 7.5 and pH 7 to 7.5 respectively;
(iii) the activities of both enzymes increase linearly to approximately 60º C, then decrease sharply and at 75º C appear to be inactive;
(iv) the double mutant form of the enzyme appears to be significantly more active at elevated temperatures compared to the wildtype, however 2mEPSPS is inactivated after 10 minutes at 60º C; and
(v) cations and anions have minor but comparable effects on respective enzyme activity.

On sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the 2mEPSPS and wildtype enzymes co-migrated with the same apparent molecular mass of 47 kDa. Western blot analysis of wildtype and modified proteins revealed a single cross-reacting polypeptide corresponding to the same molecular mass for both enzymes.

4.2 Protein characterisation

Study submitted:
1. Structural and Functional Equivalence of 2mEPSPS protein produced in *Escherichia coli* and GHB614 cotton, *Gossypium hirsutum*, T. Currier and K. Hendrickx; completed November 2006. Study ID: DQ06Q003

The 2mEPSPS protein and wildtype protein were both expressed in *E. coli* and purified to allow a direct comparison of their enzymatic properties (section *4.1.2* above). A range of analytical techniques was then used to determine the identity as well as the physicochemical and functional properties of the plant-produced 2mEPSPS protein isolated from cotton line GHB614 compared with the *E. coli*-produced form (Table 2). The *E. coli*-produced protein was used as a reference standard for these analyses.

Table 2: Criteria and methodologies for demonstrating equivalence between the microbially-produced and plant-produced protein in GHB614 cotton

Equivalence criteria	Methodology
Confirm identity of 2mEPSPS protein	Edman degradation
Comparable immunoreactivity	Western blot analysis
Comparable molecular mass	Mobility in SDS-PAGE
Comparable peptide masses	HPLC/Electrospray Mass Spectrometry (LC/MS) of peptides
Glycosylation profile	Staining SDS-PAGE for glycoproteins
Comparable biological activity	Enzyme activity assay

4.2.1 Characterisation of plant-produced 2mEPSPS

The 2mEPSPS protein was purified from frozen leaves of cotton line GHB614 using a combination of filtration and immunoaffinity chromatography with a covalently attached monoclonal antibody to 2mEPSPS.

Protein identity

The identity of the plant-produced 2mEPSPS was confirmed by Western blot analysis, N-terminal peptide sequencing, SDS-PAGE analysis and HPLC/Electrospray Mass Spectrometry:

(i) Western blot analysis used a monoclonal antibody to the 2mEPSPS protein. The results showed that the electrophoretic mobilities and immunoreactivities of the 2mEPSPS protein produced in *E. coli* and GHB614 cotton plants are indistinguishable.

(ii) The N-terminal amino acid sequence of the 2mEPSPS protein isolated from GHB614 cotton leaves was determined by Edman degradation. The theoretical N-terminal sequence of the 2mEPSPS protein deduced from the gene sequence is: methionine, alanine, glycine, alanine, glutamic acid, glutamic acid and isoleucine. Apart from the terminal methionine residue, the primary N-terminal amino acid residues of the 2mEPSPS from GHB614 cotton exactly matched the theoretical sequence. This result also shows that the N-terminal methionine is missing from the plant-produced 2mEPSPS protein, however this is a common finding in protein sequencing.

(iii) The microbially-produced and GHB614 cotton-produced 2mEPSPS proteins were analysed by SDS-PAGE. Following staining of the gel, the electrophoretic mobility of the protein from the two different sources was the same, and indicated an equivalent molecular weight of approximately 42 kDa (compared with the theoretical molecular weight of 47 kDa calculated from the amino acid sequence). The SDS-PAGE also showed that the protein preparations were highly pure.

(iv) Peptides from a tryptic digest of the microbially-produced 2mEPSPS protein were separated by HPLC and subsequently analysed by electrospray mass spectrometry. Expected peptides from the microbially produced 2mEPSPS protein were identified by SIM with 93% coverage of the 445 amino acids comprising the protein. The ability to identify a protein using this method is dependent on matching a sufficient number of observed tryptic mass fragments to expected (theoretical) mass fragments. The most abundant ion for each peptide from the *E. coli* 2mEPSPS protein was chosen for selected ion monitoring of the peptides produced by tryptic digestion of the 2mEPSPS protein isolated from GHB614 cotton. Peptides from the microbially-produced 2mEPSPS protein were identified in the 2mEPSPS protein from GHB614 cotton with coverage of 91.5% of the protein. The data showed that the calculated masses for the detected peptides from both proteins were identical, which confirms the equivalence of the microbially-produced and plant-produced 2mEPSPS proteins.

Glycosylation analysis

Glycoprotein staining was used to assess whether post-translational glycosylation of the plant-produced 2mEPSPS protein was present.
As prokaryotic organisms lack the capacity for protein glycosylation, the *E. coli*-produced 2mEPSPS protein would not be expected to yield a positive result in this analysis. Standard control proteins consisted of a mixture of glycosylated and non-glycosylated proteins. Only the glycosylated standard proteins showed a strong signal with the glycoprotein stain; both the *E. coli*- and plant-produced 2mEPSPS were barely detectable. This analysis indicates the absence of glycosylation in the 2mEPSPS protein from GHB614 cotton and confirms that it is equivalent to the *E. coli*-produced protein in terms of its lack of glycosylation.

Enzyme assay

In the shikimate pathway in plants, chorismate is formed via seven enzymatic steps. The reaction catalysed by EPSPS is the reversible transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to shikimate-3-phosphate, leading to formation of 5-enolpyruvyl-3-shikimate phosphate (EPSP) and the release of inorganic phosphate. The EPSPS activity assay can be measured according to a published colorimetric method described in Forlani *et al* (1994).

The enzymatic activity of the purified 2mEPSPS protein preparations from *E. coli* and GHB614 cotton leaves was measured in the forward direction using shikimate-3-phosphate and PEP as substrates. The amount of inorganic phosphate released during the reaction was determined using the malachite green dye method, with minor modifications. Both protein preparations generated free phosphate, indicating that 2mEPSPS from either the microbial or plant source showed the expected biological activity. This result confirms that the proteins from the two sources were present in the correct conformation.

4.2.3 Conclusion

Numerous studies have been conducted on the 2mEPSPS protein to confirm its identity and physicochemical and functional properties as well as to determine its equivalence to *E. coli*-produced 2mEPSPS. These studies have demonstrated that the novel protein expressed in GHB614 cotton conforms in size and amino acid sequence to that expected and also exhibits the expected enzymatic activity. The *E. coli*-produced protein was also shown to be equivalent to the plant-produced protein in terms of size, amino acid sequence, physicochemical properties, and enzyme activity. The *E. coli*-produced 2mEPSPS protein was therefore a valid substitute for the plant-produced protein for safety assessment purposes.

4.3 Protein expression levels

4.3.1 Protein expression in greenhouse grown cotton

Studies submitted:
1. 2mEPSPS protein content in leaf, stem, root, square, apex and pollen tissues during the life cycle of the glyphosate-tolerant cotton event GHB614, R-J van der Klis and K. De Pestel; completed October 2006. Report No. 2006-GHB614-EPC-017

A validated Enzyme Linked Immunosorbent Assay (ELISA) method was used to quantify the levels of the 2mEPSPS protein in tissues from GHB614 cotton grown in the greenhouses of Bayer BioScience N.V. (Astene, Belgium). Seeds were planted and at the 1-2 leaf stage (V1-V2), the transgenic plants were sprayed with glyphosate herbicide (0.7% glyphosate and 50 ml/square metre). The non-transgenic parental line was grown in the greenhouses at the same time as the test plants.

Samples from 6 different tissues of cotton line GHB614 were harvested separately covering four different growth stages of the plant (samples collected at 16, 33, 51 and 68 days after planting). Transgenic and non-transgenic plants were chosen randomly out of a starting population of 240 plants. In the first growth stage (V2-V3) leaf specimens from 15 plants were taken; in the other growth stages separate specimens per tissue were harvested from 10 plants. In the first growth stage (V2-V3) and the third growth stage (pre-flowering), young leaf tissue was sampled.

In the second and fourth growth stages (V4-V6 and flowering) stem, root and young leaf tissues were sampled. In the fourth growth stage also square, apex and pollen tissues were sampled. Identical samples were taken from the control cotton line.

In order to analyse the presence of 2mEPSPS protein in these tissues, samples were crushed, extracted and the Total Extractable Protein (TEP) content was determined using the Bradford method (Bradford, 1976). The amount of 2mEPSPS in the total protein extracts was measured using a quantitative ELISA developed by Strategic Diagnostics Inc. (SDI, Newark, DE, USA). However, this 2mEPSPS ELISA also detects traces of endogenous EPSPS protein in plants. To measure the sensitivity of the ELISA, the limit of detection (LOD) was determined for each tissue before the analysis was performed (Table 3). The LOD was defined per tissue as the concentration of (2m)EPSPS protein producing an absorbance that is statistically different from the background absorbance of the non-transgenic cotton line. The LOD was expressed as the concentration of 2mEPSPS per unit of fresh weight (µg/g).

Monoclonal antibodies to 2mEPSPS (produced in bacteria) were used as the capture antibodies; polyclonal detection antibodies were linked to a horseradish peroxidase conjugate. All samples were analysed in duplicate.

Table 3: LOD for the 2mEPSPS protein ELISA in different cotton plant tissues

Tissue	LOD $x10^{-3}$ µg/g
Leaf	4.47
Stem	8.34
Root	27.3
Square	27.3
Apex	8.10
Pollen	16.1

The levels of 2mEPSPS protein in the various tissues obtained from glasshouse-grown GHB614 cotton plants are summarised in Table 4. The protein was detected in all plant tissues. In leaf tissue, the levels of 2mEPSPS decreased over time, whereas the levels in stem tissue remained constant.

Table 4: Average 2mEPSPS protein content in different plant tissues of GHB614 cotton grown in the glasshouse

	Average 2mEPSPS protein levels in GHB614 cotton tissues µg/g fresh weight ± SD			
Tissue Type	Growth Stage 1	Growth Stage 2	Growth Stage 3	Growth Stage 4
Leaf	11.16 ± 3.73	7.94 ± 2.87	6.52 ± 7.20	0.45 ± 0.22
Stem	ND	1.94 ± 0.61	ND	1.58 ± 0.96
Root	ND	0.99 ± 1.00	ND	4.04 ± 1.71
Square	NA	NA	NA	5.35 ± 0.25
Apex	ND	ND	ND	5.47 ± 0.22
Pollen	NA	NA	NA	0.16 ± 0.01

ND: not determined; NA: not applicable

4.3.2 Protein expression in field-grown cotton

Studies submitted:
1. Production of RAC (Fuzzy Seed) Samples of GlyTol Cotton and the Non-transgenic Counterpart, USA, 2005, W.J. Kowite; completed October 2006. Study No. DQ05B001
2. Analyses of Raw Agricultural Commodity (Fuzzy Seed) of Cotton GHB614 for 2mEPSPS Protein, USA, 2005, T.C. Currier; completed October 2006. Study No. DQ06Q002
3. Residue Analysis of GlyTol Cotton Processed Fractions, USA, 2006, W.J. Kowite; completed November 2006. Study No. DQ06Q005

The purpose of these studies was to determine the amounts of 2mEPSPS protein in fuzzy cottonseed of transgenic line GHB614, grown in field trials under agricultural conditions typical of the commercial cultivation of cotton. GHB614 cotton and its non-transgenic parental line (Coker 312) were grown within individual plots established at each of nine field trial sites in southern USA. At each site, six plots were planted with transgenic cotton and three plots planted with the non-transgenic control. Three of the transgenic plots were sprayed three times with glyphosate herbicide at the level of 840 g/hectare, and three transgenic plots were untreated.

Because the cottonseed (fuzzy seed) had been ginned but not delinted, it could not be ground into a homogeneous material. A procedure was developed to effectively remove the lint and the associated seed coat. This created two fractions, which were designated 'kernel' and 'lint coat'. The kernel could be easily ground to homogeneity; the lint coat fraction was a relatively homogeneous matrix of intertwined cotton fibres and broken fragments of seed coat. These fractions were analysed separately for 2mEPSPS protein and total extractable protein and the respective values added to give values for the fuzzy seed as received from the field.

The 2mEPSPS protein was found in all fractions of transgenic fuzzy seed (kernel and lint coat). As expected, more than 99.5% of the novel protein was found in the kernel samples. The lint coat generally contained less than 0.5% of the 2mEPSPS protein, and some samples were below the limit of detection. The levels of 2mEPSPS protein varied between different trial sites and between treatments with glyphosate. On a fresh weight basis, the 2mEPSPS protein content in fuzzy seed of GHB614 cotton, not sprayed with glyphosate, ranged from about 15.8 µg/g to 25.5 µg/g fresh weight, with an overall average value of 19.2 ±3.1 µg/g. On a fresh weight basis, the fuzzy seed from GHB614 cotton plants, sprayed with a conventional herbicide regime, contained 2mEPSPS protein in the range 16.2 µg/g to 30.5 µg/g, with an overall average value of 21.2 ± 4.0 µg/g. Using the average values for the amount of novel protein in unsprayed and sprayed fuzzy seed relative to the amount of crude protein, the 2mEPSPS protein comprised an average of 0.0093% ± 0.0018% and 0.0100% ± 0.0019% of the total crude protein respectively.

In a separate field study, cotton line GHB614 and the conventional line Coker 312 were grown under typical agricultural conditions to evaluate the levels of the novel protein 2mEPSPS in eight fractionated agricultural products of cottonseed. The transgenic plot was sprayed three times with glyphosate herbicide equivalent to 0.75 pounds active ingredient per acre. The results of these analyses are presented in Table 5.

Table 5: Levels of 2mEPSPS protein in fuzzy seed and processed fractions of cotton line GHB614 as detected by ELISA

Sample	Average 2mEPSPS protein levels in tissues µg/g fresh weight ± SD	
	GHB614 treated with glyphosate	Conventional Coker 312
Kernel	16.4 ± 3.1	ND
Lint coat	0.67 ± 0.24	ND
Fuzzy seed	6.99	ND
Lint	ND	ND
Linters	ND	ND
Delinted seed	102 ± 2	ND
Seed hulls	6.93 ± 0.40	ND
Meal	0.26 ± 0.10	ND
Toasted meal	ND	ND
Crude oil	ND	ND
Refined, bleached, deodorised oil	ND	ND

ND: not detected

These results show that 2mEPSPS protein was not at detectable levels in cottonseed lint, toasted meal, crude oil and refined/bleached/ deodorised oil.
The highest levels of 2mEPSPS occurred in delinted seeds. The amount of 2mEPSPS protein was greatly reduced by processing delinted seeds into meal and toasted meal.

4.4 Potential toxicity of novel proteins

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish whether the novel protein will behave like any other dietary protein, based on a weight of evidence approach. The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; and structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a reason for further investigation.

4.4.1 History of use

EPSPS enzymes

EPSPS is the sixth enzyme in the shikimate pathway, the metabolic pathway for the biosynthesis of aromatic compounds found in plants and in microorganisms (bacteria and fungi). As such, EPSPS enzymes are ubiquitous in nature and are present in foods derived from all plant and microbial sources. Although differences in amino acid sequence occur naturally, depending on the source of the enzyme, it is apparent that this family of proteins has a long history of safe use as normal constituents of human food and animal feed.

The 2mEPSPS enzyme shows a high amino acid sequence identity to the naturally occurring EPSPS from maize (>99.5%), and to other EPSPS enzymes found in crops with a similarly long history of human consumption (e.g. rice 86%, grape 79%, lettuce 77%, tomato 75% and oilseed rape 75%) or in microbial food sources such as baker's yeast. The EPSPS enzymes present in these and other plant- or microbially-derived foods are all commonly consumed proteins within a normal human diet, and are not associated with any adverse health effects.

The 2mEPSPS has been used in other crops previously assessed by FSANZ. Food derived from glyphosate-tolerant corn line GA21 was approved in Australia and New Zealand in 2000 (Application A362). Corn line GA21 expressing the 2mEPSPS protein was developed in the 1990's and has been assessed and approved in other countries including Japan, Canada, European Union, Korea, Mexico, Argentina, South Africa, China, Taiwan and the USA. It is therefore likely to have been widely distributed in corn based foods.

4.4.2 Similarities with known protein toxins

The complete amino acid sequence of the 2mEPSPS protein expressed in GHB614 cotton is known from the molecular characterization studies. Bioinformatic analyses were done to assess the 2mEPSPS enzyme for any amino acid sequence similarity with known protein toxins. This *in silico* study was carried out by comparing the complete sequence of 445 amino acids of the 2mEPSPS protein with all protein sequences present in the following large reference databases:
Uniprot_Swissprot, Uniprot_ TrEMBL, PIR, NRL-3D, DAD and GenPept. Using the BLASTP (Standard Protein-protein Basic Local Alignment Search Tool) program, the key indicator for this study was a 35% identity with a known protein toxin over a window of 80 amino acids.

The extent of similarity was evaluated using visual inspection of the aligned sequences, the calculated percent identity, and *E* score. The *E* score reflects the degree of amino acid similarity between a pair of sequences and can be used to evaluate the significance of the alignment. A larger *E* score indicates a lower degree of similarity. Typically, alignments between two sequences will need to have an *E* score of less than 1×10^{-5} to be considered as significant homology.

The results of the overall homology search with the 2mEPSPS protein showed no amino acid identity with known toxins. As expected, the search revealed homology only with other EPSPS proteins from various sources. These analyses did not demonstrate any significant similarity between the 2mEPSPS protein and other proteins that may potentially be toxic to humans or other animals.

4.4.3 Acute oral toxicity studies

Two independent acute oral toxicity studies using purified 2mEPSPS protein have been conducted by different Applicants. FSANZ has previously considered the results of an acute oral toxicity study in mice submitted as part of Application A362[8]. The current Applicant has submitted a similar but smaller study using a higher dose of test substance. The findings of both studies are presented below.

In the previously evaluated study (Application A362), the modified EPSPS (2mEPSPS) protein was administered by a single oral gavage dose to ten male and ten female CD-1 mice, at target doses of 5, 15 and 50 mg/kg bodyweight. In this study, this corresponded to actual doses of 3.7, 11.8 and 45.6 mg/kg respectively. A control group of ten mice/sex was administered only the carrier substance without 2mEPSPS. An additional control group of ten mice/sex was administered Bovine Serum Albumin (BSA) in the same carrier substance at the highest target dose (50 mg/kg). At defined stages throughout the duration of the study, clinical observations were performed for mortality and signs of toxicity, and body weights and food consumption measured. At the termination of the study (day 13-14), animals were sacrificed, examined for gross pathology and numerous tissues were collected.

The results of the study showed no statistically significant differences in group mean body weights, cumulative weight gains or food consumption in either males or females at any level of either the BSA control or test material, when compared with the respective carrier control group. All animals survived to the end of the study, and there were no clinical signs observed that could be related to the test material.

A unilateral corneal opacity was noted in one male mouse at the high dose level of the test material, but this finding was not considered to be treatment related. The study concluded that there was no evidence of toxicity in mice following a single oral dose of 45.6 mg/kg modified EPSPS (2mEPSPS) protein.

Study submitted by Applicant:
2mEPSPS Protein – Acute Toxicity by Oral Gavage in Mice, completed August 2006. Report of Study: SA06175, Study Director: D. Rouquie. Performing Laboratory: Bayer CropScience, France. Sponsor: Bayer CropScience, Germany.

In this study, the 2mEPSPS protein was administered by a single oral gavage dose of 2000 mg protein/kg bodyweight to 5 female OF1 mice. A second group of female mice received the same dose of bovine serum albumin as a negative control. All animals were observed for clinical signs daily for fifteen days and body weights were measured weekly. At termination, all animals were subjected to necropsy including macroscopic examination.

There were no clinical signs, mortalities or treatment related effects on bodyweight in female OF1 mice observed during this study. Based on these findings, it was concluded that no oral toxicity was demonstrated in mice at a very high dose of 2000 mg/kg bodyweight.

[8] Studies previously assessed were conducted by Monsanto:

1. T.C. Lee *et al*., 1997. Preparation and Confirmation of Doses for an Acute Oral Toxicity Study (ML-97-195) with Modified Maize 5-Enolpyruvylshikimate-3-Phosphate Synthase (mEPSPS) Protein in Albino Mice.
2. M.W. Naylor, 1997. Acute Oral Toxicity Study with Modified Maize 5-Enolpyruvylshikimate-3-phosphate Synthase (mEPSPS) Protein in Albino Mice.

4.5 Potential allergenicity of novel proteins

The potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on: the source of the novel protein; any significant amino acid sequence similarity between the novel protein and known allergens; the structural properties of the novel protein, including susceptibility to digestion, heat stability and/or enzymatic treatment; and specific serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity with a known allergen. Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

4.5.1 Source of novel protein

The source of the 2mEPSPS enzyme present in GHB614 cotton is the maize EPSPS enzyme with two defined amino acid changes that reduce the binding and inactivation of the enzyme by glyphosate. Corn is not regarded as a major food allergen. In addition, humans have been exposed to a suite of EPSPS enzymes through a normal diet containing plants and edible microorganisms. Consumption of a large number of EPSPS enzymes with similar function but different degrees of amino acid homology is therefore usual for humans, and has never been identified with food allergenicity.

4.5.2 Similarity to known allergens

The bioinformatic analyses described above in *4.4.2* also assessed whether the 2mEPSPS protein demonstrated any overall amino acid sequence similarity with known allergens, gliadins or glutenins. Further bioinformatic analysis was applied to the sequence to identify the presence of potential epitope homology by comparing the amino acid sequence of the 2mEPSPS protein, subdivided into 8 amino acid blocks, with known allergens compiled in a large reference database.

The Allergen database of 1433 sequences (release 3.2, 19 April 2006) was built by assembling relevant allergens described in the five large protein databases used in the previous bioinformatic study. The criterion indicating potential allergenicity was a 100% identity with an allergenic protein using a sliding window of eight contiguous amino acids. Segments of eight amino acids were chosen because this is considered to be the smallest number of amino acids that will identify immunologically relevant matches. Searches using smaller segments (e.g. 6 or 7 amino acids) lead to high rates of false positive matches and therefore have little predictive value. In this study, the algorithm used for identifying epitope homology was FindPatterns (GCG package).

No identity between the 2mEPSPS protein analysed in this way and known allergens was identified. These sequence homology searches establish that the 2mEPSPS protein does not share any theoretical structural similarity with known allergenic proteins.

4.5.3 Potential glycosylation sites

Glycosylation of proteins is known to promote proper protein folding and confer enhanced protein stability, particularly for proteins secreted from the cell or associated with membranes.

Glycosylation is also associated with many allergenic proteins. Consideration of potential glycosylation sites using an *in silico* approach may therefore be useful for considering the potential allergenicity of novel proteins for which the patterns of N-glycosylation may differ from their wildtype counterpart.

The bioinformatics study of the 2mEPSPS protein for assessment of potential toxicity and allergenicity also considered the potential N-glycosylation sites in the protein by searching for a described consensus sequence as found in known allergenic proteins. Using this approach, two potential glycosylation sites (at amino acid positions 118 and 394) were identified in the 2mEPSPS sequence. Both of these potential sites are downstream from the two amino acid changes introduced into the maize EPSPS protein at amino acid positions 102 and 106. As neither of these substitutions is within the potential glycosylation sites, the N-glycosylation profile of the 2mEPSPS protein can reasonably be expected to be identical to that of the naturally occurring wildtype EPSPS enzyme.

Furthermore, the 2mEPSPS protein is specifically targeted to the chloroplast where the shikimate biochemical pathway operates in plant cells. It is widely accepted that nuclear-encoded proteins destined for intracellular compartments are not glycosylated in plants. This information provides further weight to the *in silico* analyses indicating that 2mEPSPS in GHB614 cotton is not glycosylated.

4.5.4 Digestibility

Studies submitted:
1. 2mEPSPS Protein – *In vitro* Digestibility Study In Simulated Gastric Fluid, D. Rouquie; completed August 2006. Report of Study SA06101.
2. 2mEPSPS Protein – *In vitro* Digestibility Study In Simulated Intestinal Fluid, D. Rouquie; completed July 2006. Report of Study SA06102.

One of the criteria for assessing potential allergenicity is to determine the stability of novel proteins in conditions that simulate human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response. It should be noted that ordinarily an ingested protein would first be exposed to pepsin-mediated hydrolysis in the acidic environment of the stomach before being subject to further proteolytic digestion in the small intestine.

The 2mEPSPS protein was subjected to digestibility studies using simulated human gastric fluid (SGF) containing pepsin and simulated intestinal fluid (SIF) containing porcine pancreatin, which is a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. A pepsin digestibility assay protocol has been standardised in a multi-laboratory evaluation published by Thomas *et al.* (2004), and these studies followed the protocols described in that reference.

Because it was not possible to purify sufficient quantities of 2mEPSPS from GHB614 cotton for use in these studies, *E. coli*-produced 2mEPSPS was used as the test substance in both the SGF and SIF studies. The equivalence of the *E. coli*- and cotton-produced 2mEPSPS proteins was established using a range of biochemical methods including Western blot analysis and enzyme activity assay (see Section 4.2).

Digestibility in SGF was measured by incubating 2mEPSPS protein at 37ºC in reaction mixtures (pH 1.2) with and without pepsin, taking samples at selected time points (0, 0.5, 2, 5, 10, 20 30 and 60 minutes) and subjecting these to SDS-PAGE. The two control proteins, horseradish peroxidase (unstable reference protein) and ovalbumin (stable reference protein), were treated with pepsin under identical incubation conditions to the test substance. Proteins were visualized by staining the gel with Coomassie blue prior to scanning. In the absence of pepsin, the 2mEPSPS protein band was equally visible in the zero and 60 minute incubation samples. In SGF (with pepsin), there was no full length or partially degraded 2mEPSPS protein observed at 30 seconds and at subsequent time points. In the same experiment, the intensity of the ovalbumin band was undiminished at incubation times up to and including five minutes, but at subsequent time points showed a gradual reduction in staining intensity. A band corresponding to intact ovalbumin remained faintly visible after 60 minutes, indicating that digestion of this protein was not complete within 1 hour. The horseradish peroxidase band was not visible after 30 seconds incubation with pepsin, demonstrating that the experimental conditions were appropriate in this study for measuring *in vitro* digestion patterns.

Digestibility in SIF was measured by incubating 2mEPSPS protein at 37ºC in reaction mixtures (pH 7.5), with and without pancreatin, taking samples at selected time points (0, 0.5, 2, 5, 10, 20 30 and 60 minutes) and subjecting these to SDS-PAGE. Degradation of a standard protein (azoalbumin) under identical digestion conditions was used as the control. Proteins were visualized by staining the gel or by transferring the protein to a nitrocellulose membrane for Western blot analysis. The 2mEPSPS protein band was visible in the zero and 60 minute incubation samples without pancreatin, with no decrease in stain intensity over the 60 minutes. In SIF, the 2mEPSPS protein band was only faintly visible after scanning the gel even at time zero. At all subsequent incubation times, there was no full length or partially degraded 2mEPSPS protein observed. Further, the authors reported that digestibility was dramatically increased by pre-heating (data not supplied).

4.6 Conclusion

GHB614 cotton expresses one novel protein, 2mEPSPS, which retains enzyme activity in the presence of glyphosate. The protein is expressed at relatively low levels in cottonseed, but is not detected in processed oil fractions or linters. The average concentration for 2mEPSPS in fuzzy seed was approximately 21 μg/g fresh weight.

A large number of studies have confirmed the identity and physicochemical and functional properties of the 2mEPSPS protein as expressed in GHB614 cotton, and examined its potential to be either toxic or allergenic in humans when present in foods. These studies have demonstrated that the protein conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation, and also demonstrates the expected enzymatic activity.

In terms of its potential toxicity and allergenicity, it is worth noting that the 2mEPSPS protein has been evaluated previously as the novel protein present in glyphosate-tolerant corn line GA21 which was approved in Australia and New Zealand in 2000. This modified enzyme is derived from the native EPSPS enzyme in maize (99.5% amino acid homology), and is closely related to other EPSPS enzymes from plants and microorganisms which are natural constituents of human diets.

Bioinformatic studies with the 2mEPSPS protein sequence has confirmed the absence of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that the protein would undergo rapid degradation in the digestive tract, similar to other dietary proteins. Acute oral toxicity studies in mice have confirmed the absence of toxicity. The weight of evidence shows that the 2mEPSPS protein is not toxic and unlikely to be allergenic in humans.

5. COMPOSITIONAL ANALYSES

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

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

In the case of cottonseed, the key components that should be considered in the comparison include protein, fat, carbohydrate, amino acids, fatty acids, vitamins, minerals and the anti-nutrients, gossypol and cyclopropenoid fatty acids (OECD 2004). Cottonseed oil (refined, bleached and deodorized) typically contains 27% saturates, 18% monounsaturates and 55% polyunsaturates. Refined cottonseed oil is free of gossypol (Gunstone *et al.* 1994). The total tocopherol (α- and β-tocopherol) content of cottonseed oil is about 60 mg/100 ml (NCPA, 2000). Cottonseed meal, whole cottonseed or delinted cottonseed are typically used as animal feed.

Studies submitted:

1. Currier, T.C. (2007). Statistical Analysis of Compositional Data of Fuzzy Seed from First Year Field Trials of Glytol Cotton, event GHB614, USA 2005. Bayer CropScience Study ID: DQ06Q008-2, completed September 2007.
2. Kowite, W.J. (2006). Composition of Processed Commodities (FRAC) of Transgenic GlyTol Cotton and the Non-transgenic Counterpart, USA 2006. Bayer CropScience Study No. DQ06Q006, completed November 2006.
3. Haas, C.L. (2005). Composition of RAC (Ginned Seed) of GlyTol Cotton and the Non-transgenic Counterpart, USA 2005. Bayer CropScience Study No. DQ06Q004, completed October 2006.

5.1 Study design and conduct

To determine whether unexpected changes have occurred in the composition of GHB614 cotton as a result of the modification, and to assess its nutritional adequacy, compositional analyses were done on fuzzy seed collected from GHB614 cotton and the non-GM counterpart, Coker 312, grown in field trials typical of commercial agricultural production.

Nine field trials were conducted in 2005 at sites representing primary cotton-growing regions of the south-eastern United States. At each test site, six plots of transgenic event GHB614 cotton and three non-transgenic plots of Coker 312 were planted. Three of the six plots containing GHB614 cotton were sprayed three times with glyphosate herbicide. Each application of glyphosate herbicide was at a rate of 0.75 pounds of active ingredient (glyphosate acid equivalent) per acre.

Ginned cottonseed (fuzzy seed) samples were collected from each trial. Replication was provided from the triplicate plots of each planted regimen, rather than from multiple samples from each plot. Each sample was representative (a composite) of cotton bolls harvested from multiple areas within the plot. Ginning was carried out at the field trial locations with small research scale cotton gins. A total of 135 samples were generated for analysis. Following compositional analysis, the results were statistically analysed using ANOVA (analysis of variance) at a significance level of 0.01.

Compositional analysis of the cottonseed samples included proximates (protein, fat, ash and moisture), acid detergent fibre (ADF), neutral detergent fibre (NDF), minerals (calcium, iron, magnesium, phosphorus, potassium and zinc), amino acids, fatty acids, vitamin E (alpha tocopherol) and carbohydrates by calculation. In addition, three known anti-nutrients found in cotton (gossypol, cyclopropenoid fatty acids and phytic acid) were analysed. Methods of analysis were based on internationally recognised procedures (e.g. AOAC International methods) or other published methods. The results of the combined site comparisons are presented in Tables 6–11. The results from individual trial sites were also evaluated but are not presented in this Report.

Table 6: Combined Mean Proximate Composition of Ginned Cottonseed of Cotton Line GHB614 and Control Line Coker 312 (percentage dry matter)

Analyte	Non-GM Control Coker 312	GHB614 cotton unsprayed	GHB614 cotton sprayed
Crude fat (%)	17.71 ± 1.46	17.15 ± 1.49	17.09 ± 1.37
Crude protein (%)	23.47 ± 2.51	23.16 ± 2.70	23.42 ± 2.55
Ash (%)	4.25 ± 0.31	4.26 ± 0.36	4.24 ± 0.34
ADF (%)	40.81 ± 3.43	41.00 ± 2.77	40.66 ± 2.31
NDF (%)	50.06 ± 3.10	50.20 ± 3.53	49.66 ± 2.68
Carbohydrate (calc.)	54.58 ± 2.66	55.43 ± 3.01	55.25 ± 2.41
*Moisture (%)	9.63 ± 3.42	9.42 ± 2.11	8.92 ± 1.39

Data represent an average of three replicate samples at nine field test sites.
* Moisture is expressed as % fresh weight.

Table 7: Combined Mean Mineral and Vitamin E Composition of Ginned Cottonseed of Cotton Line GHB614 and Control Line Coker 312 (percentage dry matter)

Analyte	Non-GM Control	GHB614 cotton unsprayed	GHB614 cotton sprayed
Calcium (%)	0.14 ± 0.03	0.14 ± 0.03	0.13 ± 0.04
Phosphorus (%)	0.62 ± 0.08	0.63 ± 0.06	0.63 ± 0.06
Iron (%)	0.0058 ± 0.002	0.0058	0.0064
Magnesium (%)	0.38 ± 0.03	0.38 ± 0.02	0.38 ± 0.02
Potassium (%)	1.18 ± 0.08	1.18 ± 0.08	1.18 ± 0.08
Zinc (%)	28.3 ± 5.2	29.2 ± 4.7	29.2 ± 5.4
α-Tocopherol (ppm)	106 ± 18	105 ± 13	103 ± 13
Total Tocopherol (ppm)	153 ± 23	154 ± 22	155 ± 24

Table 8: Combined Mean Amino Acid Composition of Ginned Cottonseed of Cotton Line GHB614 and Control Line Coker 312 (percentage dry matter)

Amino Acid	Non-GM Control	GHB614 cotton unsprayed	GHB614 cotton sprayed
Alanine	0.96 ± 0.09	0.96 ± 0.12	0.96 ± 0.11
Arginine	2.60 ± 0.34	2.68 ± 0.48	2.62 ± 0.46
Aspartic Acid	2.27 ± 0.25	2.31 ± 0.30	2.30 ± 0.29
Cystine*	0.36 ± 0.04	0.35 ± 0.04	0.37 ± 0.06
Glutamic Acid	4.78 ± 0.56	4.86 ± 0.77	4.85 ± 0.68
Glycine	0.96 ± 0.10	0.98 ± 0.13	0.98 ± 0.12
Histidine	0.64 ± 0.07	0.65 ± 0.10	0.64 ± 0.09
Isoleucine	0.69 ± 0.09	0.70 ± 0.12	0.70 ± 0.10
Leucine	1.34 ± 0.14	1.35 ± 0.20	1.35 ± 0.17
Lysine	1.03 ± 0.10	1.04 ± 0.13	1.04 ± 0.11
Methionine*	0.38 ± 0.04	0.37 ± 0.04	0.39 ± 0.05
Phenylalanine	1.24 ± 0.14	1.26 ± 0.21	1.26 ± 0.18
Proline	0.86 ± 0.10	0.88 ± 0.10	0.88 ± 0.07
Serine	1.02 ± 0.10	1.05 ± 0.13	1.04 ± 0.13
Threonine	0.76 ± 0.07	0.78 ± 0.10	0.78 ± 0.09
Tryptophan	0.31 ± 0.04	0.32 ± 0.03	0.32 ± 0.03
Tyrosine	0.59 ± 0.06	0.61 ± 0.09	0.61 ± 0.07
Valine	0.97 ± 0.12	0.99 ± 0.17	1.00 ± 0.15

* Statistically significant differences for site and treatment (see Section 5.3)

Table 9: Combined Mean Fatty Acid Composition of Ginned Cottonseed of Cotton Line GHB614 and Control Line Coker 312 (relative per cent)

Fatty Acid	Non-GM Control	GHB614 cotton unsprayed	GHB614 cotton sprayed
Saturated			
Myristic (C14:0)	0.76 ± 0.09	0.75 ± 0.09	0.75 ± 0.10
Palmitic (C16:0)	24.28 ± 0.93	24.21 ± 1.00	24.30 ± 1.00
Stearic (C18:0)	2.35 ± 0.10	2.24 ± 0.12	2.24 ± 0.13
Arachidic (C20:0)	0.30 ± 0.02	0.29 ± 0.02	0.29 ± 0.03
Behenic (C22:0)	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
Unsaturated			
Palmitoleic (C16:1)	0.62 ± 0.05	0.64 ± 0.05	0.65 ± 0.05
Oleic (C18:1)	15.10 ± 0.85	14.33 ± 0.84	14.38 ± 0.91
Polyunsaturated			
Linoleic (C18:2)	54.94 ± 1.82	56.14 ± 1.87	55.99 ± 2.04
Linolenic (C18:3)	0.61 ± 0.04	0.45 ± 0.05	0.46 ± 0.04
Other components	0.97 ± 0.17	0.87 ± 0.13	0.66 ± 0.11

Table 10: Combined Mean Cyclopropenoid Fatty Acid Composition of Ginned Cottonseed of Cotton Line GHB614 and Control Line Coker 312 (relative)

Analyte	Non-GM Control Coker 312	GHB614 cotton unsprayed	GHB614 cotton sprayed
Sterculic	0.163 ± 0.066	0.119 ± 0.037	0.125 ± 0.037
Malvalic	0.204 ± 0.124	0.145 ± 0.070	0.156 ± 0.074
Dihydrosterculic	0.152 ± 0.022	0.092 ± 0.012	0.090 ± 0.000

Data represent an average of three replicate samples at nine field test sites. Some individual analyses returned a value <0.10, and were changed to 0.09 for inclusion into the statistical calculations.

Table 11: Combined Mean Antinutrient Composition of Ginned Cottonseed of Cotton Line GHB614 and Control Line Coker 312

Analyte % dry matter	Non-GM Control Coker 312	GHB614 cotton unsprayed	GHB614 cotton sprayed
Gossypol free	0.50 ± 0.07	0.48 ± 0.08	0.50 ± 0.08
Gossypol total	0.66 ± 0.09	0.67 ± 0.08	0.67 ± 0.09
Phytic Acid	1.70 ± 0.08	1.69 ± 0.21	1.67 ± 0.18

5.2 Natural toxicants and antinutrients

Cotton is not considered harmful to humans, however the plant does produce several key anti-nutrients relevant to food safety including gossypol and the cyclopropenoid fatty acids (OECD 2004). The levels of these antinutrients in GHB614 cotton were compared with the levels in conventional cotton and the results (Tables 10 and 11) discussed in Section 5.3.

Gossypol is a terpenoid compound naturally occurring throughout the cotton plant, including seeds. It is an important source of plant resistance to damage by herbivores and insects. The levels of gossypol in food and feed products derived from cottonseed must be minimised in order to avoid toxic effects. Gossypol is in the free state in whole cottonseed and is bound to lysine or other components during processing into meal. Once bound in this way, the gossypol is not generally available to animals that consume cottonseed, however sensitivity to gossypol is considerably different between animal species.

The amount of free gossypol has been considered as the guide used by many nutritionists in making recommendations on feeding of cottonseed products to humans, as free gossypol is toxic. As noted previously, refined cottonseed oil is free of gossypol (Gunstone et al., 1994).

The cyclopropenoid fatty acids, sterculic (C:19) and malvalic (C:18) acids, are unique to cotton (0.1–1.3% of cottonseed oil). Their presence in foods can result in adverse health effects and therefore levels must be minimised for food and feed safety. These fatty acids are largely deactivated or removed from cottonseed oil by hydrogenation or during deodorisation at 230-235ºC.

In addition to the key antinutrients, the Applicant measured the levels of phytic acid in GHB614 cottonseed to demonstrate its safety as animal feed as it is often mixed with corn. Phytic acid is considered an anti-nutrient in cottonseed meal used for animal feed, since it can significantly reduce the bioavailability of essential divalent minerals calcium, iron and zinc (Liener, 1994). Phytic acid can be present at levels around 3-4% in cottonseed flour, depending on the type of cotton, glandless or glanded (Wozenski and Woodburn, 1975).

5.3 Statistical Analyses

The compositional data for all analytes obtained from all sites were analysed by ANOVA using a significance level of 0.01 ($\alpha = 0.01$ or 1%). Non-GM control plants (Coker 312) were designated as Treatment A, unsprayed transgenic GHB614 plants as Treatment B and sprayed GHB614 plants as Treatment C. Independent variables evaluated were the site and treatment as above.

The null hypothesis states that there are no differences between the values of an analyte (dependent variable) due to the independent variables.

A small probability (p-value) means that an observed difference is unlikely to occur by chance, so the null hypothesis should be rejected. A low p-value (< 0.01) suggests that there is a significant difference caused by the effect analyzed. The α-level was chosen at .01 to increase the power of each test. The Applicant used this level to reduce the chance of generating a false positive to less than 50%.

T-test comparisons at a significance level of 0.01 (α = 1%) were performed using the analyte values from the following sets of data:

1. non-transgenic samples (Treatment A) and unsprayed transgenic samples (Treatment B)
2. non-transgenic samples (Treatment A) and sprayed transgenic samples (Treatment C)

5.3.1 Discussion of results

Compositional data were generated for 81 samples of ginned cottonseed (9 samples from each of 9 field trials), from the total number of 135 samples collected. This corresponds to 27 samples from each of the three treatment groups: non-GM control Coker312 cotton (A), GHB614 cotton not sprayed with glyphosate (B), and GHB614 cotton sprayed three times with glyphosate (C). The fresh weight compositional data reported by the principal analytical laboratory, Eurofins Scientific, were adjusted as appropriate to a weight percent of dry matter basis.

For each analyte tested, p-values were generated from the ANOVA to indicate significant differences for site, for treatment, and for the interaction of site and treatment. The summary ANOVA data showed that iron was the only analyte that did not give a significant difference for site; all other analytes showed a highly significant difference for site.

The results for crude fat, fibre (neutral detergent), valine, phytic acid and a number of fatty acids (palmitoleic, stearic, oleic, linoleic, linolenic and arachidic acids) were significantly different for the interaction of site and treatment. When the interaction between site and treatment was significant (using α = 0.01), these variables were considered to be affecting each other. In this situation, the p-values for each of them are not accurate and any apparent significance due to treatment alone is not regarded as meaningful.

The amino acids cysteine and methionine, and one fatty acid (behenic acid, C22:0) were the only key analytes that showed a significant difference for the treatment variable. For all three analytes however, the p-value for the interaction of site and treatment was very close to 0.01 (0.0167 for cysteine, 0.0222 for methionine and 0.0194 for behenic acid). Due to the highly significant difference in these analytes observed between sites ($p<0.0001$ for all three analytes), the Applicant considered that the interaction between site and treatment was most likely to have caused these analytes to show a significant difference in treatment alone.

Table 12 lists the p-values from the t-tests and indicates the significance of the analyte value generated for the following sets of data:

1. non-transgenic samples (Treatment A) and unsprayed transgenic samples (Treatment B)
2. non-transgenic samples (Treatment A) and sprayed transgenic samples (Treatment C)

In this data comparison, the T-tests comparing the analyte values for non-GM control samples and GHB614 cotton samples, derived from either sprayed or unsprayed plants, showed no significant difference for the majority of analytes tested.

As shown in Table 12, statistically significant differences between non-transgenic control and the transgenic line (sprayed and unsprayed) were noted for three fatty acids (stearic, oleic and linolenic acids) and dihydrosterculic acid, a key antinutrient in cotton (see Section 5.3.2 below). Based on the mean values obtained for fatty acids for each treatment (Tables 9 & 12), it can be seen that the magnitude of the difference in each case was very small; levels of stearic, oleic and linolenic acids slightly lower in the transgenic line compared to Coker 312. The levels of all fatty acids measured were within the range of natural variation for conventional cotton varieties.

5.3.2 Cyclopropenoid fatty acids

Statistically significant differences were observed in the levels of sterculic and dihydrosterculic acids between GHB614 cottonseed and Coker 312 (Table 12). For both of these analytes, the levels were slightly decreased in the transgenic line. The Applicant noted that the measured levels for dihydrosterculic acid were very close to the limit of quantification. The levels of all three cyclopropenoid fatty acids were within the range of natural variation reported for conventional cotton varieties (OECD 2004)

Table 12: Sample t-test analyses for Control (non-transgenic) vs. Transgenic, not sprayed, and for Control (non-transgenic) vs. Transgenic, sprayed.

Variable	Treatment[a]			A vs. B		A vs. C	
	A (n=27)	B (n=27)	C (n=27)	p-value	Signif.	p-value	Signif.
Alanine	0.959	0.964	0.964	0.8743	-	0.8712	-
Arginine	2.604	2.676	2.621	0.5252	-	0.8826	-
Aspartate	2.268	2.308	2.310	0.6030	-	0.6598	-
Cysteine	0.355	0.350	0.374	0.6212	-	0.1623	-
Glutamate	4.780	4.863	4.846	0.6541	-	0.6982	-
Glycine	0.963	0.976	0.975	0.6850	-	0.6794	-
Histidine	0.638	0.648	0.645	0.6849	-	0.7672	
Isoleucine	0.691	0.697	0.704	0.8350	-	0.6171	-
Leucine	1.336	1.352	1.351	0.7373	-	0.7204	-
Lysine	1.029	1.043	1.039	0.6692	-	0.7423	-
Methionine	0.383	0.369	0.394	0.2160	-	0.3889	-
Phenylalanine	1.241	1.264	1.256	0.6440	-	0.7324	-
Proline	0.858	0.878	0.879	0.4646	-	0.3864	-
Serine	1.021	1.046	1.039	0.4361	-	0.5769	-
Threonine	0.765	0.779	0.776	0.5243	-	0.5968	-
Tryptophan	0.313	0.321	0.317	0.4263	-	0.7004	-
Tyrosine	0.593	0.614	0.606	0.3043	-	0.4577	-
Valine	0.973	0.986	0.996	0.7602	-	0.5405	-
Ash	4.248	4.257	4.242	0.9240	-	0.9446	-
Carbos, calc.	54.58	55.43	55.25	0.2731	-	0.3367	-
Crude fat	17.71	17.15	17.09	0.1673	-	0.1139	-
ADF	40.81	41.00	40.66	0.8227	-	0.8524	-
NDF	50.06	50.20	49.66	0.8732	-	0.6129	
Protein, combust[n]	23.47	23.16	23.42	0.6712	-	0.9493	-
Calcium	0.137	0.137	0.132	0.9931	-	0.5168	-
Iron	0.006	0.006	0.006	0.9456	-	0.4963	-
Magnesium	0.377	0.381	0.381	0.5855	-	0.5662	-
Phosphorous	0.622	0.634	0.626	0.5388	-	0.8447	-
Potassium	1.176	1.180	1.182	0.8509	-	0.7591	-
Zinc	28.34	29.21	29.20	0.5235	-	0.5513	-

Variable	Treatment[a]			A vs. B		A vs. C	
	A (n=27)	B (n=27)	C (n=27)	p-value	Signif.	p-value	Signif.
Gossypol (total)	0.660	0.670	0.670	0.6755	-	0.6719	-
Free gossypol	0.501	0.484	0.505	0.4236	-	0.8660	-
A-tocopherol	106.24	104.53	102.89	0.3043	-	0.4577	-
Phytic acid	1.699	1.687	1.675	0.8161	-	0.6276	-
Palmitic acid C16:0	24.276	24.211	24.301	0.8053	-	0.9253	-
Palmitoleic acid C16:1	0.624	0.642	0.646	0.1757	-	0.1041	-
Stearic acid C18:0	2.345	2.243	2.241	0.0011	+	0.0017	+
Oleic acid C18:1	15.100	14.328	14.382	0.0015	+	0.0043	+
Linoleic acid C18:2	54.944	56.137	55.990	0.212	-	0.0524	-
Linolenic acid C18:3	0.606	0.454	0.454	<0.0001	++	<0.0001	++
Arachidic acid C20:0	0.298	0.287	0.290	0.0865	-	0.2682	-
Behenic acid C22:0	0.149	0.143	0.143	0.0559	-	0.1157	-
Malvalic acid	0.205	0.145	0.156	0.0341	-	0.0875	-
Sterculic acid	0.163	0.119	0.125	0.0040	+	0.0119	-
Dihydrosterculic acid	0.152	0.092	0.090	<0.0001	++	<0.0001	++

[a]A= non-transgenic; B= transgenic, unsprayed; C= transgenic, sprayed.
++ indicates p-value <0.001. Variable is very significantly different.
+ indicates p-value <0.01. Variable is significantly different.
- indicates p-value greater than 0.01. Variable is not significantly different.

5.4 Composition of processed fractions

Seed samples from cotton line GHB614 and the non-GM parental line Coker312, grown in the field trials outlined above, were used to produce processed cottonseed products including linters, delinted seed, meal, toasted meal, hulls, crude oil and deodorized, bleached refined oil. Compositional data were obtained from one sample from each of the transgenic sprayed and non-transgenic processed cottonseed samples for each fraction giving a total of 16 samples analysed.

Proximates, amino acids and mineral analyses of ginned and delinted cottonseed, cottonseed meal and toasted cottonseed meal were determined using published AOAC methods. Detailed fatty acid analyses were carried out on the crude oil and deodorized, refined cottonseed oil and the results are presented in Table 14. The results from other compositional analyses of the crude and refined cottonseed oils are presented in Table 13. No statistical analysis of the compositional results for the crude and refined cottonseed oils was provided due to the sample number. Overall, the results presented in Tables 13 and 14 do not show any differences in oil composition between Coker 312 (parental line) and GHB614 cotton.

Table 13: Compositional Analyses of Crude Oil and Deodorized, Bleached Refined Oil Samples of Cottonseed from Cotton Line GHB614 and Control Line Coker 312

Analyte	Crude Oil		Deodorised, refined Oil	
	Non-GM Coker 312	GHB614 cotton sprayed	Non-GM Coker 312	GHB614 cotton sprayed
α – tocopherol (mg/100 g)	21.7	21.5	29.1	26.8
β – tocopherol (mg/100 g)	<2.00	<2.00	<2.00	<2.00
δ – tocopherol (mg/100 g)	<2.00	<2.00	<2.00	<2.00
γ – tocopherol (mg/100 g)	<2.00	<2.00	<2.00	<2.00
Total vitamin E (tocopherols) (mg/100 g)	21.7	21.5	29.1	26.8
Free gossypol	NA	NA	NA	NA
- gossypol (%)	0.36	0.38	<0.01	<0.01
+ gossypol (%)	0.47	0.47	<0.01	<0.01
Gossypol – Total (%)	0.83	0.85	<0.02	<0.02
Dihydrosterculic acid (%)	0.185	<0.100	0.162	<0.100
Malvalic acid (%)	0.634	0.423	0.430	0.357
Sterculic acid (%)	0.395	0.281	0.301	0.280

5.5 Conclusion

Compositional analyses were done to establish the nutritional adequacy of GHB614 cotton, and to compare it with conventional cotton varieties. The components analysed were proximates, fatty acids, amino acids, vitamin E, minerals, gossypol, phytic acid and the cyclopropenoid fatty acids.

No differences of biological significance were observed between GHB614 cotton and its conventional counterpart. Statistically significant differences in some of the key constituents were noted, however the differences observed were minor, and in each case the levels observed were within the range of literature values reported for conventional cotton varieties. The composition of cotton is known to vary significantly with the site, agricultural conditions and season of production, and differences reported here most likely reflect normal biological variability. Food from GHB614 cotton is therefore considered to be compositionally equivalent to food from conventional cotton varieties.

Table 14: Compositional Analyses of Crude Oil and Deodorized, Bleached Refined Oil Samples of Cottonseed from Cotton Line GHB614 and Control Line Coker 312

Fatty Acid Profile % relative	Crude Oil		Deodorised, refined Oil	
	Non-GM Coker 312	GHB614 cotton sprayed	Non-GM Coker 312	GHB614 cotton sprayed
Octanoic (C8:0)	<0.10	<0.10	<0.10	<0.10
Decanoic (C10:0)	<0.10	<0.10	<0.10	<0.10
Undecanoic (C11:0)	<0.10	<0.10	<0.10	<0.10
Dodecanoic (C12:0)	<0.10	<0.10	<0.10	<0.10
Tridecanoic (C13:0)	<0.10	<0.10	<0.10	<0.10
Myristic (C14:0)	0.63	0.65	0.63	<0.10
Myristoleic (C14:1)	<0.10	<0.10	<0.10	<0.10
Pentadecanoic (C15:0)	<0.10	<0.10	<0.10	<0.10
Pentadecanoic (C15:1)	<0.10	<0.10	<0.10	<0.10
Palmitic (C16:0)	23.63	24.12	23.62	24.00
Palmitoleic (C16:1)	0.58	0.61	0.54	0.58

Fatty Acid Profile % relative	Crude Oil		Deodorised, refined Oil	
	Non-GM Coker 312	GHB614 cotton sprayed	Non-GM Coker 312	GHB614 cotton sprayed
Hexadecadienoic (C16:2)	<0.10	<0.10	<0.10	<0.10
Hexadecatrienoic (C16:3)	<0.10	<0.10	<0.10	<0.10
Hexadecatetraenoic (C16:4)	0.16	0.14	<0.10	<0.10
Heptadecanoic (C17:0)	<0.10	<0.10	<0.10	<0.10
Heptadecanoic (C17:1)	<0.10	<0.10	<0.10	<0.10
Stearic (C18:0)	2.62	2.52	2.61	2.51
Oleic (C18:1)	15.57	15.08	15.47	14.98
Linoleic (C18:2)	54.74	55.21	55.06	55.39
Linolenic (C18:3)	0.56	0.38	0.47	0.39
Octadecatetraenoic (C18:4)	<0.10	<0.10	<0.10	<0.10
Arachidic (C20:0)	0.29	0.30	0.29	0.29
Eicosenoic (C20:1)	<0.10	<0.10	0.12	0.11
Eicosadienoic (C20:2)	<0.10	<0.10	<0.10	<0.10
Eicosatrienoic (C20:3)	<0.10	<0.10	<0.10	<0.10
Arachidonic (C20:4)	<0.10	<0.10	<0.10	<0.10
Eicosapentaenoic (C20:5)	<0.10	<0.10	<0.10	<0.10
Heneicosapentaenoic (C21:5)	<0.10	<0.10	<0.10	<0.10
Behenic (C22:0)	0.13	0.13	0.12	0.12
Erucic (C22:1)	0.24	0.18	0.17	0.14
Docosadienoic (C22:2)	<0.10	<0.10	<0.10	<0.10
Docosatrienoic (C22:3)	<0.10	<0.10	<0.10	<0.10
Docosatetraenoic (C22:4)	<0.10	<0.10	<0.10	<0.10
Docosapentaenoic (C22:5)	<0.10	<0.10	<0.10	<0.10
Docosahexaenoic (C22:6)	<0.10	<0.10	<0.10	<0.10
Lignoceric (C24:0)	<0.10	<0.10	<0.10	<0.10
Nervonic (C24:1)	<0.10	<0.10	<0.10	<0.10
Other components	0.85	0.68	0.90	0.84

6. NUTRITIONAL IMPACT

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

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

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

In this case, GHB614 cotton is the result of a simple genetic modification to confer tolerance to glyphosate herbicide, with no intention to significantly alter nutritional parameters in the food. In addition, extensive compositional analyses have been undertaken to demonstrate the nutritional adequacy of food derived from GHB614 cotton and these indicate the GM cotton is equivalent in composition to its non-GM counterpart. The Applicant has however submitted a feeding study comparing the nutritional performance of GHB614 cottonseed meal with that derived from the conventional variety when added to animal feed. This study is evaluated below as additional supporting information.

6.1 Feeding study in broiler chickens

Study submitted:
1. Broiler Chicken Feeding Study with Glyphosate-tolerant GHB614 Cotton, J.M. Stafford; completed June 2007. Springborn Smithers Study No. 13798.4115

The growing broiler is very sensitive during the first 40 days of life to changes in nutrient quality in its diet, as the birds increase body weight approximately 45-fold during this period. Broiler chicks are thus often used as a model to assess the wholesomeness of feed components, including GM commodities such as corn, cotton and soybean.

The purpose of this study was to compare the wholesomeness of GHB614 cotton (herbicide treated) to its conventional (non-GM) counterpart, as well as to a commercial cotton variety. The study was conducted using rapidly growing broiler chicks (Ross #708). Effects on health, survival, weight gain, feed consumption, feed conversion and marketable carcass quality (muscle: breast, thigh, leg and wing), and abdominal fat pad weights and yields were evaluated.

Cottonseed meal varieties used in the diets were toasted and analysed prior to shipment to the testing laboratories. Using the analytical information, a poultry nutritionist devised formulations for Starter (birds aged 0-7 days), Grower (birds aged 8-21 days), and Finisher (birds over 21 days old) growth phase diets for each cottonseed variety. The prescriptions were designed to be equivalent in energy/calories and protein, and as similar as possible in terms of limiting amino acids with respect to the cottonseed variety and the growth phase.

For each of the three treatment groups (GHB614 cotton, non-transgenic counterpart variety, non-transgenic commercial variety) there were 140 broilers in 14 pens (7 pens of males and 7 pens of females), for a total of 420 birds housed as 10 broilers/pen. Birds were randomised to treatment groups and received one of the three test diets immediately at cage assignment and throughout the 42 days of the study. The lighting regime was adapted from that currently used in regional, commercial, broiler chicken operations to reduce certain metabolic disturbances associated with rapid growth and extended photoperiod. Temperature and humidity were monitored daily. Water and feed were generally provided *ad libitum* throughout the study, however birds were fasted in darkness for a minimum of 8 hours prior to measurements of body weight on days 21, 35 and 42 (study termination).

All birds were monitored at least once a day for health status, overt signs of toxicity, and mortality. Body weights were recorded initially and at days 7, 21, 35 and 42. Feed consumption was measured for each pen on a weekly basis and used to calculate feed conversion ratios. Carcass and tissue weights were recorded for 126 of the 420 broilers in this study (21 birds/gender/treatment group). Statistical analysis was conducted on performance, carcass yield and meat quality parameters.

Chick mortality (14 birds across the three treatment groups, equivalent to 3% in this study) was considered to be on the low side of normal for the species and study conditions and was not related to treatment. The reduced mortality was attributed to a less extreme daylight schedule applied in this study which reduced commonly observed light-related metabolic abnormalities. Overall, including the 14 deaths, 29 birds showed clinical signs that are typically seen in feeding studies of this type and were not related to the dietary treatments.

All data on the following parameters were statistically analysed: feed consumption, body weight and total weight gain, feed conversion, chilled carcass weight, abdominal fat pad weight, leg weight, thigh weight, wing weight and breast weight.

The statistical analyses indicated significant differences among the treatment groups for several test (dependent) variables however most of the differences were between the two non-transgenic control groups.

As expected, there were significant differences in feed consumption between genders during all weeks as males consumed more than females, however there were no significant treatment/gender interactions over the study period. At the end of the study, the two factor ANOVA indicated a significant mean weight difference between the treatment groups; mean male body weight in the non-transgenic control group tended to be lower than that in males in the non-transgenic commercial variety. None of the body weight differences identified among the treatment groups could be related to the dietary treatment itself.

There were no significant differences in mean leg weight, thigh weight or wing weight across the treatment groups for the broilers fed diets containing GHB614 cottonseed, the control or commercial cottonseed. There were statistically significant differences in mean abdominal fat pad weight and breast weight between treatments and genders, with the non transgenic control group recording a mean fat pad weight significantly lower than either the GHB614 cotton group or the commercial variety. Similarly, mean female breast weight was lower in the control group than either the GHB614 cotton or the commercial variety. As expected, mean breast weight was significantly higher in male birds than in female birds across treatments. The results showed no differences among the three diets in the percentage of moisture, protein, and fat in the thigh and breast meat of broilers.

In conclusion, no biologically relevant differences were observed in the parameters measured between broilers fed the GHB614 cotton diet and the control diet. For the individual treatment comparisons, broilers in general had similar performance values and carcass yield and meat composition, regardless of whether the diets contained cottonseed meal from GHB614 cotton, the conventional counterpart or commercial cotton hybrids.

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Attachment 3

SUMMARY OF PUBLIC SUBMISSIONS

Submitter	Comments
Colette Connolly, Elisa Clarke, Laurel Buxton, Tessa Reimers, Victoria Lane, Jennifer Raco, Melissa Quigley, Michelle Parker, Jane Barrett, John Watson, Kim Healy, Glynis Gilkes, Helen Bell, Merrilyn and Garry Craig, Mary Gazzo, Pam Gladstones, Allison Rocher, Trevor Archibald, Danielle Romaine, Janine Asano, Lynne Turnbull, Jenni Bourke, Tobias Koberle, Adam Breasley, M. Rucco, Carol and Keith Keller, D. Hellale, Lesley Irving, Christine and Peter Villiers, Arthur Vasiliou, Jenifer Sharp, Mary Rainsbury, Jason Blake, Leony Malak, Janet Riches, Francine Moore, Diana Cole, Anna Rosenburg, Helen Ericson, J.S. Roszkowski, Margaret Wynne, Amy Jennings, Sarah Aitken, Michele Smith, Kelly Madden, Russell Langfield, Don and Liz Bretherton, Bryan Sait, Berenice Jamieson, Gaye de Lisle, Neville and Jeannette Dowling, Herbert Gerig, James Harris, Nick Maas, Meredith Stanton, Michael Dunne, Sarah Burnell, John Richards, Wendy Adamis, Rebecca Clark, Aram Joukadjian, Yeliz Surucu, Matthew Boyes, Rosslyn Evans, Richard Bouwmeester.	Comments received as a campaign letter: ▪ Opposed to the approval of all GM foods until such time that foods derived from GM cottons are labelled as being derived from a GM source.
Dr Anna Lavelle, CEO AusBiotech Ltd represents approximately 3000 members in the health, medical device, environmental and agricultural sectors.	▪ Supports approval of food derived from glyphosate tolerant cotton line GHB614 on the grounds that the safety assessment found no cause for concerns. ▪ Approximately 90% of Australian cotton is genetically modified. ▪ The TGA and APVMA assess company data in conjunction with published studies. The public process undertaken by FSANZ is a strong peer-review and critiquing process and concerns expressed about this are not justified. ▪ One way of controlling costs associated with testing would be to require 'GM-free' claims to be verified by testing, 'non-GM' and 'contains GM' claims to be justified by paper audits. This would provide information to consumers while allowing producer and market choice. ▪ Current labelling requirements are satisfactory to allow choice in the market place.
Madeleine Love representative for Mothers Are Demystifying Genetic Engineering (MADGE)	▪ Particularly concerned with the allergenicity of novel proteins. ▪ Refined oil has been repeatedly demonstrated to contain protein, and cottonseed meal may be consumed by humans. All novel proteins must therefore be included in the assessment.

Submitter	Comments
	▪ Various lengths of the chloroplast transit peptide (CTP) may be left behind on the 2mEPSPS protein as was reported in RoundUp Ready canola line GT73, where 4 amino acids from the CTP remained. Only one of the possible proteins has been assessed however a range of possible proteins may be present. ▪ FSANZ should request a full protein analysis of the seed products from this plant identifying all that may have originated from the T-DNA. ▪ It is not known whether the monoclonal antibodies used to detect the 2mEPSPS protein would have detected the range of possible 2mEPSPS proteins that may have been produced. ▪ FSANZ should obtain independent verification of the findings reported by the Applicant. ▪ Novel cross-border transcriptions may play a potential role in the plant including initiating the production of unintended novel toxins and allergens. ▪ The 2mEPSPS protein is synthetic, not natural. ▪ Six amino acid segment lengths was suggested as indicating allergen binding sites in a WHO/FAO consultation in 2001. The FSANZ assessment relies on eight amino acid segment lengths. ▪ Glycosylation data are irrelevant in the determination or otherwise of potential allergenicity. The published literature suggests that many cross-reactive proteins are not glycosylated. ▪ In vitro digestibility studies are not good indicators of potential allergenicity.
Ivan Jeray	▪ Strongly opposed to the approval of cotton line GHB614 on the grounds that the long term effects of GM foods are not known, and no tests are conducted on humans. ▪ GM food will induce antibiotic resistance. ▪ GM crops require greater amounts and stronger herbicides that poison the earth and induce resistant weeds and insects. ▪ GM food cannot be segregated and contamination of non-GM food has occurred, destroying the credibility of organic certification. ▪ Too many foods escape GM food labelling, and labelling is not policed. ▪ FSANZ Notification Circular did not state that A614 is a GM food application.
Christine Bennett	▪ Strongly opposed to the Application because of unknown and proven safety hazards. ▪ Labelling should clearly cite all GM substances in products marketed for human or animal consumption. ▪ The Applicant's GM food is an aberration of forced genetic transmutation. Humans cannot thrive on aberrant substances; only food produced by nature is biologically balanced. ▪ GM foods contravene social expectations of restorative and regenerative health benefits from food.

Submitter	Comments
	▪ Most Australians do not want GM foods. There was no referendum to ask the public if GM foods are wanted. ▪ The application process should be disseminated in all forms of media and be highly visible. ▪ Where are the peer-reviewed [safety] studies? ▪ Every person has the moral right to protection from material interests resulting from any scientific production: GM foods are unethical. ▪ Aberrant GM substances potentially endanger mental health. ▪ There are major documented adverse health effects including organ deformity, unnatural immune responses, nerve damage, skin problems, airway and lung inflammation and fatalities caused by ingesting GM substances. ▪ There is evidence that GM substances are teratogenic.
New Zealand Food Safety Authority (NZFSA)	▪ The Institute of Environmental Science and Research Limited (ESR) reviewed the FSANZ safety assessment for the NZFSA. Two issues require clarification: (i) the presence of 2mEPSPS protein in cotton linters, and (ii) the FAR should clearly present the compositional data that support the conclusions. The data presented in the assessment are hard to follow.
The Food Technology Association of Australia	▪ Supports approval of this Application. ▪ Strongly objects to the use of the same gene in multiple plant species. ▪ GM labelling should be opened to debate and review; GM ethical considerations should be part of the review.
Sally Wylie representative for Consumers Against GM (WA)	▪ More comprehensive GM food labelling based on the production process is needed to protect consumer choice, as applies in Europe.
Queensland Health	▪ Advances in science and technology should not be impeded if shown to be safe for humans and the environment. ▪ The use of GM crops and GM foods is considered on a case-by-case basis with particular consideration and relevance to human health. ▪ Updates on the regulatory status of COT67B in other countries should be provided in the FAR. ▪ Further consideration should be given to the fate of ingested recombinant DNA. ▪ FSANZ has used mostly data provided by the Applicant.
Joan Kennedy	▪ Opposed to the Application. All foods derived from a GM source should be labelled to give consumers the right to decide whether to buy the GM product.

Submitter	Comments
Australian Food and Grocery Council (AFGC)	▪ Supports approval of glyphosate tolerant cotton line GHB614. ▪ Notes concerns of jurisdictions on the costs of enforcement. Suggests an auditing system for products to ensure that manufacturers have assessed whether they are using GM products and are labelling them correctly. ▪ Strongly rejects suggestions that broader GM labelling is required because this would not address a demonstrated consumer safety concern, would increase costs, and would be inconsistent with the Ministerial Council's policies.
Rhys Brown representing family (WA)	▪ Oil from GM cotton lines is not safe and should not be used in foods. Long term safety studies have not been undertaken. ▪ GM processes are not the same as plant breeding. ▪ GM cotton is a textile crop, and should not be used for human consumption. ▪ GM labelling should include cottonseed products if present in foods.
Paul Elwell-Sutton	▪ Strongly opposed to this Application due to concerns about safety. ▪ Herbicide residues are higher in GM crops. For example, permitted glyphosate residues in food have been increased in New Zealand by a factor of 200. ▪ Glyphosate based herbicides contain other substances which may be toxic contaminants, such as compounds in the dioxin family that do not degrade readily and therefore could end up as residues in food. ▪ GM food labelling does not inform the consumer when the process has been used to produce food. This deprives consumers the right and ability to make an informed choice when purchasing foods.